DAIRY PRODUCT QUALITY – A NEW VISION FOR THE TWENTY FIRST CENTURY

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The quality of dairy products relies on the implementation of best practices from the pre-harvest environment throughout the processing and distribution chain, requiring a grass-to-glass approach. The dairy industry has long used a number of parameters to determine the quality of dairy products, both at the farm level and the processing level. Milk quality at the farm has historically focused on somatic cell counts (SCC), which are indicators of udder health, while processors primarily focus on testing finished product for coliforms as indicators of hygienic processing conditions. The dairy industry has managed and tested for these parameters for decades, basing quality premiums and performance targets on them, developing rapid testing methods and investing in control strategies. While SCC and coliforms are indeed measures of raw and pasteurized product quality, respectively, research on contemporary dairy products reveals that these measures may not be adequately assessing the major influencers of dairy product quality and shelf-life, specifically in fluid milk. In fact, there are two primary causative groups of bacterial contaminants that lead to product spoilage in high-temperature, short-time (HTST) processed fluid milk in the United States, namely psychrotolerant sporeforming bacteria and Gram-negative bacteria. Psychrotolerant sporeforming bacteria enter the fluid milk continuum at the farm where they are ubiquitous in the environment. These organisms survive pasteurization

in spore form and subsequently germinate and grow, reaching the Pasteurized Milk Ordinance (PMO) bacterial limit of 20,000 CFU/mL approximately 14-17 days after pasteurization. Studies have implicated sporeforming bacteria as the causative agent in approximately 50% of fluid milk reaching the PMO limit. In contrast, Gram-negative bacteria are eliminated by HTST pasteurization so their presence in finished product indicates that there has been re-contamination after pasteurization, also known as post-pasteurization contamination (PPC). Gram-negative bacterial contaminants, primarily *Pseudomonas*, grow rapidly at refrigeration temperatures, reaching the PMO limit 7-10 days after pasteurization. Our research indicates that approximately 50% of fluid milk reaches the PMO limit due to these Gram-negative bacterial contaminants. Importantly, coliforms, the traditional indicator organism in Grade "A" fluid milk are also a group of Gram-negative bacteria, but our work suggests that they account for a minor proportion of the total causative agents of PPC. Driving quality improvements in the dairy industry will require a new approach to defining quality parameters and addressing factors that influence those parameters in contemporary dairy products.

BIOGRAPHICAL SKETCH

Nicole H. (Woodcock) Martin was born in Elmira, NY in 1982 and lived in the Southern Tier of the great state of New York until she graduated from Addison High School in 2001. Nicole attended Cornell University, earning a Bachelor's degree in Food Science in 2006. Upon graduating Nicole took a technical position with the Milk Quality Improvement Program (MQIP), under the supervision of Kathryn J. Boor, where she primarily studied the quality of fluid milk. In 2007 Nicole began a M.S. degree in Food Science in the MQIP through the Cornell employee degree program. Nicole earned her M.S. in January, 2011 and began supervising the MQIP laboratory. She continued to pursue her research interests, which included the transmission of spoilage microorganisms from environmental sources into raw and pasteurized dairy products. In 2014 Nicole began a Ph.D. in Food Science under the mentorship of Dr. Martin Wiedmann, again through the Cornell employee degree program. Throughout her time as an employee and student in the MQIP Nicole has given numerous presentations and seminars at industry meetings and conferences, including at the U.S. Dairy Industry Spore Conference in 2014, 2016 and 2018; the International Symposium on Dairy Cow Nutrition and Milk Quality in 2015; the American Dairy Science Association Annual meeting in 2015, 2017 and 2018, and; the International Association for Food Protection in 2015 and 2018. Nicole is a frequent speaker at Cornell Dairy Foods Extension workshops and has been invited to present webinars for Dairy Australia every year since 2014. Further, Nicole regularly works with and consults for dairy industry stakeholders throughout the United States at both the farm and processing levels on matters relating to dairy product quality and safety.

This dissertation is dedicated to my grandmother, Lilya Kohl-Bennet, who as the mother of three young daughters in the 1960's returned to school and earned her degree in Registered Nursing. May I inspire our future generations the way you have inspired me.

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CHAPTER 1

Introduction

Parameters used to evaluate raw and finished product quality have changed little in the century since the widespread adoption of pasteurization. Raw milk quality is primarily evaluated using somatic cell counts (SCC) and standard plate count (SPC) (Boor et al., 1998), while finished product quality is primarily evaluated using SPC and coliform count (CC) (Martin et al., 2012). Yet research on contemporary dairy product quality has identified that these parameters may not reflect the bacterial groups that are driving dairy product quality in the twenty first century. For example, none of the standard tests currently used for monitoring raw milk quality detect sporeformers, which have emerged as an important cause of dairy quality issues across different products, from fluid milk, to cheese, and dairy powders (Murphy et al., 2016). Specifically, sporeforming organisms are present ubiquitously in natural environments including in dairy farm environments, are capable of surviving processing conditions (e.g., pasteurization) in spore form and subsequently grow in finished dairy products including fluid milk, cheese and in reconstituted products manufactured using dry dairy powders. Due to the impact of spores on finished dairy products, processors are increasingly interested in obtaining low spore raw milk. While spores present in raw milk are becoming progressively more important as causes of dairy product quality issues, post-pasteurization contamination (PPC) remains a major cause of dairy products quality issues and can have particularly negative impacts in fluid milk quality, where approx. 50% of spoilage issues are due

to PPC (Alles et al., 2018, Reichler et al., 2018). While further improvements in reducing PPC will require industry investment into equipment and infrastructure, the lack of easy-to-use rapid tests for PPC detection remains a major hurdle that impacts processor's ability to address this issue.

Based on the issues outlined above, the goal of the work presented in this dissertation is to (i) demonstrate the need for a comprehensive, systems wide approach to dairy product quality that acknowledges the impact of farm and processing level factors and to (ii) generate new knowledge that will provide new tools that can be used to improve microbial milk quality from farm to table and grass to glass. Combined, this knowledge will help the dairy industry to implement and utilize appropriate new tools that move beyond SCC, SPC, and CC to improve raw milk and processed dairy product quality. Chapters Two and Three are provided in lieu of a formal literature review and represent two published review articles that (i) outline the impact of post-pasteurization contamination on finished product, and specifically fluid milk quality (Chapter Two) and (ii) detail the need for new tests beyond CC to detect post-pasteurization contamination in a range of different dairy products from fluid milk to fermented products such as yogurt and cheese (Chapter Three). Chapter Three not only summarizes the current knowledge of different quality tests and suggests microbial tests that should be used for different dairy products, but also details additional research that needs to be pursued to further define the most appropriate indicator tests for certain products, such as butter.

The subsequent 3 chapters represent primary research that has been completed to address key knowledge and research gaps with regard to microbiological milk

quality; these three chapters move from farm level issues (e.g., on-farm spore sources; Chapter Four), to assessment, development, and of new microbiological methods to monitor finished products for (i) sporeformers (Chapter Five) and (ii) organisms responsible for PPC (Chapter Six). Specifically, Chapter Four focuses on understanding where spores are found in dairy farm environments and what factors at the farm level are important for the resultant spore level in bulk tank raw milk. This study showed that spores are abundant in the dairy farm environment and that environmental spore levels and management factors are important for the presence and levels of spores in bulk tank raw milk. The study presented in Chapter Four is an important step toward providing dairy farmers with the knowledge and tools to produce low-spore raw milk and will be used to develop future spore intervention studies to test the hypotheses generated from this work. Chapter Five outlines the examination of various methodologies for enumerating spores in dry dairy powders, with the goal of understanding how spore test parameters (e.g., heat treatment) impact the resultant spore count. This study found that spore counts varied significantly based on what parameters were used and demonstrated the need for further standardization of spore test methods in the dairy industry. Finally, Chapter Six is an examination of methodologies aimed at evaluating a set of contemporary fluid milk samples to rapidly detect PPC in fluid milk. Outcomes from the study described in Chapter Six not only supported that *Pseudomonas* is the primary PPC organism of concern in fluid milk but also identified a method that provided accelerated detection of PPC when compared with traditional methods (i.e., Moseley keeping quality test).

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CHAPTER TWO

Impact of post-pasteurization contamination on fluid milk quality

ABSTRACT

Fluid milk quality in the US has improved steadily over the last two decades, in large part due to the reduction in post-pasteurization contamination (**PPC**). Despite these improvements, some studies suggest that nearly 50% of fluid milk still shows evidence of PPC with organisms that are able to grow at 6°C, even though PPC may be much less frequent in some facilities. A number of Gram-negative bacteria when introduced as PPC can grow rapidly at refrigeration temperatures around 6°C and can lead to bacterial levels above 20,000 CFU/ml (the regulatory limit for bacterial numbers in fluid milk in the US) and spoilage that can be detected sensorially within 7-10 days of processing. Importantly however, storage temperature can have a considerable impact on microbial growth and fluid milk stored at 4°C and below may show considerably delayed on-set of microbial growth and spoilage as compared to samples stored at what may be considered mild abuse (6°C and above). Notable organisms that cause PPC and grow at refrigeration temperatures include psychrotolerant Enterobacteriaceae and coliforms as well as *Pseudomonas*. These organisms are known to produce a variety of enzymes that lead to flavor, odor and body defects that can ultimately impact consumer perception and willingness to buy. Detecting PPC in freshly pasteurized HTST fluid milk can be challenging because many times PPC occurs sporadically and at low levels. Additionally, indicator organisms typically used in fluid milk (i.e., coliforms) have been shown to represent

only a fraction of the total PPC. Recent studies indicate that coliforms account for less than 20% of the total Gram-negative organisms introduced into fluid milk post-pasteurization. In contrast, *Pseudomonas*, which is not a coliform and therefore is not detected using coliform media, is the most commonly isolated genus in PPC fluid milk. In order to reduce PPC, processors must (i) use testing methods that can both detect coliforms as well as non-coliform Gram-negatives (i.e., *Pseudomonas*) in order to understand true contamination rates and patterns and (ii) establish cleaning and sanitation protocols, as well as a plant culture that target persistent and transient PPC organisms.

INTRODUCTION

Raw milk, even when produced under ideal circumstances, has a diverse bacterial ecology that is a reflection of the life style of the animal and environment in which that commodity is produced. A wide range of Gram-positive and Gram-negative bacteria, pathogens, spoilage bacteria and organisms that are commensal with the animal or cause animal disease are all found in raw milk. Fortunately, pasteurization, which was widely adopted in the US in the 1940's reduces levels of many of these organisms by up to six orders of magnitude (Villamiel and de Jong, 2000). Certain heat resistant or thermoduric bacteria (e.g., *Micrococcus*) are capable of surviving pasteurization conditions (e.g. 72°C/15s) in vegetative form, but these organisms are typically not able to grow under refrigeration (Gleeson et al., 2013). Additionally, sporeforming bacteria can survive pasteurization in spore form; importantly, a number of aerobic sporeformers that can grow under refrigeration conditions have been identified in both raw milk and HTST-pasteurized fluid milk (Ivy et al., 2012). When post-pasteurization contamination (**PPC**) with organisms that can grow at refrigeration temperatures occurs, the Gram-negative organisms introduced will typically cause spoilage and reach levels above the Pasteurized Milk Ordinance (**PMO**) limit of 20,000 CFU/ml before growth of psychrotolerant sporeformers occurs and appear to also outcompete these sporeformers. In the absence of PPC with Gram-negative psychrotolerant organisms, aerobic psychrotolerant sporeformers present in raw milk typically grow to spoilage levels after 14d at 6°C (Ranieri and Boor, 2009). The predominant sporeforming bacteria capable of growing at refrigeration temperatures are certain strains of *Paenibacillus* and *Viridibacillus*

along with *Bacillus weihenstephanensis* (Ivy et al., 2012). One may expect that much of the fluid milk supply would be spoiled by these aerobic sporeforming bacteria that originate in raw milk and survive pasteurization, yet nearly 50% of the fluid milk supply shows evidence of contamination with heat-labile Gram-negative bacteria that originate from the processing facility environment and re-contaminate fluid milk after pasteurization.

Post-pasteurization contamination of fluid milk with psychrotolerant spoilage bacteria plays a significant role in limiting the quality and shelf-life of conventionally pasteurized fluid milk. From the earliest days of pasteurization, re-contamination of fluid milk after pasteurization has been identified as a problem. In 1920, milk inspectors were urged by Russel S. Smith of the Department of Agriculture to "not rest assured of a safe product because of the mere presence of a milk pasteurizing plant in their city. Special attention must be given to the operation of such a plant in view of the fact that unless it is properly operated it may become a chance source of infection" (Smith, 1920). Smith goes on to outline locations within the processing facility that should receive particular attention in order to prevent recontamination, namely pumps, bottling machines, bottles and milk cans (Smith, 1920). Despite the passage of nearly a century since the above advice to the dairy industry, PPC remains an important cause of fluid milk spoilage. This review focuses on the effects of PPC on fluid milk quality with special attention paid to the organisms commonly responsible for PPC, where they are typically introduced into fluid milk and diagnostic tools for detecting and tracking them in product and processing environments.

Pseudomonas is the primary causative agent of post-pasteurization contamination in fluid milk

Pasteurization is designed to reduce the populations of the most heat resistant vegetative pathogen found in milk, *Coxiella burnetii*, to levels that would not pose a public health risk (Holsinger et al., 1997). The resultant pasteurization parameters, a minimum of 72°C for 15 seconds for high temperature, short time (HTST) processing has been reported to deliver a considerable reduction in psychrotolerant Gram-negative bacteria (Champagne et al., 1994), and at least a 6 log reduction in some species of *Pseudomonas* (Villamiel and de Jong, 2000). Therefore, the presence of *Pseudomonas* and other Gram-negative bacteria in pasteurized fluid milk is typically an indication that there has been a contamination event post-processing. However, pasteurization failures and presence of high levels of Gram-negative bacteria (with subsequent survival of pasteurization of some bacterial cells) in raw milk may also be responsible for presence of Gram-negative bacteria in finished HTST products. The PMO limits total bacterial counts in Grade 'A' commingled raw milk to 300,000 CFU/mL (FDA, 2015). However, if this raw product is held for an extended period of time or at an elevated temperature prior to pasteurization, bacterial numbers may reach concentrations where even a 6 log reduction would result in residual bacterial cells in pasteurized finished product. While these deviations (i.e., pasteurization failure and very high pre-pasteurization bacterial levels) are less likely to be an issue in countries with well-developed and sophisticated dairy industries, in countries that lack effective on-farm cooling practices and an effective farm-to-processing plant refrigeration chain, high levels of Gram-negative bacteria in raw milk are not unusual.

There are four primary groups of psychrotolerant bacteria of importance in PPC of fluid milk (Table 2.1); (i) *Pseudomonas*; (ii) coliforms; (iii) non-*Pseudomonas*, non-coliform Gram-negatives and (iv) Gram-positive sporeforming bacteria. *Pseudomonas* is by far the most commonly reported organism responsible for PPC of HTST fluid milk in the US (Ranieri and Boor, 2009, Martin et al., 2011b) and globally, including Sweden (Ternström et al., 1993, Eneroth et al., 1998), Australia (Juffs, 1973, Deeth et al., 2002), the UK (Schröder, 1984a, Stevenson et al., 2003) and others. A number of factors contribute to the success of *Pseudomonas* as an agent of PPC, the first being its ability to grow rapidly at low temperatures (Ternström et al., 1993). Ranieri and Boor (2009) reported that samples of HTST fluid milk contaminated with *Pseudomonas* had significantly higher bacterial numbers by 7 days post-pasteurization than samples lacking PPC, with those samples contaminated with *Pseudomonas* reaching the PMO limit of 20,000 CFU/mL on average at day 8 post-pasteurization as opposed to day 15 post-pasteurization for samples with no PPC, but with presence of psychrotolerant sporeformers. Trmcic et al. (2015) demonstrated that various *Pseudomonas* strains were capable of growing more than 4 log CFU/mL over 21d at a slightly stressed refrigeration temperature (i.e., 6°C). Additionally, *Pseudomonas* are known to be particularly adept at outcompeting other spoilage microorganisms due in part to the ability of many strains to produce antibacterial and antifungal agents and siderophores (Gram et al., 2002) which are excreted into the growth medium where they bind and solubilize iron. *Pseudomonas* produce a variety of siderophores, notably pyoverdin (Brown and Luke, 2010). *Pseudomonas fluorescens* has been described as the predominant (Law, 1979, Dogan and Boor,

2003, Brown and Luke, 2010) and most important species of *Pseudomonas* found in

Table 2.1. Key groups of spoilage bacteria known to contaminate pasteurized fluid milk and their characteristics

Group	Examples of genera/species	Overview of key phenotypic characteristics relevant to fluid milk spoilage	Importance in PPC
Pseudomonas	*P. fluorescens* *P. fragi* *P. lundensis* *P. putida*	Many strains can grow at 4° and below, many strains can produce extracellular enzymes (e.g., proteases and lipases), some strains produce pigmented siderophores (e.g., pyoverdin)	Primary cause of PPC fluid milk
Coliforms	*Enterobacter* *Klebsiella* *Citrobacter* *Hafnia* *Serratia*	Many strains can grow at 4°C and below, many strains can produce extracellular enzymes (e.g., proteases and lipases)	Indicator used in US dairy industry; PMO does not allow for coliforms levels >10 CFU/ml
Non-coliform, non-*Pseudomonas* Gram-negatives	*Aeromonas*[1] *Flavobacterium* *Acinetobacter* Non-coliform Enterobacteriaceae (EB)	Many strains can grow at 4°C and below, many strains can produce extracellular enzymes (e.g., proteases and lipases)	Includes indicator organisms (i.e., EB) used in European dairy testing
Gram-positive sporeformers	*Bacillus cereus* group members *Paenibacillus*	Some *B. cereus* group linages (e.g., *B. weihenstephanensis*) and a number of *Paenibacillus* strains can grow at temperatures as low as at least 6°C; some strains produce extracellular enzymes (e.g., proteases and lipases)	Difficult to differentiate between contamination resulting from spores in raw milk and PPC

[1] PPC = Post-pasteurization contamination; PMO = Pasteurized Milk Ordinance

[2] *Aeromonas* strains have been shown to ferment both lactose and glucose thereby testing positive on coliform and EB media.

HTST pasteurized milk. Other species that have been detected in fluid milk include *P. fragi*, *P. lundensis* and *P. putida* (Ternström et al., 1993, Eneroth et al., 2000b, Brown and Luke, 2010).

Another common cause of PPC in fluid milk is due to coliforms (Kaloianov and Gogov, 1977, Wessels et al., 1989, Martin et al., 2012). Coliforms are defined not by taxonomic relationships, but by common phenotypic characteristics (Martin et al., 2016). Specifically, coliforms are a group of aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that are capable of fermenting lactose to produce gas and acid within 48h at 32-35°C (Davidson et al., 2004). Most coliforms are within the Enterobacteriaceae family, but at least one organism, *Aeromonas*, can produce a positive reaction on coliform media and is in the family Aeromonadaceae (Abbott et al., 2003). Coliforms have been used by the dairy industry for nearly a century as indicators of hygienic conditions in fluid milk (Tortorello, 2003), although there has been some discussion on whether coliforms are the best indicators to use in fluid milk (Martin et al., 2016). In the US, coliforms are limited in Grade 'A' pasteurized milk to no more than 10 CFU/mL (FDA, 2015).

Coliforms, because of their method defined nature, are a very diverse group of microorganisms. A study of microbiological quality of pasteurized fluid milk in New York state from 2001 to 2010 reported that 7.6 to 26.6 % of samples were positive for coliform in a given year (Martin et al., 2012). In a study of coliform contaminants found in HTST-pasteurized milk from 21 processors in the northeast US, Masiello et al. (2016) found that *Enterobacter* was the most prevalent coliform, compromising 42% of isolates collected. *Hafnia*, *Citrobacter*, *Serratia*, *Raoultella, Buttiauxella,*

Cedecea, Kluyvera, Leclercia, Pantoea, and *Rahnella* were also found. Another group found *Enterobacter*, *Klebsiella* and *Citrobacter* to be the predominant coliform genera in fluid milk and other dairy products in South Africa (Wessels et al., 1989).

In addition to *Pseudomonas*, other non-coliform Gram-negative bacteria linked to fluid milk spoilage are known to contaminate HTST fluid milk including *Aeromonas*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter* and others (Sørhaug and Stepaniak, 1997). Like *Pseudomonas* and coliforms, this group of bacteria has been shown to include a number of species and strains that can grow at low temperatures and produce a variety of enzymes that lead to fluid milk degradation (Michener and Elliott, 1964). Non-coliform Enterobacteriaceae are an important group of bacteria in this category, and include organisms such as *Proteus* (Hervert et al., 2016) which are less frequent contaminants in pasteurized fluid milk. Also, in this group are bacteria belonging to genera that include both strains that ferment lactose (coliforms) and those that do not (non-coliform). A recent study that surveyed growth of Enterobacteriaceae (**EB**) and coliforms isolated from dairy products on EB and Coliform Petrifilm, showed that even some strains from genera well known as coliforms do not ferment lactose and therefore do not meet the criteria to be coliforms (Hervert et al., 2016). For example, the authors report that of 10 *Rahnella* isolates tested, only 6 were able to ferment lactose (Hervert et al., 2016). *Rahnella* has been implicated in a smoky/phenolic defect in chocolate milk as a result of guaiacol production (Jensen et al., 2001). These strain variations highlight one reason some groups advocate using EB or total Gram-negative testing as more comprehensive indicators of PPC.

Gram-positive bacteria, including aerobic Gram-positive sporeformers, are

also capable of contaminating milk after pasteurization, however there are a number of Gram-positive bacteria that survive pasteurization, either in vegetative (e.g., *Micrococcus*) (Gleeson et al., 2013) or spore form (e.g., *Paenibacillus*) (Postollec et al., 2012), making it more complicated to determine whether these types of organisms, when found in finished products, originated from raw milk or PPC. A study conducted in Brazil showed that the same subtypes of *Bacillus cereus*, as determined by ribotyping, were found in finished product and on equipment swabs downstream from the pasteurizer (Salustiano et al., 2009). A similar study used randomly amplified polymorphic DNA (**RAPD**) to assess subtypes of *Bacillus* found in fluid milk along various points in two processing facilities in Sweden and in corresponding sealed consumer packages. The authors found that some RAPD types were found in consumer packages and in samples after the pasteurizer, but not in samples taken prior to the pasteurizer, concluding that these types were evidence of PPC (Eneroth et al., 2001). While these studies do not necessarily provide proof that *Bacillus* was introduced by PPC in these cases, these studies at least raise the possibility of PPC with Bacillus and other sporeformers and point to the importance of additional studies on occurrence and importance of PPC with sporeforming bacteria that cause fluid milk spoilage. If PPC with aerobic Gram-positive sporeformers is suspected, the subtyping tools used by these authors (i.e., ribotyping and RAPD), and others, discussed below, may be useful in determining if the contamination occurred at the farm or processing plant.

Post-pasteurization contamination has a significant impact on bacterial levels and sensorial properties of fluid milk

It has been extensively reported that fluid milk with reduced shelf-life (i.e., <10-14d) is virtually always characterized by presence and growth of microorganisms introduced by PPC (Schröder et al., 1982, Griffiths et al., 1988, Ranieri and Boor, 2009, Martin et al., 2012). In these studies shelf-life was either defined microbiologically (number of days under refrigerated storage to reach the PMO limit of 20,000 CFU/mL) or by milk defect judging or sensory evaluation. In the absence of PPC, the limiting biological agents in fluid milk are aerobic psychrotolerant sporeforming bacteria that originate in the farm environment, enter the fluid milk continuum on the farm, survive pasteurization in spore form, then subsequently grow at refrigeration temperatures (Huck et al., 2008). Fluid milk reaching spoilage levels due to aerobic sporeforming bacteria typically have shelf-lives of greater than 14d (Ranieri and Boor, 2009) in contrast to those with PPC which routinely reach spoilage levels after 7-10 days of refrigerated storage at around 6°C (Ranieri and Boor, 2009). Martin et al. (2012), in a survey of fluid milk over a ten-year time period in New York State indicated that samples with PPC, specifically with coliform bacteria, showed significantly higher total bacteria counts at 14 days post-processing than samples with no coliform contamination. Another study showed that many psychrotolerant coliform strains are capable of growing more than 5 log in refrigerated fluid milk at 6°C over 10 days (Masiello et al., 2016). Others have shown that even at refrigeration temperatures of 4°C and at temperatures below 0°C, *Pseudomonas* and other psychrotolerant post-pasteurization contaminants are capable of growing in and spoiling (i.e., producing

degradative enzymes) pasteurized fluid milk (Michener and Elliott, 1964, Sørhaug and Stepaniak, 1997). While some spoilage microorganisms can grow in milk even at temperatures of 4°C and below, storage temperature is a known factor affecting growth rates of *Pseudomonas* and other Gram-negative contaminants (e.g., coliforms) in pasteurized fluid milk (Schröder et al., 1982). Temperature may also play a role in the relative populations of psychrotolerant contaminants in fluid milk over shelf-life (Schröder et al., 1982). For example, Schröder et al. (2009) found that the predominant psychrotolerant organisms present in pasteurized fluid milk from four processors in the UK after storage at 5°C were Gram-negative rods, while after storage at 11°C the predominant organisms detected were psychrotolerant sporeformers. However, another study found that when PPC was present, there were no major differences in the populations found in pasteurized milk held at 6°C or held at 10°C (Griffiths et al., 1988). More research is needed in order to better understand the effect of storage temperature on specific population changes that occur in fluid milk over shelf-life.

In addition to growth to high numbers during storage, a number of PPC organisms (e.g., *Pseudomonas*) also produce a variety of enzymes that lead to sensorial defects in fluid milk. Production of proteases and lipases that break down milk components have been described in *Pseudomonas* (Corrêa et al., 2011), a variety of coliform bacteria (Masiello et al., 2016) and in psychrotolerant sporeforming bacteria (Trmcic et al., 2015). For example, Dogan and Boor (2003) report that of a total of 338 *Pseudomonas* isolates, representing 42 unique ribotypes collected from processed milk, raw milk and dairy plant environments, 51% were protease positive,

47% were lecithinase positive and 67% were lipase positive. They further report that enzyme production appeared to be strain dependent, with the majority (69%) of *P. fluorescens* positive for all three enzymes and 87.5% of *P. putida* negative for all three enzymes. Another group assessed proteolytic and lipolytic activity of 37 *P. fluorescens* isolates from pasteurized milk and reported that all of the isolates were positive for protease and lipase activity (Rajmohan et al., 2002). Species and strain variations are consistent with other studies that report that different *Pseudomonas* species produce different sensory defects in skim and whole milk (Hayes et al., 2002). Specifically, Hayes et al. (2002) found that *P. putida* produced fruity fermented odors while *P. fluorescens* did not. Reports indicate that most enzyme production by *Pseudomonas* occurs when bacterial concentrations reach approximately 10^6 CFU/mL or higher. However, some strains are known to produce these enzymes at much lower concentrations (i.e., 10^4 CFU/mL) (Law, 1979, Schröder et al., 1982, Sørhaug and Stepaniak, 1997). Psychrotolerant coliforms have also been reported to vary in their ability to produce lipolytic and proteolytic enzymes. Masiello et al. (2016) reported that of 10 *Buttiauxella* isolates collected from pasteurized fluid milk, none were positive for lipolysis, while all *Serratia* isolates (n=17) from the same study were positive for lipolysis. Similar to the variation seen between different *Pseudomonas* species, strains within the same genera of psychrotolerant coliforms also show varying levels of enzyme production. For example, among the 17 *Serratia* isolates characterized by Masiello et al. (2016), 4 were negative for proteolytic activity, 6 had moderate activity and 7 were highly proteolytic. Wessels et al. (1989) observed a similar variation in capacity to produce proteolytic and lipolytic enzymes in strains of

coliforms isolated from various dairy products in South Africa. Specifically, they found that some strains of *Enterobacter* and *Klebsiella* were proteolytic at 7°C, while some strains of *Enterobacter*, *Klebsiella* and *Serratia* showed lipolytic activity at 30°C (Wessels et al., 1989).

The flavor and odor defects resulting from the production of extracellular enzymes are varied. Hayes and colleagues (Hayes et al., 2002) investigated the odor defects produced by six strains of *Pseudomonas* (two strains each of *P. fluorescens, P. fragi* and *P. putida*), and found that odor defects such as fruity, barny, rotten, cheesy and others were produced and differed by strain, milk fat level (e.g., skim or whole) and time of storage. The accumulation of small peptides resulting from bacterial proteolysis have also been reported to cause bitterness (Bodyfelt et al., 1988, Ma et al., 2000) and astringency (Harwalkar et al., 1989). Lipolytic activity, causing the release of free fatty acids, may cause rancidity (Shipe et al., 1978), unclean and soapy flavors (Dogan and Boor, 2003), all of which are common defects in fluid milk contaminated after pasteurization with psychrotolerant Gram-negative bacteria.

In addition to flavor and odor defects generated by PPC organisms in fluid milk, some cause severe body defects as well. A major defect associated with the growth of organisms introduced after pasteurization is coagulation, which can occur via two major pathways, first, acid production as a byproduct which destabilizes the protein matrix. Many common PPC organisms, including some strains of *Pseudomonas* and many psychrotolerant coliforms produce acid and thereby coagulate milk (Komagata, 1961). Another cause of coagulation is via proteolytic activity and is also commonly associated with psychrotolerant Gram-negative bacteria such as

Pseudomonas (NÖRnberg et al., 2010). This defect, typically called "sweet-curdling" because it occurs in the absence of acidification, is also known to be caused by some Gram-positive sporeforming bacteria (Collins, 1981). Another body defect associated with PPC is "ropiness", which is the production of exopolysaccharides which cause the product to develop a slimy consistency. This defect is caused by a number of organisms, including *Klebsiella* and other common post-pasteurization contaminants (Cheung and Westhoff, 1983). Finally, certain PPC organisms are known to produce pigments that may cause color defects in fluid milk (Palleroni, 1984). For example, a study by Evanowski et al. (Evanowski et al., 2017) describes a gray pigment defect in conventionally pasteurized fluid milk that was contaminated with *Pseudomonas azotoformans*. This organism is closely related to *Pseudomonas fluorescens* which has been implicated in a number of color related defects in dairy products (Martin et al., 2011a, Nogarol et al., 2013).

Processors committed to reducing post-pasteurization contamination will use total Gram-negative testing

In the US and many countries, indicator organisms are used to determine hygienic quality of pasteurized milk. US standards require total plate counts of less than 20,000 CFU/mL and coliforms no greater than 10 CFU/mL in Grade 'A' pasteurized fluid milk (FDA, 2015). In Europe, Enterobacteriaceae are the primary indicators used for pasteurized milk and milk products (European Communities Regulation, 2010). Importantly, total plate counts do not provide indication of PPC as high counts with this method could be due to bacteria surviving HTST (as detailed

above) or due to PPC. Methods approved for coliform enumeration in Grade 'A' pasteurized fluid milk include (i) coliform plate count on violet red bile agar (**VRBA**); (ii) Petrifilm Coliform Count and/or High Sensitivity Coliform Count; (iii) TEMPO CC-Coliform Count and; (iv) Peel Plate E. coli and Coliform and/or Peel Plate E. coli and Coliform High Volume Sensitivity (FDA, 2015). These methods, while approved for coliforms, are not able to detect all PPC because they do not detect lactose non-fermenters (e.g., *Pseudomonas*) which are known to compromise the bulk of PPC. Van Tassell et al. (2012) specifically demonstrated that VRBA, Petrifilm Coliform Count plates and MacConkey agar were ineffective at recovering a panel of 12 dairy associated *Pseudomonas* isolates. However, pour plating with crystal violet tetrazolium agar (**CVTA**) showed the highest detection efficiency for the presence of PPC (determined by end of shelf-life testing for Gram-negative bacteria) as compared to a non-selective standard plate count agar (R^2=0.95). Another recent study has shown that plating pasteurized fluid milk on CVTA following an enrichment step (21°C/18h) resulted in significantly higher detection of PPC than other methods (e.g., plating on coliform media following the same enrichment protocol (Alles et al., 2016)). The primary driver of the increased sensitivity for CVTA based methods is the ability of this media to detect total Gram-negative bacteria including *Pseudomonas*, which represented ~50% of the isolates identified in milk with PPC by the study cited above (Alles et al., 2016), as well as traditional indicators (i.e., coliforms).

The use of CVTA for detection of total Gram-negative bacteria is outlined in the Standard Methods for the Examination of Dairy Products (Frank et al., 1992). Crystal violet has been shown to inhibit Gram-positive bacteria while not significantly

suppressing Gram-negative bacteria (Smith and Witter, 1979) and has been used for detecting PPC since the 1960's (Thomas, 1969). Despite the half century since this method was first used in the dairy industry, very little additional methodological development, in particular in the area of rapid and automated methods, has occurred for detecting total Gram-negative bacteria in fluid milk. This is in stark contrast to the numerous methods that have been developed and are widely used for detecting total viable organisms and coliforms in fluid milk including dehydrated film media (Ginn et al., 1985), flow cytometry (Loss et al., 2012), and optical based detection methods (Firstenberg-Eden et al., 2002). The limited availability of rapid and automated methods for detection and enumeration of total Gram-negative bacteria in fluid milk, is a major barrier to the dairy industry's ability to quickly identify and resolve contamination events and ultimately deliver the highest quality product to consumers. Further research and development both at the academic and diagnostic industry level is needed to fill this gap.

Fillers are a major source of post-pasteurization contamination, but tracking post-pasteurization contamination sources requires discriminatory molecular subtyping methods

Many factors contribute to the occurrence of PPC in fluid milk including problems with hygienic design of equipment, cleaning and sanitization procedures, preventative maintenance, control of plant air and prevention of cross contamination. In order to identify and resolve PPC events processors must perform root-cause analysis that includes establishing if the contamination is persistent or transient in

nature, as this will inform the necessary steps to resolve the contamination. Persistent contamination occurs when an organism is introduced into, and continues to live in, the facility or equipment over time without being removed by cleaning and sanitation. A common vehicle of persistent contamination is biofilms, which are communities of bacteria that attach to processing equipment and are resistant to cleaning and sanitation, leading to continued contamination of the product over time (Marchand et al., 2012). Many organisms have been found inhabiting biofilms in dairy processing facilities, including Gram-positive (e.g., *Bacillus*) and Gram-negative (e.g., *Escherichia coli*) bacteria (Salustiano et al., 2009, Shi and Zhu, 2009, Simões et al., 2010, Cherif-Antar et al., 2016). Biofilms are likely to occur when cleaning and sanitation, and preventative maintenance programs are ineffectively designed or implemented. This may be in the form of dead ends in equipment, incorrect concentrations of cleaning and sanitizing chemicals and cracked or pitted rubber filler components. Even correctly used clean-in-place systems may allow development of biofilms in dairy processing equipment which cannot be subsequently removed (Simões et al., 2010). However, persistent bacterial communities may not necessarily have to represent biofilms; sanitary design issues with equipment and facilities may also provide niches where bacteria are protected from sanitizer and survive in "non biofilm communities". For example, it is conceivable that pipe dead ends may contain planktonic bacterial communities or sessile bacteria without the extracellular matrix that is typical for biofilms.

Filling equipment has been identified by a number of studies as a primary source of persistent PPC in fluid milk. For example, Eneroth and others (1998) took

samples of HTST fluid milk at various sites along the processing continuum (e.g., silo tank, immediately preceding and following the pasteurizer, buffer tank, filler and consumer package) and found the majority of the PPC was occurring at the filling step. Similarly, another study used molecular subtyping tools, specifically ribotyping, to track the source of *Pseudomonas* PPC of HTST pasteurized fluid milk to filler nozzles, which had cracks and other evidence of deterioration upon manual compression that were not evident in the nozzles during cleaning and sanitation (Ralyea et al., 1998). A study conducted in the UK showed that while there were instances of PPC originating from milk storage tanks, the majority of PPC originated at the filling step and occurred at low levels (1-50 psychrotolerant Gram-negative bacteria per 100mL) (Schröder, 1984b). Gruetzmacher and Bradley (1999) in their study also found that the filling equipment was a major source of PPC. The authors sampled milk just prior to flowing through the filling machine head and just after, finding that the milk that had not passed through the filling equipment had a 20 d longer shelf-life at 7°C (Gruetmacher and Bradley, 1999).

Transient contamination occurs when an organism that is present in the equipment or facility is introduced onto food contact surfaces or directly into the product but is subsequently removed with effective cleaning and sanitation. Primary modes of transient contamination are through employee contact, especially when proper handwashing frequency and technique are not adhered to (Montville et al., 2002) as well as via biological aerosols (Kang and Frank, 1989). Aerosols are a suspension of microscopic solid or liquid particles in air or gas (Kang and Frank, 1989), and when carrying bacteria, fungi or other microorganisms, are considered

biological aerosols. In the case of biological aerosols, contamination that may be present in non-food contact areas (e.g., floors or drains) are aerosolized by a variety of mechanisms including hose use during production (Kang and Frank, 1989). One study found that hose use, drains and personnel activity were all associated with an increase in total aerobes and staphylococci found in dairy processing facility air (Ren and Frank, 1992). In addition to hose use causing potential PPC through aerosolization, direct use of water on fillers in dairy plants has been found to be associated with higher levels of PPC (Eneroth et al., 2000a).

Determining the source, type (i.e., persistent vs. transient) and causative agents of PPC is not achievable with traditional microbiological methods alone. Molecular subtyping tools, which have been used in source tracking in foodborne disease outbreaks for decades (Sabat et al., 2013), provide a sensitive tool for the dairy industry to track and resolve PPC. Previously used subtyping techniques for tracking PPC include pulsed field gel electrophoresis (PFGE) (Martin et al., 2011a), ribotyping (Martin et al., 2011a), multilocus sequence typing (MLST) (Andreani et al., 2014), randomly amplified polymorphic DNA (RAPD) (Eneroth et al., 2000a) and DNA based sequencing techniques (e.g., rpoB allelic typing) (Huck et al., 2007). For example, Martin and others (2011) used DNA sequencing, ribotyping and PFGE as molecular subtyping tools to track PPC causing a blue discoloration in a fresh, low-acid cheese product and identified environmental sources that were responsible for the finished products contamination. Ultimately, PFGE was found to be sufficiently discriminatory to distinguish between *P. fluorescens* capable of causing the defect and those that did not, as well as to determine that the source of the organism was an

agitator track above a cheese vat. Likewise, RAPD was used to identify the primary sources of Gram-negative contamination in three dairies in Sweden (Eneroth et al., 2000b). The authors found that the same persistent RAPD types, most of which were identified as *Pseudomonas*, were found in condensed water on the filling nozzles, in waste-water at the bottom of the filling machine and in the air surrounding the filling machine as were found in pasteurized packaged milk (Eneroth et al., 2000b).

Finally, in the case of suspected PPC with organisms that can both originate in raw milk as well as potentially contaminate product after pasteurization (i.e., Gram-positive sporeformers such as *Paenibacillus*), these highly sensitive molecular subtyping tools are necessary to distinguish the source of the contamination. A 2007 study (Huck et al., 2007) demonstrated that Gram-positive sporeforming bacteria can be tracked from the farm throughout a HTST fluid milk processing facility, and that certain rpoB allelic types (AT) appear to be introduced at various points throughout the process. However while single gene seeing methods (such as rpoB allelic typing) provide for good characterization and identification, these methods typically show limited discriminatory power and hence may not always provide the best source tracking tool Interestingly, another study showed that unique RAPD subtypes of Gram-positive sporeformers (*Bacillus cereus* s.l. and *Paenibacillus odorifer*) were detected over time in extended shelf-life (ESL) fluid milk products processed in Germany, but were never isolated from raw bulk tank milk (Doll et al., 2017); while this may suggest that contamination with these organisms represented PPC, it may also be due to difficulties detecting low levels of these organisms in raw milk. These studies highlight the need for sensitive and discriminatory subtyping tools in order to

better identify and characterize PPC sources and transmission of both Gram-positive and Gram-negative spoilage organisms. While application of emerging whole genome sequencing tools likely will provide valuable tools for these purposes, the importance of well-designed sampling plans and schemes can also not be under-emphasized.

CONCLUSIONS

While PPC remains an important cause of fluid milk spoilage, some processing facilities have been highly successful at minimizing PPC (Martin et al., 2012), indicating that effective control of PPC is possible, even though contamination of a milk container with a single organism that can grow at refrigeration temperatures is sufficient to cause product spoilage over shelf life. Efforts to develop and deploy more effective tools to detect PPC, trace it to a source, and ultimately prevent PPC are however, essential to improve the quality and shelf-life of HTST-pasteurized fluid milk. Specific areas of need include (i) development of better methods for detection and trace-back of PPC, (ii) validation and implementation of improved procedures to prevent PPC (e.g., SSOPs, procedures for mid-shift clean-up) and (iii) sanitary equipment design. Lessons learned from the control of environmentally transmitted foodborne pathogens (foremost *L. monocytogenes*) maybe translatable to improved control of PPC. For example, use of "seek and destroy" type approaches developed for *Listeria* control (Malley et al., 2015) could be modified to identify and eliminate environmental sources of PPC. Similarly, data that indicates that mid-shift clean-up actually increases the risk of *Listeria* contamination could be applied to PPC and spur further studies on the effectiveness of different in process cleaning strategies that may

currently be used in HTST plants.

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CHAPTER THREE

The Evolving Role of Coliforms as Indicators of Unhygienic Processing Conditions in Dairy Foods

ABSTRACT

Testing for coliforms has a long history in the dairy industry and has helped to identify raw milk and dairy products that may have been exposed to unsanitary conditions. Coliform standards are included in a number of regulatory documents (e.g., the U. S. Food and Drug Administration's Grade "A" Pasteurized Milk Ordinance). As a consequence, detection above a threshold of members of this method-defined, but diverse, group of bacteria can result in a wide range of regulatory outcomes. Coliforms are defined as aerobic or facultatively anaerobic, Gram negative, non-sporeforming rods capable of fermenting lactose to produce gas and acid within 48 hours at 32-35°C; 19 genera currently include at least some strains that represent coliforms. Most bacterial genera that comprise the coliform group (e.g., *Escherichia*, *Klebsiella* and *Serratia*) are within the family Enterobacteriaceae, while at least one genus with strains recognized as coliforms, *Aeromonas*, is in the family Aeromonadaceae. The presence of coliforms has long been thought to indicate fecal contamination, however, recent discoveries regarding this diverse group of bacteria indicates that only a fraction are fecal in origin, while the majority are environmental contaminants. In the US dairy industry in particular, testing for coliforms as indicators of unsanitary conditions and post-processing contamination is widespread. While coliforms are easily and rapidly detected and are not found in pasteurized dairy

products that have not been exposed to post-processing contamination, advances in knowledge of bacterial populations most commonly associated with post-processing contamination in dairy foods has led to questions regarding the utility of coliforms as indicators of unsanitary conditions for dairy products. For example, *Pseudomonas* spp. frequently contaminate dairy products after pasteurization, yet they are not detected by coliform tests. This review will address the role that coliforms play in raw and finished dairy products, their sources and the future of this diverse group as indicator organisms in dairy products.

INTRODUCTION

In microbiological testing, an "indicator organism" is defined as a marker that reflects the general microbiological condition of a food or environment (Chapin et al., 2014). In contrast, an "index organism" is a marker that reflects the possible presence of ecologically similar pathogens, suggesting a potential public health risk (Chapin et al., 2014). For nearly a century, coliforms have been used as indicator organisms, first in evaluating water for fecal contamination and later in identifying unsanitary conditions in pasteurized dairy products and other foods. Indeed, coliform testing of pasteurized milk was recommended by the U.S. Public Health Service in the earliest edition of the Grade "A" Pasteurized Milk Ordinance (PMO) published in 1924 (Tortorello, 2003). Currently, the PMO limits coliforms in Grade "A" pasteurized milk and milk products to 10 or fewer CFU per mL (FDA, 2015). Coliforms, defined as aerobic or facultatively anaerobic, Gram-negative, nonspore-forming rods capable of fermenting lactose with

the production of acid and gas at 32°C-35°C (Davidson et al., 2004), were originally considered to represent only strains from the genera *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella*. Classification of coliforms has been a difficult issue for decades. Coliform differentiation was originally primarily based on the fermentation of sucrose and dulcitol, production of indole and acetylmethylcarbinol, and gelatin liquefaction. Later, Parr established the IMViC formula, which involved indole production, methyl red reaction, Voges-Proskauer test, and citrate utilization (Parr, 1938). Even with these methodological improvements, some strains were still not detected as part of the coliform group.

As taxonomic classification methodologies have improved over the decades, it has become clear that coliforms, as defined solely by the method used to detect them, are a much broader and more diverse group of bacteria (Leclerc, 2001). Currently, 19 genera have member strains that fall into the coliform group, mostly encompassed in the family Enterobacteriaceae, however strains of *Aeromonas*, in the family Aeromonadaceae, also have been identified as coliforms (Abbott et al., 2003) because of their ability to ferment lactose to form gas and acid within 48h at 32-37°C, although it should be noted that there is some disagreement regarding whether *Aeromonas* should be considered a coliform. Importantly, because of the method-defined nature of this group, it is not uncommon for some species or strains within a genus to be coliform-positive while others are coliform-negative. Such variability within genera complicates classification and understanding of these microorganisms.

In an effort to increase functional differentiation within the diverse coliform group, Leclerc et al., (2001) proposed three categories of coliforms based on

taxonomic and physiological traits: “thermophilic,” which include *E. coli* of fecal origin; “thermophilic and ubiquitous” and; “psychrotrophic,” which are purely environmental. Of the “thermophilic” coliforms, which are characterized by their ability to grow and ferment lactose at 44-45°C, the only reliable indicator of fecal contamination is *Escherichia coli*. This organism does not survive well in environments outside of the intestinal tract of warm-blooded animals, hence, it is not an environmental contaminant. However, while others in this group, including some species of *Klebsiella*, *Enterobacter* and *Citrobacter*, may originate from fecal matter, they also can originate from environmental sources, making them unreliable indicators of fecal contamination. In contrast, “psychrotrophic” environmental coliforms have the ability to grow and ferment lactose at refrigeration temperatures, but generally do not grow above 38°C, which distinguishes them from the thermophilic group. Members of the genera *Klebsiella*, *Enterobacter*, *Serratia* and others are considered environmental coliforms (Leclerc, 2001). Finally, “thermophilic and ubiquitous” coliforms originate from various natural environments including soil, water, vegetation, insects, farm produce, wooden reservoirs, grass, silages, and fresh vegetables (Seidler et al., 1975). Members of this group of “ubiquitous” coliforms are found within the genera *Klebsiella*, *Enterobacter* and *Citrobacter*.

As a consequence of the improved understanding of the environmental sources of many microorganisms that test positive as coliforms, many industries have moved away from using detection of total generic coliforms for food and water testing (Leclerc, 2001; Busta et al., 2006) as they are poor indicators of fecal contamination and overall hygienic conditions. However, coliform testing remains a cornerstone of

microbial testing in the U.S. dairy industry, from raw milk testing to processed dairy product testing. Recent studies provide evidence that coliform testing should be reconsidered as a marker for unsanitary conditions in the dairy industry as further understanding of this diverse group of microbes is achieved.

Coliforms represent a common raw milk contaminant that originates from various environmental and fecal sources

Coliforms are among the many groups of microorganisms that are normally present in raw milk, i.e., 96% of all bulk tank milk samples collected during a 2002 study in the U.S. were coliform-positive (Van Kessel et al., 2004). California has established the only regulatory limit for coliforms in raw milk intended for Grade "A" dairy products in the U. S. (not to exceed 750 CFU/mL; CDFA, 2016). Reported coliform levels in raw milk vary greatly, with mean coliform counts for milk sampled in the US ranging from 31 CFU/mL (Boor et al., 1998) to 2,570 CFU/mL (Jayarao and Wang, 1999). Similar results have been reported by others (D'Amico et al., 2008; Pantoja et al., 2011; Jackson et al., 2012). Common coliform genera in raw milk include *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella* (Jayarao and Wang, 1999), which can originate from a variety of sources in the dairy farm environment including water, plant materials, equipment, dirt, and fecal sources (Kagkli et al., 2007). High levels of coliforms (e.g., >1,000 CFU/mL) in raw milk may indicate unsanitary practices on the farm, inadequate refrigeration, or the presence of coliform mastitis (Jayarao and Wang, 1999, Hogan and Smith, 2003, Pantoja et al., 2011). Additionally, certain management practices at the farm level, including milking

machine wash failures, rate of cluster washes and rate of milking unit fall-off during milking also correlate to variations in levels of coliforms in raw milk (Pantoja et al., 2011).

Milking mastitic cows can introduce coliforms into bulk tank raw milk, hence somatic cell counts (SCC) also can be correlated with the presence of coliform bacteria. Coliform genera recognized as causing mammary infections include *Escherichia*, *Klebsiella*, *Enterobacter* and *Serratia* (Hogan and Smith, 2003). The cow may become exposed to mastitis pathogens through manure, bedding, soil and water (Hogan and Smith, 2003). Pantoja et al., (2011) found that in-line coliform counts increased 6.3% for every 10% increase in in-line SCC, which could reflect as little as the milk from one mastitic cow being milked into the bulk tank.

Despite there being no federal coliform regulation for raw milk being processed into U. S. Grade "A" dairy products, many states that allow the sale of raw milk for direct human consumption have regulatory limits for coliforms. For example, in California, raw milk "shall contain not more than 15,000 bacteria per milliliter or [not] more than 10 coliform bacteria per milliliter" (California Food and Agriculture Code, 2016). According to a Raw Milk Survey conducted by the National Association of State Departments of Agriculture (NASDA; Ehart) in 2011, 30 states allowed raw milk sales. Five of the thirty states had special regulations for raw milk, including "cow-share" agreements, in which the consumer "owns" all or part of a cow, and therefore, can have access to its milk, or limit raw milk sale to specific markets. Among the 30 states, twelve allow the consumer to access milk at both the farm where the milk is produced and at retail stores that can be separate from the farm. The

remaining thirteen states restrict legal sales of raw milk only to the farm where the milk is produced. Of the 30 states that allow sale of raw milk for human consumption, coliform limits of ≤10 CFU/mL to ≤100 CFU/mL are imposed in 20 states (Table 3.1; Ehart, 2011).

Table 3.1. Summary of coliform standards for raw milk sold for human consumption

	No. of states allowing raw milk sales[1]		
Coliform standard[2]	On-farm sale	Retail milk sale	Cow-share/Other [3]
No limit	8	0	2
≤10 CFU/mL	4	9	2
≤25 CFU/mL	0	1	0
≤50 CFU/mL	0	2	0
≤100 CFU/mL	1	0	1
Total	13	12	5

[1] For details on raw milk regulations by state, see Ehart (2011); as state-level raw milk regulations change frequently, states are not listed here to avoid mis-leading or out-of-date information

[2] CFU = colony forming units

[3] A cow-share is an agreement entered into by individual(s), who pay a farmer a fee for boarding and milking the cow(s) that they own. After the cows are milked, the individual(s) obtain the milk from the farmer. Technically, these arrangements are not considered "raw milk sales."

While the use of coliforms as indicator organisms for the presence of unsanitary conditions in milk handling is increasingly under scrutiny, it is clear that coliforms are not appropriate index organisms for the presence of public health hazards in dairy products. For example, Jackson et al., (2012) examined levels of coliform bacteria in raw silo milk in correlation to the presence and levels of four pathogens of interest (*Bacillus cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp.). The study concluded that there were no significant increases in

coliform levels in pathogen-positive samples as opposed to pathogen-negative samples. Similarly, no significant differences existed in coliform counts from samples with zero, one, two, three or four pathogens detected. These results illustrate that coliform counts are not an index of the presence of these four pathogens, and that coliform testing of raw milk intended for human consumption cannot be used to reliably identify raw milk that presents a public health risk. This is also consistent with other studies (D'Amico et al., 2008) that detected pathogens in raw milk samples that had very high microbiological quality and low coliform counts.

Coliform contamination in pasteurized fluid milk leads to high total bacteria counts and low sensory scores

Coliform testing has been used to indicate hygienic condition of dairy products for nearly a century. Coliforms are common contaminants in fluid milk (Carey et al., 2005; Martin et al., 2012), cheeses (Wolfe et al., 2014; Trmcic et al., 2016) and other dairy products. Recent studies have shown post-processing contamination (PPC) with coliforms in 7.6% to 26.6% of U. S. fluid milk samples tested between 2001 and 2010 (Martin et al., 2012). Pasteurized fluid milk samples that were contaminated with coliforms had significantly higher bacterial counts and significantly lower overall sensory scores (Martin et al., 2012) over shelf-life than samples that tested negative for coliforms. The PMO limits the number of coliforms in pasteurized grade "A" milk to no more than 10 CFU/mL throughout shelf-life (FDA, 2015). In general, due to the heat labile nature of these organisms, the presence of coliforms and other Gram-negative bacteria in pasteurized fluid milk indicates: (i) PPC of the product; or (ii)

pasteurization failure. Many coliforms in pasteurized fluid milk products are psychrotolerant, and thus able to grow to high levels at refrigeration temperatures (Carey et al., 2005; Ranieri and Boor, 2009; Martin et al., 2011, Masiello et al,. 2016).

A recent study of coliform bacteria in pasteurized fluid milk indicated that species of *Enterobacter*, *Hafnia*, *Citrobacter*, *Serratia* and *Raoultella* represented the majority of the coliform population (Masiello et al., 2016). Of the coliform isolates collected by Masiello and colleagues (2016), the majority showed the ability to grow substantially (i.e., > 5 log growth) over 10 days at refrigeration temperatures. This robust growth, accompanied by the ability of many psychrotolerant coliforms to produce lipolytic and proteolytic enzymes (Wessels et al., 1989; Nornberg et al., 2009; Masiello et al., 2016) which are capable of causing flavor, odor and body defects in fluid milk, make the presence of coliforms in fluid milk detrimental to quality and consumer acceptance.

Prevention of PPC with coliforms and other microorganisms remains a major hurdle for some dairy processors in the U. S. (Ralyea et al., 1998; Ranieri et al., 2009; Martin et al., 2011). In many cases, contamination can be traced back to the presence of biofilms in processing equipment. Many types of bacteria are capable of forming biofilms in equipment, especially in cracks, dead ends and gaskets. Biofilms, which have been described as a functional consortium of microorganisms attached to a surface and embedded in the extracellular polymeric substances produced by the microorganisms (Costerton et al., 1987), allows colonization of populations of microorganisms and provides protection for the microbes from cleaning and sanitization procedures. As the biofilm matures, cells slough off and can contaminate

product during processing (Kumar and Anand, 1998). In dairy processing, in particular, the use of clean-in-place (CIP) systems may unintentionally lead to biofilm formation because such systems may fail to remove accumulated microorganisms and organic materials effectively (Kumar and Anand, 1998). The formation of the biofilm begins with a process known as conditioning which begins 5-10 seconds after milk processing begins (Marchand et al., 2012). In particular in processes where temperatures are high enough to begin to denature whey proteins (i.e., 65°C), adherence of this layer to the surface alters the surface properties and improves the ability of bacterial contaminants to adhere (DeJong, 1997). Continuation of the process of biofilm formation, namely bacterial adhesion, bacterial growth and biofilm expansion (Marchand et al., 2012) leads to biofilms that are resistant to removal, especially using CIP systems. Stringent cleaning and sanitation practices along with attention to sufficient preventative maintenance, hygienic design and employee training are essential to minimize formation of biofilms and prevent PPC.

Coliforms in cheese represent a diverse group of organisms

Coliforms are widely found in many cheeses (Khayat et al., 1988; Brooks, et al., 2012). However, in contrast to the presence of these microbes in raw and pasteurized fluid milk, and even in some other cultured products (e.g., yogurt), the presence of coliforms in cheese may not necessarily be negative. The vast variety of types of cheese manufactured contributes to the complexity of fully understanding the role of coliforms in cheese quality and safety. Cheese product characteristics, including moisture content, pH, salt content, ripening conditions, age of product and

culture all influence potential levels of and roles for coliforms and other microorganisms in the final product (Wolfe et al., 2014; Trmcic et al., 2016). A survey of raw milk cheeses by Brooks et al., (2012) found that 5 of 41 commercially available raw milk cheese samples had detectable coliforms (i.e., >10 CFU/g). In a similar study, Trmcic and colleagues (2016) surveyed 273 raw and pasteurized cheeses from the U. S. and other countries and found that 75 of those samples were positive for coliforms in concentrations above 10 CFU/g.

Many individual states in the U. S. have limits of 10 or 100 CFU/g for coliforms in cheese. In the European Union (EU), where microbiological specifications are regulated by the European Commission (EC), there are no regulations concerning coliforms (EC No 2073/2005) for cheese products. Regulations, instead, are focused on *Salmonella*, coagulase positive *Staphylococci* and *E. coli*. Additionally, regulations set forth by the EC are categorized by type of product (i.e., cheese made from raw milk or from thermized milk, soft cheese, fresh cheese and other cheeses), thus acknowledging the need for a scientific approach to assessing the hygienic conditions and microbial food safety hazards associated with cheeses.

Sources of coliforms in cheese products can vary depending on the product. Due to the nature of raw milk cheeses, the presence of coliforms is not unexpected as coliforms are common in raw milk. However, in pasteurized cheese products, coliforms present in raw milk should have been eliminated by pasteurization, implying that any coliforms present in the finished product resulted from PPC. Recontamination can occur in the processing or aging facility through cheese contact with contaminated

water, humans, air and biofilms on equipment (Lilly, 1972; Dancer et al., 1997; Hughes, 2003; Kilb et al., 2003).

High levels of coliforms in pre-cultured milk intended for cheese making may have deleterious effects on cheese production, specifically if acid development by the lactic acid bacteria (LAB) occurs more slowly than desired. Growth of coliforms early in cheese production may lead to early blowing, or gas production defects in the product (Farkye, 2000; Ledenbach and Marshall, 2009). Additional effects and byproducts of coliform growth early in cheese production can be reduction of desirable formation of diacetyl (Ledenbach and Marshall, 2009), lactic acid, acetic acid, formic acid, succinic acid, ethanol and 2,3-butyleneglycol (Farkye, 2000).

The growth or death of coliforms in cheese products depends on a variety of parameters including cheese pH, age, moisture content, salt content, free fatty acid content and others. Nunez and others (1985) found that Manchengo cheese products made with cooked curd had higher levels of coliforms than those made with uncooked curd. This difference was attributed to lower pH in the uncooked curd (due to superior growth of LAB). Nunez and others (1985) also found that the temperature of ripening had a significant effect on the reduction of coliforms, concluding that an aging temperature of 15°C was the optimum temperature to achieve reduction in coliforms (and other unwanted bacteria) and also to protect desired sensory attributes. Coliforms are typically inactivated and/or inhibited by the drop in pH during cheesemaking acidification. If pH increases during aging (due to proteolysis, typically in surface ripened cheese), however, conditions may exist to support coliform growth (Ledenbach and Marshall, 2009). Finally, Trmcic et al., (2016) reported that

pasteurization, pH, water activity, milk type (e.g., cow milk), and rind type were cheese factors that significantly influenced detection of coliforms in cheese. They also report that water activity is significantly associated with the final concentration of coliforms in cheese; suggesting more than 0.5 log CFU/g higher average final concentration of coliforms for every 0.01-unit increase in water activity.

Proteolytic and lipolytic enzyme production varies greatly in the coliform group (Wessels et al., 1989). Enzyme production is largely dependent on product storage temperature. Proteolytic and lipolytic enzymes can contribute both desirably and undesirably to flavor and texture characteristics of cheese. The proteolytic activities of some strains of coliforms have been studied (Nornberg et al., 2009; Macedo and Malcata, 1997); some are highly proteolytic. To date, studies have primarily focused on the negative impact of enzymatic activity from coliform origin on dairy product quality, but some work has examined possible advantageous impacts that coliform enzymatic activity may have on ripening and flavor development of certain cheeses (Macedo and Malcata, 1997). The notion of coliforms as possible desirable contributors to the complex ecosystem of cheeses, particularly farmstead and artisan cheeses, is supported by studies suggesting that coliforms may be part of the natural microflora of at least some cheeses (Quigly et al., 2011). Further, as different Gram-negative bacteria are being identified as having a high potential for production of aroma compounds during cheese production, new bacterial cultures are being developed to utilize this potential. Some of the species used in these new bacterial cultures (e.g., *Hafnia alvei*) are members of coliforms/Enterobactriaceae in which case the use of these bacterial groups as indicators would not be appropriate (Morales et al.,

2003; Deetae et al., 2009).

In the U. S., testing dairy products for coliforms (beyond fluid milk and cheese) is required by the PMO. Coliform limits in cultured products (e.g., yogurt), ice cream, nonfat dry milk and others are set at ≤10 CFU/ml or g (FDA, 2015). Current standard methods recommend testing yogurt for coliforms within 24h of production to obtain meaningful results (Duncan et al., 2004). However, enumerating *Enterococcus* may provide a more reliable hygiene indicator than coliforms because they are more likely to survive in the low pH environment (Frank and Yousef, 2004). There is little research on the use of Enterococci as indicators in high acid dairy products, however, Birollo et al., (2001) concluded that Enterococci have little industrial use as hygiene indicators in yogurt processing. While the pH of yogurt has long been considered too low to allow survival of coliforms, limited evidence exists to support this conventional wisdom. A recent study by Hervert (2016) evaluated a variety of common coliforms, *Enterobacteriaceae* (EB) and non-EB Gram-negatives (e.g., *Pseudomonas*) for their abilities to survive in commercial yogurt products. The study showed that, in general, coliform and EB organisms were capable of surviving and, sometimes, even growing under conditions encountered in commercial yogurt products, while non-EB Gram-negative bacteria showed rapid die-off. The authors concluded that testing for EB provided the most comprehensive approach for monitoring hygiene indicators in yogurt as opposed to testing for coliform and total Gram-negative bacteria.

Coliform contamination in ice cream has not been widely or recently studied in the U. S., although surveys from other countries indicate that coliform levels range from less than detectable to $>10^4$ CFU/g (Massa et al., 1989; Warke et al., 2000; M-E-

Elahi et al., 2002; El-Sharef et al., 2006). The storage conditions of ice cream are generally thought to inhibit growth of bacterial contaminants, including coliforms. As a heat-treated product, the presence of coliforms in ice cream and other frozen dairy products is an indicator of PPC. However, because contaminated ingredients (e.g., nuts, fruits, etc) may be added to the product after pasteurization, there is considerable opportunity for bacterial contamination that does not originate from unhygienic conditions, per se, in the processing facility (Duncan et al., 2004).

A century of coliform testing -- time to rethink our indicator organisms in the dairy industry?

As the landscape of the global and U. S. food industries changes and responds to new requirements to ensure a safe food supply, there is reason to review traditional methods of evaluating dairy product hygiene and safety. Because of their heat-labile nature, coliforms long have been used in the dairy industry as indicators of PPC. Certainly, in general, coliforms are undesirable in processed dairy products (e.g., fluid milk). However, while coliforms do represent PPC and can cause flavor, odor and body defects in many dairy products, in some dairy products, detection of this group of microbes is insufficient for identifying unhygienic conditions.

Recent work indicates that testing for EB or total Gram-negative bacteria offers a distinct advantage to coliform testing when detecting common PPC organisms in dairy products (Hervert et al., 2016). EB is a taxonomic group of microorganisms that encompasses almost all of the coliform group (Hervert et al., 2016) with the exception of *Aeromonas*, and has been used as a hygiene indicator broadly in Europe

(European Communities Regulation, 2010). A benefit of testing for EB over coliforms is increased sensitivity for detecting PPC because of the broader range of contaminants detected (Hervert et al., 2016). Although the EB group includes some pathogenic bacteria (e.g., *Salmonella*), EB are considered indicators as opposed to index organisms. In general, their presence in some food products has no correlation with the presence of pathogens (Johnson, 1996), although this has not been studied specifically in dairy foods. Recent work has identified that the EB group is superior as a hygiene indicator in yogurt products because they are capable of surviving, and even growing, under conditions encountered in that product (Hervert, 2016).

Testing for total Gram-negative bacteria as an indicator of unsanitary conditions in certain dairy products (e.g., fluid milk) offers a distinct advantage over coliform or EB testing (Table 4.2). *Pseudomonas*, which lacks the ability to ferment lactose and is therefore not a coliform, has been described as the major contributor to PPC in the U. S. fluid milk industry (Ranieri and Boor, 2009; Martin et al., 2012). *Pseudomonas* readily forms biofilms in processing equipment (Ralyea et al., 1998) and, according to a survey of fluid milk across the U. S., accounts for ~70% of fluid milk spoilage from PPC in the U. S. (Ranieri and Boor, 2009). However, coliform tests do not detect *Pseudomonas* and other non-coliform Gram-negative bacteria that commonly contaminate fluid milk post-processing. Van Tassell et al., (2012) found that crystal violet tetrazolium agar (CVTA) was the most effective selective medium for detecting a diverse group of *Pseudomonas* commonly associated with PPC in fluid milk, whereas commonly employed coliform media (e.g., violet red bile agar) had limited ability to detect *Pseudomonas*. Therefore, coliform testing is not an effective

Table 3.2. Proposed hygiene indicator tests for different dairy products

Product	Proposed microbial hygiene indicator test[2]	Justification	Key references
Fluid milk	Total Gram-negative bacteria	Key hygienic issues in pasteurized fluid milk are (i) PPC and (ii) pasteurization failure. Both can be detected more reliably with a test that detects all GN bacteria (rather than coliform or Enterobacteriaceae [EB] tests)	Ranieri and Boor, 2009; Martin et al., 2012
Fermented dairy products (e.g., yogurt, kefir, etc)	Entero-bacteriaceae (EB)	Non-EB Gram-negative bacteria decline rapidly at the pH encountered in fermented dairy products while EB generally survive in these conditions making it possible to detect them as indicators of unhygienic conditions.	Hervert et al., 2016; Hervert, 2016
Aged cheeses	Targeted risk-based pathogen testing [1]	No suitable tests are currently available, specific pathogen tests are recommended based on risks associated with specific cheese characteristics (e.g., pH, A_w, etc).	Schvartzman et al., 2014; Trmcic et al., 2016
Fresh cheeses	EB and/or E. coli (additional research needed) [2]	Currently coliforms and EB are commonly used as hygienic indicators in fresh cheeses.	
Dairy powders	EB and/or targeted risk-based pathogen testing (additional research needed) [2]	Currently coliforms and EB are commonly used as hygienic indicators, but testing for selected pathogens is typically required for dairy powders that are used in infant formula	
Ice cream	Total Gram-negative bacteria (additional research needed)[2]	Currently coliforms and EB are commonly used as hygienic indicators in ice cream.	
Butter	Total Gram-negative bacteria (additional research needed) [2]	Currently coliforms, EB and proteolytic bacteria are commonly used as hygienic indicators.	

[1] Testing for target pathogens of concern may be appropriate for all products (or required under some jurisdictions), even if not specifically mentioned in this Table

[2] Proposed indicator tests for these four products (fresh cheese, dairy powders, ice cream, butter) are based on product characteristics, processing parameters and research findings from other dairy products; additional research is needed for these specific products to make more definitive recommendations regarding best practices for microbial hygiene indicator tests.

approach for detecting fluid milk exposed to PPC. As dairy plants strive to reduce PPC, the ability to identify contamination occurrences and to rapidly respond is critical to improving the quality of fluid milk products. Based on the current understanding of the ecology of PPC in fluid milk and the inability of coliform testing to identify the majority of these contaminants, exclusive use of coliform testing for this purpose ironically may prevent the fluid milk industry from detecting and rapidly resolving contamination issues.

Further, in the cheese industry, there is growing concern that coliform testing, especially in raw milk cheeses, provides little in the way of indicating hygienic conditions. Some research suggests that certain members of the coliform group, in fact, may be advantageous microorganisms in certain types of cheese (Macedo and Malcata, 19977; Quigly et al., 2011), and that coliforms serve no scientifically valid function as an index organism (i.e., for suggesting pathogen contamination). At best, coliform testing in cheese may provide insight into potential PPC, depending on the product. At worst, coliform testing may provide a false sense of security when public health risks from pathogenic contaminants are present. Trmcic et al., (2016) assessed the association between coliform detection in raw and pasteurized cheeses and the presence of *Salmonella*, *Staphylococcus aureus*, Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes* and other *Listeria* species. This study found no association between pathogen presence and coliform detection, despite an association between *Listeria monocytogenes* with washed rind style cheeses. Other groups have also found that cheese characteristics (e.g., pH) are associated with the presence of pathogens in the product (Schvartzman et al., 2014). This is not surprising given the

association between cheese characteristics and overall microbial diversity in cheese (Wolfe et al., 2014). The lack of association between the presence of pathogens and coliform detection, as well as the evidence that cheese characteristics are associated with pathogen prevalence, suggests that a model whereby products are categorized by their inherent characteristics and tested for organisms that are likely to cause a public health threat in those particular products provides a more effective approach to assuring public health than coliform testing (Table 3.2).

CONCLUSIONS

Testing for the presence of coliform bacteria, a method-defined group, has long been practiced in the U. S. dairy industry, from raw milk to processed products. Coliform testing is rapid and has long been used as a primary indicator test for hygienic conditions associated with dairy products. However, recent advances in taxonomy and understanding of coliforms has led to questions regarding the suitability of testing for this diverse group of organisms as indicators for unhygienic conditions in dairy products. From fluid milk, where coliforms represent a minor proportion of PPC, to cheese products, where coliforms do not accurately represent public health risks, it is time to rethink the relevance of this century-old indicator group as a means for protecting public health. We propose implementation of appropriate pathogen testing (e.g., *Listeria* testing in washed rind cheeses) or testing for a comprehensive group of all organisms linked to PPC (e.g., total Gram-negative testing in fluid milk) to ensure a high quality and safe dairy food supply.

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CHAPTER FOUR

Environmental and cow hygiene factors play an important role in the transmission of sporeforming bacteria from farm environments into bulk tank raw milk

ABSTRACT

Sporeforming bacteria are responsible for the quality deterioration of a number of dairy products including fluid milk, cheese and products manufactured using dried dairy powders as ingredients. Sporeforming bacteria represent a considerable challenge for the dairy industry because they primarily enter the dairy product continuum at the farm, survive processing hurdles and subsequently grow in finished products. The resultant spoilage contributes to the $6.4 billion fluid milk loss that occurs every year in the United States. As such, strategies to reduce spoilage due to this group of bacterial contaminants have focused on understanding the impact of farm level factors on the presence of spores in bulk tank raw milk with the goal of reducing spore levels in raw milk, as well as understanding processing contributions to spore levels and outgrowth in finished products. The goal of the current study was to investigate sources of spores in the farm environment along with surveying farm management practices in order to identify variables that affect the presence and levels of spores in bulk tank raw milk. To this end, environmental samples including feed, bedding, manure, soil, water, etc., and bulk tank raw milk were collected twice from 17 Upstate New York dairy farms over a 19-month period and the presence and levels

of various spore types (e.g., psychrotolerant, mesophilic, thermophilic, highly heat resistant thermophilic, specially thermoresistant thermophilic and anaerobic butyric acid bacteria) were assessed. Multimodel inference was used to determine variables (i.e., management factors, environmental spore levels and meteorological data from each sampling) that were important for each spore type in bulk tank raw milk. Analyses indicated that variables of importance for more than one spore type included the residual level of spores in milk from individual cows after thorough teat cleaning and forestripping, udder hygiene, clipping or flaming of udders, spore level in feed commodities, spore level in parlor air, how often bedding was topped up or changed, the use of recycled manure bedding and the use of sawdust bedding. These results improve our understanding of how spores transfer from environmental sources into bulk tank raw milk and provide information that can be used to design intervention trials aimed at reducing spore levels in raw milk.

INTRODUCTION

Processed dairy product microbiological quality can broadly be determined by factors that occur at the farm level and factors that occur at the processing level. While raw milk is known to contain a variety of bacterial contaminants, only a very select group of those contaminants go on to influence finished product quality, namely sporeforming bacteria. Sporeforming bacteria are in and of themselves a diverse group whose primary reservoir has traditionally been considered soil (Nicholson, 2002, Hong et al., 2009, Carlin, 2011, Heyndrickx, 2011b). Indeed, spores are found

ubiquitously in soil and other natural environments and are known to contaminate various food products pre- and post-harvest (Carlin, 2011, Heyndrickx, 2011a, Postollec et al., 2012), including raw milk (Huck et al., 2008, Masiello et al., 2014, Miller et al., 2015a, Miller et al., 2015b, Ortuzar et al., 2018).

Sporeforming bacteria present a particular challenge to dairy products because they can survive typical processing hurdles (e.g., pasteurization) in spore form and are capable of growing in a variety of resultant finished products (Overcast and Atmaram, 1974, Muir et al., 1986, Scheldeman et al., 2006, Ranieri and Boor, 2009, Oliveira et al., 2016, André et al., 2017). For example, a number of studies have shown that psychrotolerant sporeforming bacteria are responsible for approximately 50% of fluid milk samples reaching the Pasteurized Milk Ordinance (PMO) bacterial limit of 20,000 CFU/mL during refrigerated shelf-life (Ranieri and Boor, 2009, Alles et al., 2018, Reichler et al., 2018). A recent study of the ability of sporeforming bacteria and other dairy relevant organisms to produce lipolytic and proteolytic enzymes demonstrated that sporeforming bacteria are capable of producing a number of enzymes that have the potential to degrade dairy product quality (Trmcic et al., 2015). For example, *Bacillus weihenstephanensis*, a psychrotolerant sporeformer in the *Bacillus cereus* group, is known to produce proteolytic enzymes in fluid milk causing a defect known as sweet-curdling (Overcast and Atmaram, 1974, Meer et al., 1991, Gopal et al., 2015). Further, a group of anaerobic sporeforming bacteria, butyric acid bacteria (**BAB**), are known to produce gas and butyric acid under conditions found during aging of certain styles of cheese (e.g., Gouda) and ultimately causing a defect known as “late-blowing” (Vissers et al., 2006). This defect represents a significant

economic burden for processors of hard and semi-hard aged cheeses, as the resultant product is not saleable (Klijn et al., 1995). Finally, mesophilic and thermophilic sporeforming bacteria are prevalent in dried dairy products (Scott et al., 2007, Yuan et al., 2012, Miller et al., 2015b) and some are even known to survive processing conditions associated with ultra-high temperature (UHT) pasteurization (e.g., *Bacillus sporothermodurans*) (Scheldeman et al., 2006). Spores found in dried dairy products may go on to cause quality deterioration in end-product applications and therefore are often subject to strict quality specifications (Watterson et al., 2014).

Due to the widespread implications of sporeforming bacteria in dairy products there is a considerable amount of interest in controlling their entry into raw milk on the farm as well as technological strategies for spore removal which include centrifugation (Torres-Anjel and Hedrick, 1971, Gésan-Guiziou, 2010) and microfiltration (Fernández García et al., 2013, Doll et al., 2017). A number of research studies have examined various management practices at the farm level that may influence the presence and levels of certain types of spores in bulk tank raw milk. For example, Magnusson and colleagues (2007) found that levels of *Bacillus cereus* in raw milk were associated with increased levels of spores in bedding materials and feed and proposed that feeding silage with >100,000 spores/g will increase the risk of raw milk contamination. Similarly, Vissers and colleagues (2006) modeled the contamination of bulk tank raw milk with BAB spores and found that BAB spore level in silage was the most important factor, further suggesting that silage containing more than 5 $\log_{10}$ BAB spores/g should not be fed in order to reduce the risk of bulk tank raw milk contamination. To date, however, there has been no comprehensive evaluation of

primary spore sources in the dairy farm environment for a diverse set of dairy-relevant sporeforming bacteria. To this end, the goal of this study was to further our understanding of primary spore sources on dairy farms and factors that affect the presence and levels of a diverse set of spores in bulk tank raw milk, which may serve to identify opportunities for the disruption of spore transmission from environments into bulk tank raw milk.

MATERIALS AND METHODS

Farm selection and characteristics

A longitudinal study of spore levels in dairy farm environments on 17 Upstate New York (NY) farms was conducted between April 2014 and October 2015. Sample collection occurred twice on each of the 17 farms during the study period. Three producer cooperatives with member farms in Upstate NY generated lists of producers willing to participate in this environmental sampling project. From these master lists a subset of 20 farms were selected randomly by assigning each farm a numerical value and then using a random number generator to select the farms with the first 20 random numbers. After initial contact with each producer, three producers chose not to participate in the study resulting in a total of 17 farms enrolled in the study.

Farms were located across Upstate NY and varied in size and management styles. Herds ranged from 144 to 3,200 lactating cows with a mean lactating herd size of 929. Annual average production per cow ranged from 10,660 kg to 14,900 kg (23,500 lb to 32,850 lb) per year with a mean of 12,615 kg (27,812 lb) per year. All 17 farms housed lactating cows in freestalls with 7 farms bedding with sand, 3 farms

bedding with sawdust, 2 farms bedding with recycled manure, 2 farms bedding with a combination of materials, 1 farm bedding with recycled sand, 1 farm bedding with recycled paper, and 1 farm using mattresses with no bedding.

Sample collection

Primary environmental sample collection included samples taken from soil, feed components (i.e., alfalfa, aminomax, canola, chocolate, citrus pulp, corn silage, cornmeal, cottonseed, dry hay, dry shelled corn, flaked corn, grain pellets, ground canola, haylage, high mix, liquid whey, low mix, molasses, protein mix and straw), bedding, manure, filter socks, drinking water, air sedimentation, teat end swabs, individual cow milk, towels used during udder preparation, wash water, equipment swabs, and bulk tank milk.

Soil samples were collected with sterile plastic scoops (15 mL) from soil within 3 m of the lactating cow housing barn. Soil was obtained by collecting 10 to 15 scoops from a depth of up to 8 cm, these subsamples were commingled in 1,625 mL Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Grab samples were taken using 1,625 mL Whirl-Pak® bags from; (i) each of the individual feed components (e.g., silage, cornmeal, etc.) included in the lactating cow rations and from; (ii) fresh, unused, bedding directly from storage areas. Manure samples were taken using sterile plastic scoops (15 mL) from 5 freshly fallen manure piles and commingled in Whirl-Pak® bags (1,625 mL).

From each farm, a drinking water sample (~250 mL) was collected from one drinking trough in the lactating cow housing area in a 295 mL vial. Air sedimentation samples were collected by transferring 99 mL of sterile phosphate buffered saline

(**PBS**) into a sterilized metal pan and placing it at the end of the milking parlor furthest from the holding area and exposing the PBS to the air for a period of 15 minutes. Following the exposure, the PBS was collected in a 295 mL vial. Teat ends from four individual teats on four different cows were collected using 3M QuikSwabs (3M, St. Paul, MN) by swabbing the teat end prior to any teat cleaning procedures. Following standard milking preparation (e.g., pre-dipping and wiping) by farm personnel, each of the teats was further cleaned using 70% ethanol and forestripped (e.g., removal of 4-5 streams of milk from each teat). Milk (~250 mL) from each of the prepared teats on these individual cows was then manually collected into a sterile vial (295 mL). Laundered towel samples were collected immediately after laundering and stored in a Whirl-Pak® bag. Filter sock samples were collected at time of replacement on each farm and stored in a Whirl-Pak® bag. Samples of the water (~250 mL) used to hose down the milking parlor and wash equipment was collected in a 295 mL vial. Prior to collection, the hose was soaked in 70% ethanol and water was flushed from the hose for ~1 min. Equipment swabs were taken from the rim of the bulk tank drop down pipe using a 3M hydrated sponge with neutralizing buffer. Finally, a bulk tank raw milk sample (~250 mL) was collected at the end of a milking shift from the bulk tank after 5 min of agitation and using a dipper sanitized in 200 ppm chlorine. Samples were transported on ice in hard sided coolers by Milk Quality Improvement Program (**MQIP**) personnel to the MQIP laboratory (Department of Food Science, Cornell University, Ithaca, NY). Samples arrived at or below 6°C and were stored at 4°C until further analysis, which began within 24 h of sample collection.

Survey design and administration

The survey design was based on a previously used survey (Masiello et al., 2014). Survey questions focused on farm level characteristics (e.g., number of lactating cows, average production per cow, etc.), housing characteristics (e.g., housing type, bedding type, etc.), cleanliness factors (e.g., hygiene scores, housing area cleanliness, etc.), parlor characteristics (e.g., holding area cleaning, scraping of parlor deck, etc.) and bulk tank characteristics (e.g., total bulk tank capacity, bulk tank cleaners and sanitizers, etc.).

Surveys were administered by MQIP personnel during each of the two visits conducted during the sampling period. Farm personnel interviewed include farm owners or herd managers.

Microbiological analysis

Liquid samples (i.e., individual cow milk, bulk tank raw milk, air sedimentation, drinking water and wash water) were shaken in accordance with Standard Methods for the Examination of Dairy Products (**SMEDP)** (Duncan et al., 2004) prior to transferring 3 x 30 mL aliquots into individual sterile screw capped glass tubes, which were stored on ice until heat treatment. Solid samples (i.e., soil, feed components, manure, towels, equipment swabs, and filter socks) were weighed (30 g for soil, feed components and manure) or transferred (for swabs and filter sock) into filter Whirl-Pak® bags with 270 mL of phosphate buffer and were either manually agitated for samples with large particles (e.g., straw) or stomached at 270 rpm for 1 min. A 3 x 30 mL portion of the liquid from diluted solid samples were

transferred into three individual sterile screw capped glass tubes, which were stored on ice until heat treatment.

Samples were heat treated to eliminate vegetative bacteria and activate spore germination using three treatments including; (i) 80°C for 12 min (Frank and Yousef, 2004); (ii) 100°C for 30 min (Kent et al., 2016), and; (iii) 106°C for 30 min (Kent et al., 2016) then cooled immediately on ice until temperature control samples reached 6°C. A 50 µL aliquot of re-suspended soil, feed, bedding, manure, filter sock buffer and drinking water samples were spiral plated (Autoplate 4000, Advanced Instruments, Norwood, MA) on pre-poured brain heart infusion (**BHI**) agar plates in duplicate. A 1 ml aliquot of air sedimentation buffer, teat end swab buffer, individual cow milk, towel buffer, wash water, equipment swab buffer and bulk tank raw milk samples were pour plated in BHI agar in duplicate. Agar plates from samples heat treated at 80°C for 12 min were incubated at either 6°C, 32°C or 55°C for psychrotolerant spore count (**PSC**), mesophilic spore count (**MSC**) and thermophilic spore count (**TSC**), respectively for 48 h prior to enumeration. Agar plates from samples heat treated at 100°C for 30 min and 106°C for 30 min were incubated at 55°C for 48 h for highly heat resistant thermophilic spore count (**HHR TSC**) and specially thermoresistant spore enumeration (**STSE**), respectively. Enumeration was performed on an automated colony counter (Q-count, Advanced Instruments, Norwood, MA).

Samples were also examined for butyric acid bacteria (e.g., *Clostridium tyrobutyricum*) using a most probable number (**MPN**) technique as outlined previously with slight modifications (Brändle et al., 2018). Briefly, 3 x 1 mL of each raw milk,

wash water, air sedimentation buffer, towel buffer, teat end swab buffer and equipment swab buffer samples were distributed into previously sterilized tubes of 9 mL Bryant and Burkey (**BB**) broth. Additionally, for re-suspended soil, feed, bedding, manure, filter sock buffer and drinking water a 9 tube MPN consisting of 9 mL BB tubes was prepared with 3 tubes each of; (i) 1 mL of undiluted sample, (ii) 1:10 dilution of each sample, and; (iii) 1:100 dilution of each sample. Paraffin wax was melted and approximately 2 cm plug of the liquid wax was poured on top of each tube to create an anaerobic environment. All BB tubes were heat treated at 75°C for 15 min to eliminate vegetative cells. Tubes were incubated at 35°C for 6 days and checked every 48 h for gas production. Tubes were scored as positive or negative for gas production.

Isolate selection and storage

Bacterial isolates (n = 5,433) were selected from each sample/heat treatment/incubation combination by visually inspecting colony morphologies. A representative of each unique morphology was selected and streaked for isolation on BHI agar and then incubated for 24 h at either 32°C for PSC and MSC tests or 55°C for TSC, HHR TSC and STSE tests. BAB isolates were selected from positive BAB MPN tubes by using a sterile loop to substreak from the positive tube onto BHI agar and then incubated anaerobically for 24 h at 35°C. Following incubation BAB isolates were examined for colony morphology and representatives of each unique morphology from each sample were selected and streaked for isolation on BHI agar followed by incubation as described above.

Isolated colonies were all inoculated into BHI broth and incubated for 18 h at the appropriate temperature and condition (e.g., anaerobic or aerobic) for the test of origin as described above. Isolates were then frozen and stored in 15% glycerol at -80°C. Further isolate information can be found at Food Microbe Tracker (http://www.foodmicrobetracker.com).

Data handling and statistical analysis

Data was stored in a Microsoft Access database (Microsoft Access, Redmond, WA). All statistical analyses were performed in R (Team, 2014). Data handling was performed with the dplyr package (Wickham et al., 2016). Mean spore counts for each bedding type were compared using ANOVA with Tukey's HSD when appropriate in R using agricolae (De Mendiburu, 2014).

Multimodel inference (Burnham and Anderson, 2004) was performed to identify environmental samples, farm management practices and meteorological factors with important relationships to bulk tank milk spore counts. For each test (i.e., heat treatment/incubation combination), mixed-effect linear models were fitted using lme4 (Bates et al., 2007) to the log-transformed spore count of the bulk tank milk, with farm as a random effect and up to four environmental or survey factors as fixed effects. All linear models also included the factors; (i) average annual production per cow; (ii) number of milking cows, and; (iii) how often is each cow milked per day. All such models were fitted and retained using the glmulti package (Calcagno and de Mazancourt, 2010). Relative variable importance scores and (unconditional) averaged models based on Akaike weights (Burnham and Anderson, 2004) were computed

using the MuMIn package (Barton, 2009). Relative variable importance scores are an estimate which is calculated by summing the Akaike weights across all models where that variable appears. The variables can then be ranked by this weighed sum, which allows for interpretation of relative importance (Burnham and Anderson, 2004). For example, a variable with a relative importance value of 1.0 would be present in 100% of all the fitted models and can be interpreted as having high relative importance. An arbitrary relative variable importance cutoff was set at 0.2 for this study.

RESULTS

Spores are abundant in the dairy farm environment, yet are found in low levels in bulk tank raw milk

Spore counts in environmental sources varied widely by spore test and sample type. Overall, proportion of samples with detectable SP PSC, SP MSC, SP TSC, HHR TSC, STSE ranged from 13 to 100%, 53 to 100%, 41 to 100%, 8 to 100%, and 3 to 100% respectively (Table 4.1). Mean SP PSC ranged from a low of -0.52 $\log_{10}$ CFU/mL in equipment swabs to a high of 5.04 $\log_{10}$ CFU/mL in soil (Table 4.1). Mean SP MSC ranged from a low of -0.15 $\log_{10}$ CFU/mL in equipment swabs to a high of 5.87 $\log_{10}$ CFU/g in manure (Table 4.1). Mean SP TSC ranged from a low of -0.20 $\log_{10}$ CFU/mL in equipment swabs to a high of 5.22 $\log_{10}$/g in manure (Table 4.1). Mean HHR TSC ranged from a low of -0.50 $\log_{10}$ CFU/mL in equipment swabs to a high of 4.35 $\log_{10}$ CFU/g in manure (Table 4.1). Mean STSE ranged from a low of -0.55 $\log_{10}$ CFU/mL in equipment swabs to a high of 3.68 $\log_{10}$ CFU/g in manure

(Table 4.1).

Table 4.1. Aerobic psychrotolerant, mesophilic, thermophilic, highly heat resistant thermophilic and specially thermoresistant spore counts for dairy farm environmental samples and raw milk samples

Sample Type (n)[2]	SP PSC[1]			SP MSC[1]			SP TSC[1]			HHR TSC[1]			STSE[1]		
	% Positive Samples	Mean (log cfu/ml or g)[3]	SD	% Positive Samples	Mean (log cfu/ml or g)[3]	SD	% Positive Samples	Mean (log cfu/ml or g)[3]	SD	% Positive Samples	Mean (log cfu/ml or g)[3]	SD	% Positive Samples	Mean (log cfu/ml or g)[3]	SD
Air Sedimentation (31),	17	-0.46	0.39	90	0.64	0.80	84	0.52	0.68	48	-0.20	0.60	23	-0.45	0.39
Bedding (36)	80	2.89	1.94	97	4.62	1.49	92	3.80	1.83	72	2.51	2.42	49	1.54	2.51
Bulk Tank Milk (34)	42	-0.24	0.50	91	0.50	0.56	88	0.36	0.51	53	0.05	0.77	47	-0.23	0.49
Commodity (127)	63	1.46	1.70	95	3.50	1.47	87	2.78	1.75	57	1.28	1.76	30	0.26	1.37
Drinking Water (36)	50	0.34	1.02	94	2.09	0.82	89	1.64	0.94	50	0.36	1.05	14	-0.38	0.65
Equipment (32)	13	-0.52	0.21	53	-0.15	0.55	44	-0.20	0.54	16	-0.50	0.29	9	-0.55	0.18
Filter Sock (31)	70	1.16	1.40	97	3.01	1.01	100	2.57	0.77	71	1.31	1.35	39	0.31	1.25
Inidividual Cow Milk (33)	47	-0.26	0.44	91	0.42	0.58	76	0.29	0.63	55	-0.03	0.62	48	-0.21	0.52
Manure (34)	100	4.23	0.66	100	**5.87**	0.58	100	**5.22**	0.71	100	**4.35**	0.86	100	**3.68**	0.88
Silage (80)	39	0.75	1.85	86	2.91	1.64	79	2.43	1.77	8	-0.39	0.78	3	-0.54	0.41
Soil (33)	100	**5.04**	0.55	100	5.68	0.40	100	4.61	0.86	84	3.43	1.86	82	2.68	1.77
Teat End Swabs (32)	55	0.08	0.82	100	1.24	0.61	100	1.00	0.53	58	0.11	0.81	25	-0.32	0.58
Towel (30)	55	0.01	0.66	93	1.56	0.73	93	1.12	0.60	83	0.45	0.78	37	-0.32	0.51
Wash Water (34)	21	-0.32	0.64	53	0.26	1.09	41	-0.03	0.83	18	-0.44	0.44	12	-0.53	0.21

[1] SP PSC = Spore Pasteurized Psychrotolerant Spore Count; SP MSC = Spore Pasteurized Mesophilic Spore Count; SP TSC = Spore Pasteurized Thermophilic Spore Count; HHR TSC = Highly Heat Resistant Thermophilic Spore Count; STSE = Specially Thermoresistant Spore Enumeration.

[2] n = number of samples for each sample type. Sample types with more than 34 samples are a result of more than one representative sample per farm (e.g., farms using multiple beddings for lactating cows). Sample types with fewer than 34 samples (e.g., filter sock) are a result of laboratory error or sample not being available at the time of sample collection.

[3] Bolded cells indicate sample with highest spore count within individual spore test type (e.g., SP PSC). Left censored data (i.e., samples with below detectable bacterial counts) was accounted for by adding a small value to each data point (i.e., -0.6 log) calculating the mean and standard deviation. Mean spore counts for air sedimentation, bulk tank raw milk, drinking water, equipment swab buffer, filter sock buffer, individual cow milk, teat end swab buffer, towel buffer and wash water presented in log cfu/mL, while bedding, commodity, manure, silage and soil samples are presented in log cfu/g.

Air sedimentation buffer samples generally had low levels of spores, with four of the spore tests (SP PSC, HHR TSC, STSE and BAB) having means of less than 1.00 spore/mL (0.00 $\log_{10}$ CFU/mL), while the mean SP MSC and mean SP TSC were 0.64 and 0.52 $\log_{10}$ CFU/mL, respectively (Table 4.1). Equipment swab buffer and wash water samples also had very low mean spore counts, with all of the equipment swabs having less than 1.00 spore/mL (0.00 $\log_{10}$ CFU/mL; Table 4.1) and only mean SP MSC for wash water above that level (0.26 $\log_{10}$/mL; Table 4.1).

In contrast to the low spore levels found in air, equipment and wash water samples, the majority of environmental samples had considerably higher spore counts. Manure was found to have the highest mean spore counts for all spore tests except SP PSC (Table 4.1), with mean SP PSC, SP MSC, SP TSC, HHR TSC and STSE of 4.23, 5.87, 5.22, 4.35 and 3.68 $\log_{10}$ CFU/g, respectively (Table 4.1). Similarly, soil also had high levels of spores, with SP PSC, SP MSC, SP TSC, HHR TSC and STSE of 5.04, 5.68, 4.61, 3.43 and 2.68 $\log_{10}$ CFU/g, respectively (Table 4.1).

Bedding, silage and other feed component samples (e.g., cornmeal, cottonseed, etc.) also had generally high mean spore levels. Overall mean bedding spore levels for SP PSC, SP MSC, SP TSC, HHR TSC and STSE were 2.89, 4.62, 3.80, 2.51 and 1.54 $\log_{10}$ CFU/g, respectively (Table 4.1). When bedding spore counts were assessed by type of material, the spore counts varied. For example, spore counts in manure solids bedding (n=4) were consistently higher for all spore types than in new sand bedding (n=18) and sawdust bedding (n=8) (Table 4.2) yet were only significantly higher ($p<0.05$) for SP TSC and STSE (Table 4.2). Mean silage spore counts for SP PSC, SP MSC, SP TSC, HHR TSC and STSE were 0.75, 2.91, 2.43, -0.39 and -0.54 $\log_{10}$

CFU/g, respectively (Table 4.1). Mean feed commodity spore counts were found to be 1.46, 3.50, 2.78, 1.28 and 0.26, respectively for SP PSC, SP MSC, SP TSC, HHR TSC and STSE (Table 4.1).

Table 4.2. Aerobic psychrotolerant, mesophilic, thermophilic, highly heat resistant thermophilic and specially thermoresistant spore counts in unused bedding samples from 17 farms in New York State

Bedding Type (n)[2]	SP PSC[1]		SP MSC[1]		SP TSC[1]		HHR TSC[1]		STS E[1]	
	Mean (log cfu/g)[3]	SD	Mean (log cfu/g)[3]	SD	Mean (log cfu/g)[3]	SD	Mean (log cfu/g)[3]	SD	Mean (log cfu/g)[3]	SD
Manure Solids (4)	3.93[a]	0.53	5.64[a]	0.41	5.46[a]	0.26	4.85[a]	0.46	3.95[ab]	0.14
Paper (4)	3.15[a]	2.59	5.83[a]	1.32	6.30[a]	1.24	5.18[a]	2.69	4.95[a]	2.31
Sand (18)	2.69[a]	2.01	4.04[a]	1.67	2.74[c]	1.59	1.52[ab]	1.83	0.33[c]	1.49
Recycled Sand (2)	4.57[a]	0.55	5.69[a]	1.06	5.68[a]	2.70	5.22[a]	3.13	3.17[abc]	5.34
Sawdust (8)	2.39[a]	1.93	4.52[a]	0.77	3.83[b]	0.65	1.55[ab]	1.88	0.78[bc]	2.10

[1] SP PSC = Spore Pasteurized Psychrotolerant Spore Count; SP MSC = Spore Pasteurized Mesophilic Spore Count; SP TSC = Spore Pasteurized Thermophilic Spore Count; HHR TSC = Highly Heat Resistant Thermophilic Spore Count; STSE = Specially Thermoresistant Spore Enumeration

[2] All 17 farms housed lactating cows in freestalls with 7 farms bedding with sand, 3 farms bedding with sawdust, 2 farms bedding with recycled manure, 2 farms bedding with a combination of materials, 1 farm bedding with recycled sand, 1 farm bedding with recycled paper, and 1 farm using mattresses with no bedding.

[3] Spore counts within test type (e.g., SP PSC) with different letters represent significant differences ($P<0.05$) between bedding type

Mean spore counts in drinking water from the housing area for SP PSC, SP MSC, SP TSC, HHR TSC and STSE were 0.34, 2.09, 1.64, 0.36 and -0.38 $\log_{10}$ CFU/mL, respectively (Table 4.1). Towels used during milking time preparation had overall mean spore levels for SP PSC, SP MSC, SP TSC, HHR TSC and STSE of 0.01, 1.56, 1.12, 0.45, and -0.32 $\log_{10}$ CFU/mL, respectively (Table 4.1). Further, spore levels in filter sock samples for SP PSC, SP MSC, SP TSC, HHR TSC and STSE were 1.16, 3.01, 2.57, 1.31 and 0.31 $\log_{10}$ CFU/mL, respectively (Table 4.1).

Despite high concentrations of all spore types in many environmental locations, bulk tank raw milk, raw milk from individual cows and teat end swabs all had relatively low spore levels. Teat end swabs had mean spore counts of 0.08, 1.24, 1.00, 0.11 and -0.32 for SP PSC, SP MSC, SP TSC, HHR TSC and STSE, respectively (Table 4.1). Raw milk samples from individual cows had mean SP PSC, SP MSC, SP TSC, HHR TSC and STSE of -0.26, 0.42, 0.29, -0.03 and -0.21 $\log_{10}$ CFU/mL, respectively (Table 4.1). Finally, mean spore levels in bulk tank raw milk for SP PSC, SP MSC, SP TSC, HHR TSC and STSE were -0.24, 0.50, 0.36, 0.05 and -0.23 $\log_{10}$ CFU/mL, respectively (Table 4.1).

BAB presence and levels in environmental samples were evaluated using MPN as opposed to direct enumeration. Proportion of samples below detection for BAB ranged from a low of 0.0% for manure and soil to a high of 93% for wash water (Figure 4.1). Conversely, samples above the detection limit for BAB MPN ranged from a low of 0% for air sedimentation, bulk tank raw milk, individual cow milk, towel samples and wash water to a high of 87% in filter sock samples (Figure 4.1).

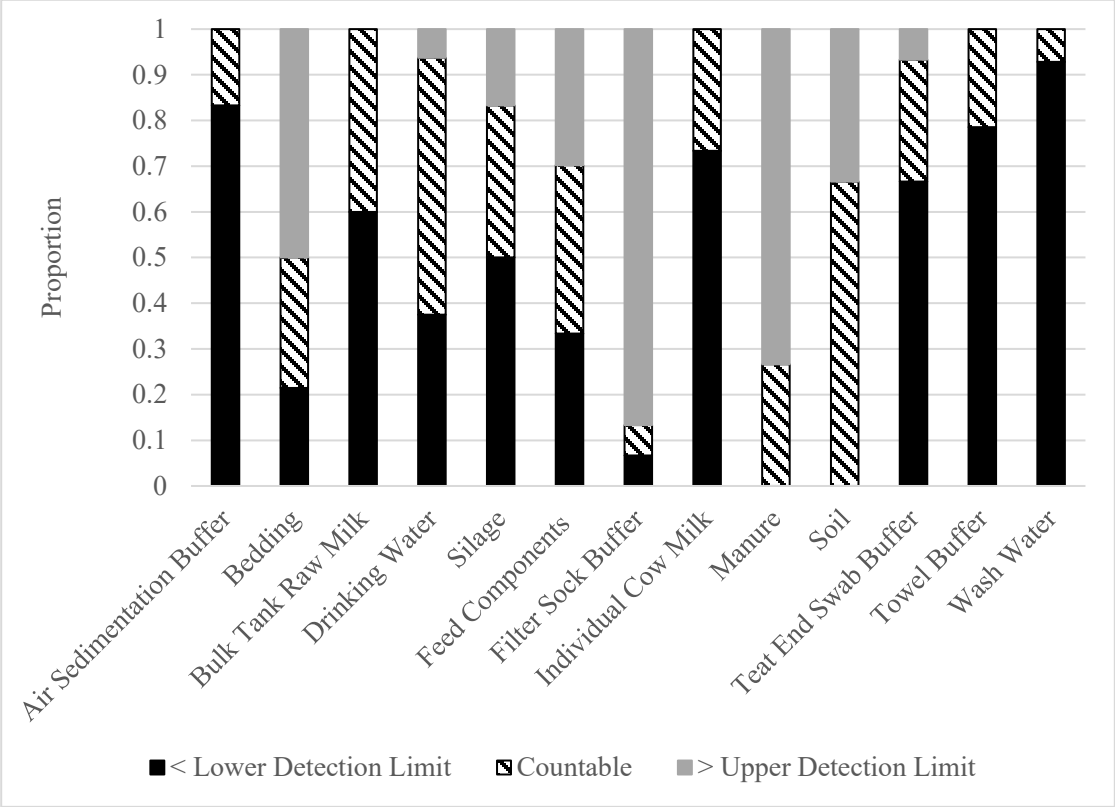


Figure 4.1. Bar graph depicting the proportion of samples with butyric acid bacteria (BAB) most probable number (MPN) levels below the lower detection limit (black bars), within the countable range (hashed bars), and above the upper detection limit (gray bars) for thirteen environmental and milk sources from 17 New York State dairy farms. Lower and upper detection limit for raw milk (including bulk tank milk and individual cow milk), wash water, air sedimentation buffer, towel buffer, teat end swab buffer, filter sock buffer, liquid feed components (e.g., whey) and drinking water was 0.3 and 1.1 MPN/mL, respectively. Lower and upper detection limit for soil, solid feed components, bedding and manure was 3.0 and 1,100 MPN/g, respectively.

Bedding type and practices along with cow level hygiene factors play an important role in bulk tank raw milk spore levels

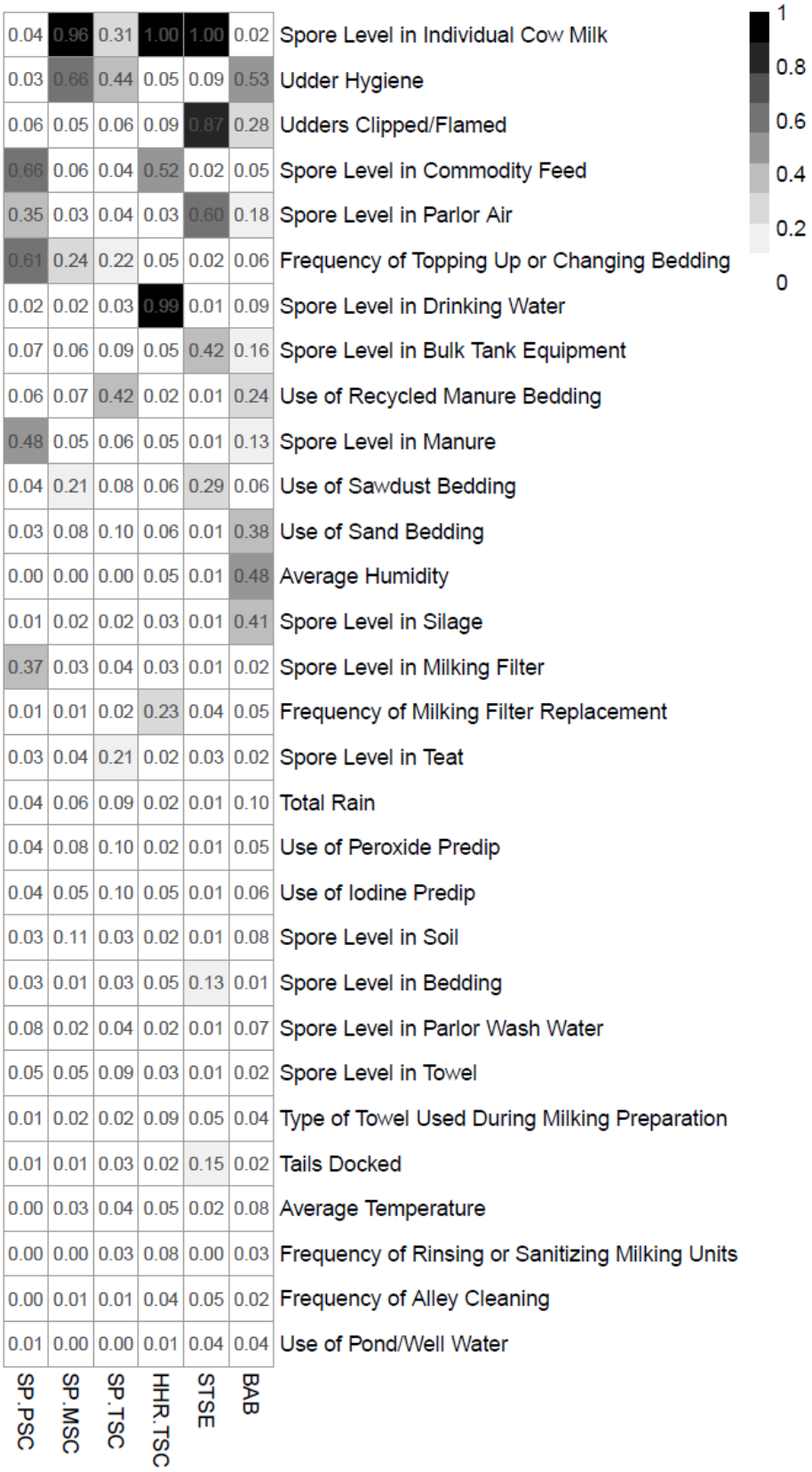
Environmental spore counts along with meteorological data and survey data were analyzed to determine relative factor importance for resulting bulk tank spore levels by spore type (e.g., SP PSC, SP MSC, etc.). Variables that were found to be important across spore types (variable importance >0.2 for two or more spore types) included bedding type, bedding management, udder hygiene, individual cow milk spore level, if udders were clipped/flamed, spore level in air and spore level in feed commodities (Figure 4.2).

The use of certain bedding types for lactating cows had high variable importance including; sawdust (SP MSC variable importance = 0.21 and STSE variable importance = 0.29), recycled manure (SP TSC variable importance = 0.42 and BAB variable importance = 0.24) and sand (BAB variable importance = 0.38) (Figure 4.2). How often bedding was topped up or changed per week was also identified in the top variables of importance for SP PSC (variable importance =0.61), SP MSC (variable importance = 0.24) and SP TSC (variable importance = 0.22) (Figure 4.2).

Cow level factors found in the top variable importance included spore levels in individual cow milk, which was identified as the most important variable associated with bulk tank raw milk SP MSC (variable importance = 0.96), HHR TSC (variable importance = 1.00) and STSE (variable importance = 1.00) and was also one of the top three variables for SP TSC (variable importance = 0.31). Udder hygiene, which was identified in the top variables of importance for SP MSC (variable importance = 0.66),

SP TSC (variable importance = 0.44) and BAB (variable importance = 0.53) (Figure 4.2). Further, whether udders were clipped or flamed was found to be a variable of importance for STSE (variable importance = 0.87) as well as BAB (variable importance = 0.28) (Figure 4.2). Importantly, individual cow milk was the only variable in the data set that had a significant parameter estimate (in the MSC MMI), with a positive effect of 0.52 ($p<0.05$). Udder hygiene (in the MSC MMI) and spore levels in commodities (in the PSC MMI), while not significant ($p=0.23$ and $p=0.22$, respectively) were the only other variable with p-values below 0.25, both with positive effects of 0.33 and 0.16, respectively.

Figure 4.2. Heat map of variable importance based on Multi-Model Inference (MMI) for environmental spore counts, meteorological data and survey data for various bulk tank spore levels by spore type. Matrix color represents variable importance from 0.0 (least important; white) to 1.0 (most important; black). SP PSC = Spore Pasteurized Psychrotolerant Spore Count; SP MSC = Spore Pasteurized Mesophilic Spore Count; SP TSC = Spore Pasteurized Thermophilic Spore Count; HHR TSC = Highly Heat Resistant Thermophilic Spore Count; STSE = Specially Thermoresistant Spore Enumeration, and; BAB = Butyric Acid Bacteria. Meteorological factors (e.g., average humidity, total rain and average temperature) represent the average or total of the hourly data for the 24 h prior to each sample collection.


0.04 0.96 0.31 1.00 1.00 0.02 Spore Level in Individual Cow Milk
0.03 0.66 0.44 0.05 0.09 0.53 Udder Hygiene
0.06 0.05 0.06 0.09 0.87 0.28 Udders Clipped/Flamed
0.66 0.06 0.04 0.52 0.02 0.05 Spore Level in Commodity Feed
0.35 0.03 0.04 0.03 0.60 0.18 Spore Level in Parlor Air
0.61 0.24 0.22 0.05 0.02 0.06 Frequency of Topping Up or Changing Bedding
0.02 0.02 0.03 0.99 0.01 0.09 Spore Level in Drinking Water
0.07 0.06 0.09 0.05 0.42 0.16 Spore Level in Bulk Tank Equipment
0.06 0.07 0.42 0.02 0.01 0.24 Use of Recycled Manure Bedding
0.48 0.05 0.06 0.05 0.01 0.13 Spore Level in Manure
0.04 0.21 0.08 0.06 0.29 0.06 Use of Sawdust Bedding
0.03 0.08 0.10 0.06 0.01 0.38 Use of Sand Bedding
0.00 0.00 0.00 0.05 0.01 0.48 Average Humidity
0.01 0.02 0.02 0.03 0.01 0.41 Spore Level in Silage
0.37 0.03 0.04 0.03 0.01 0.02 Spore Level in Milking Filter
0.01 0.01 0.02 0.23 0.04 0.05 Frequency of Milking Filter Replacement
0.03 0.04 0.21 0.02 0.03 0.02 Spore Level in Teat
0.04 0.06 0.09 0.02 0.01 0.10 Total Rain
0.04 0.08 0.10 0.02 0.01 0.05 Use of Peroxide Predip
0.04 0.05 0.10 0.05 0.01 0.06 Use of Iodine Predip
0.03 0.11 0.03 0.02 0.01 0.08 Spore Level in Soil
0.03 0.01 0.03 0.05 0.13 0.01 Spore Level in Bedding
0.08 0.02 0.04 0.02 0.01 0.07 Spore Level in Parlor Wash Water
0.05 0.05 0.09 0.03 0.01 0.02 Spore Level in Towel
0.01 0.02 0.02 0.09 0.05 0.04 Type of Towel Used During Milking Preparation
0.01 0.01 0.03 0.02 0.15 0.02 Tails Docked
0.00 0.03 0.04 0.05 0.02 0.08 Average Temperature
0.00 0.00 0.03 0.08 0.00 0.03 Frequency of Rinsing or Sanitizing Milking Units
0.00 0.01 0.01 0.04 0.05 0.02 Frequency of Alley Cleaning
0.01 0.00 0.00 0.01 0.04 0.04 Use of Pond/Well Water
SP.PSC
SP.MSC
SP.TSC
HHR.TSC
STSE
BAB
1
0.8
0.6
0.4
0.2
0

DISCUSSION

Spores in dairy farm environments likely originate from soil, yet transfer and concentration of spores on dairy farms may occur through various pathways

The transmission of spores into food distribution channels has been widely reported on (Hong et al., 2009, Carlin, 2011, Postollec et al., 2012), and sources have been proposed to be primarily soil (Nicholson, 2002, Heyndrickx, 2011b), decaying plant material (Siala et al., 1974) and as commensal communities within insects and animals (Nicholson, 2002, Hong et al., 2009). The study described here found that aerobic spores capable of growing under a number of temperature conditions (e.g., psychrotolerant, mesophilic, etc) are found in high concentrations in a variety of sources on conventional dairy farms, including soil, manure, bedding and feed (Table 4.1). These sources may simply allow for persistence of these bacteria in spore form, but ongoing work suggests that there are multiple locations in natural environments where sporeforming bacteria can not only persist, but live out their full vegetative-sporulation-germination life cycles (Heyndrickx, 2011a, Gauvry et al., 2017), thereby increasing in numbers over time in those niches. The expectation that sporeforming bacteria will be present in both spore form as well as vegetative form in the dairy farm environment is also reasonable considering the wide range of conditions (e.g., pH, moisture content, temperature, etc) that they would encounter in those environments. For example, Borreani and colleagues (2013) describe the proliferation and subsequent sporulation of *Paenibacillus* in aerobically deteriorated silage on dairy farms, leading

to increasing *Paenibacillus* spore levels in silage over time (Borreani et al., 2013). Regardless of whether spores present in the dairy farm environment are growing or simply persisting, a number of studies have demonstrated that high concentrations of spores can be found throughout the dairy farm. A study in The Netherlands examined both spores of *Bacillus cereus* as well as total aerobic mesophilic spores on 7 farms and found similar levels of contamination in soil (5.52 log CFU/g), manure (5.20 log CFU/g), unused bedding (4.08 log CFU/g), teat end swabs (2.82 log CFU/g) and bulk tank raw milk (1.20 log CFU/g) (Slaghuis et al., 1997) as was found in the current study. Similarly, Huck et al. (2008) examined spores in bedding, feed, manure, soil and water samples on one farm in NY state and found spore levels ranged from 2.57 to 6.08 log CFU/g or mL (Huck et al., 2008). A survey of spores in various lactating cow bedding materials on dairy farms across 18 states in 2016-2017 found that mesophilic and thermophilic spores levels, particularly in organic bedding materials (e.g., manure solids), ranged from 3.21 to 6.03 and 2.79 to 5.41, respectively (Murphy, et al., unpublished).

In the study described here, manure had the highest spore counts of all environmental locations tested in 4 out of 5 aerobic spore tests (Table 4.1) which would seem to indicate that spores are either, (i) being concentrated in manure as the animal consumes and digests feed containing spores, (ii) spores are geminating in the gastrointestinal track, increasing in number through vegetative growth and subsequently re-sporulating, or (iii) a combination of these mechanisms. Previous work supports these hypotheses, as many researchers have proposed that soil contamination may lead to spore transfer into bedding and feed during harvest and

processing (Ortuzar et al., 2018). The use of spores as dairy cow probiotics seems to support that sporeforming bacteria are capable of germinating and proliferating in the gut of the animal, (Qiao et al., 2010, Sun et al., 2013, Souza et al., 2017), demonstrating that the gastrointestinal track is a favorable environment for vegetative growth of at least some sporeforming bacteria.

Factors affecting spore transmission from the farm environment into bulk tank raw milk

Variables that were found to be of importance across spore tests in the current study included cow environmental factors (e.g., type of bedding used) and sources (e.g., spore levels in feed) as well as cow level factors (e.g., udder hygiene) and sources (spores in individual cow milk samples). These results improve our understanding of the mechanisms driving spore transmission from the spore-rich dairy farm environment into bulk tank raw milk. These points appear to be centered in two primary areas, including; (i) critical factors involved in the exposure of the animal, and specifically the exposure of the teat and udder, to spores from the wider environment (e.g., spore levels in bedding and bedding management factors), and; (ii) the residual number spores present in and on the udder at the time of milking and related factors.

Our study identified that spore levels in certain types of bedding (e.g., recycled manure, sand, etc.) were variables of importance for the presence and levels of various spores in bulk tank raw milk leading to the hypothesis that the exposure of the dairy cow to high levels of environmental spores is a critical control point in the transmission of spores into bulk tank raw milk. Our findings are consistent with

previous research that has identified the role of environmental factors and sources in bacterial spore transmission into bulk tank raw milk. For example, Magnusson and colleagues (2007) reported that used bedding and contaminated feed were primary sources of *Bacillus cereus* on five farms in Sweden (Magnusson et al., 2007). Miller et al., (2015) reported that the use of certain beddings, in particular, sawdust and sand beddings, were associated with lower bulk tank mesophilic spore counts in farms across New York State. That same study also found that the use of straw bedding was associated with lower incidence of thermophilic spore counts in bulk tank raw milk (Miller et al., 2015b). Similarly, a recent study of spore levels in new (unused) and used bedding and bulk tank raw milk across 18 states found that mesophilic and thermophilic spore levels in new bedding directly and indirectly impacted the mesophilic and thermophilic spore levels in bulk tank raw milk (Murphy et al., unpublished). The study also found that bedding management practices, such as how often bedding was topped up or changed was significantly associated with spore levels in bulk tank raw milk, further supporting our findings. Given that dairy cows spend 12-14 h each day lying down (Gomez and Cook, 2010), with direct contact between teat and udder surfaces and bedding, it is unsurprising that spores in bedding and bedding management are important variables in the transmission of spores into bulk tank raw milk.

In the current study udder cleanliness and the level of residual spores in the teat canal after udder preparation and forestripping were found as the two variables of most importance for a number of spore types (Figure 4.2). Importantly, the only variable among the dataset that was found to be significant ($p<0.05$) after averaging

over 31,000 models was the spore level in individual cow milk. This points to the critical nature of the level of spores in the teat canal for controlling the transmission of spores into bulk tank raw milk. Previous work has identified that udder and teat hygiene scores were significantly reduced (e.g., indicating cleaner teats and udders) in cows housed in freestalls with automatic alley scrapers (Magnusson et al., 2008). Masiello and colleagues (2017) also found that housing area cleanliness was associated with the levels of psychrotolerant spores in bulk tank raw milk. We hypothesize that this association between housing area cleanliness (e.g., alley floor scraping, bedding topped up or changed) and udder and teat hygiene represents a fundamental pathway for the transmission of spores from environmental sources into bulk tank raw milk. Specifically, we hypothesize that spores originating from environmental sources in the housing area, and in particular in bedding and manure, are able to enter the teat canal upon completion of milking when the teat sphincter muscle, which is one of the primary defense mechanisms against bacterial contamination of the teat canal, remains open for a period of approximately two hours (Zecconi et al., 2002). It is during this time period that bacterial contaminants can enter the teat canal and accumulate until the next milking (Braem et al., 2013) through contact with environmental bacterial sources (e.g., bedding). Factors affecting the ability of the teat sphincter muscle to adequately close, and therefore prevent bacterial contamination from environmental sources, include formation of callouses (de Pinho Manzi et al., 2012, Paduch et al., 2012), over milking (Zecconi et al., 2002) and stage of lactation (Shearn and Hillerton, 1996, Neijenhuis et al., 2001). Indeed, Braem et al., (2013) reported that sporeforming bacteria, including *Bacillus*, *Lysinibacillus*,

Oceanobacillus, *Paenibacillus* and *Brevibacillus*, are contaminants in the teat canal and at the teat apex in healthy lactating cows (Braem et al., 2013), further supporting our hypothesis that spores are entering the teat canal between milking.

CONCLUSIONS

The current study highlights role of environmental factors (e.g., spore levels in bedding) as well as cow level factors (e.g., udder hygiene and spore levels in raw milk from individual cows) in the presence and levels of various spores in bulk tank raw milk. Based on these results, dairy farmers who want to reduce spores in bulk tank raw milk should ensure that bedding and housing area cleanliness are well managed, which will reduce the exposure of dairy cows to high levels of environmental spores. Further, there should be a comprehensive approach to udder hygiene as well as teat condition and cleanliness by controlling key risk factors (e.g., overstocking, overmilking, etc.). Future studies should focus on testing intervention strategies, developed based on the outcomes of this study for reducing spore levels in bulk tank raw milk.

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CHAPTER FIVE

Spore test parameters matter: mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method

ABSTRACT

US dairy industry exports have steadily risen in importance over the last ten years, with dairy powders playing a particularly critical role. Currently, approximately half of US produced nonfat dry milk and skim milk powder are exported. Reaching new and expanding existing export markets in part relies on the control of endospore-forming bacteria in dairy powders. This study reports baseline mesophilic and thermophilic spore counts and spore populations from 55 raw material samples (primarily raw milk) and 33 dairy powder samples, from dairy powder processors across the US. Samples were evaluated using various spore testing methodologies and included initial heat treatments of: i) 80°C for 12m; ii) 100°C for 30m and; iii) 106°C for 30m. Results indicate that significant differences in both the level and population of spores were found, for both raw milk and dairy powders, with the various testing methods. Additionally, on average, spore counts were not found to increase significantly from beginning to the end of dairy powder processing, most likely related to the absence of biofilm formation by processing plant associated sporeformers (e.g., *Anoxybacillus* sp.) in the facilities sampled. Finally, in agreement with other studies, *Bacillus licheniformis* was found to be the most prevalent sporeformer in both raw materials and dairy powders, highlighting the importance of this organism in developing strategies for control and reduction of spore counts in dairy powders.

Overall this study emphasizes the need for standardization of spore enumeration methodologies in the dairy powder industry.

INTRODUCTION

Aerobic endospore-forming bacteria of the *Bacillaceae* family have been recognized as major contributors to dairy product quality issues over the past two decades (Ralyea et al., 1998; Huck et al., 2007b; Ranieri and Boor, 2009). In spore form, these organisms are capable of surviving environmental stresses including low pH, high temperatures, exposure to sanitizers, high pressure, and others (Logan and Devos, 2009). These qualities combined with sporeformers' ubiquitous presence in natural environments (Carlin, 2011) leads to interest in controlling their entry into the dairy product continuum, on the farm (Vissers et al., 2006; Masiello et al., 2014; Miller et al., 2015a), in the transportation chain (Huck et al., 2008), and in the processing environment (Flint et al., 1997; Scott et al., 2007). In recent years the presence of mesophilic and thermophilic spores in dairy powders have gained increasing attention as specifications for these microorganisms in powders have become progressively more stringent (Watterson et al., 2014).

Mesophilic spores have been shown to be the most prevalent sporeformer found in bulk tank raw milk (Miller et al., 2015b). Organisms such as *Bacillus licheniformis* and *Bacillus pumilus* predominate in raw bulk tank milk (Ivy et al., 2012; Miller et al., 2015b) and appear to originate primarily from the dairy farm environment (te Giffel et al., 2002; Huck et al., 2008). In contrast, thermophilic spores are more prevalent in dairy powders (Watterson et al., 2014). Studies across the globe have consistently identified *Bacillus licheniformis*, *Anoxybacillus* sp. and *Geobacillus* sp. as the three primary sporeformers present in dairy powders (Ronimus et al., 2003; Ruckert et al., 2004; Scott et al., 2007; Yuan et al., 2012). While *Anoxybacillus* sp. and

Geobacillus sp. are considered obligate thermophiles (i.e., optimum growth temperatures of 50-62°C and 55-65°C, respectively; Pikuta, 2009; Logan et al., 2009) and are generally associated with the dairy processing environment (Flint et al., 1997; Scott et al, 2007), *Bacillus licheniformis* is capable of growing at both mesophilic temperatures as well as thermophilic temperatures and is found throughout the dairy production and processing continuum (Ivy et al., 2012).

Strategies for reducing the prevalence and levels of spores in dairy powders include reducing their entry into raw milk (Masiello et al., 2014; Miller et al., 2015a) and controlling their presence and growth in processing environments (Flint et al., 1997; Scott et al., 2007). The success of these approaches is evaluated based on results of spore testing in the final product (i.e, reducing spore counts). Unfortunately, this seemingly straightforward process is more complicated than it would seem due to the lack of standardization in spore testing methodologies. While a host of testing methodologies have been devised for enumerating spores in dairy products (Murphy et al., 1999; Hill et al., 2004; Scheldeman et al., 2005; ISO-IDF, 2009), there is little in the way of standardization when it comes to spore tests. Initial heat treatments to eliminate vegetative cells and select for spores range from 80°C to 125°C for 10 to 30 minutes; combined with incubation temperatures to select for mesophilic (i.e., 30-32°C) or thermophilic (i.e., 55°C) spores and various plating media, this leads to the potential for hundreds of unique spore test combinations. This makes national and global benchmarking and comparison nearly impossible. Additionally, while some spore tests are designed to target specific groups of sporeforming bacteria, in general, little is known regarding the effects of different spore treatments on the population of

spores that will be detected.

To this end, the objective of this study was to utilize various commonly employed spore enumeration methodologies to compare baseline mesophilic and thermophilic spore levels and populations in raw milk and dairy powders sourced from across the US, and to test the specific hypotheses that; i) increasing spore counts throughout a processing run would indicate the presence of in-plant associated sporeforming bacteria (i.e., *Anoxybacillus*) and; ii) spore testing parameters affect both the level and types of spores recovered from dairy powders. Results of this study will enable the US and global dairy industry to; i) compare and reference spore levels in both raw milk and dairy powders and; ii) define standard methods for enumeration of spores in dairy powder products, thereby allowing for targeted efforts to reduce spore levels in these products.

MATERIALS AND METHODS

Dairy Powder Processing Plants

Eleven dairy powder processing plants located either in the east (Plants A, F, E, B, I and K) or the west regions (G, H, J, C and D) of the US participated in the survey study. Each of the 11 dairy plants manufactures one of the following finished powder (**FP**) products: Plant A; Whey protein concentrate (**WPC**), Plants B, C, D, E, F and K; Non-fat dry milk (**NFDM**), Plants G and H; Skim milk powder (**SMP**), and Plants I and J; Whole milk powder (**WMP**). All plants made milk powders from raw material (**RM**) primarily raw milk, except for plant A and plant E which used cheese whey and condensed milk, respectively. The length of the production runs for each of

the 11 dairy plants varied between 6.5 h for the shortest and 44 h for the longest.

Sample Collection

In total, 5 RM (representing RM used during the entire processing run) and 3 FP samples (representing the beginning [within 1 h of processing start-up], middle [within +/- 1 h of projected mid-point of processing run] and end [within 1 h of shut-down] of the processing run) were collected by plant personnel once from each of the 11 dairy plants over the 10 month sampling period (July 2013 – April 2014). Detailed sampling instructions and checklists for sample collection, storage and shipping were provided to plant personnel. Fluid samples and powder samples were aseptically collected in 10 oz. Capitol Plastics locking vials and 24 oz. Whirl-Pak bags, respectively, and held at or below 6 °C until tested within 24 h of arrival at the Milk Quality Improvement Program (MQIP) laboratory (Cornell University, Ithaca, NY).

Spore Treatment and Enumeration

Aerobic spores were enumerated using methods described previously (Watterson et al., 2014). Briefly, 11 g of FP samples were rehydrated in 99 ml of phosphate buffer solution (**PBS**) with magnesium chloride under aseptic conditions. Five different spore tests were performed on 100 ml each of the RM and rehydrated FP samples, each test comprising of a heat treatment to inactivate vegetative bacterial cells followed by spread plating in duplicate on brain heart infusion (**BHI**) agar and incubation to recover viable spores. The methods used were; (i) spore pasteurized mesophilic spore count (**SP-MSC**; 80 °C for 12 min followed by incubation at 32 °C for 48 h); (ii) spore pasteurized thermophilic spore count (**SP-TSC**; 80 °C for 12 min followed by incubation at 55 °C for 48 h); (iii) highly heat resistant mesophilic spore

count (**HHR-MSC**; 100 °C for 30 min followed by incubation at 32 °C for 48 h); (iv) highly heat resistant thermophilic spore count (**HHR-TSC**; 100 °C for 30 min followed by incubation at 55 °C for 48 h), and; (v) specially thermoresistant spore enumeration (**STSE**; 106 °C for 30 min followed by incubation at 55 °C for 48 h). Condensed milk and WPC samples which thickened during heat treatment were diluted 2:1 in PBS post-heat treatment to enable plating.

Additionally, an enrichment step was performed on all heat treated samples to enable detection of spores present in low levels. Thirty ml aliquots from each spore test were incubated for 48 h alongside the plates. For those samples where the final count after direct plating was below the detection limit (i.e. no colonies present on BHI plate), a 10 µl aliquot of the enriched sample was streaked on BHI and then incubated under corresponding (i.e., 32°C or 55°C) conditions for 24 h.
Individual colonies were selected from samples treated with different spore tests (SP-MSC, SP-TSC, HHR-MSC, HHR-TSC and STSE) following direct plating or enrichment, selecting one for each unique colony morphology. Isolates were streaked for purity on BHI and frozen for further characterization in 15 % glycerol (wt/vol) at -80 °C.

rpoB Gene Sequencing for Identification of Bacterial Spores

Isolates were characterized as previously reported (Huck et al., 2007a). Briefly, cultures from frozen stocks were streaked onto BHI agar, followed by incubation at either 32°C or 55°C for 24 - 48 h. A single colony was picked with a sterile toothpick and resuspended in 100 µl of distilled water followed by heating in a microwave for 3

min at maximum power. PCR amplification was performed using a touchdown PCR method with primer pairs, *rpoB*-V3F (5'-AARYTNGGHCCDGARGAAAT-3') and rpoB-V3R (5'- TGNARYTTRTCRTCRACCATGTG-3'), amplifying a 740-nucleotide rpoB gene fragment. PCR reagents and thermocycling conditions used were as described by Durak et al. (2006). PCR products confirmed by 1.5% agarose gel electrophoresis were then purified using an ExoSAP method (Dugan et al., 2002), submitted for a bidirectional sequencing using the same primer pair at the Cornell Biotechnology Resource Center (Cornell University), and analyzed as described previously (Ivy et al., 2012). Each isolate was assigned an rpoB allelic type (**AT**) as described previously (Huck et al., 2007b; Ivy et al., 2012). Each rpoB AT represents a unique sequence within a specific 632-nucleotide region of the amplified gene fragment. For isolates that could not be identified by their rpoB AT sequence, a 700 nucleotide segment of the 16S gene was sequenced for species identification as previously described (Huck et al., 2007b) and compared to 16S sequences from all Bacillales type strains obtained from the Ribosomal Database Project (Cole et al., 2014). Separate multiple alignments for the rpoB and 16S sequences were generated and trimmed using MUSCLE (Edgar, 2004) and AliView (Larsson, 2014), respectively. All sequences were edited using Sequencher software 5.0 (Gene Codes Corporation, Ann Arbor, MI). For each alignment, a maximum-likelihood phylogenetic tree was generated using the rapid maximum-likelihood algorithm RAxML (Stamatakis, 2006) with rapid bootstrapping (100 bootstrap replicates). These trees were used to provide genus and species identification as described previously (Ivy et al., 2012).

Statistical Analysis

All analyses were performed in the R Statistical Programming Environment. Spore counts were modeled using a multilevel Poisson mixed-effect regression with observation-level random effects with the 'lme4' package. Sample material (i.e., raw material and powder) and spore test method were entered as fixed effects, while sample and plant were entered as random effects, with samples nested within plants. Post-hoc least- squares means tests were performed with the 'lsmeans' package, using the Tukey method for multiple testing correction. Spore population similarities were examined with the analysis of similarity (ANOSIM) method, using the 'vegan' package.

RESULTS

Spore counts in raw milk and dairy powders are significantly affected by spore test parameters

Overall, of the 55 raw material samples (45 raw milk, 5 cheese whey and 5 condensed milk) collected from 11 US dairy powder processors across the US, 100%, 98%, 80%, 84 % and 29% were positive either on direct plating or after enrichment for SPMSC, SPTSC, HHRMSC, HHRTSC and STSE tests, respectively (Table 5.1). Log mean spore counts in raw materials intended for powder production were 1.60 log CFU/mL, 1.20 log CFU/mL, 0.90 log CFU/mL, 1.80 log CFU/mL and 0.20 log CFU/mL for SPMSC, SPTSC, HHRMSC, HHRTSC and STSE, respectively (Figure 5.1). Additionally, of the 33 finished powder samples tested, 100%, 100%, 82%, 100% and 85% were positive for SPMSC, SPTSC, HHRMSC, HHRTSC and STSE, respectively (Table 5.1). Log mean spore counts in powder samples were 2.30 log CFU/g, 3.40 log CFU/g, 1.10 log CFU/g, 3.20 log CFU/g and 2.50 log CFU/g for SPMSC, SPTSC, HHRMSC, HHRTSC and STSE, respectively (Figure 5.1).

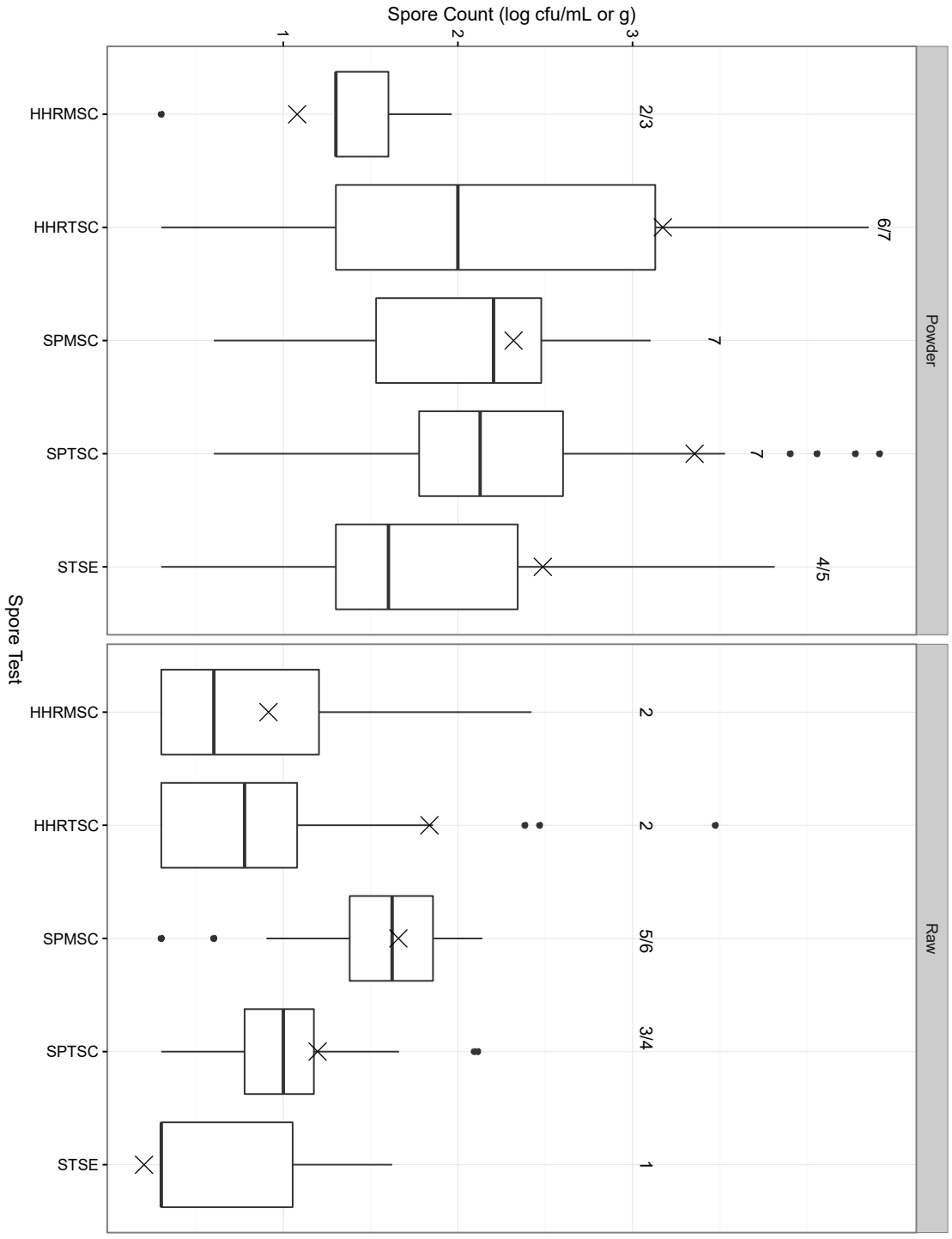
Of the five spore count methods employed in this study, STSE resulted in significantly lower spore counts in raw materials than SPMSC, SPTSC, HHRMSC and HHRTSC ($p<0.0001$, <0.0001, $=0.0024$ and <0.0001, respectively). HHRMSC and HHRTSC, while not significantly different from each other ($p=0.5157$), were significantly lower than SPMSC (both comparisons $p<0.0001$) and SPTSC ($p<0.0001$ and $=0.0010$, respectively). Finally, SPMSC in raw materials were significantly higher than SPTSC ($p=0.0027$).

Table 5.1. Summary of the mesophilic and thermophilic spore counts in raw ingredients and dairy powders obtained from each of the 11 dairy powder processing plants located across the US

		Treatments[1]									
		SP				HHR				STSE	
		MSC[2]		TSC[2]		MSC		TSC		TSC	
Plant	Sample Type[3]	After direct plating (Log mean cfu/ml or /g)	After enrichment	After direct plating (Log mean cfu/ml or /g)	After enrichment	After direct plating (Log mean cfu/ml or /g)	After enrichment	After direct plating (Log mean cfu/ml or /g)	After enrichment	After direct Plating (Log mean cfu/ml or /g)	After enrichment
A	R	3 (0.2)	2	2 (-0.1)	3	1 (0.0)	1	0	5	0	0
	FP	3 (1.5)	0	3 (2.4)	0	2 (1.5)	NP[6]	3 (1.9)	0	1 (1.1)	1[6]
B	R	5 (1.9)	0	5 (1.2)	0	3 (0.3)	2	3 (0.5)	2	0	2
	FP	3 (2.4)	0	3 (2.7)	0	1 (0.8)	2	3 (2.8)	0	3 (2.3)	0
C	R	5 (1.5)	0	5 (0.9)	0	0	5	2 (-0.1)	2	0	0
	FP	3 (2.4)	0	3 (2.3)	0	1 (0.8)	2	1 (1.3)	2	0	1
D	R	5[5] (1.7)	0	5 (1.4)	0	4 (1.8)	1	4 (2.8)	1	1 (-0.1)	4
	FP	3 (2.7)	0	3 (2.5)	0	1 (1.1)	2	3 (2.5)	0	2 (1.3)	1
E	R[4]	5 (2.0)	0	5 (1.3)	0	5 (1.2)	0	5 (1.0)	0	0	1
	FP	3 (2.2)	0	3 (1.4)	0	0	3	3 (2.7)	0	1 (1.8)	2
F	R[4]	5 (1.6)	0	5 (0.8)	0	4 (0.5)	1	2 (0.3)	3	1 (-0.3)	1
	FP	3 (2.0)	0	3 (1.7)	0	1 (0.8)	2	2 (1.1)	1	3 (1.7)	0
G	R	5 (1.6)	0	5 (1.2)	0	3 (0.3)	2	3 (0.1)	2	2 (-0.1)	0
	FP	3 (2.2)	0	3 (4.2)	0	1 (0.8)	1	3 (3.5)	0	3 (2.6)	0
H	R	5 (1.8)	0	5 (1.8)	0	2 (-0.1)	3	5 (2.1)	0	3 (1.2)	0
	FP	3 (2.4)	0	3 (3.8)	0	3 (1.4)	0	3 (4.0)	0	3 (3.4)	0
I	R	5 (1.5)	0	4 (0.8)	NP[6]	1 (-0.4)	NP[6]	2 (0.2)	NP[6]	0	NP[6]
	FP	3 (1.0)	0	3 (1.8)	0	0	NP[6]	3 (1.3)	0	2 (0.3)	NP[6]
J	R	5 (1.6)	0	5 (0.9)	0	0	4	4 (0.6)	0	0	1
	FP	3 (1.5)	0	3 (0.9)	0	1 (-0.2)	2	2 (0.6)	1	1 (-0.2)	1
K	R	5 (1.0)	0	5 (0.7)	0	1 (-0.4)	1	1 (-0.4)	0	0	0
	FP	3 (2.6)	0	3 (3.2)	0	2 (1.5)	0	3 (2.8)	0	2 (0.9)	1
Total (Log mean cfu/ml or /g)	R	53 (1.6)	2	51 (1.2)	3	24 (0.9)	20	31 (1.8)	15	7 (0.2)	9
	FP	33 (2.3)	0	33 (3.4)	0	13 (1.1)	14	29 (3.2)	4	21 (2.5)	7

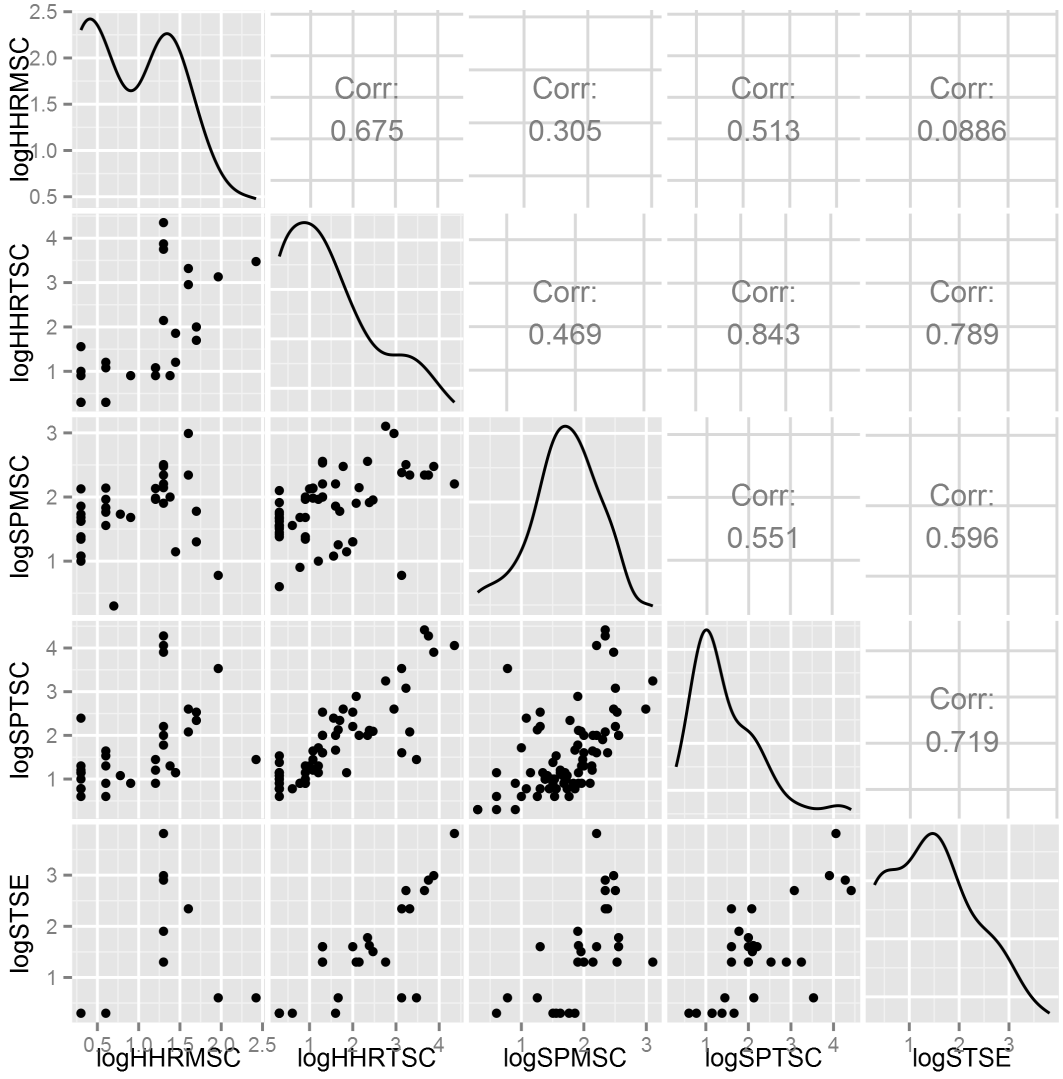
[1]Treaments: SP = spore pasteurization (80 °C for 12 min); HHR = highly heat resistant (100 °C for 30 min); STSE = specially thermo-resistant spore enumeration (106 °C for 30 min).
[2]Represents samples tested positive for mesophilic spore count (MSC) and thermophilic spore count (TSC).
[3]A total of 5 raw ingredients (R) and 3 finished powder (FP) samples were collected from each of the 11 plants.
[4]Raw ingredients collected from plant E (R = 5) and plant F (R = 1) represented condensed milk type.
[5]Includes 2 SP-treated raw ingredient samples with uncountable growth; these samples were not included in calculations of mean cfu/ml.
[6] Enrichment step was "Not performed" for the remaining heat treated samples, both raw ingredients and dairy powders.

Figure 5.1. Boxplots representing the distribution of spore counts from five spore count methods (SPMSC = spore pasteurized mesophilic spore count; SPTSC = spore pasteurized thermophilic spore count; HHRMSC = highly heat resistant mesophilic spore count; HHRTSC = highly heat resistant thermophilic spore count and; STSE = specially thermoresistant spore enumeration) from 33 dairy powder samples and 55 raw material samples (45 raw milk, 5 cheese whey and 5 condensed milk) sourced from 11 US dairy powder processors. Dark horizontal lines within the boxplot represent median spore count values, while "X" represents the log mean spore count for each respective test. Different numbers above the boxplots represent significant ($p<0.05$) differences in spore counts.


Spore Count (log cfu/mL or g)
1
2
3
Spore Test
HHRMSC
HHRTSC
SPMSC
SPTSC
STSE
2/3
6/7
7
7
4/5
2
2
5/6
3/4
1
Powder
Raw

Results showed that in dairy powders, HHRMSC was significantly lower than SPMSC, SPTSC, HHRTSC and STSE ($p<0.0001$, <0.0001, <0.0001 and $=0.0009$, respectively). STSE was significantly lower than SPMSC, SPTSC and HHRTSC ($p<0.0001$ for all three comparisons), while HHRTSC, SPMSC and SPTSC were not significantly different from each other ($p>0.2$ for all comparisons). Correlations between different spore count methods (including raw and powder samples) range from 0.0886 for log STSE vs log HHRMSC to 0.843 for log SPTSC vs log HHRTSC (Figure 5.2).

Figure 5.2. Comparison of various spore count methods (SPMSC = spore pasteurized mesophilic spore count; SPTSC = spore pasteurized thermophilic spore count; HHRMSC = highly heat resistant mesophilic spore count; HHRTSC = highly heat resistant thermophilic spore count and; STSE = specially thermoresistant spore enumeration). Bottom triangle contains scatterplots of log-transformed spore counts between different spore tests. Only samples with non-zero counts for both tests are included in each plot. Upper triangle contains the correlation of the log-transformed spore counts between different spore tests. Diagonal contains kernel density estimates for the probability distribution of each test.


logHHRMSC
logHHRTSC
logSPMSC
logSPTSC
logSTSE
Corr:
0.675
Corr:
0.305
Corr:
0.513
Corr:
0.0886
Corr:
0.469
Corr:
0.843
Corr:
0.789
Corr:
0.551
Corr:
0.596
Corr:
0.719

Mean thermophilic spore counts increased, although not significantly, from beginning through the end of a processing run

No significant increase was found in dairy powder spore counts (all methods combined) throughout a processing run ($p=0.39$), despite a trend for increasing counts from beginning to middle and end (2.19, 3.00 and 3.15 log mean CFU/g, respectively; Figure 5.3). The mesophilic spore count comparisons between the beginning and the end of processing showed a slight decrease of 0.15 log CFU/g in the HHRMSC method and a slight increase of 0.30 log CFU/g in the SPMSC method. Conversely, all of the thermophilic spore counts tended to increase from beginning to the end of processing, with an increase of 1.22, 0.90 and 1.09 for the SPTSC, HHRTSC and STSE methods (Figure 5.3). However, despite the trend for increasing counts in the thermophilic methods, none represented a significant increase ($p=0.19$).

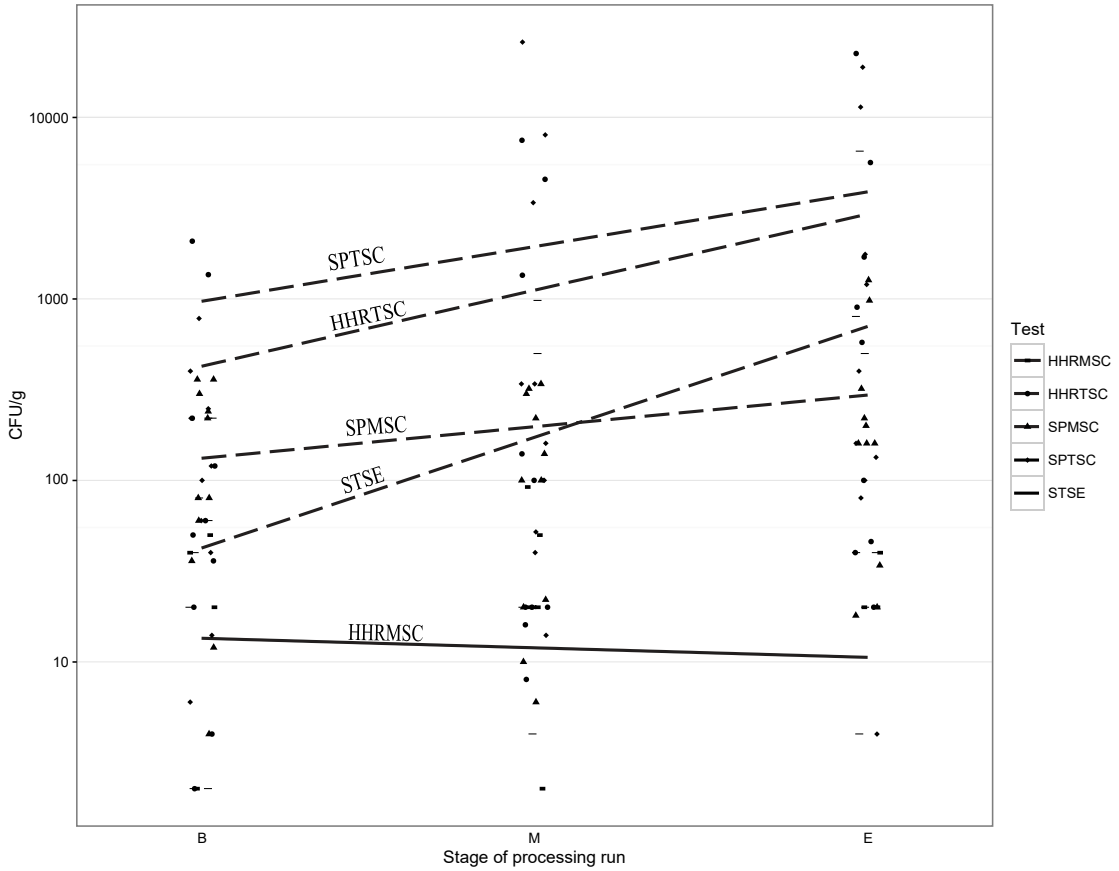
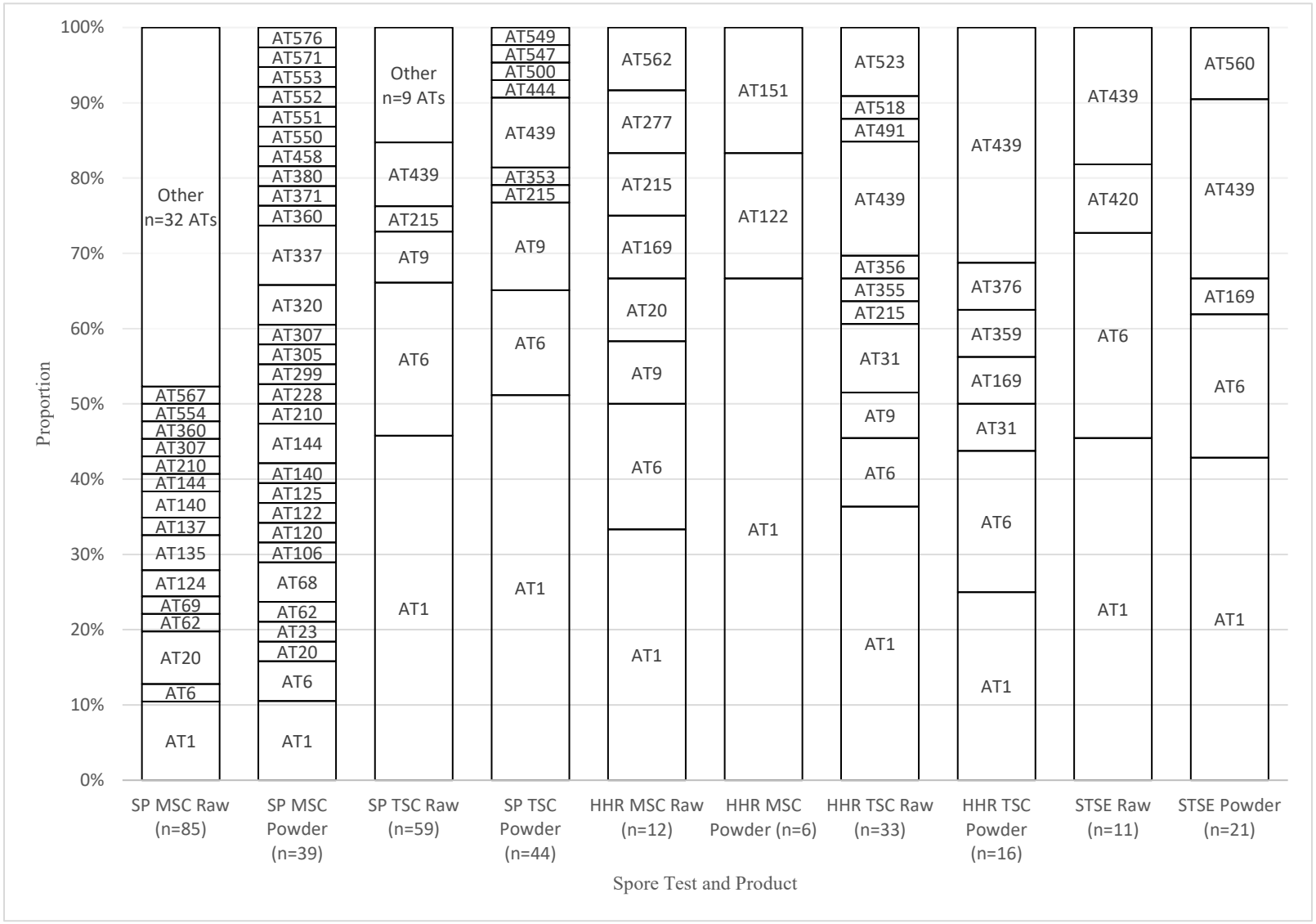


Figure 5.3. Spore counts at beginning (B), middle (M) and end (E) stages of a dairy powder processing run. Tests are represented by the following symbols; (■) = SPMSC (spore pasteurized mesophilic spore count); (+) = SPTSC (spore pasteurized thermophilic spore count); (●) = HHRMSC (highly heat resistant mesophilic spore count); (▲) = HHRTSC (highly heat resistant thermophilic spore count) and; (☒) = STSE (specially thermoresistant spore enumeration). Trend lines represent Poisson regression for each individual spore test.

Bacillus licheniformis is the predominant sporeformer isolated from raw milk and dairy powders

A total of 326 isolates were collected from 33 dairy powder samples and 55 raw material samples from 11 US dairy powder processors. Of those, 126 (39%) isolates were collected from powder, and the remaining 200 isolates were collected from raw materials (61%). Among the 126 isolates collected from powder samples, 39 (31%), 44 (35%), 6 (5%), 16 (13%) and 21 (16%) were isolated from SPMSC, SPTSC, HHRMSC, HHRTSC and STSE, respectively (Figure 5.4). Similarly, of the 200 isolates collected from raw samples, 85 (43%), 59 (30%), 12 (6%), 33 (16%) and 11 (5%) were isolated from SPMSC, SPTSC, HHRMSC, HHRTSC and STSE, respectively (Figure 5.4).

Figure 5.4. Spore population diversity as defined by rpoB allelic type (AT) for each test and product (SPMSC = spore pasteurized mesophilic spore count; SPTSC = spore pasteurized thermophilic spore count; HHRMSC = highly heat resistant mesophilic spore count; HHRTSC = highly heat resistant thermophilic spore count and; STSE = specially thermoresistant spore enumeration) from 11 US dairy powder processors. n=number of spore isolates collected from each corresponding product and test. "Other" represents ATs found at less than 2% prevalence in the corresponding product and method.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Proportion
Other n=32 ATs
AT567
AT554
AT360
AT307
AT210
AT144
AT140
AT137
AT135
AT124
AT69
AT62
AT20
AT6
AT1
AT576
AT571
AT553
AT552
AT551
AT550
AT458
AT380
AT371
AT360
AT337
AT320
AT307
AT305
AT299
AT228
AT210
AT144
AT140
AT125
AT122
AT120
AT106
AT68
AT62
AT23
AT20
AT6
AT1
Other n=9 ATs
AT439
AT215
AT9
AT6
AT1
AT549
AT547
AT500
AT444
AT439
AT353
AT215
AT9
AT6
AT1
AT562
AT277
AT215
AT169
AT20
AT9
AT6
AT1
AT151
AT122
AT1
AT523
AT518
AT491
AT439
AT356
AT355
AT215
AT31
AT9
AT6
AT1
AT439
AT376
AT359
AT169
AT31
AT6
AT1
AT439
AT420
AT6
AT1
AT560
AT439
AT169
AT6
AT1
SP MSC Raw (n=85)
SP MSC Powder (n=39)
SP TSC Raw (n=59)
SP TSC Powder (n=44)
HHR MSC Raw (n=12)
HHR MSC Powder (n=6)
HHR TSC Raw (n=33)
HHR TSC Powder (n=16)
STSE Raw (n=11)
STSE Powder (n=21)
Spore Test and Product

Overall, a total of 11 genera and 25 unique species represented by 96 unique rpoB allelic types (ATs) were collected from this study (Table 5.2). Of the 326 isolates, *Bacillus licheniformis*, represented by rpoB ATs 1, 6, 9, 31, 169, 173, 215, 549 and 560, accounted for 51% (n=166) of all isolates (Figure 5.4; Table 5.2). Additionally, *Bacillus licheniformis* (all ATs) was isolated from all spore count methods, all plants, all time points (beginning, middle and end of processing) and both raw and powder products, making it the most ubiquitous organism isolated in this study. *Bacillus licheniformis* AT1 alone accounted for just over 30% of all isolates (n=100), and was isolated from all spore count methods, all plants, all time points and both raw and powder products (Figure 5.4; Table 5.2). In total, the genus *Bacillus* accounted for 263 isolates, or 81% of total isolates collected.

Following *Bacillus*, the second most commonly isolated genus was *Geobacillus* (9%, n=30). In contrast to *Bacillus*, *Geobacillus* was only isolated from 6 of the 11 plants, with 20 of the 30 (67%) isolates coming from plant A (Table 5.2). Also in contrast to *Bacillus*, *Geobacillus* was only isolated from thermophilic tests (SPTSC, HHRTSC and STSE), and was isolated from both raw and powder products and all processing time points (Figure 5.4; Table 5.2). The remaining isolates were characterized as *Aeribacillus* (n=9), *Paenibacillus* (n=8), *Brevibacillus* (n=3), *Lysinibacillus* (n=3), *Oceanobacillus* (n=3), *Psychrobacillus* (n=2), *Sporosarcina* (n=2), *Ureibacillus* (n=2) and *Anoxybacillus* (n=1; Table 5.2).

Table 5.2. Identification and characterization of unique rpoB allelic types (ATs) isolated from eleven dairy powder processors across the US

Genus	Species	rpoB AT[1]	Frequency	Test Isolated From[2]				
				SP MSC	SP TSC	HHR MSC	HHR TSC	STSE
Bacillus	*licheniformis*	1	100	Yes	Yes	Yes	Yes	Yes
Bacillus	*licheniformis*	6	37	Yes	Yes	Yes	Yes	Yes
Geobacillus	sp.	439	26	No	Yes	No	Yes	Yes
Bacillus	*licheniformis*	9	12	No	Yes	Yes	Yes	No
Bacillus	*pumilus*	20	7	Yes	No	No	No	No
Bacillus	*licheniformis*	31	5	No	Yes	No	Yes	No
Bacillus	*licheniformis*	215	5	No	Yes	Yes	Yes	No
Bacillus	cf. *aerophilus*	135	4	Yes	No	No	No	No
Bacillus	*pumilus*	144	4	Yes	No	No	No	No
Bacillus	*pumilus*	337	4	Yes	No	No	No	No
Bacillus	*safensis*	140	4	Yes	No	No	No	No
Aeribacillus	*pallidus*	523	3	No	No	No	Yes	No
Bacillus	cf. *aerophilus*	307	3	Yes	No	No	No	No
Bacillus	*licheniformis*	169	3	No	No	Yes	Yes	Yes
Bacillus	*pumilus*	62	3	Yes	No	No	No	No
Bacillus	*pumilus*	68	3	Yes	No	No	No	No
Bacillus	*pumilus*	210	3	Yes	No	No	No	No
Bacillus	*safensis*	124	3	Yes	No	No	No	No
Bacillus	sp.	122	3	Yes	No	Yes	No	No
Bacillus	*subtilis* s.l.	360	3	Yes	No	No	No	No
Aeribacillus	*pallidus*	356	2	No	Yes	No	Yes	No
Bacillus	cf. *aerophilus*	69	2	Yes	No	No	No	No
Bacillus	cf. *licheniformis*	560	2	No	No	No	No	Yes
Bacillus	cf.*nealsonii*	458	2	Yes	No	No	No	No
Bacillus	*mojavensis*	554	2	Yes	No	No	No	No
Bacillus	*pumilus*	137	2	Yes	No	No	No	No
Bacillus	*pumilus*	320	2	Yes	No	No	No	No
Bacillus	*safensis*	106	2	Yes	No	No	No	No
Bacillus	*safensis*	305	2	Yes	No	No	No	No
Lysinibacillus	sp.	299	2	Yes	No	No	No	No
Paenibacillus	*amylolyticus* s.l.	23	2	Yes	No	No	No	No
Sporosarcina	cf.*globispora*	567	2	Yes	No	No	No	No
Aeribacillus	*pallidus*	355	1	No	No	No	Yes	No
Aeribacillus	*pallidus*	359	1	No	No	No	Yes	No
Aeribacillus	*pallidus*	376	1	No	No	No	Yes	No

Aeribacillus	*pallidus*	491	1	No	No	No	Yes	No
Anoxybacillus	sp.	353	1	No	Yes	No	No	No
Bacillus	*altitudinis*	559	1	Yes	No	No	No	No
Bacillus	*altitudinis*	576	1	Yes	No	No	No	No
Bacillus	*atrophaeus*	580	1	Yes	No	No	No	No
Bacillus	*cereus* s.l.	120	1	Yes	No	No	No	No
Bacillus	*cereus* s.l.	125	1	Yes	No	No	No	No
Bacillus	*cereus* s.l.	380	1	Yes	No	No	No	No
Bacillus	*cereus* s.l.	556	1	Yes	No	No	No	No
Bacillus	cf. *aerophilus*	176	1	Yes	No	No	No	No
Bacillus	cf. *aerophilus*	582	1	Yes	No	No	No	No
Bacillus	cf. *licheniformis*	549	1	No	Yes	No	No	No
Bacillus	*coagulans*	354	1	No	Yes	No	No	No
Bacillus	*coagulans*	546	1	No	Yes	No	No	No
Bacillus	*licheniformis*	173	1	Yes	No	No	No	No
Bacillus	*megaterium*	151	1	No	No	Yes	No	No
Bacillus	*mojavensis*	237	1	Yes	No	No	No	No
Bacillus	*mojavensis*	555	1	Yes	No	No	No	No
Bacillus	*oleronius*	552	1	Yes	No	No	No	No
Bacillus	*pumilius*	20	1	No	No	Yes	No	No
Bacillus	*pumilus*	72	1	Yes	No	No	No	No
Bacillus	*pumilus*	253	1	Yes	No	No	No	No
Bacillus	*pumilus*	548	1	Yes	No	No	No	No
Bacillus	*pumilus*	565	1	Yes	No	No	No	No
Bacillus	*pumilus*	569	1	Yes	No	No	No	No
Bacillus	*safensis*	141	1	Yes	No	No	No	No
Bacillus	*safensis*	371	1	Yes	No	No	No	No
Bacillus	*safensis*	378	1	Yes	No	No	No	No
Bacillus	*safensis*	436	1	Yes	No	No	No	No
Bacillus	*safensis*	550	1	Yes	No	No	No	No
Bacillus	*safensis*	551	1	Yes	No	No	No	No
Bacillus	*safensis*	553	1	Yes	No	No	No	No
Bacillus	*safensis*	NA	1	Yes	No	No	No	No
Bacillus	sp.	228	1	Yes	No	No	No	No
Bacillus	sp.	277	1	No	No	Yes	No	No
Bacillus	sp.	581	1	Yes	No	No	No	No
Bacillus	*subtilis* s.l.	65	1	Yes	No	No	No	No
Bacillus	*subtilis* s.l.	562	1	No	No	Yes	No	No
Bacillus	*subtilis* s.l.	570	1	Yes	No	No	No	No
Bacillus	*subtilis* s.l.	578	1	Yes	No	No	No	No
Bacillus	*thermoamylovorans*	489	1	No	Yes	No	No	No

Bacillus	*thermoamylovorans*	499	1	No	Yes	No	No	No
Bacillus	*thermoamylovorans*	500	1	No	Yes	No	No	No
Brevibacillus	*borstelensis*	518	1	No	No	No	Yes	No
Brevibacillus	*borstelensis*	519	1	No	Yes	No	No	No
Brevibacillus	cf. *parabrevis*	545	1	Yes	No	No	No	No
Geobacillus	sp.	420	1	No	No	No	No	Yes
Geobacillus	sp.	444	1	No	Yes	No	No	No
Geobacillus	sp.	561	1	No	Yes	No	No	No
Geobacillus	sp.	NA	1	No	Yes	No	No	No
Lysinibacillus	sp.	579	1	Yes	No	No	No	No
Oceanobacillus	*neutrophilis/sojae*	268	1	Yes	No	No	No	No
Oceanobacillus	sp.	290	1	Yes	No	No	No	No
Oceanobacillus	sp.	572	1	Yes	No	No	No	No
Paenibacillus	cf. *illinoisensis*	571	1	Yes	No	No	No	No
Paenibacillus	cf. *sonchi*	558	1	Yes	No	No	No	No
Paenibacillus	*odorifer*	21	1	Yes	No	No	No	No
Paenibacillus	*odorifer*	568	1	Yes	No	No	No	No
Paenibacillus	*odorifer*	575	1	Yes	No	No	No	No
Paenibacillus	sp.	577	1	Yes	No	No	No	No
Psychrobacillus	cf. *psychrotolerans*	564	1	Yes	No	No	No	No
Psychrobacillus	cf. *psychrotolerans*	566	1	Yes	No	No	No	No
Ureibacillus	*thermosphaericus*	547	1	No	Yes	No	No	No
Ureibacillus	*thermosphaericus*	563	1	No	Yes	No	No	No

[1] NA indicates no assigned AT

[2] SPMSC = Spore Pasteurized Mesophilic Spore Count; SPTSC=Spore Pasteurized Thermophilic Spore Count; HHRMSC=Highly Heat Resistant Mesophilic Spore Count; HHRTSC=Highly Heat Resistant Thermophilic Spore Count and; STSE=Specially Thermophilic Spore Enumeration

Analysis of similarity (ANOSIM) was used to determine spore population similarities, based on rpoB AT, between samples of various types. Results show that spore populations were significantly different between plant ($p=0.001$), spore count method (i.e., the combination of heat treatment and incubation temperature; $p=0.04$) and heat treatment ($p=0.015$). No significant difference was found between spore populations for product (raw and powder; $p=0.381$), processing time point (beginning, middle or end; $p=0.617$) or incubation temperature (32°C or 55°C; $p=0.817$). Figure 5.4 illustrates the reduction in AT diversity as increasing heat treatment is employed for spore count methods. For example, dairy powder samples analyzed using the SPTSC test resulted in 10 unique ATs while the same powder analyzed using the HHRTSC and STSE tests resulted in 7 and 5 unique ATs, respectively (Figure 5.4). Further, only 3 unique ATs were identified in the powder treated with the HHRMSC method, while the SPMSC method resulted in 29 unique ATs, the largest number of unique ATs per test found in powder in this study (Figure 5.4).

DISCUSSION

Benchmarking mesophilic and thermophilic spore counts in dairy powders requires standardization of spore count testing parameters

This study reports baseline spore counts, using various methods, for raw milk (and other raw materials) and dairy powders across the US. Figure 5.1 shows the distribution of spore counts by product (raw or powder) and spore count method, providing a benchmarking tool for the dairy powder industry. Previous studies have reported that spore counts in dairy powders (based on various spore count methods) ranged from below detection limit to $>10^4$ CFU/g (Muir et al., 1986; Ruckert et al., 2004; Yuan et al., 2012; Watterson et al., 2014; Buehner et al., 2015). Log mean spore counts of US dairy powders in our study ranged from 1.10 log CFU/g for STSE to 3.40 log CFU/g for SPMSC, well within, and even at the low end of the range of previously reported spore counts.

Baseline spore counts in raw materials and dairy powders in this study were found to differ significantly by spore test method (Figure 5.1). Various methods for enumerating aerobic spores in dairy products have been previously described. The standard method, outlined in Standard Methods for the Examination of Dairy Products (Frank and Yousef, 2004) includes a heat treatment for 12m at 80°C to eliminate vegetative cells followed by incubation at 32°C for 48h to enumerate mesophilic spores, or 7°C for 10 days to enumerate psychrotolerant spores. Examples of other methods include those selecting for highly heat resistant spores (heat treatment of 100°C/30m; Murphy et al., 1999; Scheldeman et al., 2005) and those selecting for specific heat resistant microorganisms (heat treatment of 106°C/30m; Hill, 2004; ISO-

IDF, 2009). All of these methods employ a similar strategy, that being a heat treatment for a specific time/temperature combination to eliminate vegetative cells, plating on a spore recovery media (i.e., brain heart infusion agar, tryptic soy agar, plate count milk agar, etc), a factor not studied here, but that may contribute to variability in spore counts, and incubation at various temperatures to select for groups (i.e., psychrotolerant, mesophilic and thermophilic) of sporeforming microorganisms. It is not surprising, given the variation among these methods, and indeed the intention for some methods to select for different groups of sporeforming microorganisms, that there were significant differences in the levels and populations of sporeforming bacteria detected among the spore tests used in this study. Watterson et al. (2014) found a similar pattern in samples from four Northeastern US dairy powder processing facilities. These authors found that only 7.3% of samples were positive on direct plating for mesophilic spores after 100°C/30m spore treatment in comparison to 80°C/12m which resulted in over 30% of samples positive for mesophilic spores. Additionally, these authors found that nearly two times more samples were positive for thermophilic spores when subjected to a 80°C/12m spore treatment than when treated at 100°C/30m (Watterson et al., 2014). Buehner et al., (2015) also found that mesophilic spore counts were significantly lower than thermophilic spore counts in dairy powders from the Midwest US with a heat treatment of 80°C for 12m used for both mesophilic and thermophilic spore counts. These significant differences among spore count and spore populations among test methods also explains why there is relatively low correlation when results from these methods are compared (Figure 5.2), leading to the conclusion that on the whole, these tests do not convey the same

information and therefore are not interchangeable.

Overall, one can quickly see how many combinations might be derived from the four parameters that constitute a spore test (i.e., heat treatment temperature, heat treatment time, media and incubation temperature), leading to literally hundreds of possible methods. This lack of standardization in spore testing methodologies leads to complications and confusion when comparing, benchmarking and interpreting spore levels in powder from the US and abroad. Our study here highlights the importance of establishing standard methods, using scientifically validated data, for the examination of spore levels in dairy powders. While results from this study do not shed light on which spore test parameters are ideal for use in the dairy powder industry, it does demonstrate that small changes in these parameters can make significant differences in outcomes. For this reason, our recommendation would be for the dairy powder industry to pursue the adoption of more than one standardized method (ie, SPMSC, HHRTSC and STSE) to capture the diversity of sporeforming bacteria commonly found in dairy powders. Different standardized spore test methods can then also be used to evaluate powders that are used in different applications (i.e., infant formula versus cheese making).

Bacillus licheniformis represents a raw to finished product hurdle to the reduction of spore counts in dairy powders

Bacillus licheniformis was by far the most frequently isolated sporeforming microorganism from both raw materials and dairy powder in this study. It was identified in all products, tests, time points and was isolated from all 11 plants enrolled

in this study. Previous work has identified *Bacillus licheniformis* as one of the principal spore contaminants in dairy powders across the globe (Ronimus et al., 2003; Ruckert et al., 2004; Reginensi et al., 2011; Miller et al., 2015b). A survey of commercial milk powders in China revealed that *Bacillus licheniformis* accounted for 27.8% of the total isolates (Yuan et al., 2012), lower than the prevalence found in our study (50.9%). Buehner et al., (2015) also found *Bacillus licheniformis* to be the most prevalent sporeformer, accounting for 63% of the isolates, in nonfat dry milk powders from the Midwestern US. . In addition to widespread prevalence in dairy powders around the world, *Bacillus licheniformis* has been described as one of the most prevalent sporeforming bacteria present in raw milk (Crielly et al., 1994; te Giffel et al., 2002; Ivy et al., 2012; Miller et al., 2015b). Further, *Bacillus licheniformis* is widespread in the dairy farm environment (te Giffel et al., 2002; Huck et al., 2008). The ubiquitous nature of *Bacillus licheniformis* from the dairy farm environment, in raw milk and in dairy powders necessitates that particular attention be paid to preventing the entry of this organism into raw milk as well as eliminating it from dairy powder processing environments in order to reduce dairy powder spore counts.

Second only to *Bacillus*, *Geobacillus* accounted for just over 9% of the total sporeformers characterized from raw materials and powder samples, although two thirds of the isolates came from just one plant (A). *Geobacillus* has frequently been reported as a common thermophilic spore contaminant of dairy powders (Ruckert et al., 2004; Scott et al., 2007; Yuan et al., 2012), however it was not widespread in this this study. Interestingly, *Geobacillus* was isolated from raw milk and cheese whey from three dairy powder processing facilities in this study (plants A, G and H), cheese

whey yielded half of the *Geobacillus* isolates (n=10) from plant A, the only facility manufacturing WPC in this study. Only one previous study has described the presence of *Geobacillus* in raw milk (Miller et al., 2015b), however there are relatively few studies that have both enumerated thermophilic spores in raw milk and subsequently identified those microorganisms (e.g., Coorevits et al., 2008), which likely accounts for the lack of data describing *Geobacillus* in raw milk. It must also be noted, that the raw milk (and other raw material) samples from this study were obtained from raw milk storage tanks at the processing facility. It cannot be ruled out, therefore, that the raw materials were contaminated in the processing facility, or even during transport from the farm to the processing facility. More work is needed to determine the prevalence of raw milk contamination with *Geobacillus* and potential farm factors that may lead to transmission of this organism into the dairy powder continuum.

Surprisingly, *Anoxybacillus*, which represented one of the most commonly isolated thermophilic sporeformers in previous studies (Ruckert et al., 2004; Scott et al., 2007; Reginensi et al., 2011; Yuan et al., 2012) was only isolated once in this study. *Anoxybacillus* has been shown to form biofilms in dairy powder processing facilities, thereby contaminating the product in increasing amounts throughout a processing run. Scott et al., (2007) found that thermophilic spore counts in powder, primarily consisting of *Anoxybacillus* and *Geobacillus*, increased nearly 4 orders of magnitude from the beginning of a processing run through the end (~18h) in one plant, due primarily to spores present in foulant sloughed off during production. Several studies have shown that *Anoxybacillus* is capable of producing biofilms in dairy

processing equipment (Flint et al., 1997; Scott et al., 2007). *Anoxybacillus*, therefore may be specific to individual processing facilities where it has managed to create biofilms. This idea is supported by the results of the current study that indicate that populations of sporeforming bacteria differ significantly between processing plants. Additionally, the lack of significant increase in spore counts throughout processing found in this and previous work (Watterson et al., 2014) may be due to the absence of biofilms containing the sporeformers previously associated with post-processing contamination (i.e., *Anoxybacillus*) in the powder facilities surveyed here.

Distinguishing between raw milk sources and in-plant sources of spores requires improved discriminatory testing methods

In this study *rpoB* allelic typing was used as a subtyping method for sporeforming bacteria. This method was developed for differentiating between closely related sporeforming bacteria in dairy products and environments (Durak et al., 2006). This method has previously been used for characterization and identification of sporeformers in raw milk (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ivy et al., 2012; Masiello et al., 2014), fluid milk (Huck et al., 2007a; Huck et al., 2007b; Ivy et al., 2012), dairy powders (Miller et al., 2015b), and the dairy farm environment (Huck et al., 2008, Ivy et al., 2012). While this method offers improved subtype discrimination over 16S rDNA sequencing (Durak et al., 2006), phenotypic differences including enzyme production and optimal growth temperature have also been described between members of the same *rpoB* allelic type (Ivy et al., 2012; Trmcic et al., 2015), including *Bacillus licheniformis rpoB* allelic type 1, which

comprises approximately 30% of the isolates recovered in this study. This phenotypic variability suggests that the *rpoB* allelic typing system has limited discriminatory power within this important dairy-associated strain.

Because aerobic sporeformers can both originate from raw milk and survive processing (Watterson et al., 2014; Miller et al., 2015b) as well as originate from the processing facility (Flint et al., 1997; Scott et al., 2007; Burgess et al., 2010), source-tracking is an invaluable tool to inform the development of and implementation of intervention strategies. Other methods of subtyping have been employed for *Bacillus* isolates drawn from dairy sources, including RAPD-, BOX-, REP-, REP-I-, and (GTG)5PCR fingerprinting (Ronimus et al., 2002; Ruckert et al., 2003; De Jonghe et al., 2007; Banyko et al., 2008; Reginensi et al., 2011), ribotyping (Andersson et al., 1999), high resolution melt analysis (Dhakal et al., 2013; Chauhan et al., 2013), multilocus sequence typing (Helgason et al, 2004; Soo Ko et al., 2004), Fourier transform infrared spectroscopy (Beattie et al., 1998), and multiparametric real time PCR (Postollec et al., 2010; Postollec et al., 2012). More work is needed to understand how the discriminatory power of these methods compares to the *rpoB* allelic typing used in this study, and whether the use of such subtyping methods is viable as a source-tracking tool in industry.

CONCLUSIONS

This study highlights the need to apply a standardized set of methods for enumeration of groups of aerobic spores in dairy products. The current lack of

standardization in testing methods has made benchmarking spore counts in dairy powders from different plants and even countries an impossibility. The results of this study indicate that one single spore test is not sufficient for determining the true concentration of spores in a dairy powders due to the changes in spore populations as a result of the various testing parameters. This point was further supported by the low correlations seen between the spore counts from various testing methods. Further, there needs to be a focus on *Bacillus licheniformis* as a major dairy continuum contaminant, and major contributor to dairy powder spore counts. Reducing spore counts in dairy powders will require a better understanding of contamination sources and transmission patterns for this sporeformer.

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CHAPTER SIX

Rapid detection and characterization of post-pasteurization contaminants in pasteurized fluid milk

ABSTRACT

Microbial spoilage of pasteurized fluid milk is typically due to either (i) post-pasteurization contamination (PPC) with psychrotolerant Gram negative bacteria (predominantly *Pseudomonas*) or (ii) growth of psychrotolerant sporeformers (e.g., *Paenibacillus*) that have the ability to survive pasteurization when present as spores in raw milk, and to subsequently grow at refrigeration temperatures. While fluid milk quality has improved over the last several decades, continued reduction of PPC is hampered by the lack of rapid, sensitive, and specific methods that allow for detection of PPC in fluid milk, with fluid milk processors still often using time-consuming methods (e.g., Moseley keeping quality test). The goal of this project was to utilize a set of commercial fluid milk samples that are characterized by a mixture of samples with PPC due to psychrotolerant Gram negative bacteria and samples with presence and growth of psychrotolerant sporeforming bacteria to evaluate different approaches for rapid detection of PPC. Comprehensive microbiological shelf-life characterization of 105 pasteurized fluid milk samples obtained from 20 dairy processing plants showed that 60/105 samples reached bacterial counts >20,000 CFU/ml over shelf-life due to PPC with Gram negative bacteria. Among these 60 samples with evidence of

Gram negative PPC spoilage over shelf-life, 100% (60/60) showed evidence of contamination with non-coliform, non-Enterobacteriaceae (EB) Gram negative bacteria (e.g., *Pseudomonas*), 20% (12/60) showed evidence of contamination with coliforms and 7% (4/60) showed evidence of contamination with non-coliform EB. Among the remaining 45 samples, 28 showed levels of Gram positive bacteria above 20,000 CFU/ml and the remaining 17 samples did not exceed 20,000 CFU/mL over shelf-life. Evaluation of the same set of 105 samples using 6 different approaches (all possible combinations of two different enrichment protocols [13°C or 21°C for 18h] and three different plating media [crystal violet tetrazolium agar (CVTA), EB Petrifilm and Coliform Petrifilm], showed that enrichment at 21°C for 18 h, followed by plating on CVTA provided for the most sensitive, accelerated detection of samples that reached >20,000 CFU/ml due to PPC with psychrotolerant Gram negatives (70% sensitivity). These results show that tests still required and traditionally used in the dairy industry (e.g., coliform testing) are not suitable for monitoring for PPC. Rather, approaches that allow for detection of all Gram negative bacteria are essential for improved detection of PPC in fluid milk.

INTRODUCTION

While research has shown that fluid milk quality has consistently improved over the last two decades (Carey et al., 2005, Martin et al., 2012), post-pasteurization contamination (**PPC**) is still a hurdle for some processors. In fact, some studies suggest that ~40-50% of conventionally pasteurized fluid milk shows evidence of PPC

(Ranieri and Boor, 2009; Reichler, unpublished). PPC has previously been associated with rapid bacterial outgrowth (Ranieri and Boor, 2009, Schröder et al., 2009, Martin et al., 2012) and unacceptable sensorial properties (Hayes et al., 2002, Martin et al., 2012), both of which often lead to premature spoilage prior to the labelled product shelf-life (defined here as the sell by date provided by the manufacturer). As premature spoilage is a contributing cause of food loss, which accounts for approximately a third of the fluid milk processed in the US, at a value of 6.4 billion dollars (Buzby et al., 2014), reducing PPC is of great importance from a business, consumer and sustainability perspective.

PPC can be introduced into the fluid milk continuum at various points, but a number of research studies indicate that the filling equipment is an area that is particularly susceptible to contamination often due to lapses in good manufacturing practices (Eneroth et al., 1998, Ralyea et al., 1998, Gruetmacher and Bradley, 1999). Additionally, while stringent cleaning and sanitation programs reduce the incidence of PPC, some contaminants that exist within resistant biofilms, or in niches that are inaccessible to cleaning and sanitizers, may not be effectively removed from processing equipment and lead to persistent PPC. Methods to detect PPC in fluid milk have primarily relied upon traditional indicator organisms used in the dairy industry. Coliforms have been used, for nearly a century, as indicator organisms in the dairy industry. For example, the US Pasteurized Milk Ordinance (PMO) specifies a coliform limit of 10 CFU/mL for Grade “A” pasteurized fluid milk by (FDA, 2015). Coliforms are heat labile and are very effectively eliminated by HTST pasteurization. Hence, coliform presence in pasteurized fluid milk can be expected to generally be due to re-

introduction of these organisms after the heat step, unless coliforms were present in very high numbers (e.g., $>10^6$ CFU/mL) in raw milk. Detection of coliforms in fluid milk thus is often considered an indication of unhygienic conditions or post-pasteurization contamination. Testing for coliforms is also relatively fast, a desirable quality for indicator organisms, with many methods (e.g., Coliform Petrifilm) taking 24 hours or less.

Despite the longstanding use of coliforms as indicators of PPC in fluid milk, many studies have identified *Pseudomonas*, a non-coliform, as the primary causative agent of PPC (Ternström et al., 1993, Eneroth et al., 1998, Deeth et al., 2002). Importantly, *Pseudomonas* and other non-coliform Gram negative bacteria are not recovered on coliform media and therefore may go undetected by current indicator tests. Testing methods that allow for the detection of coliforms, *Pseudomonas* and other Gram negative bacteria (e.g., plating on crystal violet tetrazolium agar) are not as rapid as coliform testing methods, typically requiring 48h for time to results. A variety of methods with varying complexity and time-to-result have hence been developed to detect PPC in fluid milk and other fluid dairy products including impedance measurements (Bossuyt and Waes, 1983), direct epifluorescent filter technique (DEFT) (Griffiths et al., 1984) and bioluminescence assays (Griffiths, 1993). Additionally, a number of researchers have evaluated various selective enrichment procedures for rapid enumeration of PPC (Byrne et al., 1989). While some of these methods show high correlation with the shelf-life performance of fluid milk (e.g., r=0.91for impedance methods using selective media) (White, 1993) and some have rapid time to result (e.g., <20h for ATP testing following selective enrichment),

many have high initial costs for equipment, require the use of numerous chemicals and reagents or are complex to run. Importantly, nearly all of the research that has been conducted on rapid detection of PPC in fluid milk was conducted in the 1980's when milk quality was significantly different and when milk quality issues due to PPC may have been due to different organisms (e.g., more common contamination with coliforms) and may have represented different contamination patterns (e.g., higher levels of initial contamination) (Carey et al., 2005). Therefore, the goals of this study were to determine the overall population of bacterial contaminants contributing to PPC in a set of contemporary fluid milk samples and to test the ability of various methodologies, specifically those that do not require specialized or expensive equipment and complex steps, to detect PPC in these samples.

MATERIALS AND METHODS

Sample Collection and Handling

Pasteurized milk samples (n=105) were collected from 20 fluid milk processing facilities by Milk Quality Improvement Program (**MQIP**; Cornell University, Ithaca, NY) personnel from 2014 to 2015. Processing facilities were all enrolled in the Voluntary Shelf-Life (**VSL**) Program, administered by MQIP, and were located in the northeast United States (New York, Maine, Vermont, New Hampshire, and Massachusetts). Facility size ranged from small, on-farm facilities (with processing capacities of < approx. 0.5 million kg/yr) to large facilities (> approx. 250 million kg/yr). Pasteurized fluid milk samples collected included whole fat (minimum 3.25% milk fat, n=35), reduced fat (2% milk fat, n=22), low fat (1% milk fat, n=24),

and nonfat (<0.2% milk fat, n=24) milk in 12 ounce (355 mL, n=2), pint (473 mL, n=1), quart (946 mL, n=10), half gallon (1.9 L, n=91), or gallon (3.8 L, n=1) containers. None of the processors fortified their milk with non-fat dry milk. All products were pasteurized via HTST (15 facilities; 94 samples) or vat pasteurization (5 facilities; 11 samples) and packaged in either glass bottles (n=9), high-density polyethylene jugs (n=92), or paperboard cartons (n=4). After being packed in coolers with ice packs or ice, milk samples were transported to the MQIP laboratory and stored at 4°C until the initial testing, performed within 48 h of sample collection.

Shelf-Life Analysis, Rapid Shelf-life Screening, and Bacterial Isolation

On initial day of testing, pasteurized milk samples were handled and stored as described previously (Martin et al., 2012) in preparation for microbiological and organoleptic analyses, which were performed on each test day (day initial, d 7, 10, and 14) in accordance with *Standard Methods for the Examination of Dairy Products* (Laird et al., 2004). Extended shelf-life testing (on d 17 and 21) was performed on samples from a subset of processors that have a history of manufacturing high quality product. Microbiological analyses conducted on each test day included total bacteria count on standard plate count (**SPC**) agar and total Gram negative bacteria count on crystal violet tetrazolium agar (**CVTA**) with incubation at 32°C and 21°C for 48 h, respectively. Samples were also inoculated onto 3M Enterobacteriaceae (**EB**) Petrifilm and 3M Coliform Petrifilm according to manufacturer's instructions (3M, St. Paul, MN), followed by incubation at 32°C for 24 h.

Additionally, on initial day of testing, 100 mL portions of each sample

received a preliminary incubation at (i) 13°C for 18 h with subsequent plating on CVTA, EB Petrifilm, and Coliform Petrifilm and (ii) 21°C for 18 h with subsequent plating on CVTA, EB Petrifilm, and Coliform Petrifilm, following the plating procedure detailed above.

For every sample that showed positive results on media selective and differential for different Gram negatives (i.e., CVTA, EB Petrifilm, and Coliform Petrifilm), two isolates with typical Gram negative morphologies (for each combination of test day and media) were streaked on brain heart infusion (**BHI**) agar (Difco, Franklin Lakes, NJ), followed by incubation at 32°C for 24 h. Single colonies were subsequently grown in BHI broth at 32°C for 18 h; isolates were then frozen and stored in 15% glycerol at -80°C; all pertinent isolate information is cataloged in Food Microbe Tracker (http://www.foodmicrobetracker.com). This procedure was also used to recover and preserve isolates from all SPC plates with counts >20,000 CFU/mL, selecting one colony for each unique morphology per set of duplicate plates.

Sensory Defect Analysis

For sensory defect analysis, samples were evaluated by a trained panel of 6 students and staff members from Cornell University as described in Martin et al. (2012). In order to participate on the defect judging panel, panelists were required to pass pre-screening and a test administered after completion of an initial defect judging training (representing approx. 15h of training); panelists also received re-training (approx. 1h) twice a year. On each testing day (except d 7), panelists assigned a flavor criticism(s) and flavor score to each sample, with an average overall flavor quality

score computed by Compusense 5 (Compusense Inc., Guelph, ON, Canada). Only non-flavored milk products were evaluated.

Genus Identification by DNA Sequenced-Based Methods

A total of 880 Gram negative bacterial isolates and 10 non-sporeforming Gram positive bacterial isolates were characterized by 16S rDNA PCR and sequencing as described previously (Huck et. al., 2007a). Additionally, a total of 115 sporeformer isolates were characterized by *rpoB* PCR and sequencing as described previously with subsequent sequencing of the partial 16S gene for new *rpoB* allelic types (Trmcic et al., 2015). Briefly, for both sequencing procedures, isolates were plated on BHI followed by incubation at 32°C for 24 h. For 16S rDNA PCR, 100 µL of dH_20 was inoculated with a colony and either (i) heated in a 1,200-W microwave for 30s, or (ii) heated in a thermal cycler at 95°C for 15 min. For *rpoB* PCR, lysis was performed by heating an undiluted colony in a 1,200-W microwave for 3 min. After PCR amplification, products were electrophoresed in 1.5% agarose gel at 120 V for 20 to 25 min. Upon treatment with ExoSap as previously described (Dugan et al., 2002), PCR products were sequenced using bidirectional Sanger sequencing as per manufacturer's instructions (Big Dye Sequencing Kit, Life Technologies Inc., Grand Island, NY). Partial 16S and *rpoB* sequences were used to classify isolates to the genus or genus and species level based on similarity searches against the Ribosomal Database Project database (Cole et al., 2005) or our in-house *rpoB* database (MQIP, Cornell University, Ithaca, NY), respectively, using the Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004). For *rpoB* allelic types (AT), a new AT

was assigned if the 632 nucleotide sequence differed from a type strain in the *rpoB* database by one or more nucleotides; species classification of a new *rpoB* AT included analysis of the 16S rRNA gene as described previously (Huck et al., 2007, Ivy et al., 2012).

Data Analysis

Data was managed in Microsoft Excel (version 2007, Microsoft Corp., Redmond, WA) and Microsoft Access (version 2016, Microsoft Corp., Redmond, WA). All statistical modeling was performed in R (Team, 2016) using the lme4 package. Bacterial count data were log-transformed prior to analyses. The detection limit was 1.0 log CFU/mL for SPC and CVTA and 0.0 log CFU/mL for EB and Coliform Petrifilm. A mixed-effect linear model was used to analyze the SPC data for samples with Gram negative PPC spoilage and Gram positive sporeformer spoilage (R; Team, 2016). The model included test day, spoilage type, and corresponding interaction as fixed effects, and sample nested within plant as random effects (Bates et al., 2014, Lenth, 2016, Wickham, 2016). In order to determine if sensory defect scores differed between spoilage types, a mixed-effects linear model was fitted to d14 sensory scores with a single fixed effect of spoilage type. Random effects of plant, sampling, sample, panelist, sampling/plant interaction, and sampling/panelist interaction were included to account for the structure of the experiment. Standard errors and 95% confidence intervals for the spoilage type estimates and pairwise contrasts were generated using a semiparametric bootstrap method with the bootMer function of lme4 in R.

Sensitivity was defined as the proportion of true positives that are correctly identified by a test (sensitivity = true positive / (true positive + false negative)), while specificity was defined as the proportion of true negatives that are correctly identified by a test (specificity = true negative / (true negative + false positive)) (Altman and Bland, 1994a). A positive predictive value was defined as the proportion of true positives among those that test positive (positive predictive value = true positive / (true positive + false positive)), while a negative predictive value was defined as the proportion of true negatives among those that test negative (negative predictive value = true negative / (true negative + false negative)) (Altman and Bland, 1994b).

RESULTS

The Pasteurized Fluid Milk Sample Set Used Here Represents a Range of Gram negative and Gram positive Contamination Patterns

Among the 105 fluid milk samples tested here, 84% (n=88) tested reached > 20,000 CFU/mL during shelf-life (Table 6.1). For 60 of these 88 samples, bacteria representing Gram negative genera were isolated from shelf-life samples with >20,000 CFU/ml (Spoilage classifications #1 and 3 in Table 6.1). These 60 samples were designated as “samples with Gram negative PPC spoilage”. Gram negative bacteria were also isolated after pre-incubation from four further samples with > 20,000 CFU/ml, even though these four samples yielded only Gram positive isolates from the actual shelf-life samples (Spoilage classification # 4 in Table 6.1). Therefore, a total of 64 samples showed evidence of contamination with Gram negative bacteria.

Table 6.1. Microbial spoilage pattern for 105 pasteurized milk samples as well as detection pattern for 6 preliminary incubation approaches

Spoilage Classification	Spoilage Description[1]	No. of Samples	Preliminary Incubation Parameters					
			13°C/18h			21°C/18h		
			# of Samples Positive[1] on			# of Samples Positive[1] on		
			CVTA	EB Petrifilm	Coliform Petrifilm	CVTA	EB Petrifilm	Coliform Petrifilm
1	SPC >20,000 CFU/mL during shelf-life, only Gram negative bacteria isolated from shelf-life samples	34	15	4	2	29	9	6
2	SPC >20,000 CFU/mL during shelf-life, only Gram positive bacteria isolated from shelf-life samples	24	–	–	–	–	–	–
3	SPC >20,000 CFU/mL during shelf-life, Gram negative and Gram positive bacteria isolated from shelf-life samples	26	5	–	–	13	5	3
4	SPC >20,000 CFU/mL during shelf-life, only Gram positive bacteria isolated from shelf-life samples, Gram negative bacteria isolated only from Preliminary Incubation Test	4	1	–	–	3	–	–
5	SPC <20,000 CFU/mL, Gram negative bacteria isolated from shelf-life samples	2	–	–	–	–	–	–
6	SPC <20,000 CFU/mL, No evidence of Gram negative bacteria in shelf-life samples	15	–	–	–	–	–	–
	Total	105	21	4	2	45	14	9

[1]SPC = standard plate count; CVTA = crystal violet tetrazolium agar; EB = Enterobacteriaceae. Some samples were positive on more than one media type (e.g., all 4 samples positive on EB petrifilm after 13°C/18h preliminary incubation, were also positive on CVTA).

Among these 64 samples which reached >20,000 CFU/mL and showed evidence of PPC, 100% (64/64) showed evidence of contamination with non-coliform, non-Enterobacteriaceae (EB) Gram negative bacteria (e.g., *Pseudomonas*), 19% (12/64) showed evidence of contamination with coliforms and 6% (4/64) showed evidence of contamination with non-coliform EB. Importantly, all of the samples that showed evidence of contamination with coliforms or non-coliform EB were also contaminated with non-coliform, non EB Gram negative bacteria, specifically *Pseudomonas* (See Supplemental Table S1 in Alles et al., 2018).

From the 64 samples with evidence of Gram negative contamination, a total of 880 Gram negative isolates were selected; these isolates were obtained from SPC, CVTA, EB Petrifilm, and Coliform Petrifilm plating media from both the preliminary incubation methods and shelf-life testing. Sequence characterization and analysis of a 616-bp fragment of the 16S rDNA gene resulted in a subset of non-redundant Gram negative isolates (n=160), which were classified into 23 genera. *Pseudomonas*, which was isolated from 55 of the 64 (86%) samples with evidence of Gram negative contamination, represented the dominant genus. Other Gram negative genera found in 5 or more samples with PPC, included *Acinetobacter* (22 samples), *Cedecea* (6 samples), and *Raoultella* (6 samples). Additionally, *Hafnia* was isolated from 4 samples that reached 20,000 CFU/mL during shelf-life as well as one additional sample that did not reach 20,000 CFU/mL during shelf-life but still showed evidence of Gram negative contamination (isolation of Gram-negative bacteria from shelf-life; spoilage classification 5 in Table 6.1 and See Supplemental Table S1 in Alles et al.,

2018).

For the 24 samples that exceeded 20,000 CFU/mL but did not show evidence of PPC with Gram negative bacteria either over shelf-life or in preliminary incubation samples (Spoilage classification 2 in Table 6.1), Gram positive sporeforming bacteria represented the predominant bacterial contaminants, with a total of 60 Gram positive isolates obtained from these samples. Characterization with a combination of *rpoB* and 16S rDNA sequencing identified 56 isolates as Gram positive sporeformers in the family *Bacillales*, with *Paenibacillus* (43/56) the most prevalent followed by *Bacillus* (12/56) and *Viridibacillus* (1/56). The remaining Gram-positive isolates were non-sporeforming Gram-positive bacteria identified as *Leuconostoc* (4/4). Further, another 64 Gram positive bacteria were isolated from a subset of samples (n=26) that exceeded 20,000 CFU/mL and also showed evidence of PPC (Spoilage classification #3 in Table 6.1). These Gram positive isolates were identified as *Paenibacillus* (37/64), *Bacillus* (18/64) and *Viridibacillus* (1/64). The remaining Gram positive isolates were non-sporeforming Gram positive bacteria identified as *Lactococcus* (3/64), *Leuconostoc* (4/64), and *Okibacterium* (1/64).

Fluid Milk Samples with Presence of Gram negative Bacteria Showed Significantly Reduced Shelf-life

A comparison of the mean SPC for each testing day for samples with Gram negative PPC spoilage and samples with Gram positive sporeformer spoilage over shelf-life showed that samples with Gram negative bacteria had higher ($p<0.05$) total bacterial counts at days 10, 14, 17, and 21 as compared to samples with Gram positive

sporeformers. Mean SPC for samples with Gram positive sporeformer spoilage were 2.5, 2.5, 2.7, 3.6, 4.8 and 6.4 log CFU/mL on days initial, 7, 10, 14, 17 and 21, respectively, compared with mean SPC for samples with Gram negative PPC spoilage of 1.8, 3.6, 5.6, 6.9, 7.1 and 7.8 log CFU/mL on days initial, 7, 10, 14, 17 and 21, respectively.

A subset (n=90) of the samples evaluated also underwent sensory defect analysis; samples were scored on overall acceptability on a scale from 1 to 10, with a scores of <6 designated as poor, 6 to 7 as fair, and 8 to 10 as good (Bodyfelt et al., 1988). The estimated d 14 mean sensory score for samples with Gram positive sporeformer spoilage (n=22) was 7.98 (95% CI [7.74, 8.20]) while the estimated d 14 mean sensory score for samples with Gram negative PPC spoilage (n=46) was 5.78 (95% CI [5.62, 5.93]). Samples with total bacterial counts below 20,000 CFU/mL on d 14 (n=17) had an estimated mean sensory score of 8.60 (95% CI [8.34, 8.85]), while the remaining five samples were not tasted on d 14. Our results also showed that on the final day of shelf-life (either d 14 or d 21 depending on previous shelf-life data) the predominant defects identified among the 47 samples with Gram negative PPC spoilage were “coagulated” (n=24), “lacks freshness” (n=11), and “bitter” (n=10). Comparatively, the predominant defects identified among the 22 samples with Gram positive sporeformer spoilage were “lacks freshness” (n=8) and “not clearly defined” (n=6); “coagulation” was only rarely identified among these samples (n=2) (Figure 6.1).

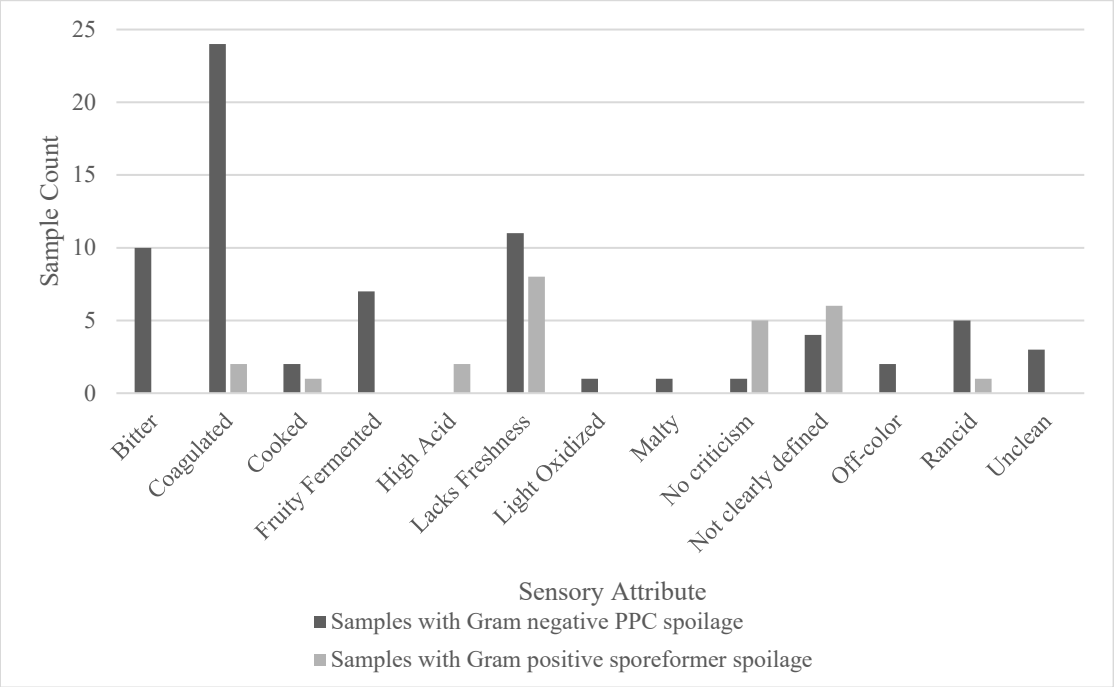


Figure 6.1. Comparison of sensory defect attributes identified by panelists for samples with Gram negative PPC spoilage (n=47) or Gram positive sporeformer spoilage (n=22) at the end of the shelf-life (d 14 or 21).

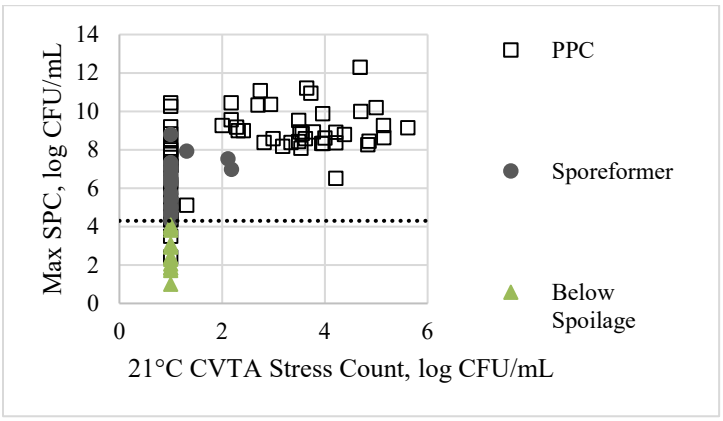
Pre-incubation at 21°C Followed by Plating on CVTA had a Sensitivity of 70% for Accelerated Detection of Gram negative Bacteria

In parallel to the shelf-life testing detailed above, the 105 milk samples were used to evaluate the ability of combinations of different preliminary incubation schemes (either 13°C or 21°C for 18 h) with subsequent plating on three different media that allow for detection of Gram negative organism groups (Coliform Petrifilm, EB Petrifilm, and CVTA) to detect PPC in commercially processed milk. The

comprehensive data shown above supported that this set of 105 samples was appropriate for evaluation of different approaches for accelerated detection of Gram negative bacteria in pasteurized milk as these samples represented a wide range of spoilage profiles and different spoilage organisms.

Among the six approaches evaluated, a preliminary incubation of 21°C for 18 h followed by plating on CVTA was the most sensitive, correctly detecting 70% (42/60) of samples with Gram negative PPC spoilage over shelf-life (Table 6.2, Figure 6.2a). This approach also detected Gram negative contamination in three additional samples that did not show evidence of Gram negative PPC spoilage over shelf-life, resulting in a specificity of 93% (42/45; Spoilage classification #4 in Table 6.1). The second most sensitive approach for detection of Gram negative PPC spoilage was a preliminary incubation of 13°C for 18 h followed by plating on CVTA; this approach correctly identified 33% (20/60) of samples with evidence for Gram negative PPC spoilage over shelf-life. This approach resulted in a single false positive (i.e., detection of Gram negative organisms with no evidence of Gram negative PPC spoilage over shelf-life), resulting in a specificity of 98% (44/45) for detection of Gram negative PPC spoilage (Table 6.2, See Supplemental Figure S1 in Alles et al., 2018). Hence, there were an overall 4 samples that yielded Gram-negatives in one of the preliminary incubation tests, but did not yield Gram-negatives in samples tested over shelf-life (these samples represent spoilage classification 4 [Table 6.1, See Supplemental Table S1 in Alles et al., 2018]); in these cases the media used after preliminary incubation correctly identified Gram-negative bacteria, but the positive tests did not correctly identify samples that spoiled due to PPC. The least sensitive test for PPC spoilage was

a preliminary incubation of 13°C for 18 h followed by plating on Coliform Petrifilm; this approach only correctly identified 3% (2/60) of samples with evidence for Gram negative PPC spoilage over shelf-life (Table 6.2, Figure 6.2b).



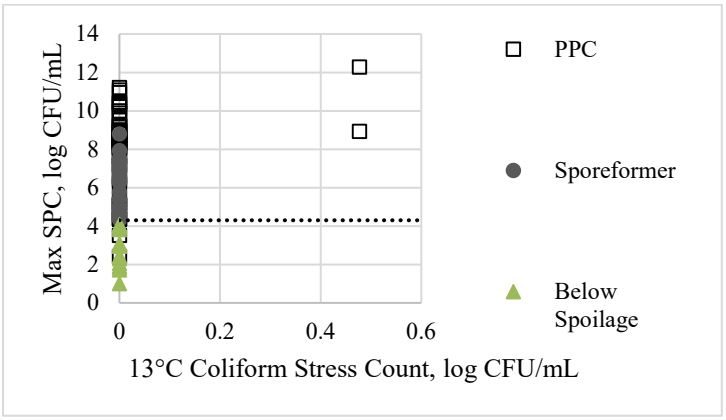


Figure 6.2. Scatter plots displaying log bacterial counts for samples plated on (a) CVTA after a preliminary incubation at 21ºC for 18 h and (b) Coliform Petrifilm after a preliminary incubation at 13ºC for 18 h with corresponding maxium SPC through shelf-life. Data points are categorized into (i) samples with Gram negative post-pasteurization contamination (PPC) spoilage ("PPC"); (ii) samples with Gram positive sporeformer spoilage ("Sporeformer"), and; (iii) samples with bacterial counts below 20,000 CFU/mL ("Below Spoilage"). The detection limit was 1.0 log cfu/mL for SPC and CVTA and 0.0 log cfu/mL for Coliforms. Data points for samples that showed no growth on selective media are shown here at the detection limit. A line at 20,000 CFU/ml (4.3 log CFU/ml) marks the US PMO regulatory limit of 20,000 CFU/ml.

Equivalent scatter plots for other media/enrichment combinations can be found in Supplemental Figure S1 (Alles et al., 2018).

Table 6.2. Sensitivity, specificity, positive predictive value and negative predictive value for 6 different approaches for accelerated detection of fluid milk samples that spoil due to post-pasteurization contaminants in 105 pasteurized fluid milk samples

Media Type[1]	Preliminary Incubation Temperature (°C)	Sensitivity[2] (%)	Specificity[3] (%)	Positive Predictive Value[4] (%)	Negative Predictive Value[5] (%)
CVTA	21	70 (42/60)	93 (42/45)	93 (42/45)	70 (42/60)
EB Petrifilm	21	23 (14/60)	100 (45/45)	100 (14/14)	49 (45/91)
Coliform Petrifilm	21	15 (9/60)	100 (45/45)	100 (9/9)	47 (45/96)
CVTA	13	33 (20/60)	98 (44/45)	95 (20/21)	52 (44/84)
EB Petrifilm	13	7 (4/60)	100 (45/45)	100 (4/4)	44 (45/101)
Coliform Petrifilm	13	3 (2/60)	100 (45/45)	100 (2/2)	44 (45/103)

[1]SPC = standard plate count; CVTA = crystal violet tetrazolium agar; EB = Enterobacteriaceae

[2]Sensitivity = TP / (TP + FN) × 100% (TP: True positive, FN: False negative)

[3]Specificity = TN / (TN + FP) × 100% (TN: True negative, FP: False positive)

[4]Positive Predictive Value = TP / (TP + FP) x 100% (TP: True positive, FP: False Positive)

[5]Negative Predictive Value = TN / (TN + FN) x 100% (TP: True positive, FP: False Positive)

The Total Gram negative Test (i.e., CVTA) Detected a Greater Diversity of Post-Pasteurization Contaminants than EB and Coliform Tests

Overall, 59 of the 60 samples (98%) that showed evidence of Gram negative PPC spoilage over shelf-life also showed bacterial growth on CVTA on at least one of the samples tested over shelf-life (See Supplemental Table S1 in Alles et al., 2018). In contrast, EB and Coliform Petrifilm were only positive for 13 (22%) and 12 (20%) of the 60 samples, respectively. Bacterial isolates from these 60 samples as well as the additional 28 samples that exceeded 20,000 CFU/mL during shelf-life due to Gram positive bacteria, were selected from SPC agar and three selective and differential Gram negative media types (i.e., CVTA, EB Petrifilm, and Coliform Petrifilm) for molecular characterization. Specifically, among a total of 1,005 Gram negative and Gram positive isolates collected from these 88 samples, a subset (n=378) of isolates was selected to represent the non-redundant diversity of isolates. Only one isolate with a given *rpoB* sequence or 16S rDNA genus obtained for each sample, media type and test (i.e., preliminary incubation or shelf-life) combination was included in this non-redundant set (to avoid over –representation of multiple identical isolates from the same test). These 378 non-redundant isolates were used to compare isolation of different genera and *rpoB* ATs from different media (Table 6.3). A total of 191 isolates representing 16 genera were isolated from SPC agar, with the predominant genera being *Paenibacillus* (82/191), *Pseudomonas* (51/191) and *Bacillus* (31/191). Among the media that detect Gram negative organisms, CVTA detected the largest

number of genera (17 genera among 126 isolates), followed by EB Petrifilm (14 genera among 34 isolates), and Coliform Petrifilm (11 genera among 27 isolates) (Table 6.3). CVTA captured 9 genera that went undetected by EB Petrifilm and 11 that went undetected on Coliform Petrifilm, with *Pseudomonas* (84/126) and *Acinetobacter* (19/126) as the predominant organisms detected on CVTA and not detected on either of the Petrifilm media. While CVTA detected the largest number of genera, EB Petrifilm and Coliform Petrifilm detected 6 and 4 genera, respectively, that were not detected by CVTA (Table 6.3). It should be noted that in addition to the ability of each media to support the recovery and growth of different bacterial genera, the isolate selection procedures used in this study may have also contributed to the observation that some genera were only recovered on some media; with characterization of a larger number of isolates from each media one would expect to see fewer genera that are only detected on a single given media (specifically EB Petrifilm and Coliform Petrifilm).

Table 6.3. Genera isolated from 88 pasteurized fluid milk samples that exceeded 20,000 cfu/mL during shelf-life on various media

Genera [2]	No. of isolates obtained on[1]				Total Isolates	% Total Isolates
	SPC	CVTA	EB Petrifilm	Coliform Petrifilm		
Pseudomonas	51	84	-	-	135	35.7
Paenibacillus	82	-	-	-	82	21.7
Bacillus	31	-	-	-	31	8.2
Acinetobacter	5	19	-	-	24	6.3
Hafnia	2	4	4	3	13	3.4
Cedecea	-	2	5	3	10	2.6
Raoultella	-	-	4	6	10	2.6
Lelliottia	2	-	4	3	9	2.4
Obesumbacterium	2	1	2	4	9	2.4
Leuconostoc	6	-	-	-	6	1.6
Aeromonas	1	2	3	-	6	1.6
Rahnella	1	-	2	2	5	1.3
Janthinobacterium	1	3	-	-	4	1.1
Citrobacter	-	-	2	2	4	1.1
Serratia	-	1	2	1	4	1.1
Lactococcus	2	1	-	-	3	0.8
Buttiauxella	1	1	1	-	3	0.8
Escherichia/Shigella	-	1	1	1	3	0.8
Klebsiella	-	1	1	1	3	0.8
Viridibacillus	2	-	-	-	2	0.5
Stenotrophomonas	1	1	-	-	2	0.5
Comamonas	-	2	-	-	2	0.5
Yersinia	-	-	2	-	2	0.5
Okibacterium	1	-	-	-	1	0.3
Brevundimonas	-	1	-	-	1	0.3
Enterobacter	-	-	-	1	1	0.3
Flavobacterium	-	1	-	-	1	0.3
Limnohabitans	-	1	-	-	1	0.3
Providencia	-	-	1	-	1	0.3
Total	191	126	34	27	378	100

[1] Numbers indicate the number of isolates collected on each medium type either from shelf-life samples or from positive stress test samples; numbers specifically represent the non-redundant diversity of isolates obtained for each sample, media type and test (i.e., stress test or shelf-life) combination (meaning only 1 isolate was counted if multiple isolates with the same 16SrDNA or rpoB ST were obtained from a given sample, media type and test combination). SPC = Standard Plate Count agar; CVTA = Crystal Violet Tetrazolium Agar; EB Petrifilm = *Enterobacteriaceae* Petrifilm

[2] Genus identification based on partial 16S rDNA and *rpoB* sequence data. *rpoB* data were used for genus identification of the majority of Gram-positive isolates.

DISCUSSION

Total Gram negative Testing Represents a Viable Indicator Test for Post-Pasteurization Contamination of Fluid Milk

Coliforms have been used in the dairy industry since the beginning of the 20th century (Tortorello, 2003) to identify milk that has been processed under unsanitary conditions and contaminated after pasteurization. Coliforms are a method defined group of organisms, primarily within the *Enterobacteriaecea* family (Davidson et al., 2004), that have been shown historically to play an important role in fluid milk spoilage (Martin et al., 2016). Testing for coliforms in fluid milk is rapid (i.e., 24-48h depending on method) and inexpensive. Despite the advantages of using coliforms as indicators of post-pasteurization contamination, current research indicates that coliforms are decreasingly prevalent in fluid milk (Carey et al., 2005, Martin et al., 2012). Carey and others (2005) found that 21-34% of fluid milk samples tested from 1991-2000 were positive for coliform, while the same group found that from 2001 to 2010 the number of samples positive for coliform on any day of shelf-life ranged from 7.6-26.6% in a given year (Martin et al., 2012). Further, a survey of fluid milk from across the US found that among 175 Gram negative isolates collected over shelf-life, only 16.5% (29/175) were Enterobacteriaceae and the remainder were non-EB Gram negatives, with *Pseudomonas* the predominant contaminant (n=122) (Ranieri and Boor, 2009). In the current study, 84% of samples reached spoilage level of bacteria (defined here as >20,000 CFU/mL), due to coliforms, non-coliform EB, non-EB Gram negative bacteria and Gram positive bacteria, with many samples yielding multiple

genera over shelf-life. Coliforms represented one of the least common groups of contaminants in this study (second only to non-coliform EB, which were only detected in 4 samples) (See Supplemental Table S1 in Alles et al., 2018).

Similarly to previous studies, *Pseudomonas* was found in over 86% of the samples tested here that showed evidence of Gram negative contamination (See Supplemental Table S1 in Alles et al., 2018). *Pseudomonas*, in the family *Pseudomonadaceae*, is not a coliform and therefore not detected using coliform test methods but represents a major post-pasteurization contaminant in this study and others (Ternström et al., 1993, Eneroth et al., 1998, Deeth et al., 2002). The presence of *Pseudomonas* in fluid milk not only indicates that PPC has occurred, but also typically has a dramatic effect on the shelf-life of the product. Many *Pseudomonas* species are not only capable of growing rapidly at refrigeration temperatures (Ternström et al., 1993, Ranieri and Boor, 2009), but also express a number of enzymes, including proteases and lipases, that degrade the quality of fluid milk (Dogan and Boor, 2003, Nörnberg et al., 2010).

Rapid Detection of Low Level Gram negative Contamination that Leads to Product Spoilage Remains a Challenge

Rapid detection of indicator organisms in fluid milk and accurate shelf-life prediction have long been goals of the dairy industry. The Moseley keeping-quality test (Moseley, 1980, Duncan et al., 2004), often considered the gold standard, has shown good correlation with fluid milk shelf-life with a study by Bishop and White (1986) reporting a correlation coefficient of -0.77; however, this test takes 7 - 9 days to obtain results. Other tests to predict fluid milk shelf life and detect PPC include a

variety of preliminary incubation methods (e.g., selective enrichment using crystal violet tetrazolium in milk incubated for 18h at 21°C) (White, 1993), which typically rely on selective agents to restrict the growth of Gram positive bacteria either in the enrichment or on the plating media (Griffiths et al., 1984). The current standard method for the "preliminary incubation" (PI) method, as detailed in the *Standard Methods for the Examination of Dairy Products* (Duncan et al., 2004), relies on enrichment of 10 mL of pasteurized milk at 21°C for 18 h followed by plating on standard methods agar, using no selective agents during enrichment or plating (Duncan et al., 2004). Use of selective agents however represents a valuable approach that can be used to inhibit the growth of one group of organisms, while still allowing for growth of a targeted set of organisms (e.g., Gram-negatives), facilitating simple screening approaches that do not require molecular or other advanced techniques for organism identification. The current study hence tested variations of the standard "PI" approach on a set of contemporary milk samples which represent current predominant bacterial contaminants, examining different incubation temperatures (i.e., 13°C and 21°C) and the use of selective and differential media (i.e., CVTA, EB Petrifilm and Coliform Petrifilm), all with pre-enrichment of a higher volume of milk (i.e., 100 mL) than the standard method. A larger sample volume was specifically used to enhance the sensitivity of the PI method to potentially 1 bacterium per 100 ml, while being realistic about the ability to routinely use large milk volumes in a PI test on larger samples sets. The variations of the PI tests evaluated here showed substantial differences in the sensitivity and specificity of the tests, with regard to early identification of samples that spoiled due to PPC with Gram-negative bacteria. Despite

the sizeable improvement in sensitivity for detection of Gram negative PPC spoilage by using enrichment at 21°C for 18h followed by plating on CVTA (70% sensitivity) over the other methods tested (e.g., sensitivity of 3% for preliminary incubation at 13°C for 18h followed by enumeration on Coliform Petrifilm), there were still a number of samples (n=18) that showed PPC over shelf-life, but were not identified with this modified PI test (Table 6.2, See Supplemental Table S1 in Alles et al., 2018). We hypothesize that the primary reason for the lack of detection in some samples is a low initial contamination levels (around or below 1 cell per 100 ml) where 100 ml sub-samples are not consistently positive. We specifically speculate that this was the case for four of the samples tested here (Spoilage classification #4 in See Supplemental Table S1 in Alles et al., 2018), which allowed for detection of Gram-negative bacteria from the enrichment vial (containing 100 ml of milk), but did not test positive for a Gram negative organism throughout shelf-life. Importantly, while freshly pasteurized milk has been reported to frequently have a total Gram negative count below 1 CFU/ml (the typical detection limit for direct plating) (Schröder, 1984), over the course of refrigerated storage even contaminants at these lower levels can grow to spoilage levels. In theory, as few as one bacterial cell introduced after pasteurization per container could result in premature spoilage (Schröder, 1984). In addition to challenges with detection of low levels of Gram-negative bacteria, CVTA may also not allow for detection of some Gram negative organisms, as demonstrated in previous work (Hervert et al., 2017); our study here however suggests that this may not be a common issue.

The study reported here also clearly demonstrated that, among the media tested

here, CVTA recovered the most comprehensive set of Gram negative organisms (Table 6.3). This is consistent with the predominance of *Pseudomonas*, which will not grow on EB or Coliform Petrifilm, in fluid milk samples that spoil due to PPC. Van Tassel and colleagues (Van Tassell et al., 2012) also found that CVTA outperformed Coliform Petrifilm, violet red bile agar and MacConkey agar when enumerating common dairy associated *Pseudomonas*. Yet, even CVTA has been shown in other studies not to detect certain Gram negative organisms. Specifically, Hervert et al., (2016) found that of a set of 211 previously characterized dairy associated Gram negative bacteria including EB and non-EB strains, 37 failed to grow in pure culture on CVTA. Importantly, Hervert, et al. (2016) also found that all of the selective media (CVTA, Coliform and EB Petrifilm and violet red bile glucose agar) used to evaluate indicator organisms relevant to dairy products showed reduced recovery compared with the non-selective media, brain heart infusion agar. Hence, despite the ability of CVTA to detect a more comprehensive set of dairy associated Gram negative post-pasteurization contaminants, as compared to coliform or EB media, further research is needed to identify alternative media solutions that will detect a broader range of Gram negative organisms associated with contemporary fluid milk PPC, which would improve sensitivity of cultures-based tests with regard to detection of PPC.

CONCLUSIONS

Reducing PPC and thereby providing consumers with the highest quality fluid milk is not only important for the dairy industry to retain customers, but would also play a role in reducing food loss, which is a major issue in the US. The current study

shows that relying on coliform testing is insufficient to detect PPC and leads to underreporting of PPC. In order to reduce PPC, processors must be able to rapidly and accurately detect PPC events, a goal that clearly is not achievable with current industry testing practices (i.e., coliform testing), particularly as the organisms responsible for post-pasteurization contamination of milk continue to shift from coliforms to primarily *Pseudomonas*. The test presented here (i.e., enrichment of 100 mL of fluid milk at 21°C for 18h followed by enumeration on CVTA) not only allows for detection of PPC with a range of organisms, including *Pseudomonas*, but also is more rapid (allowing for completion in less than 3 full days) relative to traditional methods such as the Moseley keeping quality test, which takes 7 to 9 days. While the preliminary incubation test detailed above already provides a valuable tool for industry, further research is needed to continue to develop PPC screening methods that have improved sensitivity, increased speed, and increased ease of use; ease of use is a particular concern as the PI method detailed here requires CVTA plates. Briefly, an ideal PPC indicator method should; (i) detect low level contamination, which may require testing of larger volumes of product; (ii) detect a comprehensive set of PPC organisms, in particular *Pseudomonas*; (iii) have a short time to result, ideally less than 24h, but certainly less than 48h, in order to improve processor response time to contamination events, and; (iv) be accessible to processors of all sizes and resource levels (favoring methods that are affordable and easy to use).

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CHAPTER SEVEN

Conclusions

The dairy industry of the 21st century is presented with a number challenges regarding dairy product quality that the preceding generations have not faced. These challenges include the changing landscape of dairy production and processing, informed and connected consumers who have endless product choices at their disposal and a wealth of new and emerging knowledge about what causes product deterioration. Despite these changes and challenges the dairy industry still predominantly relies on a few classical tests and tools (e.g. raw milk SSC and SPC, finished product SPC and coliform tests) to monitor and improve dairy product quality. Unfortunately, these tests do not effectively assess raw milk for sporeformer presence and levels complicating efforts to reduce spore-relate dairy product quality challenges, including production of high quality extended shelf life fluid milk products (i.e., product with shelf–life >21 to 24 days), which are highly desirable for large retail chains and new on-line distribution channels. Similarly, current tools for assessing finished product quality (coliform and SPC tests, but also Enterobacteriacae (EB) tests) have limited ability to detect post-pasteurization contamination (PPC), particularly since *Pseudomonas* spp., the main cause of PPC, are not detected with these tests. Reducing PPC is also essential to produce high quality dairy products, particularly since fluid milk products with PPC spoil rapidly (typically within 14 days of processing), often developing flavor, odor and body defects. Notably, *Pseudomonas*

is known to cause pigment defects in fluid milk whereby the product develops gray discoloration. These types of spoilage events are particularly detrimental to the dairy industry as a whole because in the age of social media these types of unusual defects are often shared widely within these networks.

The thesis presented here provides two review articles (Chapters Two and Three) that not only summarize currently available information in two important dairy quality related areas (e.g., PPC and microbiological methods for monitoring finished product quality), but also represent important resources that can be used for development and implementation of industry training. Future efforts may involve development of shorter trade magazine articles and other training materials based on these review articles to help with (i) improved control of PPC and (ii) accelerated adoption of new microbiological tests in the dairy industry. Finally, chapter three in particular provides guidance for the dairy industry with regard to using data driven decision making for the selection of indicator organisms in various dairy products. While research has been conducted on selecting appropriate indicator organisms for certain dairy products (e.g., fluid milk), further future work is needed to provide the dairy industry with recommendations for other types of processed dairy products (e.g., ice cream).

The three primary research articles included in this thesis provide important new information on (i) farm levels spore sources that appear to be the most important contributors to contamination of raw milk (Chapter Four); (ii) appropriate methods for detection and quantification of spores in dairy powders (Chapter Five); and (iii) improved methods for screening of HTST fluid milk products for PPC (Chapter Six).

While the research presented in these chapters represent important advancements in the understanding of these key groups of microbial contaminants in dairy products, further work is needed to develop specific recommendations and best-practices at the producer and processer levels. For example, the study conducted in Chapter Four with the goal of identifying spore sources in the dairy farm environment along with management practices that were important in the presence and levels of spores in bulk tank milk generated a number of hypotheses that need to be tested using intervention strategies at the farm level. An initial follow up intervention study has recently been conducted on five NY state dairy farms and results of that study indicate that through focused teat end cleaning and implementing a standardized towel washing protocol bulk tank raw milk spore levels can be significantly reduced. Further, the work presented in chapter six was conducted in NY state and therefore future research should also focus on understanding the role of environmental spore sources on bulk tank raw milk in various regions across the United States.

Additionally, further research on methods for enumerating spores in dairy products, and in particular in dry dairy products, are needed as next steps following the research presented here in Chapter Five. Follow up studies have been designed and are in progress, with the goal of understanding and reducing variation in spore test outcomes for dairy powders. These studies (i.e., Chapter Five and follow up studies) will allow for specific recommendations to the dairy industry regarding standardization of spore testing methods and ultimately should be incorporated into industry guidance documents such as Standard Methods for the Examination of Dairy Products. Finally, the work described in Chapter Six provides the dairy industry with a

simple method for accelerated detection of PPC in fluid milk. While this method offered an improvement in detection time over traditional methods (e.g., Moseley keeping quality), there are still a number of inherent challenges when detecting PPC, specifically the often low-level and sporadic nature of this type of contamination. Further work should focus on developing strategies to improve diagnostic capabilities of PPC detection methods by addressing these challenges, potentially though the use of metagenomic based approaches. While a metagenomic approach would present its own challenges, in particular the over-representation of bovine DNA in milk samples, these tools are increasingly being used in the food industry for quality and safety applications.

Overall, continuing to drive microbiological quality improvements in dairy products will present a number of opportunities to the dairy industry of the 21st century. Taking advantage of these opportunities will require acknowledging the contributions that each step along the grass-to-glass continuum makes to dairy product quality and evaluating parameters both at the farm (i.e., sporeforming bacteria) and processing levels (i.e., PPC) that are drivers of contemporary finished product quality. The work presented in this thesis provides important insights into detecting and controlling these key groups of organisms and future research will provide specific strategies to ensure that the dairy industry of the 21st century is ready to meet the demands that it faces, and ultimately provide the highest quality dairy products to consumers in the United States and across the globe.