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TRACKING HEAT-RESISTANT, SPOREFORMING BACTERIA IN THE MILK CHAIN: A FARM TO TABLE APPROACH

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by

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Sporeforming bacteria (such as *Bacillus* and *Paenibacillus* spp.) can survive pasteurization conditions (Collins, 1981) and grow in pasteurized fluid milk during refrigerated storage (Huck et al., 2008; Ivy et al., 2012), causing fluid milk spoilage and limiting the further extension of fluid milk's shelf life (Fromm and Boor, 2004; Durak et al., 2006). Moreover, *Bacillus* and related genera have been found in raw milk, pasteurized milk and environmental samples from dairy farms, indicating that these organisms are ubiquitous in nature and can enter the milk chain from different sources (Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009). The objective of this project was to evaluate the presence of *Bacillus* and related genera in a medium-size fluid milk chain in Nebraska to identify the source of these bacteria in packaged fluid milk. To achieve this, raw milk, pasteurized milk and environmental samples representing the farm-to-table continuum were collected from a dairy farm and a medium-size processing plant in Nebraska, supplied exclusively by that farm during Spring 2012, Fall 2012 and Spring 2013. Environmental and raw milk samples were heat-treated at 80°C for 12 minutes to eliminate vegetative cells. Pasteurized milk and heat-treated samples were stored at < 7°C for 21 days to enrich for psychrotrophic sporeformers. Samples were enumerated for microbial load and the plates were used for bacterial isolation throughout

storage. Isolates were characterized using *rpoB* and/or partial 16S rDNA sequencing. A vast diversity of sporeforming bacteria (i.e. 94 and 42 unique *rpoB* and 16S rDNA allelic types, respectively) and many entry points of these organisms (i.e. raw milk supply, farm and plant environment) were identified all over the milk chain. Consequently, the control of sporeformers in pasteurized fluid milk will be challenging and will require specific control strategies applied throughout the farm-to-table continuum.

To God, and to my mom and dad.

"For I know the plans I have for you, says the Lord. They are plans for good and not for disaster, to give you a future and a hope." Jeremiah 29:11

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CHAPTER 1: LITERATURE REVIEW

1. Fluid milk industry in the United States

The United States is the second single largest producer of cow's milk in the world after India (IFCN, 2012). In 2013, the US produced 201 billion pounds of milk, which accounted for an increase of 15% during the last decade, from an average of 9.2 million milk cows (NASS, 2010; NASS, 2014). The states with the higher milk production were California, Wisconsin, New York, Idaho and Pennsylvania, supplying 53% of the nation's milk. In addition, the 12 states from the Midwest region produced 34% of the milk supply (NASS, 2014), showing the significance of the dairy industry in this area.

Fluid milk consumption per capita has decreased by almost 15% due to the emergence of new products in the very competitive beverage industry during the last 20 years (Martin et al., 2012a; ERS, 2013b). From the total milk supply only about 26% is commercialized as fluid milk (whole, 1% fat, 2% fat, skim, flavored and buttermilk), while the rest is used for the production of other dairy products, such as butter, cheese, frozen dairy products, dry products, condensed and evaporated milk (ERS, 2013c). Moreover, the number of dairy operations around the country has declined during the last decade, causing an increase in the distribution times. As of 2011, the United States had 332 fluid milk bottling plants, representing a decrease of 14%, when compared to 385 plants in 2002 (ERS, 2013d). Likewise, the number of dairy cow operations drop from about 90,000 in 2003 to approximately 60,000 in 2012, representing a reduction of 33% (NASS, 2013). This tendency has triggered a rise in the number of cows per operation;

however, only about 5% of the operations have more than 500 cows (NASS, 2010). Producing fluid milk with higher quality and longer shelf life may facilitate its distribution over wider geographic regions and through new channels, to better compete in the beverage industry (Fromm and Boor, 2004).

In the next 20 years, the number of middle class consumers in emerging markets will increase, causing a rise in the demand for dairy products, especially in those regions that are unable to produce enough milk to meet their local demand. The United States along with New Zealand, the European Union and Australia are traditional key suppliers of dairy products for developing economies in Asia, Latin America and the Middle East. It is expected that there will be a shortage of global supply, especially as some of these traditional suppliers won't be able to completely meet the rising dairy demand. Consequently, the US can expand production and take advantage of this situation, still supporting growth in the domestic market (Innovation Center for U.S. Dairy, 2009). As a strategy to compete in the global market, the dairy industry needs to improve the quality of milk and reduce the losses of milk and other dairy products, mainly due to microbial spoilage.

2. HTST pasteurization and Pasteurized Milk Ordinance (PMO)

The PMO regulates the production, processing and commercialization of pasteurized milk and other dairy foods produced with Grade "A" milk. At the farm level, the PMO specifies that raw milk from a single producer and commingled raw milk should have a Standard Plate Count (SPC) of less than 100,000 and 300,000 CFU/mL, respectively. Moreover, the PMO does not specify a limit for coliform count in raw milk.

Before commercializing any fluid dairy product (i.e. fluid milk, condensed milk, cream, yogurt, cottage cheese), raw milk has to be pasteurized following the guidelines presented in the PMO. The primary goal of milk pasteurization is to kill all non-sporeforming human pathogens usually associated with raw milk, which according to the PMO is accomplished by heating milk to a minimum of 72°C for at least 15 seconds (High Temperature Short Time – HTST pasteurization) or by applying any equivalent combination of temperature and time. Grade "A" pasteurized milk should have a SPC of <20,000 CFU/mL and a coliform count of ≤10 CFU/mL during the product shelf life (FDA, 2011).

3. Shelf life and microbial spoilage of pasteurized fluid milk

In the United States, about 31% of the total milk supply destined for fluid milk is estimated to get lost along the milk chain (farm, retail and consumers), especially due to spoilage by microorganisms. Most of the losses take place at the consumer and retail levels, accounting for 20% and 10%, respectively (ERS, 2013a). A desire to further extend the shelf life of pasteurized fluid milk has risen as it would be beneficial for all milk chain levels (Fromm and Boor, 2004).

Shelf life of pasteurized fluid milk can be defined as the time that the product retains acceptable quality (flavor, odor and appearance) under recommended storage conditions (7.2° or less). The quality and shelf life of the product can be evaluated by sensory, chemical and microbiological analysis. In the United States, pasteurized fluid milk code-dating, printed as either "sell-by", "code" or "expiration" dates on retail milk packages, depends on state regulations and processors policies (Carey et al., 2005). For

example, there are states that don't have specific code date requirements (i.e. New York), while other states have specific regulations that can vary between a few days (i.e. "sell-by" 12 days in Montana) to a couple of weeks (i.e. "sell-by" 17 and 30 days in Pennsylvania and Maryland, respectively) following pasteurization (Montana Secretary of State, 2000; Northeast Dairy Foods Association, Inc., 2010; The Pennsylvania Code, 2011; COMAR, 2014). In most cases, consumers are instructed to expect an extra 2-7 days of acceptable quality after the "sell-by" dates (Carey et al., 2005). In the United States, the shelf life of pasteurized milk usually varies between 14 and 21 days (Fromm and Boor, 2004; Ranieri and Boor, 2009).

Spoilage of pasteurized fluid milk is mainly caused by sporeforming bacteria or Post-Pasteurization Contamination (PPC), such as gram-negative psychrotrophic bacteria (Dogan and Boor, 2003; Fromm and Boor, 2004; Ranieri and Boor, 2009). If both groups of organisms are initially present, then the PPC will outcompete the sporeforming bacteria and will become the dominant microflora. Implementation of improved pasteurization, sanitation and maintenance procedures has greatly reduced PPC in fluid milk, thus, contributing to the extension of milk's shelf life (Ralyea et al., 1998). However, in these processing systems, sporeforming bacteria become the main obstacle limiting the further extension of fluid milk's shelf life (Ralyea et al., 1998; Fromm and Boor, 2004; Durak et al., 2006).

4. Sporeforming bacteria: *Bacillus* and related genera

Sporeforming bacteria, such as *Bacillus* spp. and *Paenibacillus* spp., belong to the phylum Firmicutes, have low G+C content, can produce endospores, and are aerobic or

facultative anaerobic (Holt et al., 1994). These organisms are characterized as Gram-positive rods, but some strains of *Paenibacillus* spp. have showed staining patterns typical of gram-negative or gram-variable rods (Fromm and Boor, 2004; Huck et al., 2007b). There are several genera included in the *Bacilli* (Niall and Halket, 2011) and many of these have been associated with milk production systems including *Bacillus, Paenibacillus, Brevibacillus, Viridibacillus, Solibacillus, Psychrobacillus, Anoxybacillus, Geobacillus* and *Lysinibacillus* (Ruckert et al., 2004; Scott et al., 2007; Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Reginensi et al., 2011; Ivy et al., 2012). Furthermore, many of these genera contain a high number of species. In particular, *Bacillus* and *Paenibacillus* contain over 150 and 110 species, respectively (Niall and Halket, 2011).

4.1. Heat resistance: Sporulation process

Sporulation is a survival mechanism of specific bacteria (i.e. *Bacillus* and *Clostridium* genera) as a response to stress and it can be initiated by starvation, high cell density or DNA damage (Burgess et al., 2010). This process is very complex and in *B. subtilis* it has been observed to affect the regulated expression of hundreds of genes (Eichenberger et al., 2003; Piggot and Hilbert, 2004). An asymmetric division of the cell occurs during sporulation, where the mother cells engulfs the forespore. Then, multiple layers are formed around the forespore that include an inner membrane, a cell wall, a thick peptidoglycan cortex, an outer membrane, a complex protein coat and in some species an exosporium. Small, acid soluble spore proteins (SASP) are synthesized during the sporulation cycle and these compounds are the ones that mainly protect the DNA of

spores, increasing the spore's resistance and survival. The sporulation process ends with the release of the spore by lysis of the mother cell (Setlow and Johnson, 2001)

Spores are resistant to multiple environmental stresses, including heat, mechanical disruption and chemicals, thus the elimination of these organisms from dairy manufacturing environments is challenging (Cortezzo et al., 2004; Scheldeman et al., 2005; Jones et al., 2005). The resistance of *Bacillus* spores is a result of the combined action of different elements, such as spore coat, peptidoglycan cortex, SASP, dipicolinic acid (DPA), among others (Burgess et al., 2010). In particular, the heat resistance of spores is mainly associated with mineral content, low water activity and thermal adaptation (Beaman and Gerhardt, 1986). The presence of minerals, especially divalent cations (Ca^{2+}, Mg^{2+}), has been correlated with a greater heat resistance in spores of *B. sporothermodurans* (Scheldeman et al., 2005), *B. subtilis, B. coagulans* and *B. licheniformis* (Cazemier et al., 2001). Furthermore, studies have reported an enhanced heat resistance of spores formed at sporulation temperatures higher than the optimum growth temperature (Beaman and Gerhardt, 1986; Palop et al., 1999).

4.2. Spoilage of fluid milk: Germination and cold growth

Spores will remain dormant until the conditions become favorable for the change from spore to vegetative cell to take place, which usually occurs in three phases: activation, germination and outgrowth, as presented in Figure 1 (Setlow, 2003). The activation process is not completely understood, but studies have reported that heat plays an important role in activation of spores and that its effect varies within species or even strains (Kim and Foegeding, 1990; Ghosh et al., 2009). Consequently, the high

temperature used during milk pasteurization is the most likely cause of spore activation in the dairy industry (Collins, 1981; Huck et al., 2007b; Burgess et al., 2010). After spore activation, germination takes place in two phases. First, specific nutrients (i.e. amino acids, sugars, purine nucleosides) bind to receptors in the inner membrane of the spore, causing the release of Ca^{2+}-DPA and cations, which increases the core water content. This step can be also triggered by non-nutrient agents, such as lysozyme, salt, high pressure and Ca^{2+}-DPA. During the second stage, the peptidoglycan cortex is hydrolyzed and enzymes needed for SASP degradation are released from the spore core. During outgrowth, spore metabolism and macromolecular synthesis take place to convert the germinated spore into a vegetative cell (Setlow, 2003).

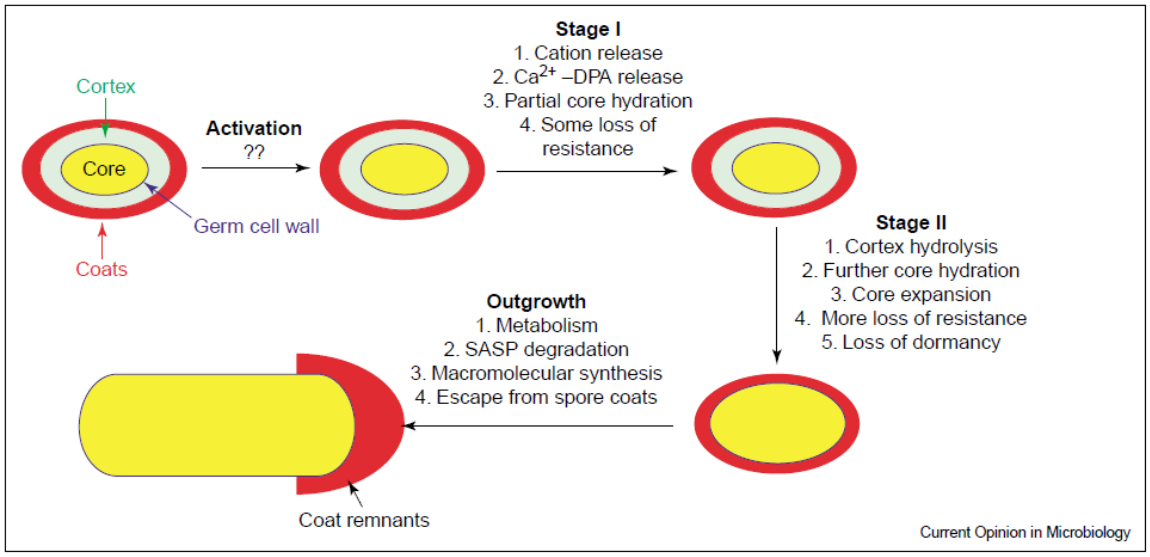

Figure 1. Spore activation, germination and outgrowth (Setlow, 2003).

Heyndrickx and Scheldeman (2002) reviewed the different aerobic sporeforming bacteria (psychrotrophic, mesophilic, thermophilic) in raw milk and throughout processing. These organisms may be present in the final product due to selection during

heating and cold storage. Pasteurized milk is stored at low temperatures, thus psychrotrophic sporeforming bacteria are the main concern in this product. Ivy et al. (2012) evaluated the cold growth potential of isolates from common clades of *Bacillus* spp. and *Paenibacillus* spp. in sterile skim milk broth. Results of this study showed that several *Paenibacillus* spp. isolates, but only one strain from the *Bacillus* clade (*B. weihenstephanensis*) and one of a genera formerly classified as *Bacillus* (*Viridibacillus* spp.), were able to grow at low temperatures. These results correlate well with reported data from other research, indicating the ability of *Paenibacillus* spp. to grow and outcompete *Bacillus* spp. during refrigerated storage (Ranieri and Boor, 2009). Through genomic comparison, several genes were identified in cold adapted *Paenibacillus* strains, encoding important features for growth in milk during refrigerated storage. For example, these strains contained genes encoding β-galactosidases, peptide transport systems and cold-adapted peptidases, besides cold growth related proteins (Moreno et al., 2014).

Aerobic sporeforming bacteria represent a quality issue for fluid milk processors, thus limiting the extension of fluid milk shelf life. As mentioned before, the pasteurization process activates the spores causing germination (Collins, 1981; Huck et al., 2007b). Then, the vegetative cells can produce extracellular enzymes such as proteases, lipases and lecithinase causing spoilage of milk and other dairy products by producing off-flavors and texture defects (Meer et al., 1991). Sweet curdling, bitter and rotten off-flavors are due to proteolytic activity (Meer et al., 1991; Heyndrickx and Scheldeman, 2002). Strains of *B. subtilis, B. cereus* group, *B. polymyxa* and *B. amyloliquefaciens* have exhibited a highly proteolytic activity. “Bitter cream” defects in pasteurized milk are cause by lecithinase, which is an enzyme produced by strains of *P.*

polymyxa and the *B. cereus* group (De Jonghe et al., 2010). Lipolytic activity can produce fruity and rancid off-flavors (Meer et al., 1991). De Jonghe et al. (2010) showed the lipolytic activity of *B. subtilis, B. pumilus* and *B. amyloliquefaciens.* Bacterial lipases are thermoresistant and stay active after the heating process even in Ultra High Temperature (UHT) milk (Janštová et al., 2006).

4.3. Spoilage of other dairy products

The quality of other dairy products such as milk powder, UHT milk and cheese can also be affected by aerobic sporeforming bacteria. In cheese, aerobic sporeforming bacteria can reduce nitrate, a preservative usually added to inhibit the growth of *Clostridium.* In a similar way to *Clostridium,* aerobic sporeformers can produce gas causing late blowing defect in cheese (Klijn et al., 1995; Quiberoni et al., 2008). De Jonghe et al. (2010) demonstrated that strains of *B. amyloliquefaciens, B. clausii, Lysinibacillus sphaericus, B. subtilis* and *P. polymyxa* can reduce nitrate. In addition, *P. polymyxa* and *P. macerans* can produce gas during lactose fermentation. Scheldeman et al. (2006) reported *B. sporothermodurans* as the predominant organism in UHT milk due to its ability to produce highly heat resistant spores and the lack of competitive flora. Furthermore, strains of *Geobacillus stearothermophilus, Anoxybacillus flavithermus, B. licheniformis* and *B. subtilis* have been identify in milk powder, being *A. flavithermus* and *B. licheniformis* the most prevalent (Ruckert et al., 2004; Scott et al., 2007; Reginensi et al., 2011). In the last decade, there has been an increased interest in the production of dry milk powder with low spore counts. Thus, the reduction of sporeforming bacteria in this product will give producers a competitive advantage in the international market, where

there is a growing interest in dry milk powder with low spore counts (U.S. Dairy Export Council, 2013).

4.4. Potential sources of sporeformers: Raw milk, farm and processing plant environments

Several studies have indicated a broad diversity of *Bacillus* and *Paenibacillus* throughout the milk chain, including dairy farms, processing plants, raw milk and pasteurized milk (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012). In particular, the same bacterial subtypes have been identified in raw and commercially pasteurized milk, suggesting that the raw milk is an important source of sporeformers (Huck et al., 2007a). Furthermore, several entry points of sporeforming bacteria have been identified at the farm level including feed concentrate, silage, bedding, manure, soil, wash water, clusters, teat cups, filter cloths, among others (Vaerewijck et al., 2001; Te Giffel et al., 2002; Scheldeman et al., 2005; Huck et al., 2008).

The processing plant has also been identified as a source of sporeforming bacteria, and there might be a potential for cross-contamination of milk due to the presence and persistence of *Bacilllus* and *Paenibacillus* spp. in processing environments (Lin et al., 1998). Huck et al. (2007b) reported the presence of some subtypes of sporeformers exclusively in pasteurized samples throughout the milk chain, suggesting the possibility of in-plant sources for these spoilage organisms. The adhesive characteristic of some sporeforming bacteria (i.e. *Paenibacillus* spp., *B. stearothermophilus, B. flavothermus, B. thermolevorans, B. coagulans, B. cereus, B.*

licheniformis, B. pumilus and *B. subtilis*) might be contributing to their persistence in the processing environment (Faille et al., 2001; Parkar et al., 2001). Biofilms are formed through the attachment of these organisms to the surfaces of pipelines and processing equipment (Andersson and Rönner, 1998).

4.5. Factors affecting microbial diversity in pasteurized fluid milk

The ubiquitous presence and persistence of sporeforming bacteria in milk production and processing systems requires the development and application of effective strategies to better control sporeformers throughout the farm-to-table continuum (Huck et al., 2007b; Huck et al., 2008). Variations in the sporeforming bacterial community within regions (Ranieri and Boor, 2009), seasons (Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994), production runs (Scott et al., 2007), pasteurization conditions (Ranieri et al., 2009; Martin et al., 2012a) and processing facilities (Huck et al., 2007b) have been described in different studies. Consequently, implementing universal strategies across the country might not be possible, and region-specific or even facility-specific strategies might need to be developed (Huck et al., 2007b). In particular, it has been observed that psychrotrophic sporeforming bacteria are present in higher numbers during summer and fall (Phillips and Griffiths, 1986; Sutherland and Murdoch, 1994), while mesophilic sporeformers predominate during the winter (Sutherland and Murdoch, 1994). Furthermore, Ranieri et al. (2009) have indicated that psychrotrophic sporeforming bacteria grow better in fluid milk when higher temperatures are used during HTST pasteurization.

4.6. Food safety

Sporeforming bacteria may become a food safety concern in milk and dairy products if the pathogen *B. cereus* is present. This pathogen can cause two types of food-borne intoxications: emetic syndrome and diarrheal syndrome due to the production of heat stable (cereulide) and heat labile toxins, respectively (Granum, 2002). The high incidence of *B. cereus* in dairy products has been reported by numerous studies (Granum et al., 1993; Becker et al., 1994; Reyes et al., 2007; Ranieri et al., 2009), but only a few outbreaks have been linked with *B. cereus* in these products (Becker et al., 1994).

There are many reasons that could explain the low number of outbreaks associated with *B. cereus* in dairy products. Probably the first reason is that many outbreaks are not reported as most people usually have mild and brief symptoms. Moreover, outbreaks can be misdiagnosed with other food poisoning organisms being indicated as the causative agent due to similarities in the symptoms. For example, *Staphylococcus aureus* and *Clostridium perfringens* cause intoxications similar to the emetic and diarrheal syndrome, respectively. In addition, foodborne diseases caused by *B. cereus* in other products have been associated with 5 to 8 logs CFU/g of food, with pathogenesis being caused by the preformed toxin (FDA, 2012). These conditions are not likely found in refrigerated pasteurized milk (Ranieri et al., 2009), because *B. cereus* is usually unable to grow or produce cereulide at temperatures below 10°C (Finlay et al., 2000).

Bacillus weihenstephanensis is a psychrotrophic species in the *Bacillus cereus* group, which has been extensively found in pasteurized milk (Ivy et al., 2012) and is

known for producing cereulide (Thorsen et al., 2006). Although this species can grow in milk under refrigeration (Ivy et al., 2012), a study reported that toxin production was inhibited at storage temperatures below 8°C for up to three weeks. This species might become a food safety concern in refrigerated pasteurized milk with extended shelf life (beyond 21 days), especially in temperature abuse situations (Thorsen et al., 2009). Thus, more research is needed concerning *B. weihenstephanensis* and other psychrotrophic sporeformers, regarding toxin production at low temperatures.

Furthermore, several studies have demonstrated the production and functionality of heat-stable toxins with similar characteristics of cereulide by several mesophilic *Bacillus* species outside the *B. cereus* group, such as *B. amyloliquefaciens, B. pumilus, B. mojavensis* and *B. subtilis* (From et al., 2005; Taylor et al., 2005; Apetroaie-Constantin et al., 2009; De Jonghe et al., 2010). Nonetheless, the toxin production by these organisms has been evaluated only at mesophilic temperatures and more research is needed before making any conclusions regarding a potential safety concern in milk and other dairy products related to them.

4.7. Identification: Molecular subtyping

As previously stated, many genera in the *Bacilli* class have been associated with milk production systems (Ruckert et al., 2004; Scott et al., 2007; Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Reginensi et al., 2011; Ivy et al., 2012). This diversity hinders the differentiation of isolates, especially, because conventional phenotypic techniques lack enough sensitivity for the differentiation of isolates throughout processing systems (Durak et al., 2006). Therefore, the implementation of

molecular-based approaches is recommended to evaluate the sporeformer's diversity in the milk chain. Sequencing of the 16S rDNA gene has been extensively used for bacterial identification. This gene is highly conserved in all bacteria as it plays an important role in cell function (Clarridge, 2004). However, 16S rDNA sequencing has limitations for identification and phylogenetic studies when dealing with closely related species (Fox et al., 1992).

During the last decade, numerous molecular methods have been developed for the identification of sporeforming bacteria beyond the traditional 16S rDNA sequencing. These methods include multi-locus sequence typing (Madslien et al., 2012), high resolution melt analysis of variable 16S rDNA regions (Chauhan et al., 2013), housekeeping genes sequencing (Da Mota et al., 2004; Drancourt et al., 2004; Durak et al., 2006), among others. In particular, Durak et al. (2006) developed a subtyping method for *Bacillus* and related genera by sequencing the *rpoB* gene, which is a highly conserved housekeeping gene that encodes for the beta subunit of RNA polymerase. The *rpoB* gene can be used as a chromosomal marker for identification of broad groups of Gram-positive bacteria because it is usually less well conserved in closely related species in comparison to the 16S rDNA gene (Yamada et al., 1999; Drancourt et al., 2004; Blackwood et al., 2004; La Duc et al., 2004).

After evaluating the efficiency of this molecular subtyping method for aerobic sporeforming bacteria isolated from fluid milk products, Durak et al. (2006) concluded that *rpoB* sequencing can be used to differentiate between subtypes and for species and genus assignment, as other studies had previously reported (Mollet et al., 1997; Dahllof et

al., 2000; Kim et al., 2003; Da Mota et al., 2004). This method offers a more economical and efficient tool for characterization of isolates to evaluate the transmission and ecology of sporeformers from raw materials to finished products (Huck et al., 2007a; Huck et al., 2007b). However, *rpoB* sequencing has its limitations, especially when trying to design universal primers as it is not conserved enough to target an entire microbial community. The identification of some species or strains can be challenging because the database for this gene is still limited (Vos et al., 2012). Nonetheless, distinct clusters have been identified with *rpoB* sequencing data, which might represent different species (Huck et al., 2007a). Several studies have also reported the need to use 16S sequencing for isolates in which specific *rpoB* primers are not able to amplify the targeted *rpoB* fragment, and for the confirmation of the genus and species of previously unreported *rpoB* subtypes (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008).

CHAPTER 2: OBJECTIVES

Overall objective

Evaluate the presence of *Bacillus* and related genera in a medium-size fluid milk chain in Nebraska to identify the source of these bacteria in the finished product.

Specific objectives

1. Evaluate the initial microbiological quality of raw and pasteurized milk samples collected throughout the milk chain.
2. Evaluate bacterial counts (Mesophilic Spore Counts – MSC or Standard Plate Counts – SPC) of heat-treated and commercially pasteurized fluid milk throughout shelf life.
3. Evaluate mesophilic spore counts (MSC) of heat-treated environmental samples from the farm and processing plant throughout 21 days of refrigerated storage.
4. Assess the bacterial diversity in milk (laboratory heat-treated and commercially pasteurized) and heat-treated environmental samples using *rpoB* and/or partial 16S rDNA sequencing.
5. Identify “potentially problematic sporeforming bacteria” (PPSB) and their sources based on their presence in laboratory heat-treated and commercially pasteurized milk samples with high bacterial counts (MSC or SPC) at the end of shelf life.
6. Compare DNA sequences from this project with other similar research projects that generated data on sporeformers isolated from milk chain in other parts of the US.

7. Propose strategies to better control sporeformers in pasteurized fluid milk based on the results in the previous objectives.

CHAPTER 3: MATERIALS AND METHODS

1. Sample collection

In order to gain an insight into the ecology and transmission of sporeforming bacteria in the milk chain, raw milk, pasteurized milk and environmental samples were collected in Spring 2012 [S12], Fall 2012 [F12] and Spring 2013 [S13] from a dairy farm (Farm A) and a medium-size fluid milk processing plant (Plant A) in Nebraska, supplied exclusively by that farm. A description of Farm A (number of cows and milk production) and the processing parameters for plant A (pasteurization conditions, average days in code, frequency of processing, average volume of milk processed and types of milk processed) for Plant A are presented in Appendix A. In addition, the average temperature of the days when the samples were collected is also presented in Appendix A (based on data from Weather Underground, Inc.).

For each season, raw milk and several environmental samples were collected from different sites on the farm. Environmental samples included rinse water from the milking lines, clean and used teat cloths, sponges of the milking clusters and teats, feed ingredients (premix, sweet bran and corn silage), mixed feed, drinking water, new and used bedding material (inorganic sand) and manure. Mixed feed, used sand and manure samples were collected directly from the barn. Table 1 shows a detailed list of all samples collected in each season.

Table 1. Milk and environmental samples from Farm A collected in Spring 2012, Fall 2012 and Spring 2013.

Milk Samples		
Codes	**Sample**	**Description**
28-S12 28-F12 A1-S13	Raw milk	Raw milk from the same sampling point, where the sample for the FDA is collected (one sample per season).
Environmental Samples*		
4,5-S12 4,5-F12 A3-S13	Rinse water from the milking parlor	Last rinse water used to clean the milking parlor.
8,9-S12 8,9-F12 A8-S13	Sponges of the milking clusters	The inside of the teat cups were rubbed with a sterile sponge in between cows.
6,7-S12 6,7-F12 A9-S13	Sponges of the teats	The cow's teats were rubbed with a sterile sponge after sanitization and before milking.
10,11-S12 10,11-F12 A10-S13	Clean towels	Clean towels used to clean the cow's teats after dipping them in an iodine solution.
12,13-S12 12,13-F12 A11-S13	Used towels	Towels that were used to clean the cow's teats.
14,15-S12 14,15-F12	Premix**	Is a feed ingredient.
16,17-S12 16,17-F12	Sweet Bran**	Is a feed ingredient.
18,19-S12 18,19-F12	Corn Silage**	Is a feed ingredient.
20,21-S12 20,21-F12 A5-S13	Mixed Feed	Feed collected from feeding containers at the barn.
2,3-S12 2,3-F12 A4-S13	Drinking water	Water collected from the pen's tanks where the cows drink.
22,23-S12	New bedding material**	Inorganic sand before being used as bedding material.
24,25-S12 24,25-F12 A7-S13	Used bedding material	Inorganic sand used as bedding material collected from the pen's floor.
26,27-S12 26,27-F12 A6-S13	Manure	Cow's manure collected from the pen's floor.

* Two samples of each kind were collected in Spring 2012 and Fall 2012, but only one sample was collected in Spring 2013.

**These samples were not collected in Spring 2013.

In addition, raw, pasteurized and environmental samples were collected at the processing plant. Samples included raw milk, pasteurized milk (skim, 1%, 2%, whole or chocolate) and environmental samples, such as rinse water (trucks, tanks and mixer) and swabs (filler's interior surface and nozzles). Samples were collected from six different sites, including [A] truck's tank outlet valves, [B] top of raw milk tanks, [C and D] in-line sampling ports right before and after HTST treatment, [E] top of pasteurized milk tanks, and [F] final packaged milk samples right after the filler. A simplified diagram of the collection points is presented in Figure 2. Aseptic samplers (QMI, St. Paul, MN) were installed at sampling points C and D, and milk samples were collected in sterile bags. All samples were transported in coolers packed with ice to the laboratory. Tables 2, 3 and 4 show a detailed list of all samples collected in each season. This processing plant uses the High Temperature Short Time (HTST) pasteurization method, thus milk is pasteurized at a temperature above 79°C for at least 27 seconds (Appendix A).

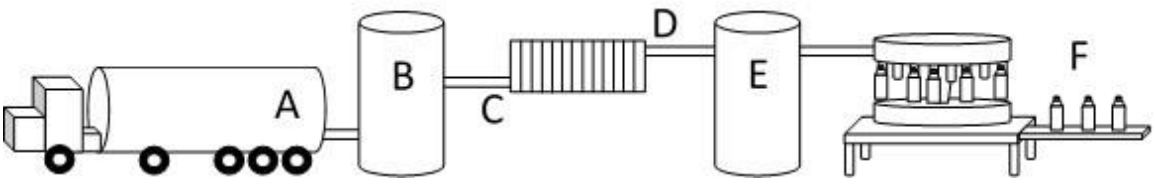

Figure 2. Simplified diagram of fluid milk sample collection points in the processing plant. Sample points include [A] truck's tank outlet valve, [B] top of raw milk tanks, [C and D] immediately before and after HTST pasteurization, [E] top of pasteurized milk tanks, and [F] packaged product taken from the line right after the filler.

Table 2. Milk and environmental samples from Processing Plant A collected in Spring 2012.

Milk Samples		
Code	**Sample**	**Description**
33-S12 34-S12	Raw milk from trucks	Raw milk samples collected from different trucks (No. 1 and No. 2) during the milk unloading at the processing plant.
35-S12 36-S12	Raw milk from tanks	Skim raw milk collected from tank No. 1 (one sample), and 2% raw milk collected from tank No. 3 (one sample).
37-S12	Raw milk before the pasteurizer	2% raw milk collected from an elbow installed before the pasteurizer.
45-S12	Pasteurized milk after the pasteurizer	2% pasteurized milk collected from an elbow installed after the pasteurizer.
43-S12 44-S12 54-S12	Pasteurized milk from tanks	2% pasteurized milk from tank No. 4 (two samples from different days), and whole pasteurized milk from tank No. 5 (one sample).
52-S12 53-S12	Bottled pasteurized milk	2% pasteurized milk collected from the filler's nozzles No. 8 and No. 11.
Environmental Samples		
46-S12 47-S12	Rinse water from tanks	Last rinse water used in tanks No. 1 (skim raw milk) and No. 5 (whole pasteurized milk).
48-S12	Rinse water from mixer	Last rinse water used in the mixer (chocolate milk)
49-S12	Filler surface	A swab was rubbed over the inside surface of the fillers. The filler's milk level was lowered before taking the sample.
50-S12 51-S12	Filler nozzles	Swabs were rubbed over the nozzle's surface of the fillers. Samples were collected from nozzles No. 3 and No. 12.

Table 3. Milk and environmental samples from Processing Plant A collected in Fall 2012.

Milk Samples		
Code	**Sample**	**Description**
33-F12 34-F12	Raw milk from trucks	Raw milk collected from one truck during the milk unloading at the processing plant. Two samples were collected from the same truck (1a and 1b), but in different days.
35-F12 56-F12	Raw milk from tanks	Skim raw milk collected from tanks No. 2 and 3.
37-F12	Raw milk before the pasteurizer	2% raw milk collected from an elbow installed before the pasteurizer.
45-F12	Pasteurized milk after the pasteurizer	2% pasteurized milk collected from an elbow installed after the pasteurizer.
43-F12 44-F12	Pasteurized milk from tanks	Whole pasteurized chocolate milk from tank No. 4, and 1% pasteurized chocolate milk from tank No. 5.
52-F12 53-F12	Bottled pasteurized milk	Skim pasteurized chocolate milk collected from the filler's nozzles No. 8 and No. 11.
Environmental Samples		
47-F12 55-F12	Rinse water from tanks	Last rinse water used to sanitize tanks No. 2 (skim raw milk) and No. 5 (1% chocolate milk).
48-F12	Rinse water from the mixer	Last rinse water used to sanitize the mixer (contained chocolate milk)
49-F12	Filler surface	A swab was rubbed over the inside surface of the fillers. The filler's milk level was lowered before taking the sample.
50-F12 51-F12	Filler nozzles	Swabs were rubbed over the nozzle's surface of the fillers. Samples were collected from nozzles No. 3 and No. 12.

Table 4. Milk and environmental samples from Processing Plant A collected in Spring 2013.

Milk Samples		
Code	**Sample**	**Description**
RA1-S13 RA2-S13	Raw milk from trucks	Raw milk collected from one truck during the milk unloading at the processing plant. Two samples were collected from the same truck (1a and 1b), but in different days.
RA3-S13	Raw milk from tanks	Skim raw milk collected from tank No. 2.
RA4-S13	Raw milk before the pasteurizer	Skim raw milk collected from an elbow installed before the pasteurizer.
PA1-S13	Pasteurized milk after the pasteurizer	Skim pasteurized milk collected from an elbow installed after the pasteurizer.
PA2-S13 PA3-S13	Pasteurized milk from tanks	2% pasteurized milk from tank No. 4, and whole pasteurized milk from tank No. 5.
PA4-S13 PA5-S13	Bottled pasteurized milk	1% pasteurized milk collected from the filler's nozzles No. 8 and No. 11.
Environmental Samples		
EA1-S13 EA2-S13	Rinse water from trucks	Last rinse water used to sanitize the trucks (1a and 1b).
EA3-S13 EA4-S13 EA5-S13	Rinse water from tanks	Last rinse water used to sanitize tank No. 2 (skim raw milk), tank No. 4 (2% pasteurized milk) and tank No. 5 (whole pasteurized milk).
EA6-S13	Filler surface	A swab was rubbed over the inside surface of the fillers. The filler's milk level was lowered before taking the sample.
EA7-S13 EA8-S13	Filler nozzles	Swabs were rubbed over the nozzle's surface of the fillers. Samples were collected from nozzles No. 3 and No. 12.

2. Sample preparation

Environmental samples, as well as raw and pasteurized milk collected in Spring 2012, Fall 2012 and Spring 2013 were prepared in the same manner. Raw and pasteurized milk samples were homogenized, and 150mL aliquots were transferred into sterile 250mL screw-capped bottles. From the samples collected in Spring 2013, sub-samples of 150mL of pasteurized milk were also transferred into sterile 250mL screw-capped

bottles. All solid environmental samples were diluted in Butterfield's phosphate buffer (BPB) using filter stomacher bags. Specific sample preparation procedures are presented in Figure 3. Diluted samples were homogenized for 1 minute using a Stomacher 400, and 100mL filtered aliquots were transferred into 250mL screw-capped bottles.

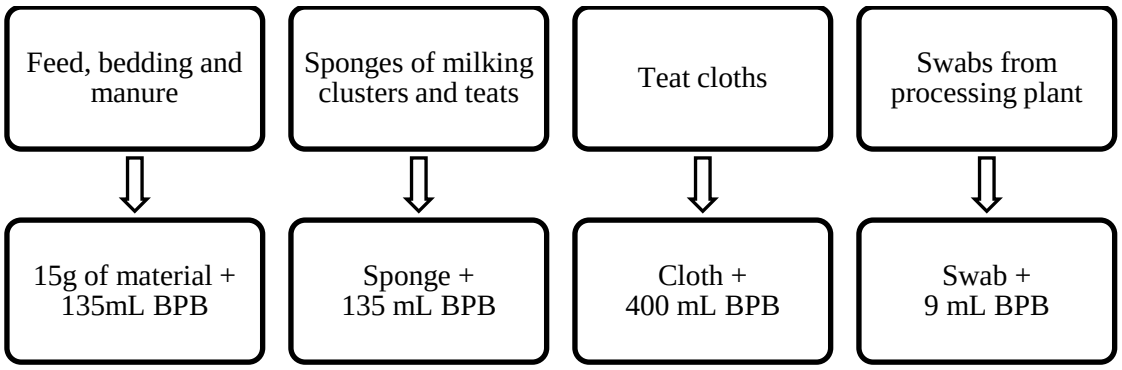

Figure 3. Preparation of environmental samples for sporeformers isolation.

3. Laboratory heat-treatment and storage

Raw milk and environmental samples were heat-treated in the laboratory at 80°C for 12 minutes to kill vegetative cells and to select for sporeforming bacteria. A water bath was used and samples were treated in groups of five based on similarities, along with temperature control. Heat-treated samples were immediately cooled on ice. Pasteurized milk samples or aliquots of thereof, were not submitted to a heat-treatment in the laboratory during Spring 2012 and Fall 2012, assuming that the pasteurization performed by the processing plant and the laboratory heat treatment would leave a similar bacterial population in the product (Huck et al., 2007a; Huck et al., 2007b). However, from the samples collected in Spring 2013, a sub-sample of pasteurized milk [HA1-S13 to HA5-S13] was heat-treated in the laboratory to evaluate the potential for post-

processing contamination on pasteurized samples. Pasteurized milk and heat-treated samples were maintained under refrigeration (<7°C) for 21 days to enrich for psychrotrophic sporeformers.

4. Microbiological analysis

The initial microbiological quality of raw and pasteurized milk samples was evaluated according to procedures defined in the Standard Methods for the Examination of Dairy Products (Frank and Yousef, 2004). The following microbiological analysis were performed: standard plate count (SPC), coliform count (CC) and *E. coli* (EC), yeast (YC) and molds count (MC), and psychrotrophic bacteria count (PBC). Furthermore, raw milk samples and a sub-sample of Spring 2013 pasteurized milk samples were heat-treated in the laboratory and mesophilic spore count (MSC) and psychrotrophic spore count (PSC) were performed in those samples.

Pasteurized milk and heat-treated samples were enumerated for aerobic mesophilic count on days (d) 1, 7, 14 and 21 post-collection or post-heat-treatment, respectively. Samples were plated by serial dilution on Standard Methods Agar (SMA) and when necessary, 1mL of each milk sample was spread-plated over 4 plates to allow bacterial enumeration in samples with low bacterial counts. Aerobic mesophilic bacteria was determined after incubation at 32°C for 48h (Frank and Yousef, 2004).

5. Bacterial isolation

Bacterial colonies present on SMA plates of all pasteurized and heat-treated samples were visually examined, and 1 to 5 colonies with different morphology were

isolated and streaked for purity on SMA at each time point. Purified isolates were frozen at -80°C in 15% glycerol for further processing.

6. DNA isolation and sequencing

Isolates obtained from samples representing the different segments of the milk chain were characterized based on their genotype, according to the DNA sequence of a 632 nucleotide fragment of their *rpo*B gene, as described by Huck et al. (2007a). DNA of 475 bacterial isolates was extracted with the QIAmp DNA Mini Kit (Qiagen Inc., Ca) following the kit protocol D with a few modifications (Appendix B). The distribution of the studied bacterial isolates based on seasons, time points and sources is presented in Table No. 5.

Subtyping was done according to the DNA sequence of a 632 nucleotide fragment of the *rpoB* gene, by using the *rpoB* PCR primers described by Drancourt et al. (2004) and the PCR conditions optimized by Durak et al. (2006) with a few modifications (Appendix B). A T100^{TM} Thermal Cycler was used and each tube contained 100 µL sample. All isolates for which the *rpoB* primers were not able to amplify the *rpoB* fragment were then characterized by partial 16S rDNA sequencing. To achieve this, primers PEU7 (Rothman et al., 2002) and DG74 (Greisen et al., 1994) were used to amplify a fragment (~700bp) of the 16S rDNA sequence, following the PCR cycling conditions described by Fromm and Boor (2004). A T100^{TM} Thermal Cycler was used and for this reaction each tube contained 50 µL sample.

PCR products were evaluated by agarose gel electrophoresis (1% agarose, 60V for 2h) and the ones yielding only one product at the appropriate base pair size were used for further analysis. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Ca) following the kits protocol with minor modifications (Appendix B). Purified PCR products were quantified using a NanoDrop ND-1000 spectrophotometer. All the products with concentrations above 20 ng/µL were sent for sequencing. If the concentration was greater than 40 ng/µL, appropriate dilutions were done with molecular biology grade water (0.3µm filtered, DNase-, RNase- and protease-free; Fisher BioReagents).

Purified PCR products were sent for bidirectional sequencing with Big Dye Terminator chemistry at Eurofins MWG Operon. PCR primers described by Drancourt et al. (2004) were used for partial *rpoB* sequencing, while primers PEU7 (Rothman et al., 2002) and DG74 (Greisen et al., 1994) were used for partial 16S rDNA sequencing. Samples were sent in 96-well plates covered with PCR cap strips at room temperature.

Table 5. Distribution of the studied bacterial isolates based on seasons, time points and sources.

Season	Day	Heat-treated raw milk	Pasteurized milk	Heat-treated pasteurized milk	Farm environment	Plant environment	Total (all sources)
Spring 2012	**1**	11	11	N/A	25	4	**51**
	7	10	7	N/A	13	4	**34**
	14	9	6	N/A	14	4	**33**
	21	10	6	N/A	27	5	**48**
	Total	**40**	**30**	N/A	**79**	**17**	**166**
Fall 2012	**1**	11	8	N/A	28	4	**51**
	7	5	4	N/A	12	3	**24**
	14	4	5	N/A	13	3	**25**
	21	17	6	N/A	28	4	**55**
	Total	**37**	**23**	N/A	**81**	**14**	**155**
Spring 2013	**1**	9	9	10	18	5	**51**
	7	6	5	5	10	0	**26**
	14	6	5	5	13	0	**29**
	21	9	9	11	16	3	**48**
	Total	**30**	**28**	**31**	**57**	**8**	**154**
Total (all seasons)		**107**	**81**	**31**	**217**	**39**	**475**

N/A – Not applicable

7. Allelic Types (AT) assignment

DNA sequences were assembled and trimmed to 632-nt *rpoB* fragments (corresponding to nt 1455 to 3086 of the 3,534-nt *rpoB* open reading frame of *B. cereus* ATCC 10987; GenBank accession number AE017194, locus tag BCE_0102) as described by Durak et al. (2006) in DNA Baser Sequence Assembler v3.x (Heracle BioSoft S.R.L Romania, 2012). Ambiguities were resolved by examination of the chromatograms and only high quality, double-stranded sequence data was used for further analyses. Different ATs were assigned manually to gene sequences that differ from each other by one or more nucleotides as described by Huck et al. (2007b).

In a similar way, partial 16S rDNA sequences were assembled and trimmed to correspond to a 616-nt fragment (nt 823 to 1438 of the 1,508-nt 16S rDNA gene in *B. cereus* ATCC 10987; GenBank accession number AE017194, locus tag BCE_5738) as described by Huck et al. (2007b) using DNA Baser Sequence Assembler v3.x (Heracle BioSoft S.R.L Romania, 2012). The forward and reverse sequences of some isolates showed the presence of two different nucleotides at a specific position due to the presence of multiple copies of rDNA operons with different sequences in a given isolate (Klappenbach et al., 2001). Thus, partial 16S rDNA sequences were assembled using nucleotide ambiguity codes as described by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. In addition, 16S rDNA Allelic Types (SATs) were assigned to partial 16S rDNA sequences that differ from each other by one or more nucleotide (Huck et al., 2007b).

8. Phylogenetic trees

DNA sequences representing each identified *rpoB* AT were aligned using Muscle in MEGA6 (Tamura et al., 2014). A phylogenetic tree was built using the Neighbor-Joining Method with 2000 bootstrap replicates in MEGA6, and *Streptococcus pyogenes* (NC_004070) was used as an outgroup as described by Huck et al. (2007b). The same methods were used to build the phylogenetic tree using partial 16S rDNA sequences. However for the 16S rDNA tree, three different *Staphylococcus* species (*Staphylococcus simiae* CCM 7213, GenBank accession number NR_043146.1; *Staphylococcus aureus* ATCC 12600, GenBank accession number D83357.1; *Staphylococcus lutrae*, GenBank accession number X84731.1) were used as an outgroup (Ivy et al., 2012).

9. Species identification

Final double-stranded partial *rpoB* and 16S rDNA sequences were used for similarity searches against the National Center for Biotechnology Information (NCBI) nucleotide sequence database (GenBank; Benson et al., 2010), using the Blast Local Alignment Search Tool (McGinnis and Madden, 2004). Genus or species assignments were based on the top matches returned by the BLAST search and on phylogenetic analysis by comparison with previous published *rpoB* and 16S rDNA sequences (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012). Information about each unique *rpoB* and 16S rDNA allelic types identified in our study is presented in Appendix C.1. and C.2., respectively.

10. Identification of "potentially problematic sporeforming bacteria" (PPSB) and their sources

Sporeforming bacteria that were present in laboratory heat-treated and commercialized pasteurized milk samples with high bacterial counts (MSC or SPC) at the end of shelf life were classified as "potentially problematic sporeformers" (PPSB). To identify the sources of these PPSB in the milk chain, the following comparisons were done: (1) the farm environment was considered as a possible source of PPSB if the same allelic types (ATs or SATs) were present in milk samples (heat-treated and commercialized pasteurized) and in the farm environment; (2) the raw milk was considered as a possible source of PPSB if the same allelic types were present in heat-treated raw milk and in heat-treated or commercially pasteurized milk; (3) the plant environment was considered a source of PPSB if the same allelic types were present in milk samples and/or environmental samples collected at the plant.

11. Comparison of DNA sequences with other similar research projects in the US

The DNA sequences obtained in this study, along with other ones obtained from similar research projects done in other parts of the US (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012, were used to build phylogenetic trees for *rpoB* as previously described. These sequences were obtained from the Food Microbe Tracker database (Vangay et al., 2013) or from GenBank (Benson et al., 2010) using the information from Table S2 of the supplemental material by Ivy et al. (2012). In particular, *rpoB* sequences from allelic types (CAT) that were identified in multiple occasions and/or that were evaluated for cold growth in these studies were

included in the analysis. Moreover, the *rpoB* allelic types (AT) that were frequently isolated and/or classified as "potentially problematic sporeforming bacteria" in the current study were also included. This tree provided insights on how the sporeformers isolated in Nebraska (current study) are related to sporeformers isolated from other regions of the US. In particular, these research projects evaluated milk and environmental samples from dairy farms and processing plants in New York State. Additionally, in one of these studies isolates were collected from pasteurized milk samples from five different regions across the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009). This information is needed to establish if nationwide or region-specific strategies will have to be developed to better control sporeforming bacteria in the US milk chain.

12. Statistical analysis

For all analyses, log-transformed bacterial count data were used. Due to the large number of different samples collected in this study, it was not possible to analyze these samples in replicates to perform a statistical analysis to assess the effect of season on the microbial quality (SPC or MSC) of milk samples. Nonetheless, samples from all seasons were organized in three groups: heat-treated raw milk, in-line pasteurized milk and packaged pasteurized milk. Analysis of Variance (Appendix D) was done to evaluate the effect of these groups on bacterial counts (SPC or MSC) throughout shelf life. One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

CHAPTER 4: RESULTS AND DISCUSSION

1. Initial microbiological quality of raw and pasteurized milk

To evaluate the initial quality of the milk samples the following microbiological analysis were performed: standard plate count (SPC), coliform count (CC) and *E. coli* (EC), yeast (YC) and molds counts (MC), and psychrotrophic bacteria counts (PBC). Also raw milk samples and a sub-sample of Spring 2013 pasteurized milk samples were heat-treated in the laboratory and the mesophilic spore count (MSC) and psychrotrophic spore count (PSC) were performed. The quality evaluation of the raw, heat-treated and processed milk collected from this milk chain in Spring 2012 [S12], Fall 2012 [F12] and Spring 2013 [S13] is presented as log-transformed bacterial counts in Table 6, 7 and 8, respectively.

Mean raw milk SPC, from 16 samples (farm, trucks and processing plant) included in this analysis comprising all seasons, was 3.15 log CFU/mL, and ranged from 2.78 to 3.79 log CFU/mL. SPC for all raw milk samples were within the regulatory limit (raw milk from one farm < 5 log CFU/mL) established in the PMO (FDA, 2011). Moreover, the mean CC for these samples was 1.44 log CFU/mL, and ranged from 0.85 to 1.97 log CFU/mL. These samples had a mean PBC of 2.78 log CFU/mL, ranging from 2.05 to 3.63 log CFU/mL. Most samples showed low (< 0.60 log CFU/mL) or no growth when plated for PSC, indicating the low initial presence of psychrotrophic sporeformers in the raw milk supply (Martin et al., 2011; Ranieri et al., 2012).

The PMO does not specify a regulatory limit for CC, PBC, MSC or PSC in raw milk (FDA, 2011). Nonetheless, high quality raw milk should have SPC and CC below 4.0 and 1.7 log CFU/mL, respectively (Murphy and Boor, 2000). All raw milk samples showed SPC below 4.0 log CFU/mL and about 81% of these samples had CC below 1.7 log CFU/mL. Thus, most of the raw milk samples analyzed in this study had high quality based on SPC and CC, and the high initial quality of raw milk was consistent along the milk chain and in every season. These results were comparable to what other studies have reported from other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009), and in most cases the values obtained for SPC were even lower in this study (Fromm and Boor, 2004; Huck et al., 2007a; Huck et al., 2008; Ranieri et al., 2009; Martin et al., 2012b).

For all seasons, mean SPC for 10 commercially pasteurized milk samples collected from in-line locations (after pasteurizer and tanks) was 1.29 log CFU/mL, and ranged from 0.30 to 1.89 log CFU/mL. Mean SPC for the 6 packaged pasteurized milk samples was slightly higher (1.74 log CFU/mL), and ranged from 1.11 to 2.31 log CFU/mL. Despite the differences that might have existed in initial SPC due to different factors (i.e. seasons, sampling points), all pasteurized milk samples were below the regulatory limit (< 4.3 log CFU/mL) established in the PMO (FDA, 2011) at the beginning of this study. These results are similar to what other studies have obtained in other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009).

Furthermore, most pasteurized milk samples showed no growth when plated for CC. Only two packaged pasteurized milk samples [PA4-S13 and PA5-S13] collected in Spring 2013 presented CC (1.29 and 1.45 log CFU/mL, respectively) above the regulatory limit (<1 log CFU/mL) established in the PMO (FDA, 2011). These samples also showed higher PBC (2.15 and 2.35 log CFU/mL, respectively) than other pasteurized milk samples, indicating PPC with coliforms and psychrotrophic bacteria. Therefore, the main recommendation for this particular milk chain based on these results would be to revise the sanitization and process protocols in the processing plant to eliminate PPC.

Although, the quality of raw milk is important for the production of pasteurized milk with higher quality and longer shelf life, some studies have indicated that plant factors (i.e. PPC and pasteurization conditions) may have a major effect on pasteurized milk quality (Fromm and Boor, 2004; Martin et al., 2011). In addition, tests commonly used to access the quality of raw milk (i.e. SPC, PBC, CC, MSC) haven't been able to predict the shelf life of pasteurized milk, in terms of its microbiological and sensory quality. These tests lack the sensitivity and specificity needed to detect all forms of sporeforming bacteria, consequently the development of tools to consistently detect PPSB in raw milk has been recommended in the literature (Martin et al., 2011).

Table 6. Quality evaluation of raw milk collected in Spring 2012, Fall 2012 and Spring 2013.

Code	Sample	log CFU/mL							
		SPC	PBC [a]	MSC	PSC [a]	CC [b]	EC [b]	YC [c]	MC [c]
28-S12	Raw milk from Farm A	3.11	3.26	0.48 est	ND	1.20	ND	2.15	ND
33-S12	Raw milk from truck No. 1 (Plant A)	3.06	2.43	1.03 est	ND	1.72	1.70	1.98	ND
34-S12	Raw milk from truck No. 2 (Plant A)	3.11	2.37	1.38 est	ND	1.44	1.24	1.58	ND
35-S12	Raw milk (skim) from tank No. 1 (Plant A)	3.09	2.32	1.08 est	ND	1.66	1.56	1.75	ND
36-S12	Raw milk (2%) from tank No. 3 (Plant A)	3.18	2.31	1.20 est	ND	1.66	1.64	1.51	ND
37-S12	Raw milk (2%) before the pasteurizer (Plant A)	3.06	2.42	0.90 est	ND	1.63	1.54	1.87	ND
28-F12	Raw milk from Farm A	3.59	3.36	1.98 est	ND	1.88	0.85	1.08	0.60
33-F12	Raw milk from truck No. 1a (Plant A)	3.45	3.15	1.81 est	ND	1.66	0.65	1.78	1.00
34-F12	Raw milk from truck No. 1b (Plant A)	3.79	3.63	2.73	ND	1.97	1.41	2.03	2.86
36-F12	Raw milk (skim) from tank No. 3 (Plant A)	2.90	2.33	1.82 est	ND	1.22	0.48	1.20	ND
56-F12	Raw milk (skim) from tank No. 2 (Plant A)	2.78	2.76	1.48 est	0.30 est	1.22	0.85	0.90	ND
37-F12	Raw milk (2%) before the pasteurizer (Plant A)	2.96	2.05	1.61 est	ND	0.93	0.40	0.60	ND
A1-S13	Raw milk from Farm A	3.33	2.76	1.66 est	0.60 est	1.35	0.85	1.45	0.90
RA1-S13 [d]	Raw milk from truck No. 1a (Plant A)	5.03	5.37	0.95 est	ND	4.02	3.96	3.50	2.81
RA2-S13	Raw milk from truck No. 1b (Plant A)	3.17	3.06	1.00 est	ND	1.52	1.38	1.79	1.38
RA3-S13	Raw milk (skim) from tank No. 2 (Plant A)	2.97	3.08	1.00 est	0.60 est	1.15	ND	1.20	1.30
RA4-S13	Raw milk (skim) before the pasteurizer (Plant A)	2.92	3.15	1.18 est	ND	0.85	ND	1.45	1.08

ND – Non detectable

a – The detection limit for Psychrotrophic Bacteria Count (PBC) and Psychrotrophic Spores Count (PSC) was 2 CFU/mL.

b – The detection limit for coliform count (CC) and *E. coli* (EC) was 1 CFU/mL.

c – The detection limit for yeast (YC) and molds count (MC) was 2 CFU/mL.

d – Quality evaluation and heat-treatment done on day 2 after collection, thus this sample was not included in the initial quality evaluation of raw milk.

est – Estimated count as plates from the lowest dilution contained less than 25 colonies.

Table 7. Quality evaluation of in-line pasteurized milk collected in Spring 2012, Fall 2012 and Spring 2013.

Code	Sample	log CFU/mL							
		SPC	PBC [a]	MSC	PSC [a]	CC [b]	EC [b]	YC [c]	MC [c]
45-S12	Pasteurized milk (2%) after the Pasteurizer (Plant A)	1.86 est	ND	NA	NA	ND	ND	ND	ND
43-S12	Pasteurized milk (2%) from tank No. 4 and truck No. 1 (Plant A)	1.26 est	ND	NA	NA	ND	ND	ND	ND
44-S12	Pasteurized milk (whole) from tank No. 5 (Plant A)	1.89 est	ND	NA	NA	ND	ND	ND	ND
54-S12	Pasteurized milk (2%) from tank No. 4 and truck No. 2 (Plant A)	0.30 est	ND	NA	NA	ND	ND	ND	ND
45-F12	Pasteurized milk (2%) after the pasteurizer (Plant A)	1.57 est	0.30 est	NA	NA	ND	ND	ND	ND
43-F12	Pasteurized milk (chocolate whole) from tank No. 4 (Plant A)	1.58 est	ND	NA	NA	ND	ND	ND	ND
44-F12	Pasteurized milk (chocolate 1%) from tank No. 5 (Plant A)	1.65 est	ND	NA	NA	ND	ND	0.30	ND
PA1-S13	Pasteurized milk (skim) after the Pasteurizer (Plant A)	0.78 est	0.30 est	0.90 est	0.60 est	ND	ND	ND	ND
PA2-S13	Pasteurized milk (2%) from tank No. 4 (Plant A)	1.23 est	ND	1.28 est	ND	ND	ND	ND	0.30
PA3-S13	Pasteurized milk (whole) from tank No. 5 (Plant A)	0.78 est	0.30 est	1.20 est	0.30 est	ND	ND	ND	ND

ND – Non detectable

NA – Not analyzed for this season, but based on results it was analyzed in Spring 2013 to evaluate post-processing contamination.

a – The detection limit for Psychrotrophic Bacteria Count (PBC) and Psychrotrophic Spores Count (PSC) was 2 CFU/mL.

b – The detection limit for coliform count (CC) and *E. coli* (EC) was 1 CFU/mL.

c – The detection limit for yeast (YC) and molds count (MC) was 2 CFU/mL.

est – Estimated count as plates from the lowest dilution contained less than 25 colonies.

Table 8. Quality evaluation of packaged pasteurized milk collected in Spring 2012, Fall 2012 and Spring 2013.

Code	Sample	log CFU/mL							
		SPC	PBC [a]	MSC	PSC [a]	CC [b]	EC [b]	YC [c]	MC [c]
52-S12	Bottled milk (2%) from nozzles No. 8 (Plant A)	1.11 est	ND	NA	NA	ND	ND	ND	ND
53-S12	Bottled milk (2%) from nozzles No. 11 (Plant A)	1.18 est	ND	NA	NA	ND	ND	ND	ND
52-F12	Bottled milk (chocolate skim) from nozzles No. 8 (Plant A)	1.79 est	ND	NA	NA	ND	ND	ND	0.30
53-F12	Bottled milk (chocolate skim) from nozzles No. 11 (Plant A)	1.86 est	0.30 est	NA	NA	ND	ND	0.60	ND
PA4-S13	Bottled milk (1%) from nozzles No. 8 (Plant A)	2.21	2.15	1.00 est	ND	1.29	1.29	ND	ND
PA5-S13	Bottled milk (1%) from nozzles No. 11 (Plant A)	2.31	2.35	0.48 est	0.30 est	1.45	1.44	ND	ND

ND – Non detectable

NA – Not analyzed for this season, but based on results it was analyzed in Spring 2013 to evaluate post-processing contamination.

a – The detection limit for Psychrotrophic Bacteria Count (PBC) and Psychrotrophic Spores Count (PSC) was 2 CFU/mL.

b – The detection limit for coliform count (CC) and *E. coli* (EC) was 1 CFU/mL.

c – The detection limit for yeast (YC) and molds count (MC) was 2 CFU/mL.

est – Estimated count as plates from the lowest dilution contained less than 25 colonies.

2. Microbiological analysis of laboratory heat-treated and commercially pasteurized milk samples throughout shelf life

As described in materials and methods, samples from all seasons were organized in three groups: heat-treated raw milk, in-line pasteurized milk and packaged pasteurized milk. All raw milk samples were heat-treated in the laboratory to evaluate their potential contamination with sporeforming bacteria. The MSC of 17 heat-treated raw milk samples (farm, trucks and plant) stored at <7°C were plotted to evaluate changes in bacterial numbers over 21 days of shelf life (Figures 4, 5 and 6).

For all seasons, MSC for heat-treated raw milk samples increased from 1.37 mean log CFU/mL (ranging from 0.48 to 2.73 log CFU/mL) on day 1 to 2.13 mean log CFU/mL (ranging from 0.30 to 5.90 log CFU/mL) on day 21. Only a few samples [1-S12, 57-F12, TA3-S13, and TA4-S13] showed increase in the counts during refrigerated storage that were superior to 2 logs (2.78, 4.42, 2.33, and 2.90 logs, respectively). These results suggested that these samples were contaminated with psychrotrophic heat-resistant sporeforming bacteria, which survived the heat-treatment in the laboratory and were able to grow under refrigeration.

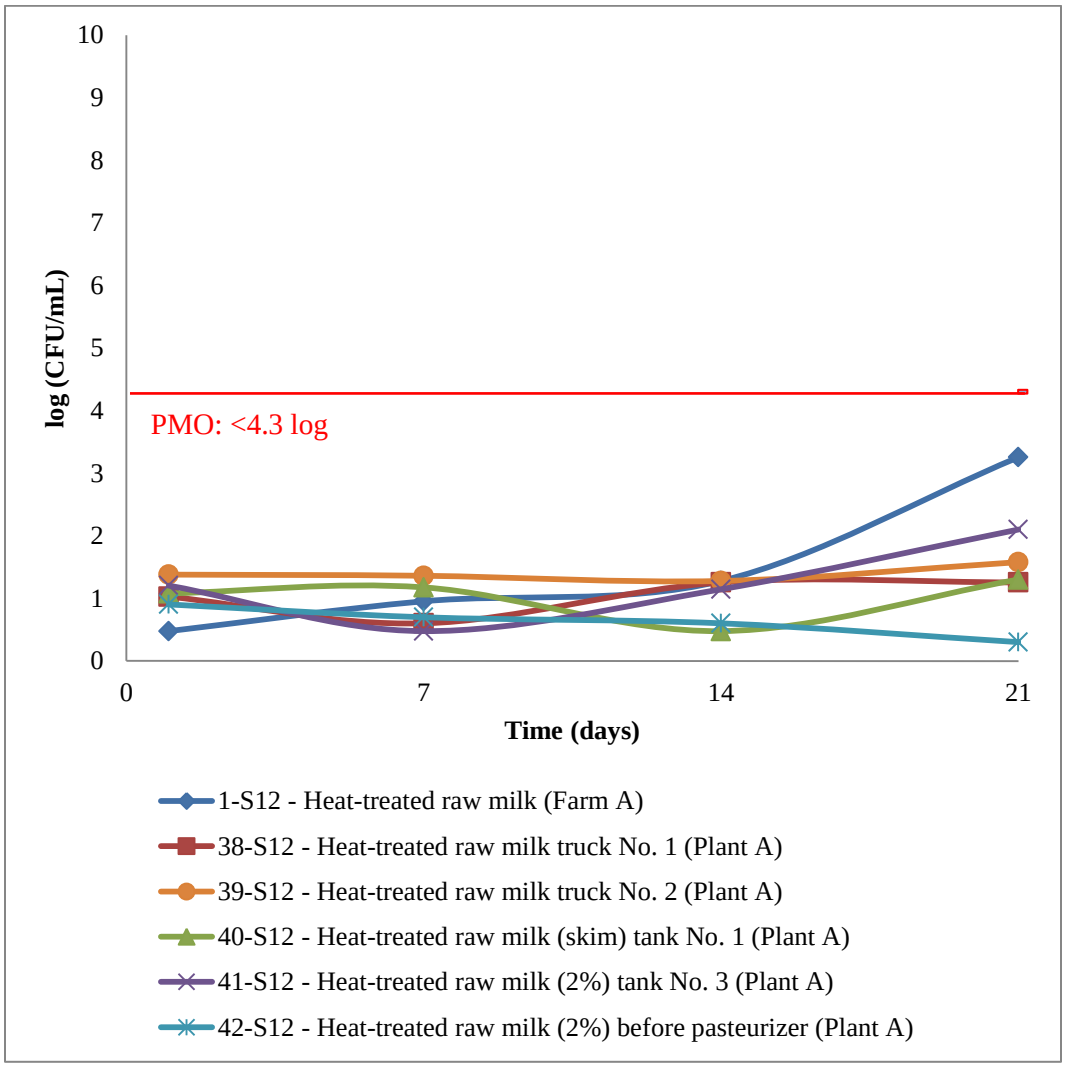

Figure 4. Mesophilic spore count (MSC) for heat-treated raw milk samples collected at Farm A and Plant A in Spring 2012. Raw milk samples were heat-treated at 80°C for 12 min, held at <7°C and plated over their shelf life.

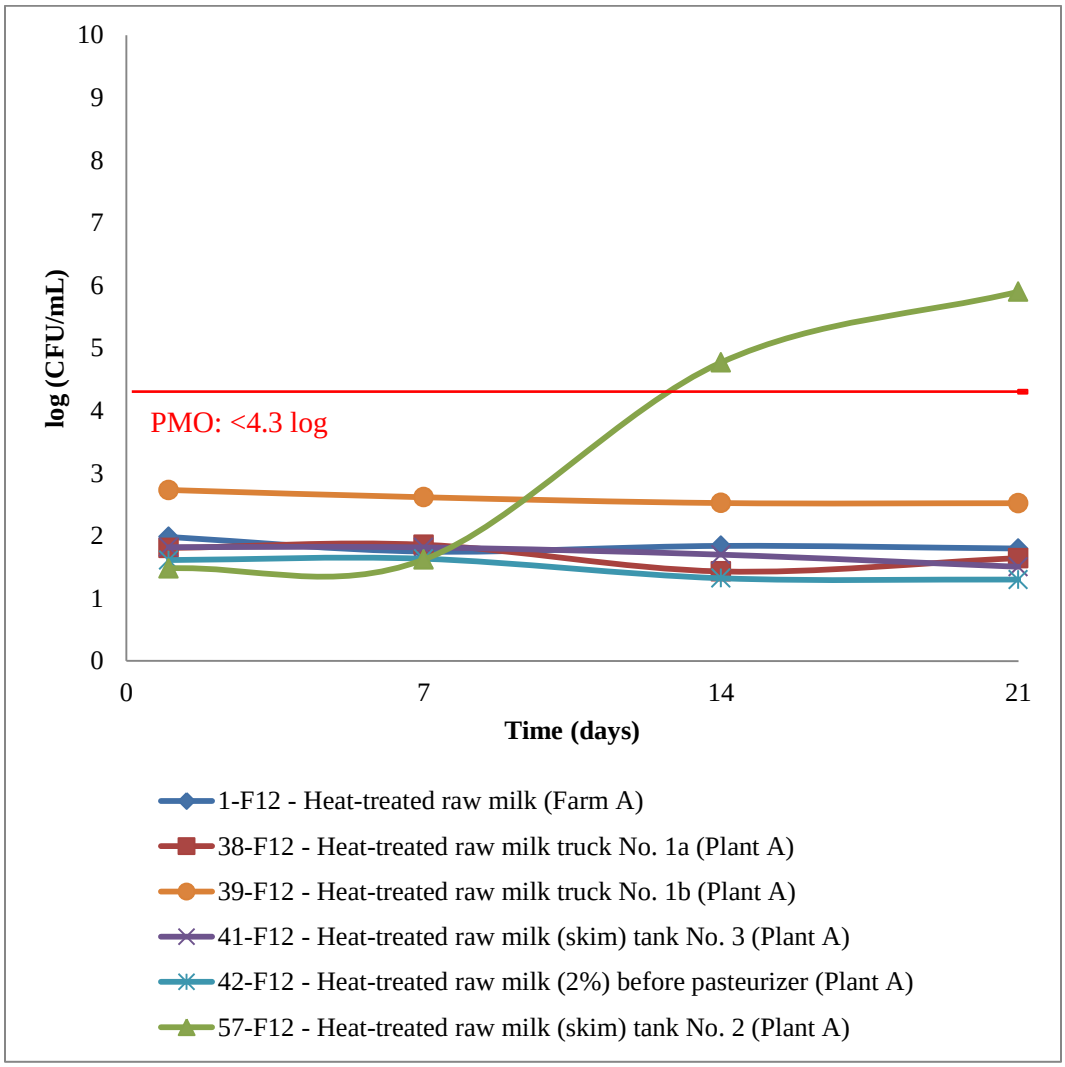

Figure 5. Mesophilic spore count (MSC) for heat-treated raw milk samples collected at Farm A and Plant A in Fall 2012. Raw milk samples were heat-treated at 80°C for 12 min, held at <7°C and plated over their shelf life.

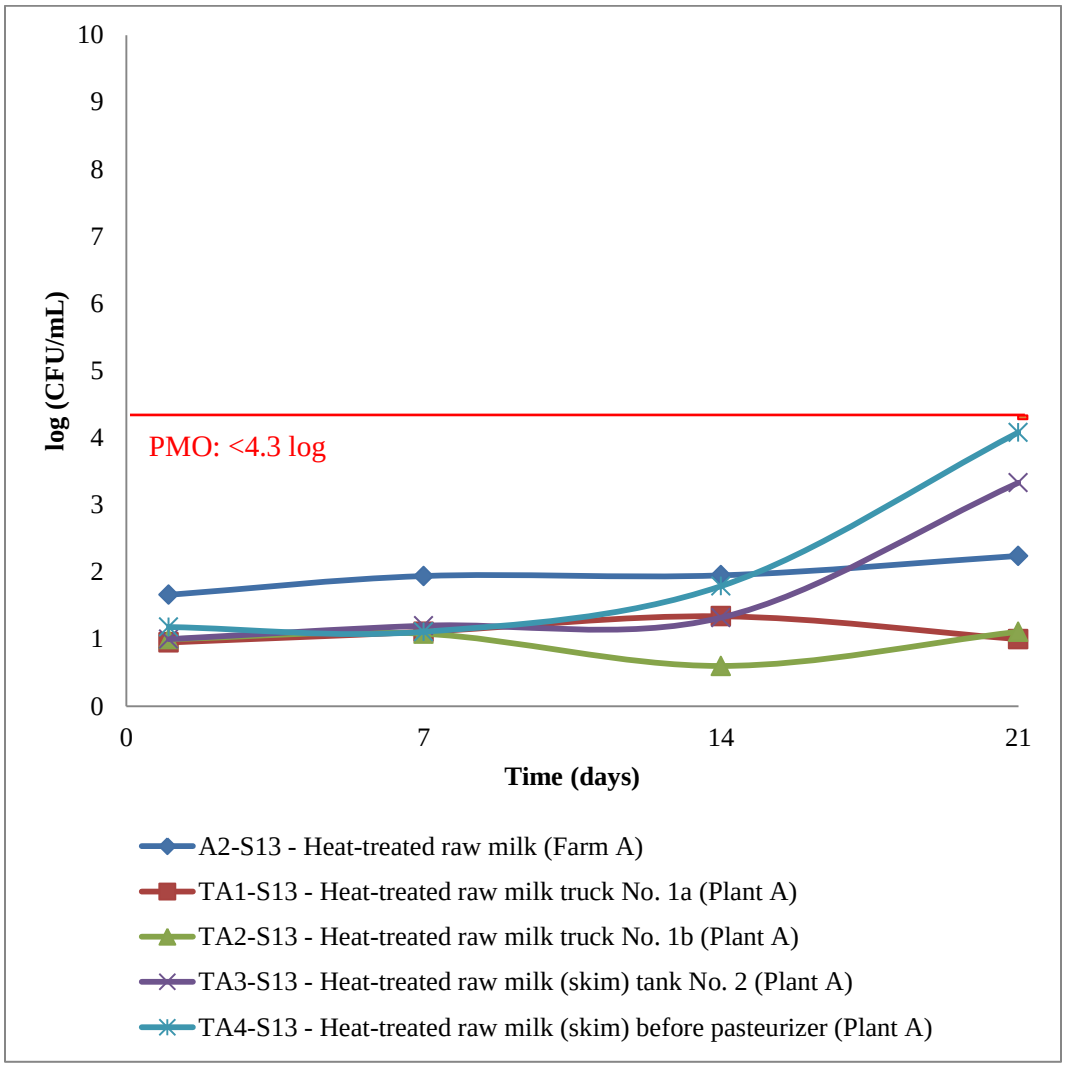

Figure 6. Mesophilic spore count (MSC) for heat-treated raw milk samples collected at Farm A and Plant A in Spring 2013. Raw milk samples were heat-treated at 80°C for 12 min, held at <7°C and plated over their shelf life.

Likewise, the SPC of 16 commercially pasteurized milk samples (10 in-line and 6 packaged products) stored at <7°C were plotted throughout shelf life (Figures 7, 8 and 9). At days 1 and 7 post-processing or post-heat-treatment, 100% of the laboratory heat-treated raw and commercially pasteurized milk (in-line and packaged) samples evaluated in this study had counts below the PMO regulatory limit of 4.3 log CFU/mL (FDA, 2011). After 14 and 21 days of storage, 94% of the laboratory heat-treated samples had counts < 4.3 log CFU/mL. On the other hand, 80% and 50% of the in-line pasteurized milk samples, and 17% and 0% of packaged pasteurized milk samples had counts below this value at days 14 and 21, respectively. Furthermore, MSC of heat-treated samples were not significantly different ($P>0.05$; ANOVA) throughout shelf life. However, a significant increase in SPC of packaged and in-line pasteurized milk samples was observed by day 14 ($P<0.05$; ANOVA) and day 21 ($P<0.05$; ANOVA), respectively. These results suggest the presence of a different microbiota among these three sets of samples (heat-treated raw, in-line pasteurized and packaged pasteurized milk). Research has shown that gram-negative bacteria (i.e. *Pseudomonas* spp.) grow faster in pasteurized milk than gram-positive sporeforming bacteria during refrigerated storage (Ranieri and Boor, 2009). Consequently, pasteurized milk samples, that showed SPC above >4.3 log CFU/mL by day 14, might not be contaminated with sporeforming bacteria, but rather with other organisms due to PPC.

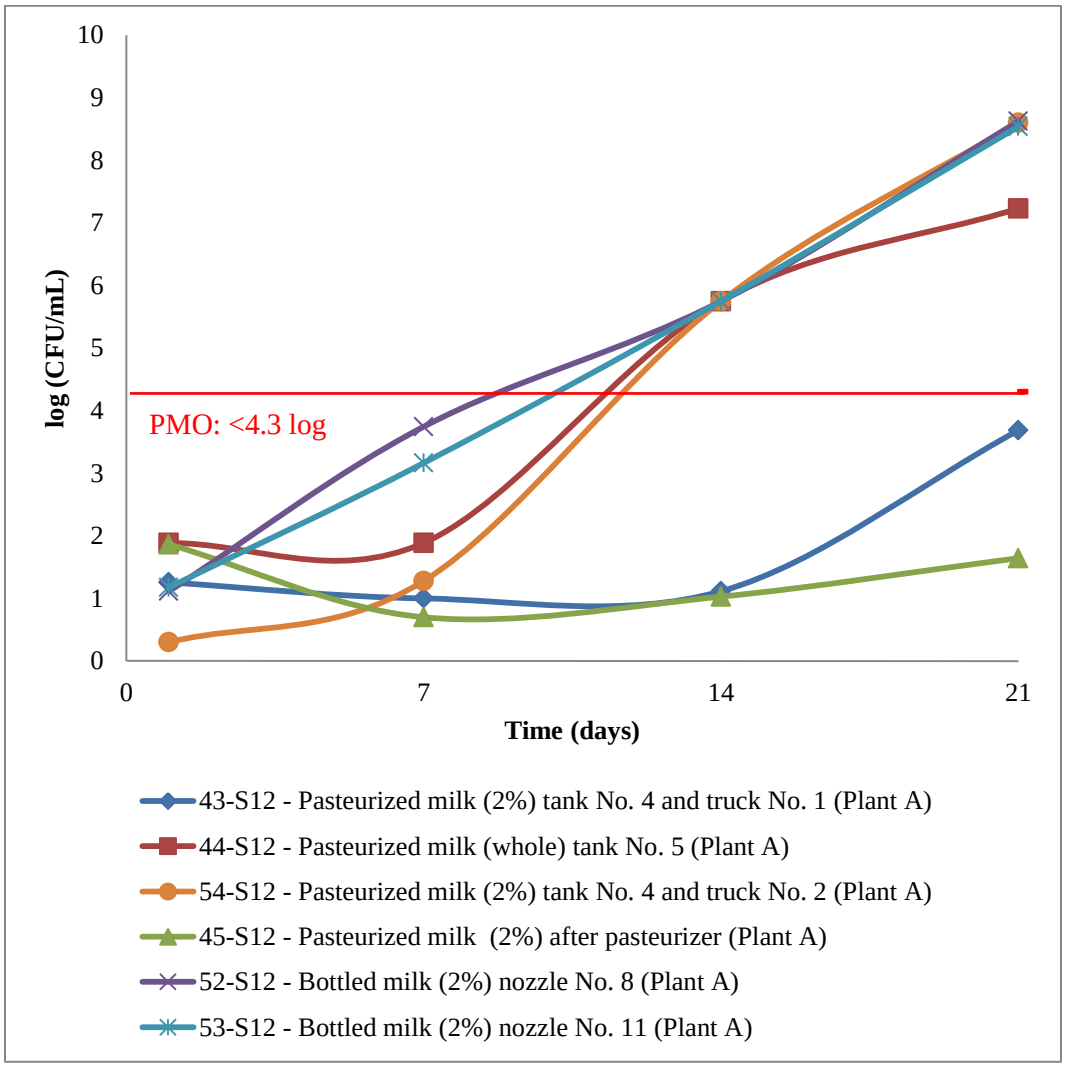

Figure 7. Standard plate count (SPC) for pasteurized milk samples collected at Plant A in Spring 2012. Pasteurized milk samples were held at <7°C and plated over their shelf life.

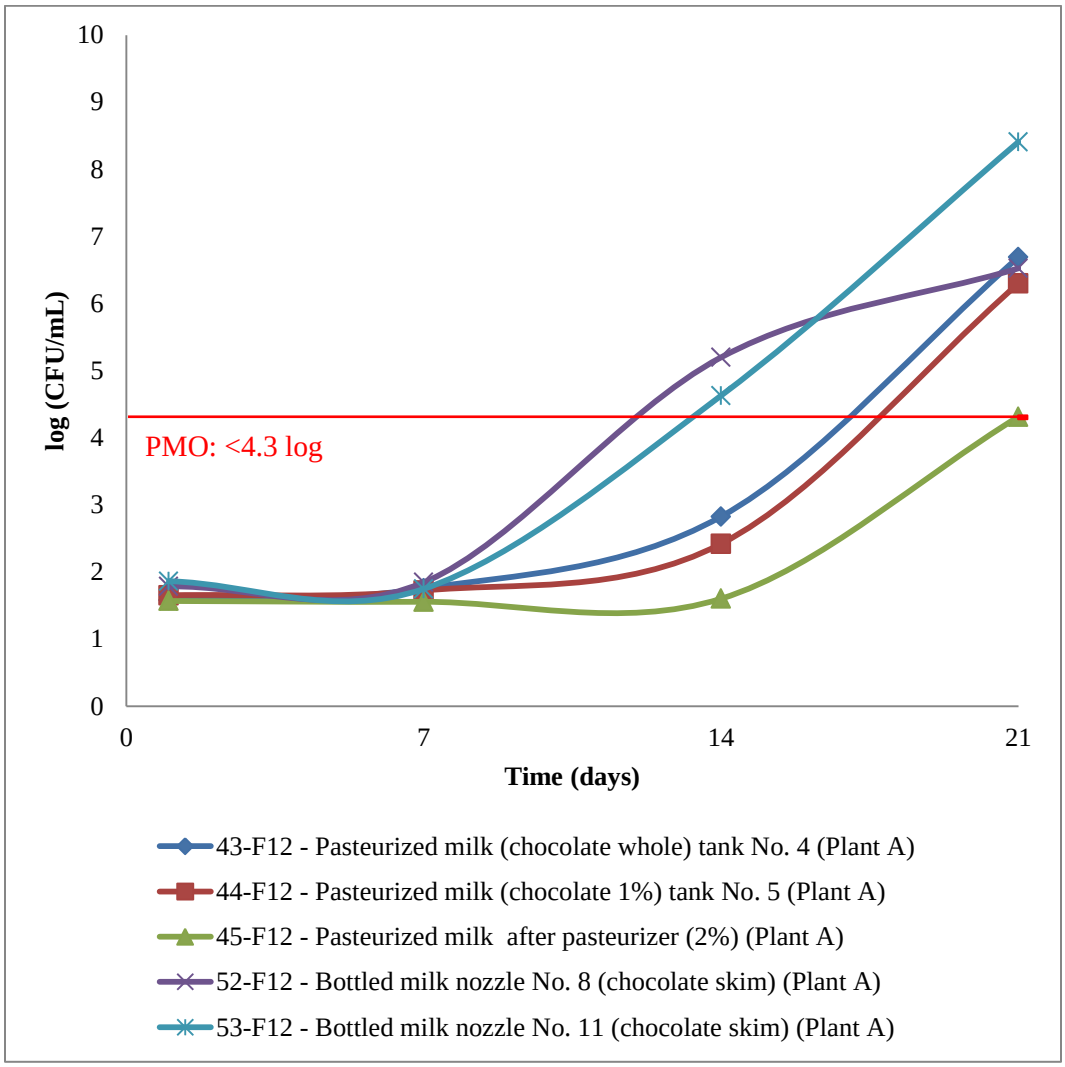

Figure 8. Standard plate count (SPC) for pasteurized milk samples collected at Plant A in Fall 2012. Pasteurized milk samples were held at <7°C and plated over their shelf life.

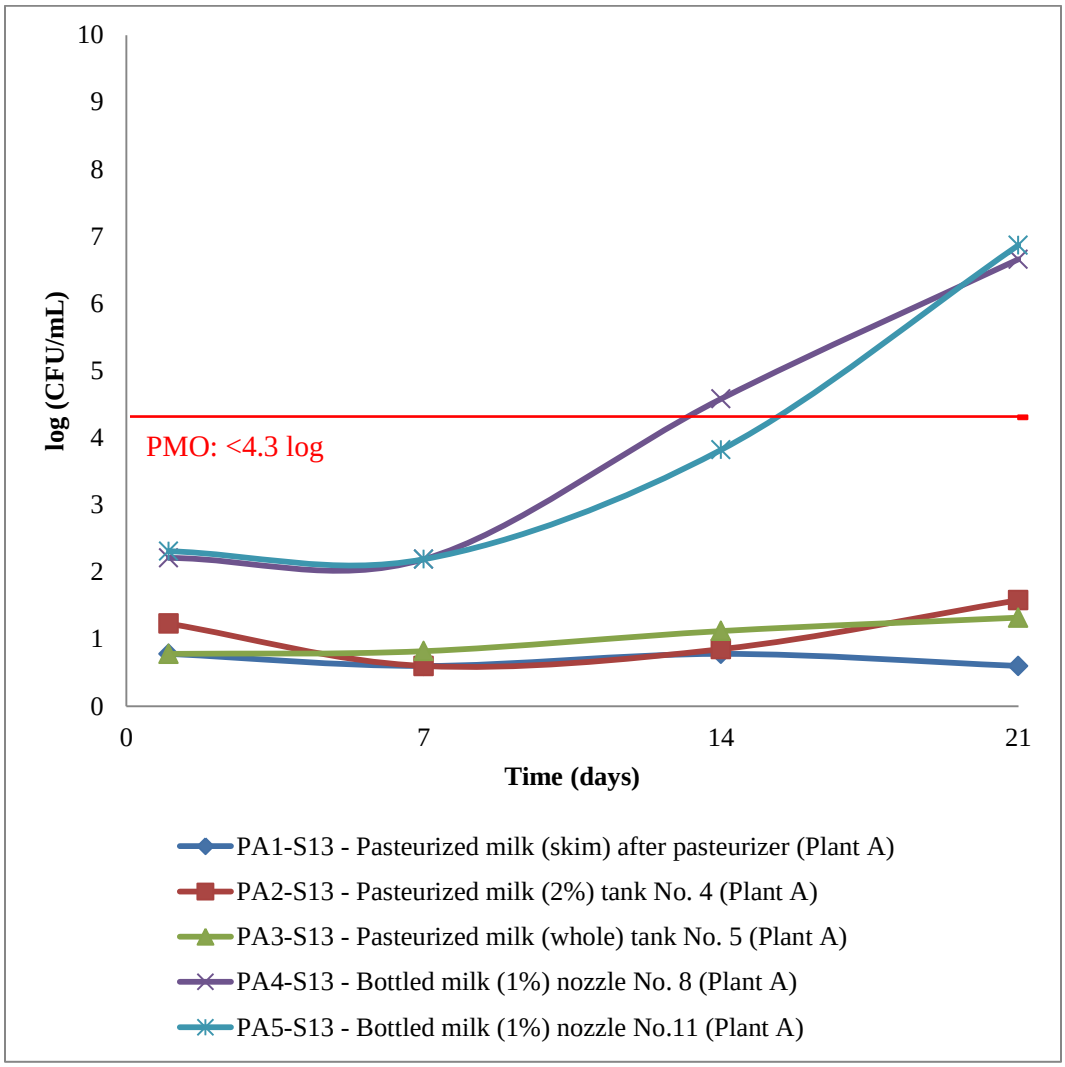

Figure 9. Standard plate count (SPC) for pasteurized milk samples collected at Plant A in Spring 2013. Pasteurized milk samples were held at <7°C and plated over their shelf life.

Bacterial counts (MSC or SPC; Figure 10) between these three groups of samples (heat-treated raw milk, in-line pasteurized milk and packaged pasteurized milk) were not significantly different ($P > 0.05$; ANOVA) at day 1. Nevertheless, at days 7, 14 and 21, SPC of packaged pasteurized milk (2.48, 4.95 and 7.60 mean log CFU/mL, respectively) were significantly higher ($P < 0.05$; ANOVA) than SPC of in-line pasteurized milk (1.19, 2.32, 4.20 mean log CFU/mL, respectively) and MSC of heat-treated raw milk (1.35, 1.57, 2.13 mean log CFU/mL, respectively). Moreover, a significant difference ($P < 0.05$; ANOVA) in bacterial counts (MSC or SPC) was observed between heat-treated raw milk and in in-line pasteurized milk until day 21.

When in-line and packaged pasteurized milk samples were evaluated throughout shelf life, most of them showed a greater than 4 log increase in SPC (Figures 7-9). As noted in Figure 10, at day 21, SPC of all packaged pasteurized milk samples (7.60 mean log CFU/mL; ranging from 6.53 to 8.62 log CFU/mL) were significantly higher ($P < 0.05$; ANOVA) than SPC of in-line pasteurized milk samples (4.20 mean log CFU/mL; ranging from 0.6 to 8.6 log CFU/mL) (Figure A). Only a few in-line samples [45-S12, PA1-S13, PA2-S13, and PA3-S13], did not show an increase in SPC during the evaluated period (Figures 7, 8 and 9). In those samples where bacterial counts increased during storage, the spoilage could have been caused by either psychrotrophic sporeforming bacteria (present in raw milk or in the processing environment) or by non-sporeforming bacteria (i.e. psychrotrophic gram-negative bacteria) due to PPC.

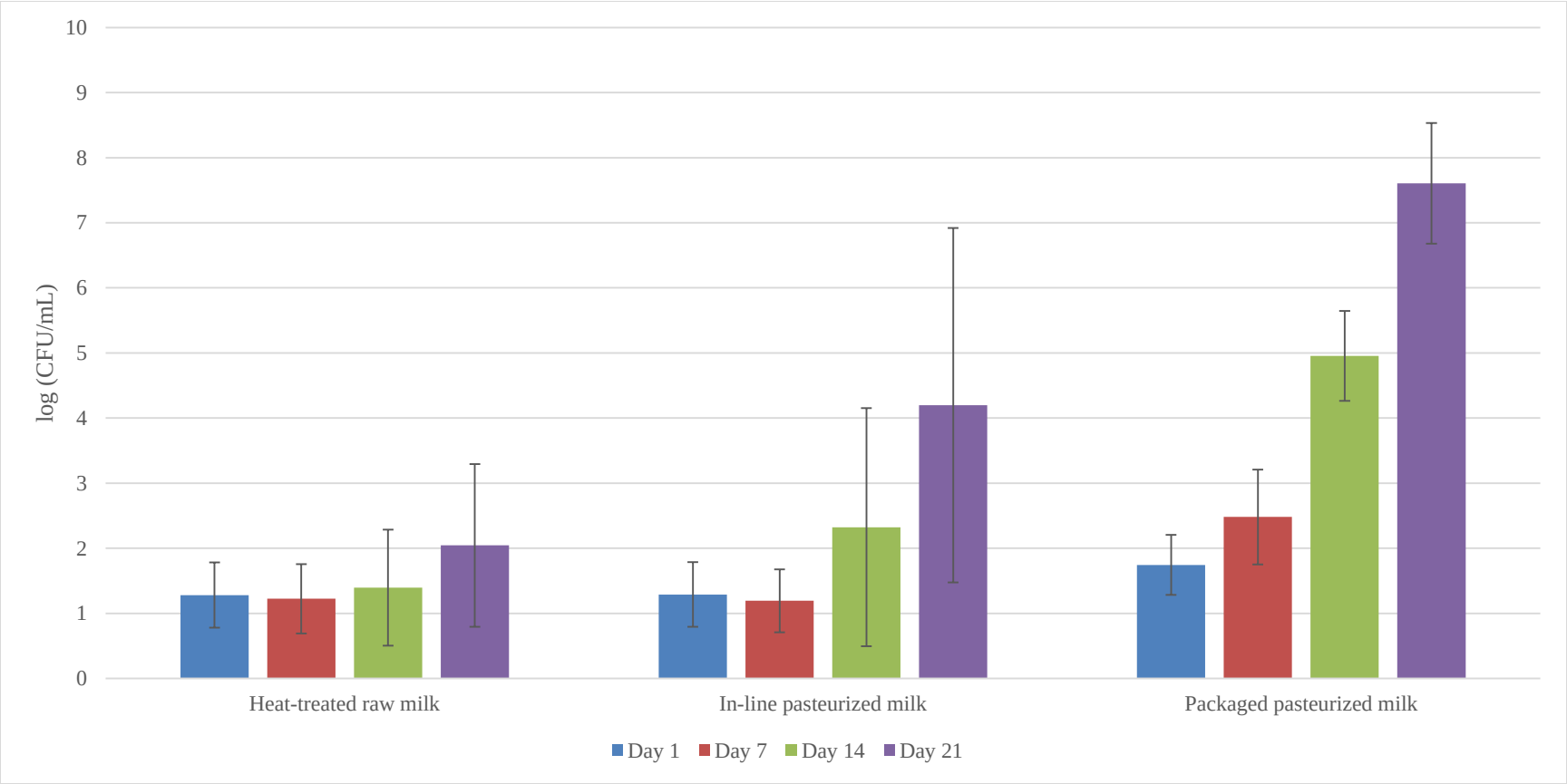

Figure 10. Bacterial counts, reported as mesophilic spore count (MSC) or standard plate count (SPC) for heat-treated raw milk, in-line pasteurized milk and packaged pasteurized milk collected at Farm A and Plant A in Spring 2012, Fall 2012 and Spring 2013. Raw milk samples were heat-treated at 80°C for 12 min. Laboratory heat-treated and commercially pasteurized milk samples were held at <7°C and plated over their shelf life. Bars indicate standard deviation.

Attempting to collect more evidence on the potential source of contamination of pasteurized milk samples, a sub-sample of those collected in Spring 2013 were heat-treated in the laboratory (Figure 11) and evaluated for the presence of sporeformers. The data shown in Figures 9 and 11 indicate that when pasteurized milk samples were heat-treated in the laboratory the presence of psychrotrophic bacteria was reduced. However, one of the samples [HA4-S13] still showed a 2.52 log CFU/mL increase in MSC during 21 days of storage. psychrotrophic sporeformering bacteria or other heat-resistant microorganisms may be contributing to the spoilage of this sample, while in the other samples from Spring 2013 non-sporeforming bacteria might have been the main spoilage organisms, as a result of PPC. In general, several studies have pointed out PPC as a recurring cause of HTST pasteurized fluid milk spoilage in the US (Fromm and Boor, 2004; Ranieri and Boor, 2009; Martin et al., 2011).

Furthermore, in-line pasteurized milk samples [PA1-S13, PA2-S13 and PA3-S13; Figure 9] and their heat-treated counterpart [HA1-S13, HA2-S13, HA3-S13; Figure 11] from Spring 2013 presented similar bacterial counts (SPC and MSC, respectively) throughout shelf life, confirming that the pasteurization performed by the processing plant and the laboratory heat-treatment leave a similar bacterial population in absence of PPC (Huck et al., 2007a; Huck et al., 2007b). Certainly the evaluation of the DNA sequences of the isolates recovered from these samples would help support these findings. Pasteurized milk samples collected in Spring and Fall 2012 (Figures 7 and 8) were not heat-treated in the laboratory, consequently, a conclusion regarding the source of contamination for those samples based solely on this data could not be made. Perhaps

the analysis of the DNA sequences of the isolates recovered from those samples could shed light on the source of contamination for those samples.

Although, a high quality raw milk supply was used for the production of HTST pasteurized milk, none of the packaged pasteurized milk samples reached a shelf life beyond 21 days (Figures 7-9). The percentage of milk samples, below the PMO specification (< 4.3 log CFU/mL; (FDA, 2011) during the shelf life study (21 days), decreased as the product moved along the milk chain, accounting for 94%, 50% and 0% of the heat-treated raw milk, in-line pasteurized milk and packaged pasteurized milk, respectively. Thus, it can be concluded that plant factors (PPC and processing conditions) might have a greater influence on pasteurized milk shelf life than raw milk quality as other studies have indicated (Fromm and Boor, 2004; Martin et al., 2011). In particular, bacterial spoilage of pasteurized milk due to PPC has been associated with the filling process (Ralyea et al., 1998).

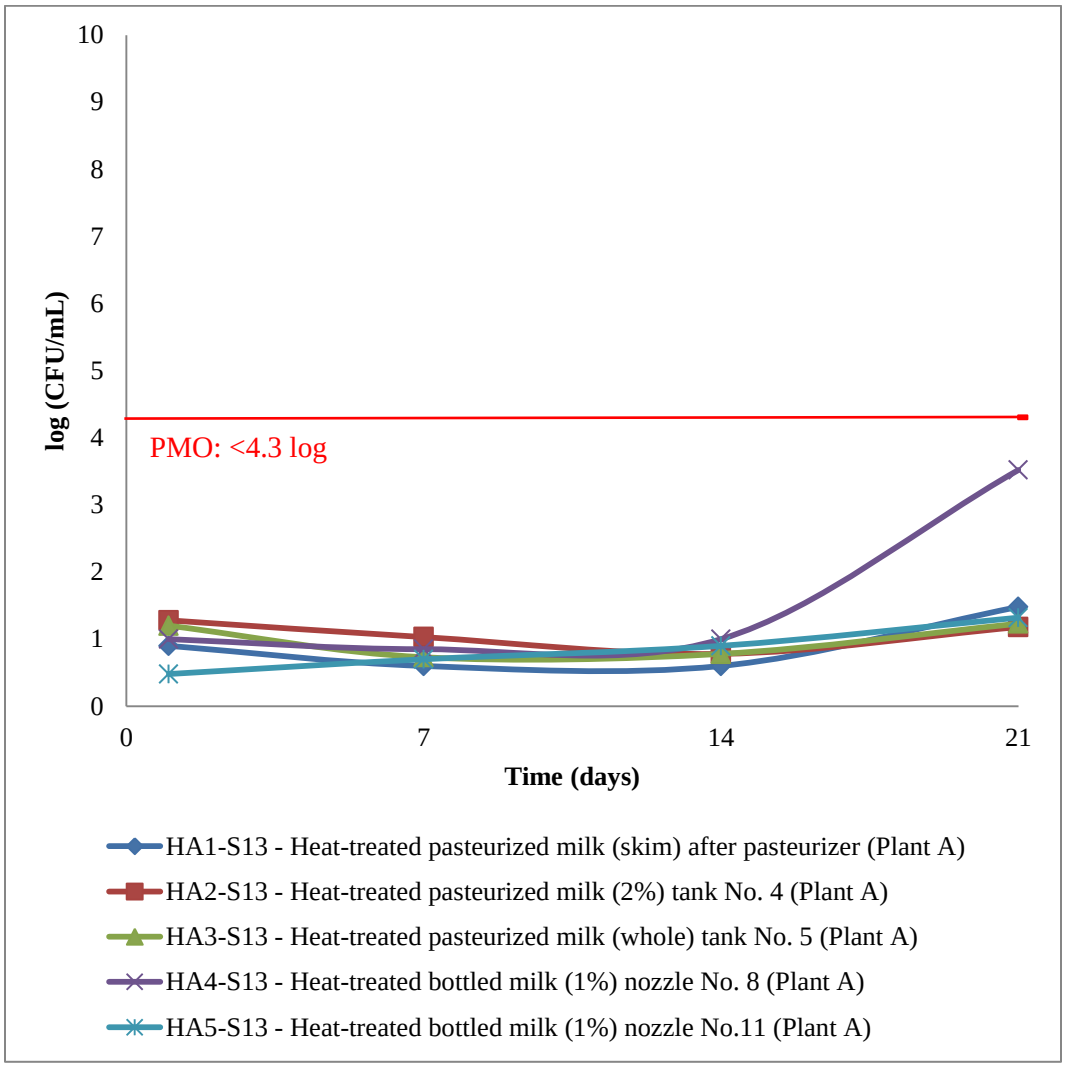

Figure 11. Mesophilic spore count (MSC) for heat-treated pasteurized milk samples collected at Plant A in Spring 2013. Aliquots of pasteurized milk samples were heat-treated at 80°C for 12 min, held at <7°C and plated over their shelf life.

3. Microbiological analysis of laboratory heat-treated environmental samples (farm and plant) throughout shelf life

On day 1, MSC of environmental samples from the farm (Figure 12) ranged from less than 20 CFU/g to 7.68 log CFU/g indicating the initial and variable presence of sporeformers from farm environment that survived laboratory heat-treatment. In general, heat-treated bedding material (inorganic sand) and manure samples (Figure 12) had the highest counts initially and throughout refrigerated storage, starting from 4.25 to 6.64 log CFU/g on day 1 and reaching from 5.43 to 8.27 log CFU/mL on day 21. Moreover, MSC in most samples increased 1-2 logs during cold storage and there was not much variation between samples. A study conducted at a dairy farm in the state of New York (Huck et al., 2008) showed that the total bacteria counts (TBC) over time increased greatly for manure samples and remained constant for fresh bedding samples (shredded paper and sawdust).

MSC of drinking water and feed samples (Figure 12) ranged from less than 20 CFU/g to 7.68 log CFU/g on day 1. Most samples did not show much variation in MSC throughout 21 days of refrigerated storage. Only two samples showed a greater than 3 log increase in MSC after storage at <7°C, including premix [14-F12] and drinking water [A4-S13]. Nonetheless, there was a high variation between and within sample types, especially in the premix and corn silage. Many studies have indicated silage as a possible source of sporeformers and have reported a great variation in MSC of these samples. For example, opened corn silage from commercially dairy farms in the Netherlands showed MSC ranging from 4.5 mean log CFU/mL in core samples to 7.7 mean log CFU/g in

samples with visible mold (Driehuis et al., 2009). Furthermore, corn silage collected at an experimental farm in Italy presented initial MSC of 2.65 log CFU/g that increased to 9.30 log CFU/g after 14 days of aerobic exposure (Borreani et al., 2013).

The set of samples collected at the milking parlor (Figure 12) showed MSC ranging from 0.30 to 4.51 log CFU/g and from less than 2 CFU/g to 6.36 log CFU/mL on day 1 and 21, respectively. There was also some variation in MSC, but mostly between sample groups. For example, the rinse water and the used towel presented the lowest (1.36 mean log CFU/g) and the highest counts (3.65 mean log CFU/g) throughout refrigerated storage, respectively. Furthermore, only one sample of the teat cups [A8-S13] and one of the teats [A9-S13] showed a 1.00 and 3.07 log CFU/g increase after storage at <7°C, respectively.

Heat-treated environmental samples from the processing plant (Figure 12) did also showed variable initial SPC, ranging from less than 2 CFU/mL to 2.53 log CFU/g on day 1. Six samples showed a 2-5 log increase in SPC after refrigerated storage, including three rinse water from tanks [47-S12, 47-F12, 55-F12], one rinse water from the mixer [48-F12], one swab from the filler surface [49-S12] and one swab from a filler's nozzle [50-S12].

All the heat-treated samples that showed increase in MSC after storage at <7°C could be the source of the psychrotrophic sporeformers that survived the pasteurization process at the plant and showed growth under refrigerated condition. However,

confirmation on these findings will only come with the analysis of the DNA sequences of the sporeformers isolated from milk and environmental samples.

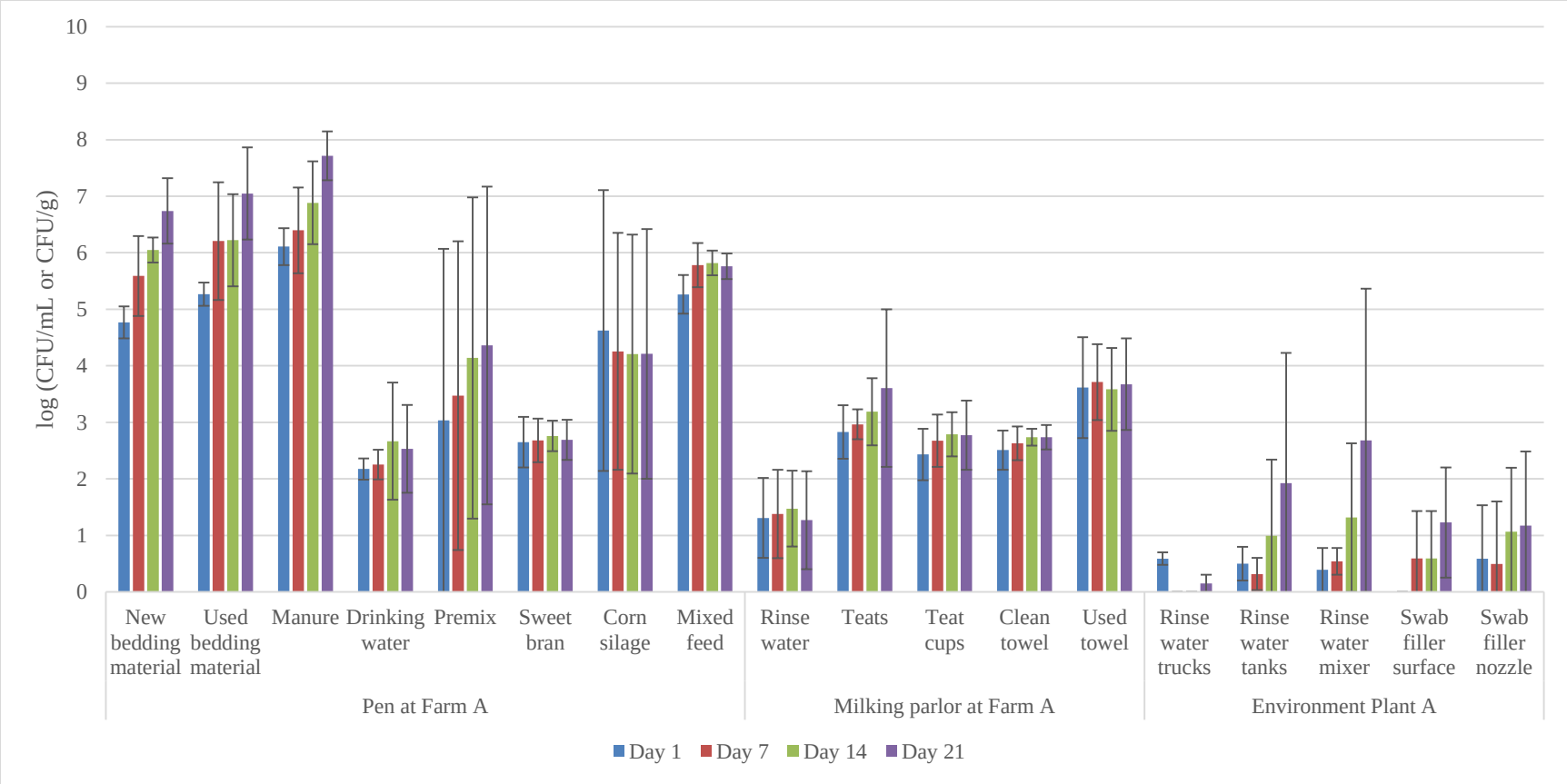

Figure 12. Mesophilic Spore Count (MSC) for all heat-treated environmental samples collected at Farm A and Plant A in Spring 2012, Fall 2012 and Spring 2013. Environmental samples were heat-treated at 80°C for 12 min, held at <7°C and plated over their shelf life. Bars indicate standard deviation. The detection limit for liquid and solid environmental samples was <2 and <20 CFU/mL, respectively.

4. *rpoB* Sequencing

A total of 475 isolates were selected from day (d) 1, 7, 14 and 21 from the bacterial counts (MSC or SPC) plates for subtyping based on their *rpoB* sequences. These included 166 isolates from Spring 2012 (d1=51, d7=34, d14=33 and d21=48), 155 isolates from Fall 2012 (d1=52, d7=23, d14=25 and d21=55) and 154 isolates from Spring 2013 (d1=51, d7=26, d14=29 and d21=48). Among these isolates, only 313 yielded *rpoB* products that could be sequenced. The other 162 isolates were characterized by partial 16S rDNA sequencing because either the *rpoB* PCR amplification yielded more than one product or the PCR primers (Drancourt et al., 2004) did not yield an *rpoB* PCR product.

From those 313 isolates that produced a single *rpoB* PCR product, a total of 94 unique *rpoB* allelic types (ATs), representing *Bacillus* spp. and closely related genera (i.e. *Lysinibacillus* spp., *Solibacillus* spp.) and *Paenibacillus* spp., were identified (Figure 13). Furthermore, 61 *rpoB* ATs were represented by only one isolate and 23 *rpoB* ATs were represented by ≥2 and ≤5 isolates. Only 10 *rpoB* ATs identified as *Bacillus licheniformis* s.l. (AT001, AT003, AT005, AT025), *B. subtilis* s.l. (AT022), *B. safensis* (AT010, AT012, AT013, AT028) and *Paenibacillus odorifer* (AT049) were represented by more than 5 isolates, which accounted for 62% of all isolates identified (Figure 14). The most frequently isolated *Bacillus* ATs were *Bacillus licheniformis* AT001 (n=105 isolates), AT003 (n=17), AT005 (n=14) and AT025 (n=15).

Figure 13. *rpoB*-based phylogenetic tree for the 94 sequences of the unique *rpoB* allelic types identified among 313 sporeforming bacterial isolates using the Neighbor-Joining Method. The bar at the bottom of the tree indicates the length representing 0.03-nt substitutions per site, the bootstrap values above 70 are shown in each branch and the value of n indicates the number of isolates representing each species.

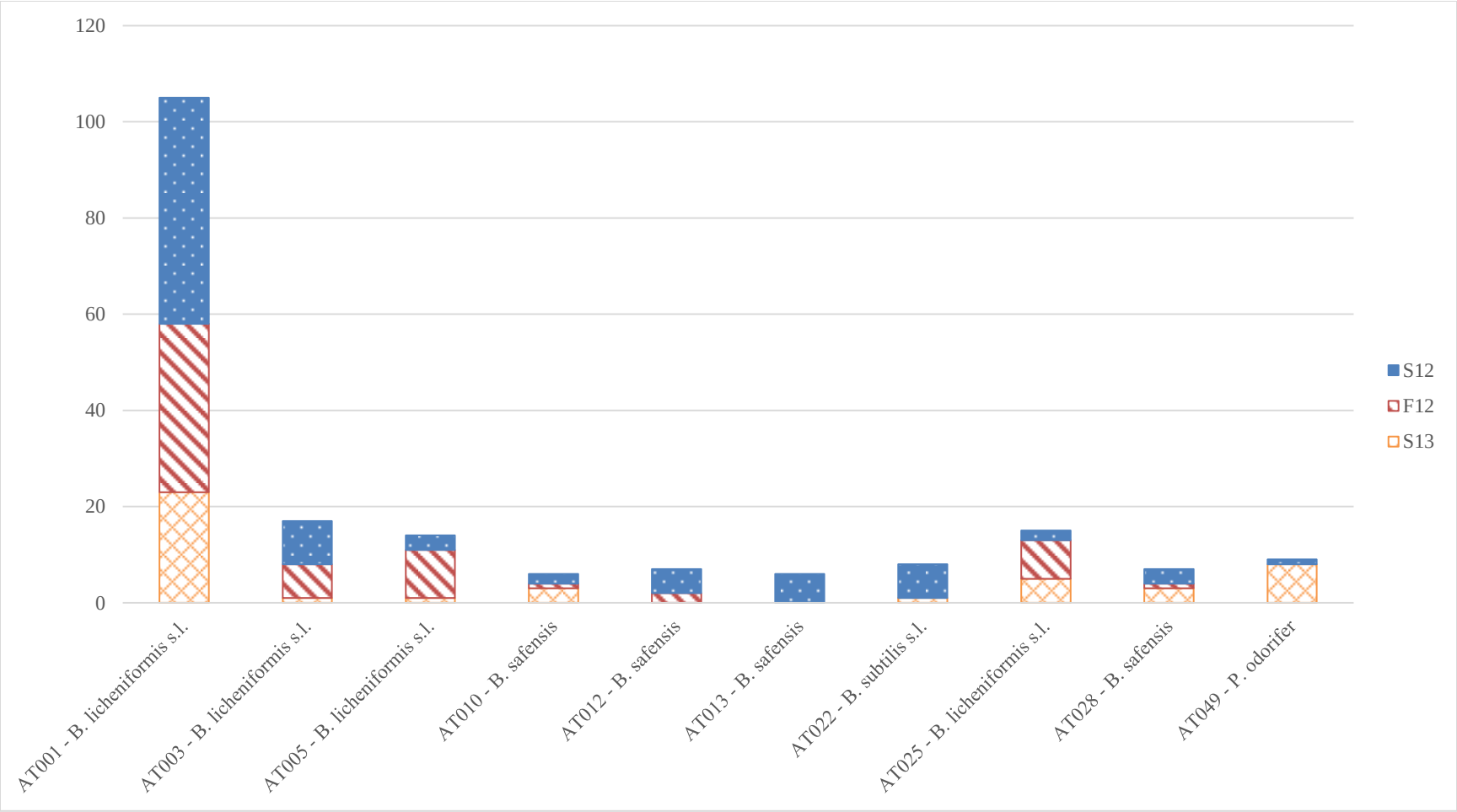

Figure 14. Distribution of the 10 most frequently isolated *Bacillus* and *Paenibacillus rpoB* allelic types (ATs) among 313 isolates obtained during Spring 2012 [S12], Fall 2012 [F12] and Spring 2013 [S13] from Plant A and Farm A. Each season is coded by color.

5. Cluster Analysis of *rpoB* ATs

The phylogenetic tree (Figure 13) obtained from the *rpoB* ATs identified in this study shows that all *rpoB* AT were classified into two distinct clusters. The first well-supported (BS, 81) phylogenetic group comprises isolates that cluster with the genus *Bacillus* and closely related genera (95% of isolates) and the second well-supported (BS, 99) group represents the genus *Paenibacillus* (5%). Previous related studies had already stated the grouping of sporeforming bacteria in these two main clusters (Huck et al., 2007a; Huck et al., 2007b; Ivy et al., 2012). In addition, the *Bacillus* clade could be further divided into four main well-supported subdivisions (BS>70), including groups I, II, III and IV. Group I is a well-supported group (BS, 82) represented by 65 different *rpoB* ATs, classified as *B. licheniformis* s.l. clades 1, 2 and 3 (156 isolates), *B. subtilis* s.l. clades 1 to 4 (36 isolates), *B. aerophilus* s.l. (15 isolates), *B. pumilus* clades 1, 2 and 3 (6 isolates), and *B. safensis* (57 isolates). Moreover, Group II (BS, 99) is represented by 4 unique ATs characterized as *B.* cf. *megaterium* (4 isolates). Group III is also a well-supported group (BS, 99) representing 5 unique *rpoB* ATs identified as *B. cereus* s.l. (4 isolates) and *B. psychrosaccharalyticus* (2 isolates). Moreover, group IV (BS, 99) is represented by 10 *rpoB* ATs that cluster together with genera distinct from *Bacillus*, including *Lysinibacillus* spp. (6 isolates), *Solibacillus* spp. (6 isolates) and *Rummeliibacillus pycnus* (1 isolate). A few small clades representing 3 unique ATs did not cluster within these major groups. Isolates in these clades were identified as *B. flexus* (1 isolate), *B. sporothermodurans* (1 isolate) and *B. nealsonii* (1 isolate).

Likewise, the *Paenibacillus* clade could be divided into two main well-supported subdivisions (BS>70), including groups V and VI. Group V is a well-supported clade (BS, 99) that includes two unique AT identified as *P. lautus* (1 isolate) and *Paenibacillus* spp. (1 isolate). Finally, group VI (BS, 99) is represented by five AT characterized as *P. odorifer* (14 isolates) and *Paenibacillus* spp. (1 isolate). In general, the sporeforming diversity based on the *rpoB* gene was characterized mainly by *B. licheniformis* s.l. (50% of isolates), *B. safensis* (18%), *B. subtilis* s.l. (12%), *B. aerophilus* s.l. (5%) and *P. odorifer* (4%), which represent 89% of all isolates (Figure 13).

6. Partial 16S rDNA sequencing

The 162 isolates that could not be characterized using the *rpoB* gene were then evaluated by partial 16S rDNA sequencing (Figures 15-17). The results obtained showed the presence of 33 unique 16S allelic types (SATs) of sporeforming bacteria among 140 isolates identified as *Bacillus* spp. (78 isolates), *Paenibacillus* spp. (30 isolates), *Brevibacillus* spp. (26 isolates), *Lysinibacillus* spp. (3 isolates), *Aneurinibacillus aneurilyticus* (1 isolate), *Terribacillus saccharophilus* (1 isolate) and *Paenisporosarcina* spp. (1 isolate). Furthermore, the other 22 isolates, that could not be sequenced using the *rpoB* gene, were represented by 11 unique 16S rDNA SATs characterized as non-sporeforming bacteria, including *Pseudomonas* spp. (8 isolates), *Acinetobacter baumanii* (2 isolates), *Exiguobacterium* spp. (5 isolates), *Pantoea* spp. (2 isolates), *Lactococcus* spp. (1 isolate), *Leuconostoc mesenteroides* (1 isolates), *Enterobacter* spp. (1 isolate), *Psychrobacter* spp. (1 isolate) and *Kurthia gibsonii* (1 isolate) These SATs were detected in pasteurized milk samples from all seasons (S12, F12 and S13; Figure 18), indicating

contamination of these samples with non-sporeforming bacteria after processing due to PPC. This elucidates the source of contamination for some of the pasteurized milk samples collected in Spring 2012 and Fall 2012. It also confirms the findings that some of the pasteurized milk samples collected in Spring 2013 were indeed contaminated with psychrotrophic non-sporeformers, since their heat-treated counterpart had their spoilage microflora eliminated by the thermal treatment (Objective 2; Figure 19).

Moreover, among the isolates that could be sequenced using the *rpoB* gene, 33 isolates were also characterized by partial 16S sequencing to confirm their identity. Although 32 unique *rpoB* ATs had been characterized among these 33 isolates, only 15 different 16S SATs of sporeforming bacteria were identified, including 9 unique SATs that had not been found among the other 140 sporeformers isolates (Figures 15 and 16). These results confirm that the *rpoB* gene is less well conserved in closely related species in comparison to the 16S rDNA gene (Yamada et al., 1999; Drancourt et al., 2004; Blackwood et al., 2004; La Duc et al., 2004), thus *rpoB* sequencing offers a greater discriminatory power (Huck et al., 2007b). However, not all the sporeformers isolates could be identified with this subtyping method, indicating the need for further optimization of PCR protocols and/or the implementation of multilocus sequence typing (MLST; Huck et al., 2007b).

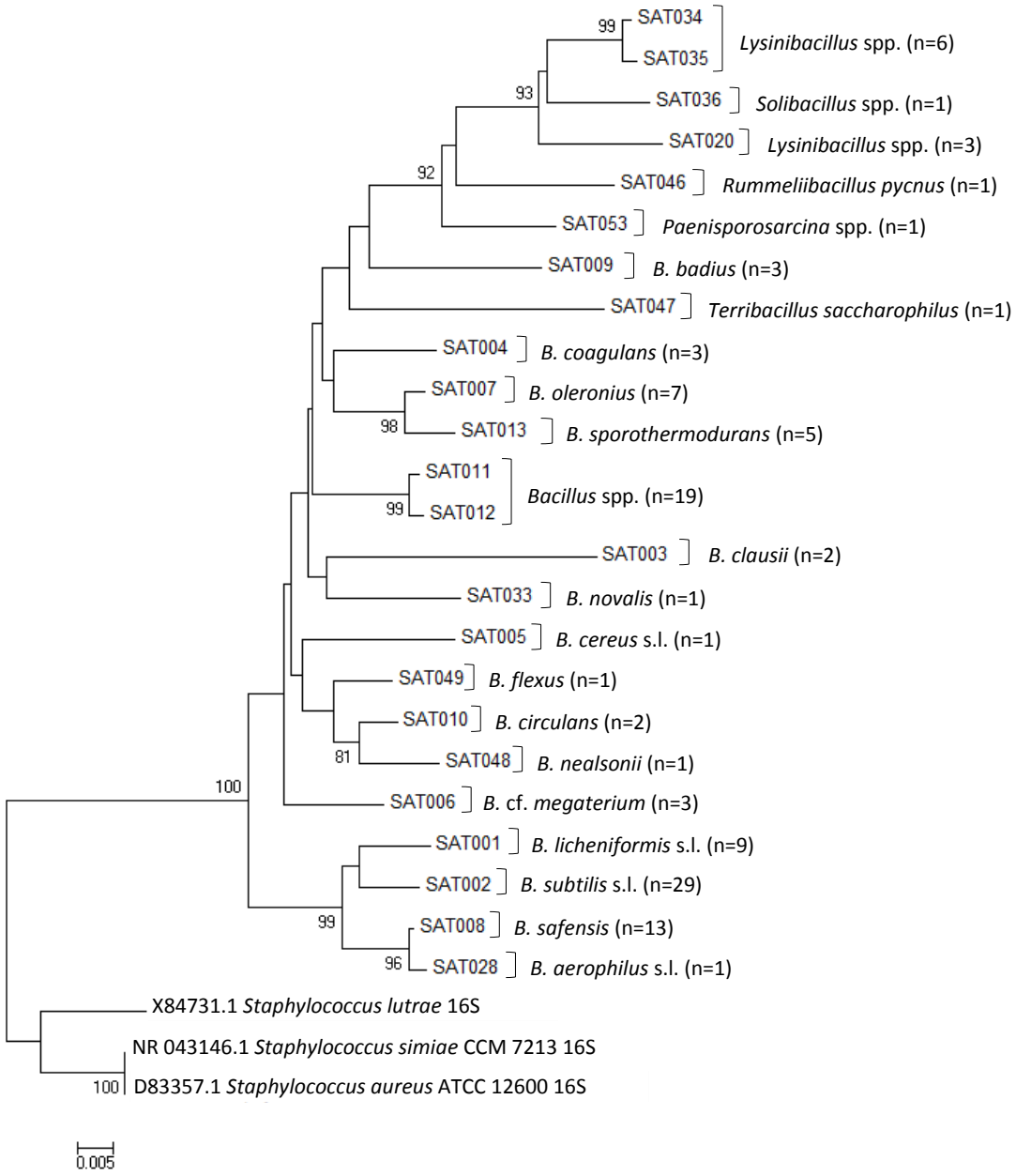

Figure 15. 16S-based phylogenetic tree for the 24 sequences of the unique 16S allelic types (SATs) of *Bacillus* spp. and related genera identified among 113 sporeforming bacterial isolates using the Neighbor-Joining Method. The bar at the bottom of the tree indicates the length representing 0.005-nt substitutions per site, the bootstrap values above 70 are shown in each branch and the value of n indicates the number of isolates representing each species.

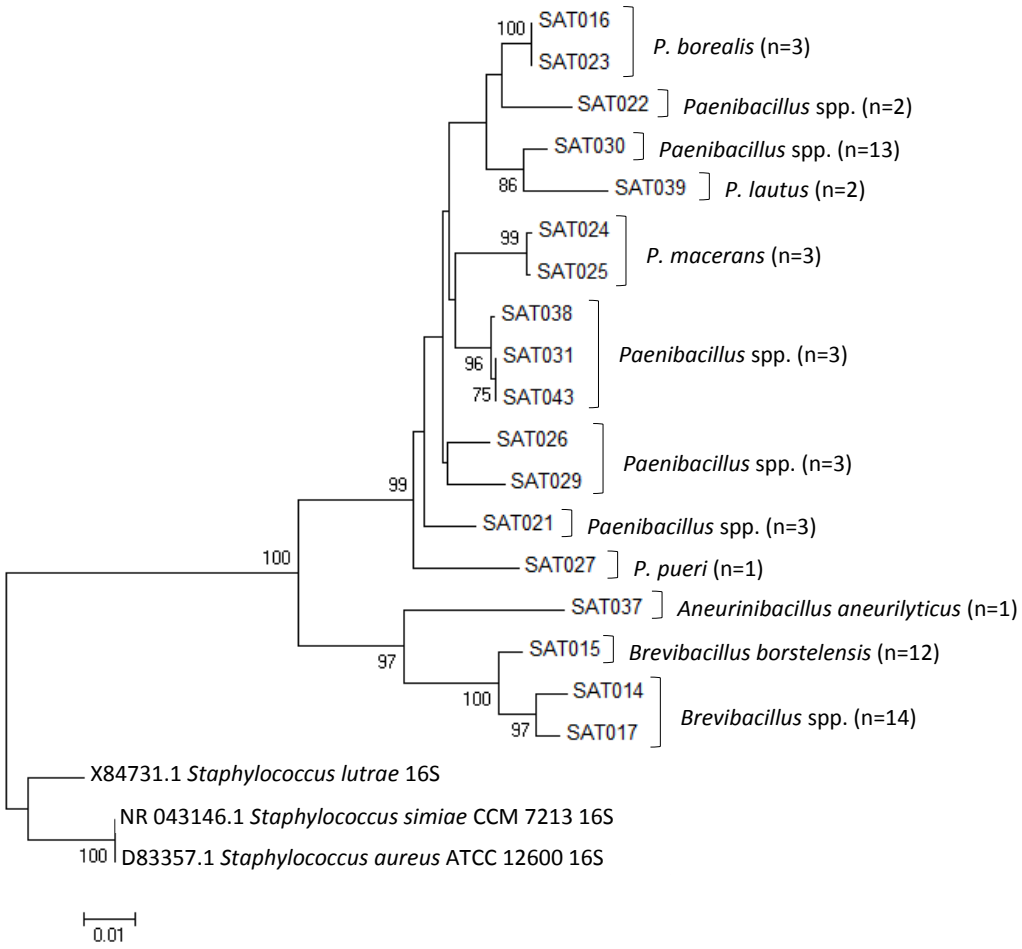

Figure 16. 16S-based phylogenetic tree for the 18 sequences of the unique 16S allelic types (SATs) of *Paenibacillus* spp. and related genera identified among 60 sporeforming bacterial isolates using the Neighbor-Joining Method. The bar at the bottom of the tree indicates the length representing 0.01-nt substitutions per site, the bootstrap values above 70 are shown in each branch and the value of n indicates the number of isolates representing each species.

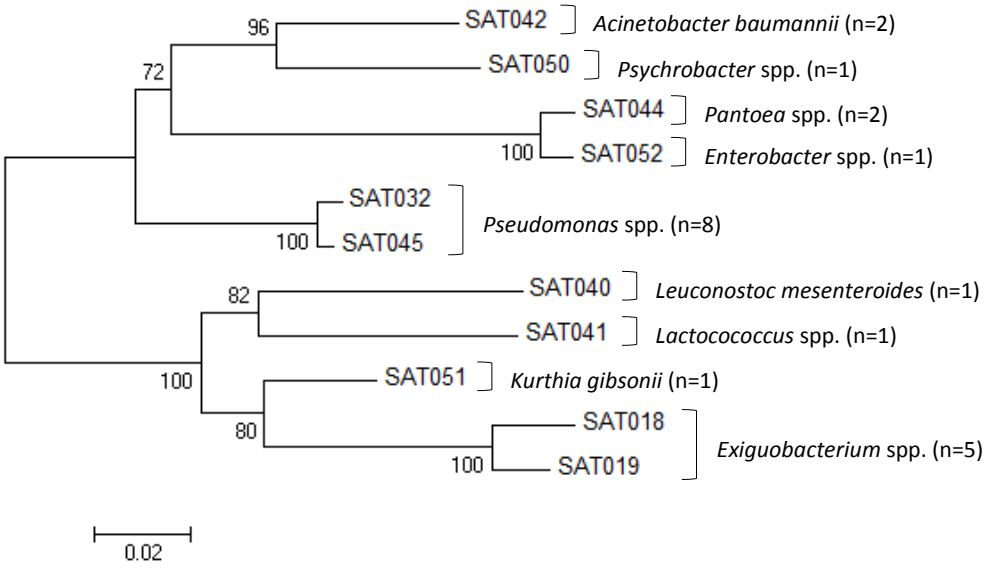

Figure 17. 16S-based phylogenetic tree for the 11 sequences of the unique 16S allelic types (SATs) of non-sporeforming bacteria identified among 22 bacterial isolates using the Neighbor-Joining Method. No outgroup was used as many diverse genera were identified. The bar at the bottom of the tree indicates the length representing 0.02-nt substitutions per site, the bootstrap values above 70 are shown in each branch and the value of n indicates the number of isolates representing each species.

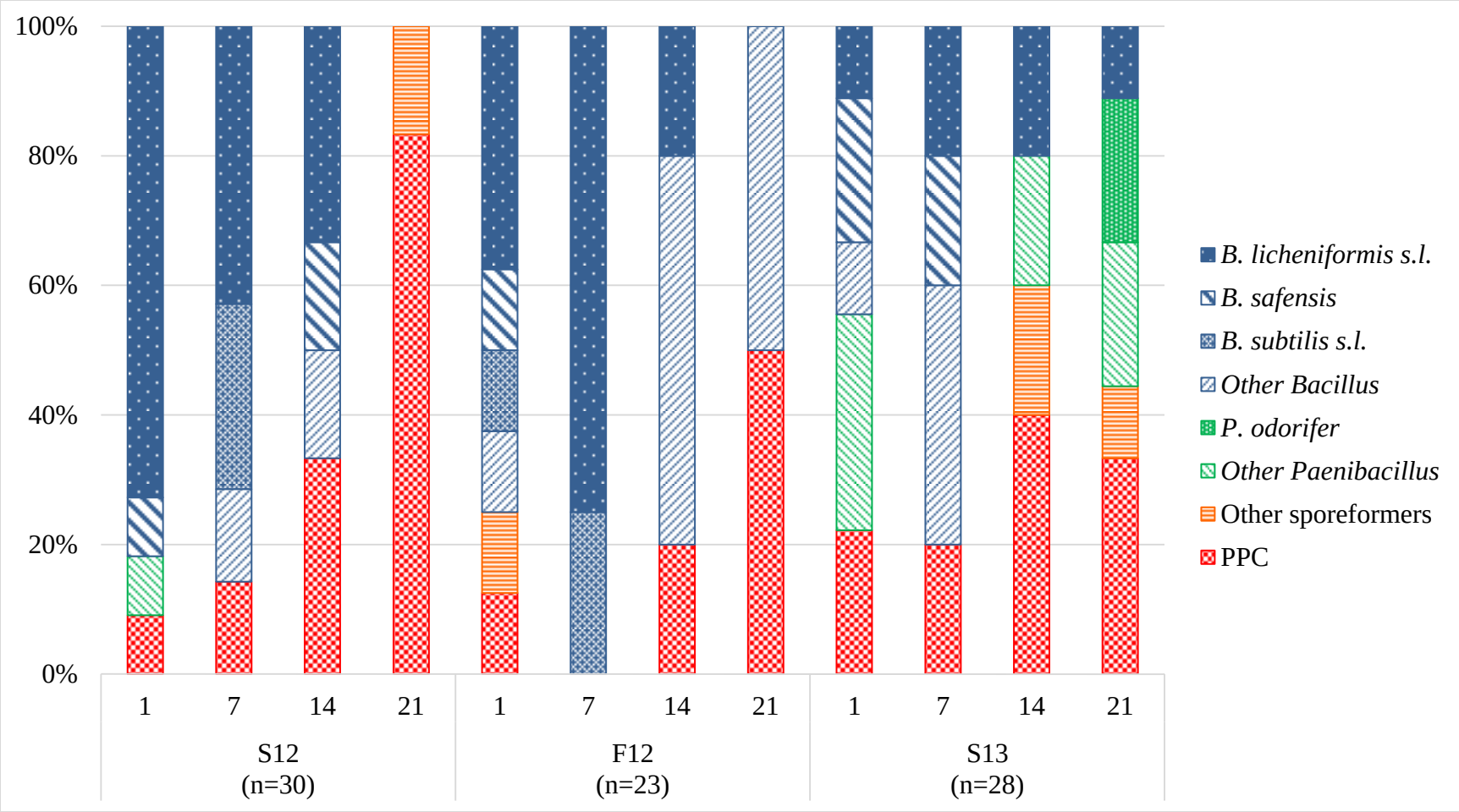

Figure 18. Microbial diversity based on *rpoB* and partial 16S sequences in pasteurized milk by time point (days 1, 7, 14 and 21) for Spring 2012 [S12], Fall 2012 [F12] and Spring [S13]. The value of n indicates the number of isolates identified in each season.

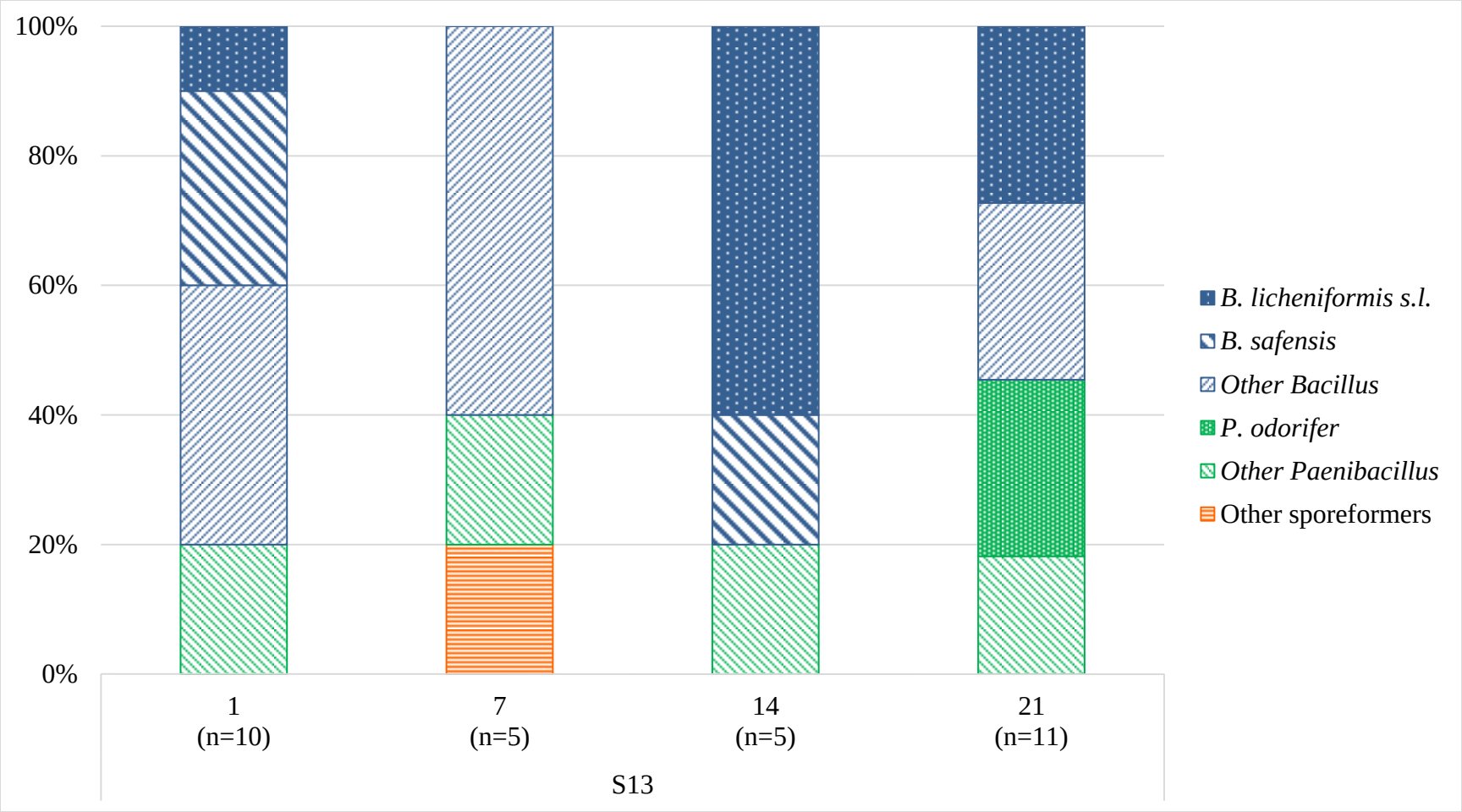

Figure 19. Microbial diversity based on *rpoB* and partial 16S sequences in heat-treated pasteurized milk by time point (days 1, 7, 14 and 21) for Spring [S13]. The value of n indicates the number of isolates identified in each time point.

7. Microbial diversity in milk and environmental samples

A substantial diversity of sporeforming bacteria was identified throughout the farm-to-table continuum as other studies had previously reported (Huck et al., 2007a; Huck et al., 2008). Furthermore, when combining the *rpoB* and partial 16S data, a variation in species and proportion of the microbial diversity was observed within seasons, sources and time points. The bacterial community in environmental samples from the dairy farm and the processing plant was dominated by *Bacillus* spp. (80% and 87% of the isolates, respectively) initially and throughout shelf life for all three seasons (Figure 20). The main *Bacillus* species that were identified in these two locations were *B. licheniformis* (30% and 28%, respectively), *B. subtilis* (20% and 18%) and *B. safensis* (16% and 8%).

Moreover, there was also a high number of isolates in heat-treated raw milk samples that were identified as *Bacillus* spp., representing 84% of the isolates collected from this source during all time points and seasons (Figure 20). The percentage of *Paenibacillus* and other sporeforming bacteria in these samples was 10% and 6%, respectively. The main species identified in heat-treated raw milk samples were represented by *B. licheniformis* (50%), *B. safensis* (16%), *B. subtilis* (5%) and *P. odorifer* (5%). In particular, most of the *Paenibacillus* spp. isolates were collected during Spring 2013 (Figure 21). In this season the *Bacillus* spp. population was more prevalent at the beginning of the study (day 1), but decreased after refrigerated storage for 21 days (from 56% to 44%). On the other hand, an increase in the *Paenibacillus* spp. (from 22 % to 44%) was observed over time.

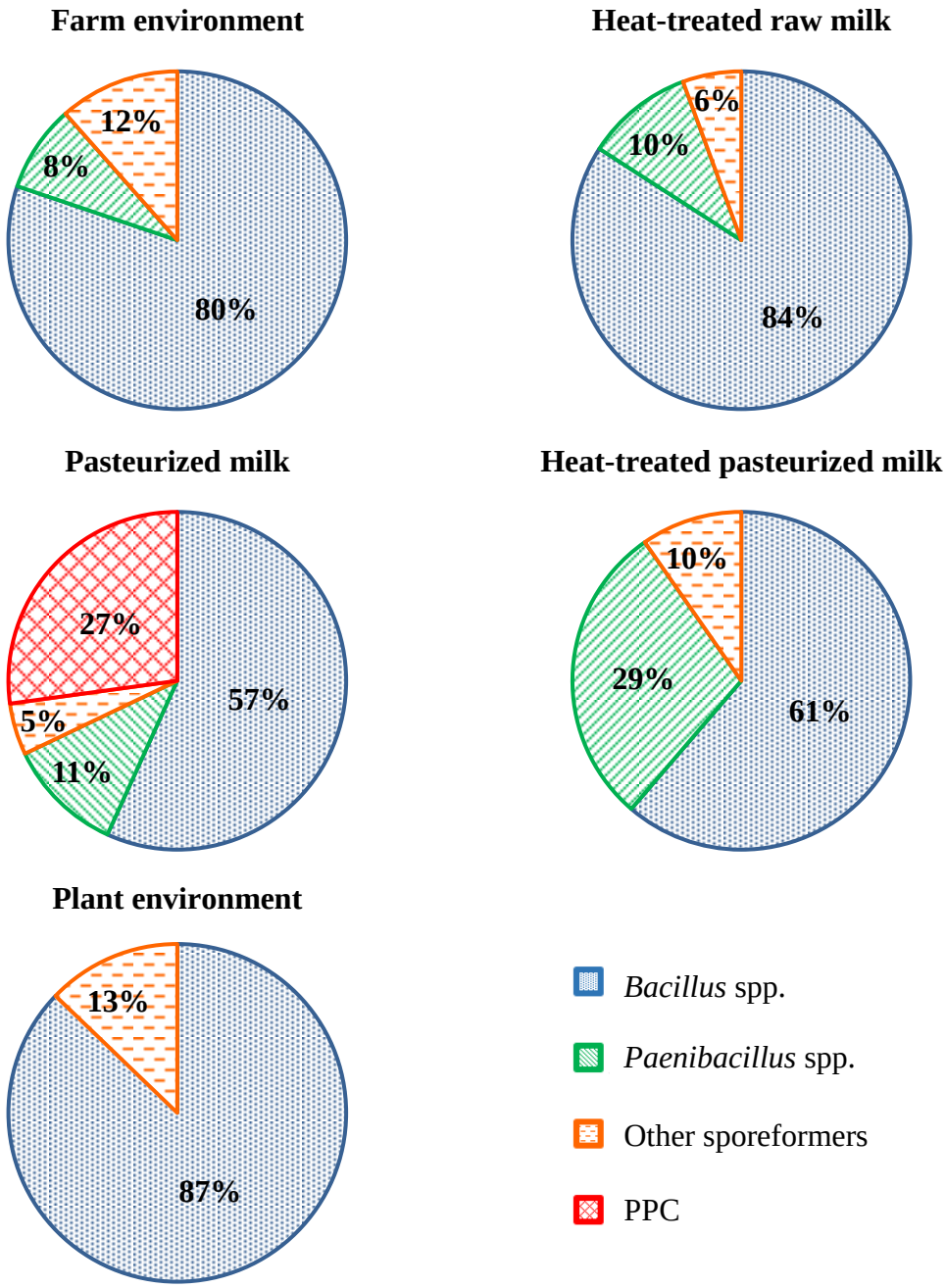

Figure 20. Distribution of *Bacillus* spp., *Paenibacillus* spp., other sporeformers and PPC isolates obtained from farm environment (n=217), heat-treated raw milk (n=107), pasteurized milk (n=81), heat-treated pasteurized milk (n=31) and plant environment (n=39) collected from Farm A and Plant A during Spring 2012, Fall 2012 and Spring 2013.

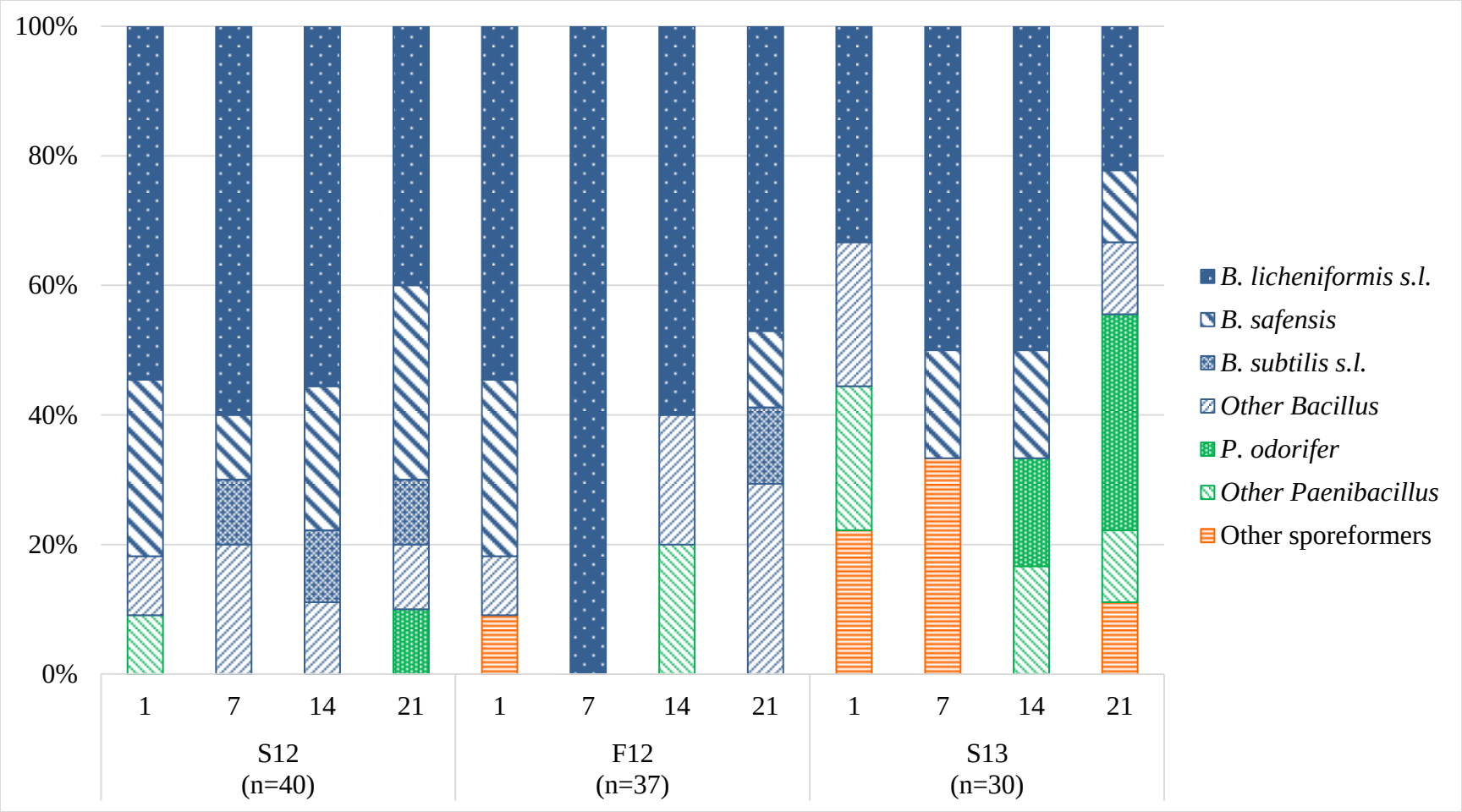

Figure 21. Microbial diversity based on *rpoB* and partial 16S sequences in heat-treated raw milk by time point (days 1, 7, 14 and 21) for Spring 2012 [S12], Fall 2012 [F12] and Spring 2013 [S13]. The value of n indicates the number of isolates identified in each season.

The microbial diversity based on *rpoB* and partial 16S sequences in pasteurized milk by time point for each season is presented in Figure 18. As noted in this figure, no tendency was observed for the change in population of pasteurized milk over shelf life during the three seasons that were evaluated. For example, the predominant organisms isolated from pasteurized milk at day 1 in Spring 2012 were *Bacillus* (82% of isolates), while non-sporeforming bacteria due to PPC (83%) represented the majority of microbes on day 21. Moreover, the predominant organisms at day 1 in Fall 2012 were also *Bacillus* (75%), but on day 21 there were equal amounts of *Bacillus* (50%) and non-sporeforming bacteria (50%). Lastly, the predominant microbes at day 1 in Spring 2013 were *Bacillus* (44%), but the initial population of *Paenibacillus* (33%) and non-sporeforming bacteria (22%) was also high. However, on day 21 the proportion of *Bacillus* (11%) decreased and more *Paenibacillus* (44%) and non-sporeforming bacteria (33%) were identified.

Previous studies have reported a shift in the predominant population of sporeforming bacteria from *Bacillus* spp. to *Paenibacillus* spp. during refrigerated storage of heat-treated raw milk and pasteurized milk (Huck et al., 2008; Ranieri and Boor, 2009). As previously described, in this study the shift was from *Bacillus* spp. to other *Bacillus* spp. and to non-sporeforming bacteria due to PPC in Spring 2012 and Fall 2012. However, Spring 2013 was the only season where a shift from *Bacillus* spp. to *Paenibacillus* spp. could be observed throughout shelf life, which can be attributed to a higher initial presence of *Paenibacillus* spp. in heat-treated raw and pasteurized milk samples (Figures 21 and 18, respectively) and to a lower incidence of PPC in pasteurized milk samples (Figure 18). This shift was even clearer when PPC was eliminated by heat-treatment of pasteurized milk samples in Spring 2013 (Figure 19). As mentioned before,

this population shift was also observed in heat-treated raw milk samples in Spring 2013 (Figure 21).

When the information presented in Figures 7, 8 and 9 (SPC of pasteurized milk samples during refrigerated storage; Objective 2) was combined with the identity of the isolates obtained from those samples, it was evident that in every season, the predominant microbial flora in packaged pasteurized milk (final product) was psychrotrophic non-sporeforming bacteria. In Spring 2012 and Fall 2012, these non-sporeformers were also found in pasteurized milk samples from some of the tanks sampled. These results indicate that pasteurized milk could have been contaminated after processing due to bacteria present in the plant environment, especially during moving the pasteurized milk into the tanks or during the filling process. In Spring 2013, this problem was only detected in the final product (packaged pasteurized milk from the filler) indicating a possible improvement of the sanitization and/or processing between the pasteurizer and the filler; however, the filler still seemed to be a problem. In conclusion, PPC was a bigger problem in Spring 2012 and Fall 2012 where final product and milk from the tanks showed contamination with these type of microorganisms; while the problem seemed to be reduced in Spring 2013 with only final product showing PPC, as discussed.

8. Contamination patterns throughout the milk chain: Identification of "potentially problematic sporeforming bacteria" and their sources

As previously described, a high diversity of sporeforming bacteria was evident along the milk chain, confirming the ubiquitous presence of these organisms (Huck et al., 2007b; Huck et al., 2008). However, only a few subtypes were frequently isolated (Figure

14) and even less were present in those samples that showed a significant increase in bacterial counts (SPC or MSC) throughout shelf life (Figures 9-13), indicating the prevalent presence of only certain psychrotrophic sporeforming bacteria in the milk chain. These organisms were classified as "potentially problematic sporeforming bacteria" (PPSB) based on their presence in laboratory heat-treated and/or commercially pasteurized milk samples with high bacterial counts (MSC or SPC) at the end of shelf life. Although PPSB are the main organisms of concern in the fluid milk industry, all other sporeforming bacteria that were identified either could become problematic if products are not stored under proper refrigeration during distribution and storage (Ivy et al., 2012) or could cause issues in other dairy industries (i.e. milk powder, condensed milk).

Tables 9-12 list all the milk samples that showed a greater than 2 log increase in bacterial counts (MSC or SPC) during 21 days of refrigerated storage, and include five *rpoB* ATs (*B. licheniformis* s.l. AT001 and AT025; *P. odorifer* AT049 and AT073; *B. safensis* AT012) and three 16S rDNA SATs (*Bacillus* spp. SAT011 and SAT012; *Paenibacillus* spp. SAT030) of PPSB. Table 13 includes environmental samples that showed a greater than 2 log increase in MSC. Among these, two plant samples [47-S12, 48-F12] had sporeformers with the same 16S sequences identified in milk samples; while two other environmental samples [47-F12, 55-F12] from the plant showed a greater than 4 log increase in MSC during storage, but those ATs (*Solibacillus* spp. AT083 and AT084) were not found in any other samples. Moreover, a 3 log increase in MSC was observed in an environmental sample from the farm [A9-S13], which contained a SAT (*Bacillus* spp. SAT011) also found in milk.

As mentioned before, and shown in Tables 9-12, 9 out of 16 pasteurized milk samples [44-S12, 52-S12, 53-S12, 54-S12, 43-F12, 52-F12, 53-F12, PA4-S13, PA5-S13] were contaminated with psychrotrophic non-sporeforming bacteria due to PPC. Consequently these samples showed very high counts in SPC after refrigerated storage, ranging from 6.53 to 8.62 log CFU/mL on day 21. Nonetheless, there were a few pasteurized milk samples [43-S12, 44-F12, 45-F12] along with heat-treated raw [1-S12, 57-F12, TA3-S13, TA4-S13] and pasteurized milk samples [HA4-S13] that showed a significant increase in SPC due to sporeforming bacteria, ranging from 3.26 to 6.30 log CFU/mL on day 21.

No isolates were recovered from one pasteurized milk sample [43-S12] on day 21. However, only sporeforming bacteria (*B. licheniformis* s.l. AT001 and AT005; *B. aerophilus* s.l. AT033) were found on day 14, thus it is unlikely that this sample was contaminated with non-sporeforming bacteria due to PPC. In addition, one heat-treated pasteurized milk sample [HA4-S13] was contaminated with yeast (based on gram-staining) beyond being contaminated with sporeforming bacteria. This yeast could have been present in this sample due to cross-contamination in the laboratory or it could be a heat-resistant yeast that survived processing.

Table 9. Microbial diversity in heat-treated raw milk samples with a higher than 2 log increase in Mesophilic Spore Count (MSC) during 21 days of refrigerated storage collected at Farm A and Plant A in Spring 2012, Fall 2012 and Spring 2013.

Code	Sample	Increase (log CFU/mL)	Sporeformer Day 21 [a]	Other sources
1-S12	Heat-treated raw milk (Farm A)	2.78 (d1=0.48 d21=3.26)	*P. odorifer* (AT049)	Heat-treated raw milk [TA2-S13, TA3-S13] Pasteurized milk [PA3-S13] Heat-treated pasteurized milk [HA1-S13] Teats [A9-S13] Teat cups [A8-S13] Manure [A6-S13]
			B. safensis (AT012)	Heat-treated raw milk [38-S12] Pasteurized milk [45-F12] Clean towel [10-S12] Manure [27-S12]
57-F12 [b]	Heat-treated raw milk (skim) tank No. 2 (Plant A)	4.42 (d1=1.48 d21=5.90)	*Bacillus* spp. (SAT012)	Pasteurized milk [45-F12] Rinse water tank [47-S12] Rinse water mixer [48-F12]
TA3-S13	Heat-treated raw milk (skim) tank No. 2 (Plant A)	2.33 (d1=1.00 d21=3.33)	*P. odorifer* (AT073)	Heat-treated raw milk [TA4-S13] Heat-treated pasteurized milk [HA2-S13]
			B. licheniformis (AT001)	In most samples (105 isolates) from farm environment, plant environment, pasteurized milk, heat-treated raw and pasteurized milk.
TA4-S13	Heat-treated raw milk (skim) before pasteurizer (Plant A)	2.90 (d1=1.18 d21=4.08)	*P. odorifer* (AT073)	Heat-treated raw milk [TA3-S13] Heat-treated pasteurized milk [HA2-S13]

a - Potentially problematic sporeforming bacteria (PPSB).

b - Heat-treated raw milk samples with counts above the regulatory limit (<20,000 CFU/mL or <4.3log CFU/mL) after 21 days of storage.

Table 10. Microbial diversity in pasteurized milk samples with a higher than 2 log increase in Standard Plate Count (SPC) during 21 days of refrigerated storage collected at Plant A in Spring 2012.

Code	Sample	Increase (log CFU/mL)	Sporeformer Day 21 [a]	Other sources	Non-sporeformer Day 21
43-S12	Pasteurized milk (2%) tank No. 4 and truck No. 1 (Plant A)	2.43 (d1=1.26 d21=3.69)	No isolates [b]		No isolates
44-S12 [c]	Pasteurized milk (whole) tank No. 5 (Plant A)	5.34 (d1=1.89 d21=7.23)			*Pseudomonas* spp. (SAT045)
52-S12 [c]	Bottled milk (2%) nozzle No. 8 (Plant A)	7.51 (d1=1.11 d21=8.62)			*Pseudomonas* spp. (SAT032)
53-S12 [c]	Bottled milk (2%) nozzle No. 11 (Plant A)	7.36 (d1=1.18 d21=8.54)			*Pseudomonas* spp. (SAT032)
54-S12 [c]	Pasteurized milk (2%) tank No. 4 and truck No. 2 (Plant A)	8.30 (d1=0.30 d21=8.60)			*Pseudomonas* spp. (SAT032)

a - Potentially problematic sporeforming bacteria (PPSB).

b - No isolates from D21, but from D14 only sporeforming bacteria were identified [*B. licheniformis* s.l. AT001 and AT005; *B. aerophilus* s.l. AT033].

c - Pasteurized milk samples with counts above the regulatory limit (<20,000 CFU/mL or <4.3log CFU/mL) after 21 days of storage.

Table 11. Microbial diversity in pasteurized milk samples with a higher than 2 log increase in Standard Plate Count (SPC) during 21 days of refrigerated storage collected at Plant A in Fall 2012.

Code	Sample	Increase (log CFU/mL)	Sporeformer Day 21 a	Other sources	Non-sporeformer Day 21
43-F12 [b]	Pasteurized milk (chocolate whole) tank No. 4 and truck No. 1 (Plant A)	5.11 (d1=1.58 d21=6.69)			*Leuconostoc mesenteroides* (SAT040)
44-F12 [b]	Pasteurized milk (chocolate 1%) tank No. 5 (Plant A)	4.65 (d1=1.65 d21=6.30)	*Bacillus* spp. (SAT011)	Pasteurized milk [53-F12] Teats [A9-S13] Clean towel [A10-S13] Manure [A6-S13]	
45-F12 [b]	Pasteurized milk after pasteurizer (2%) (Plant A)	2.75 (d1=1.57 d21=4.31)	*Bacillus* spp. (SAT012)	Heat-treated raw milk [57-F12] Rinse water tank [47-S12] Rinse water mixer [48-F12]	
52-F12 [b]	Bottled milk nozzle No. 8 (chocolate skim) (Plant A)	4.74 (d1=1.79 d21=6.53)			*Lactococcus* spp. (SAT041)
53-F12[b]	Bottled milk nozzle No. 11 (chocolate skim) (Plant A)	6.54 (d1=1.86 d21=8.41)			*Pantoea* spp. (SAT044)

a - Potentially problematic sporeforming bacteria (PPSB).

b - Pasteurized milk samples with counts above the regulatory limit (<20,000 CFU/mL or <4.3log CFU/mL) after 21 days of storage.

Table 12. Microbial diversity in pasteurized and heat-treated pasteurized milk samples with a higher than 2 log increase in Standard Plate Count (SPC) during 21 days of refrigerated storage collected at Plant A in Spring 2013.

Code	Sample	Increase (log CFU/mL)	Sporeformer Day 21 a	Other sources	Non-sporeformer Day 21
PA4-S13 [b]	Bottled milk (1%) nozzle No. 8 (Plant A)	4.45 (d1=2.21 d21=6.66)			*Exiguobacterium* spp. (SAT018)
PA5-S13 [b]	Bottled milk (1%) nozzle No.11 (Plant A)	4.56 (d1=2.31 d21=6.87)			*Exiguobacterium* spp. (SAT018/SAT019) *Psychrobacter* spp. (SAT050)
HA4-S13 [b]	Heat-treated bottled milk (1%) nozzle No. 8 (Plant A)	2.52 (d1=1.00 d21=3.52)	*Paenibacillus* spp. (SAT030)	Heat-treated raw milk [TA2-S13, TA4-S13] Pasteurized milk [PA3-S13] Heat-treated pasteurized milk [HA2-S13, HA3-S13, HA5-S13] Corn silage [18-F12] Sweet bran [17-F12]	
			B. licheniformis (AT025)	Heat-treated raw milk [40-S12, 42-F12, TA4-S13] Pasteurized milk [44-F12, 45-F12] Heat-treated pasteurized milk [HA1-S13, HA5-S13] Multiple sources at the farm environment (6 isolates) Rinse water mixer [48-F12]	
					Yeast (Gram-staining)

a - Potentially problematic sporeforming bacteria (PPSB).

b - Pasteurized milk samples with counts above the regulatory limit (<20,000 CFU/mL or <4.3log CFU/mL) after 21 days of storage.

Table 13. Microbial diversity in heat-treated environmental samples with a higher than 2 log increase in Mesophilic Spore Count (MSC) during 21 days of refrigerated storage collected at Farm A and Plant A in Spring 2012, Fall 2012 and Spring 2013.

Code	Sample	Increase (log CFU/mL)	Sporeformer Day 21 a	Other sources	Non-sporeformer Day 21
47-S12	Rinse water tank (Plant A)	2.09 (d1=0.48 d21=2.56)	*Bacillus* spp. (SAT012)	Heat-treated raw milk [57-F12] Pasteurized milk [45-F12] Rinse water mixer [48-F12]	
47-F12	Rinse water tank (Plant A)	5.40 (d1=0.85 d21=6.25)	*Solibacillus* spp. (AT084)	None	
48-F12	Rinse water mixer (Plant A)	4.58 (d1=0.78 d21=4.58)	*Bacillus* spp. (SAT012)	Heat-treated raw milk [57-F12] Pasteurized milk [45-F12] Rinse water tank [47-S12]	
55-F12	Rinse water tank (Plant A)	3.20 (d1=0.95 d21=3.20)	*Solibacillus* spp. (AT083)	None	
A9-S13	Teats (Farm A)	3.07 (d1=3.29 d21=6.36)	*Bacillus* spp. (SAT011)	Pasteurized milk [44-F12, 53-F12] Clean towel [A10-S13] Manure [A6-S13]	
			Paenibacillus spp. (SAT022)	Drinking water [A4-S13]	

a - Potentially problematic sporeforming bacteria (PPSB).

In general, bacterial counts (SPC or MSC) on day 21 were higher for milk samples contaminated with non-sporeforming bacteria than with sporeforming bacteria (Tables 9-12). In particular, milk samples with spoilage by *Pseudomonas* spp. [44-S12, 52-S12, 53-S12, 54-S12] and *Pantoea* spp. [53-F12] showed the highest SPC (7.23, 8.62, 8.54, 8.60, 8.41 log CFU/mL, respectively) on day 21. In contrast, milk samples with spoilage by sporeforming bacteria [1-S12, 57-F12, TA3-S13, TA4-S13, 43-S12, 44-F12, 45-F12, HA4-S13] showed lower bacterial counts (3.26, 5.90, 3.33, 4.08, 3.69, 6.30, 4.31, 3.52 log CFU/mL, respectively). These results confirm that gram-negative bacteria grow faster in refrigerated pasteurized milk than sporeforming bacteria, thus disguising the presence of sporeformers at the end of shelf life (Ralyea et al., 1998; Ranieri and Boor, 2009).

None of the PPSB were found exclusively in pasteurized milk samples and their potential sources were identified (Tables 9-12). First, the same allelic types (*P. odorifer* AT049 and AT073; *B. safensis* AT012; *B. licheniformis* s.l. AT001 and AT025; *Bacillus* spp. SAT012; *Paenibacillus* spp. SAT030) were identified in both raw and pasteurized milk, confirming the raw milk supply as a source of sporeforming bacteria (Huck et al., 2007a) and the ability of these organisms to survive pasteurization conditions (Collins, 1981). Consequently, the quality and shelf life of pasteurized fluid milk will depend in part on the initial quality of the raw milk supply, especially in absence of PPC (Martin et al., 2011). Producers need to control sporeforming bacteria at the farm level to supply high quality raw milk (Huck et al., 2008). Thus, the development of tools to consistently detect PPSB in raw milk has been recommended in the literature (Martin et al., 2011).

Many allelic types of PPSB listed in Tables 9-13 were present in heat-treated milk, pasteurized milk and in the farm and plant environment, indicating that these bacteria can access the milk chain from multiple sources as reported by previous studies (Huck et al., 2007b; Huck et al., 2008). In particular, the farm environment plays an important role and sporeformers can access the raw milk supply at this point (Vaerewijck et al., 2001; Te Giffel et al., 2002; Scheldeman et al., 2005; Huck et al., 2008), affecting the quality of pasteurized milk at the beginning of the milk chain. Several allelic types of PPSB were identified in milk and in environmental samples from the farm, including *P. odorifer* AT049 (found in teats [A9-S13], teat cups [A8-S13] and manure [A6-S13]), *Paenibacillus* spp. SAT030 (corn silage [18-F12] and sweet bran [17-F12]), *Bacillus* spp. SAT011 (teats [A9-S13], clean towel [A10-S13] and manure [A6-S13]) and *B. licheniformis* s.l. AT001 and AT025 (multiple sources at the farm level).

Although no PPSB were found exclusively in pasteurized milk, two allelic types of PPSB (*P. odorifer* AT073 and *Bacillus* spp. SAT012) were identified only in milk (heat-treated raw and pasteurized) and environmental samples from the processing plant, indicating a possible contamination of milk with sporeformers at plant level. Furthermore, two ATs (*Solibacillus* spp. AT083 and AT084) were identified only in rinse water from tanks at the processing plant. Even if these ATs were not found in milk samples, they were considered as PPSB, because pasteurized milk is in direct contact with these tanks. Based on these results, there is a possibility of contamination of final product after pasteurization due to sporeforming bacteria present in the processing plant environment. To better control this source of contamination, it is important to evaluate

the ability of these isolates to form biofilms and the efficacy of recommended sanitization procedures and chemicals on removing their biofilms.

The mere presence of sporeforming bacteria at high numbers in milk does not specifically mean the spoilage of the products. As there might be sporeformers that cannot grow in milk; while others may grow well in milk, but don't produce enzymes or other compounds that would render the milk unacceptable for consumption. For this reason, it is important to evaluate the spoilage potential of PPSB and redefined spoilage of pasteurized milk.

9. Comparison of DNA sequences with other studies that generated data on sporeformers isolated from milk chain in other parts of the US

A phylogenetic tree was built using the *rpoB* sequences obtained in this project, along with other ones obtained from similar research studies done in other regions of the US (Figure 22). This analysis was performed to get insight on how the sporeformers isolated in Nebraska are related to sporeformers isolated from other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Rainieri and Boor, 2009; Ivy et al., 2012).

Several studies have indicated the presence of few allelic types classified as *Bacillus licheniformis* s.l. all over the milk chain (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012). Specifically, Ranieri and Boor (2009) found CAT001 identified as *B. licheniformis* s.l. in most pasteurized milk samples collected from different processing plants at different regions across the US (i.e.

Northeast, Southeast, South, Midwest and West). In the current study, this allelic type was identified in all samples, time points and seasons and is represented by AT001. *B. licheniformis* AT001 (or CAT001) was classified in this project as PPSB. However, its status as "potentially problematic" is questionable as a cold growth study reported that this AT and other ATs classified in the *Bacillus* cluster (i.e. *B. pumilus* CAT020; *B. aerophilus* s.l. CAT135, *B. safensis* CAT141 and *B. cereus* s.l. CAT158) were not able to grow in sterile skim milk broth under refrigeration (Ivy et al., 2012).

Moreover, in this study the frequency of isolation of other subtypes that had been previously reported was low. However, the occurrence of these subtypes was also low across the US denoting their limited presence in the fluid milk chain. Most of these previously reported allelic types (ATs or SATs) were classified in the *Bacillus* clade (*B. licheniformis* s.l., *B. pumilus*, *B. safensis* and *B. aerophilus* s.l.), although there were also some *P. odorifer* (AT049, n=9; AT073, n=3; AT074, n=1; AT075, n=1).

In contrast, other ATs such as *P. odorifer* (CAT015, n=115; CAT002, n=52), *P. amylolyticus* (CAT023, n=35), *B. cereus* s.l. (CAT158, n=137), *B. weihenstephanensis* (CAT003, n=19; CAT075, n=23) and *Viridibacillus* spp. (CAT017, n=14; CAT073, n=18) were found very frequently in other regions of the US (Ivy et al., 2012), but were not found at all in the current study. Some of these allelic types of sporeforming bacteria identified in other regions have shown their ability to grow in milk during refrigerated storage, including those classified as *B. weihenstephanensis, P. odorifer, Viridibacillus* spp., *P. amylolyticus* spp., *P. graminis* and *P.* cf. *peoriae* (Ivy et al., 2012). In particular, several reports have indicated the relevance of *Paenibacillus* spp. in pasteurized milk and

their ability to grow in milk (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ranieri and Boor, 2009; Ivy et al., 2012), especially as these organisms can metabolize lactose (high levels of β-galactosidases) and are good competitors due to production of antimicrobials (Moreno-Switt et al., 2014). However, in the current study, besides *Paenibacillus* spp., previously unreported allelic types classified as *Bacillus* spp. were also considered PPSB. The cold growth profile of these organisms should be evaluated to assess if they can grow in milk at the same extent or better than *Paenibacillus* spp.

As mentioned before, some subtypes identified in this study had been previously found in other regions of the US, but many more seem to be specific to Nebraska, suggesting the need of region-specific strategies to better control sporeformers in the milk chain. Overall, 71 and 35 previously unreported *rpoB* ATs and 16S rDNA SATs of sporeforming bacteria were identified among the 453 sporeformers isolates, based on published data available from previous related studies in the BLAST database (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ivy et al., 2012). These ATs were all classified as *Paenibacillus*, *Bacillus* or related genera, and were grouped closely with sporeformers clusters previously identified by other related studies (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008; Ivy et al., 2012), making possible the assignment of genus and species.

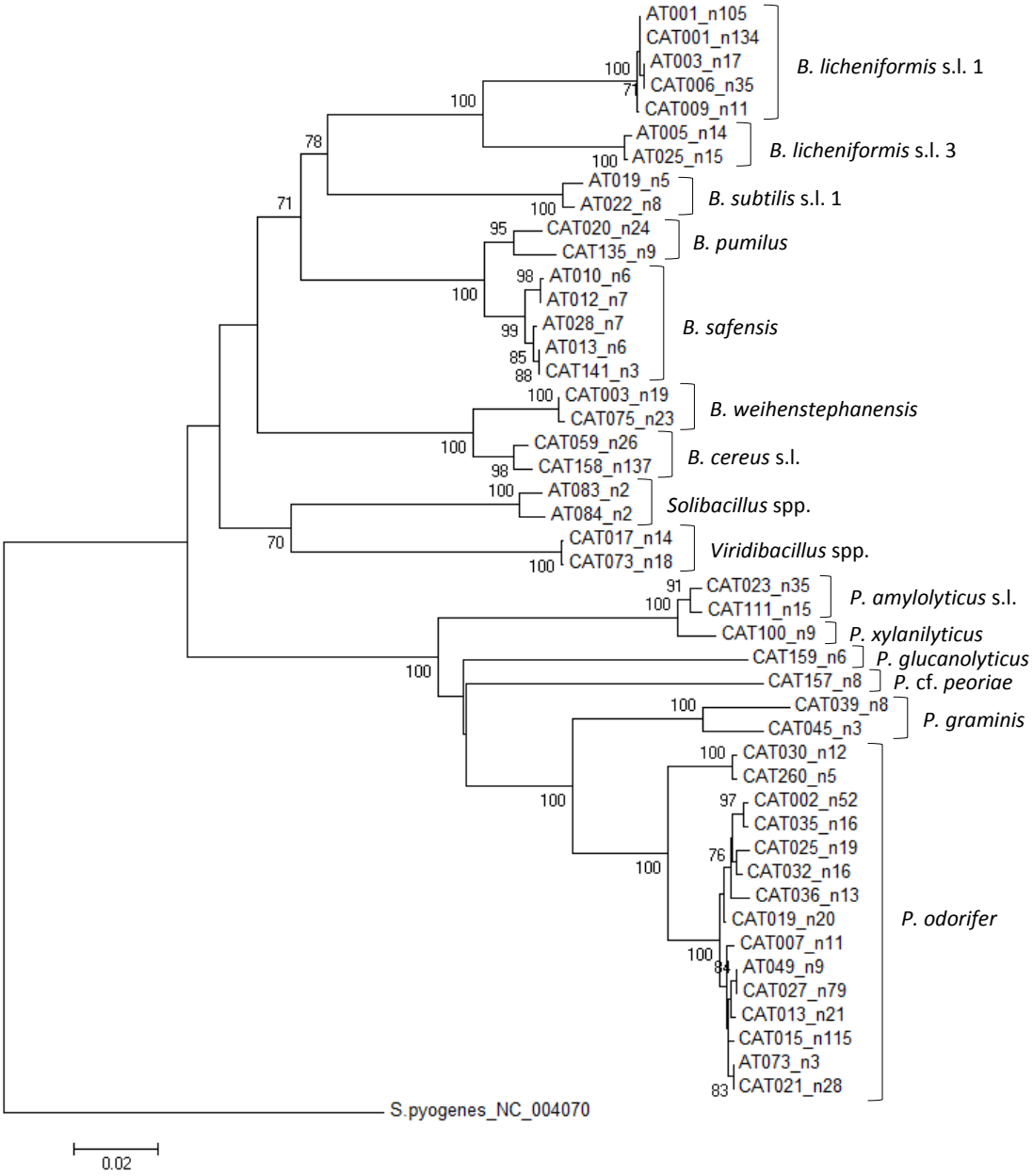

Figure 22. ***rpoB*-based phylogenetic tree for the *rpoB* allelic types mostly identified among sporeforming bacterial isolates from different parts of the United States and in this study using the Neighbor-Joining Method. The bar indicates the lengths representing 0.02-nt substitutions per site and the bootstrap values above 70 are shown and *n* indicates the number of isolates representing each species.**

10. Strategies to control sporeforming bacteria in the milk chain

A substantial diversity of sporeforming bacteria, varying in species and proportion within sources, time points and seasons, was identified throughout the farm-to-table continuum (Objective 3). This diversity hinders any efforts towards the development of universal strategies to better control sporeformers in the milk chain. Because of such diversity, most likely a systematic and comprehensive approach, including specific solutions for specific species/strains, will have to be used in the farm-to-table continuum. In addition, some subtypes identified in this study had been previously found in other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009; Ivy et al., 2012), but many more seem to be specific to Nebraska (Objective 5), suggesting the need of region-specific strategies to better control sporeformers in the milk chain.

10.1. Further evaluation of PPSB

In this study, PPSB were isolated and identified (Objective 4). Because they could be the key to the extension of fluid milk shelf life beyond 21 days, these isolates need to be further studied. Evaluations regarding their potential for biofilm formation, efficacy of sanitization procedures against these strains, heat resistance profiles, and their growth profile under refrigerated conditions commonly found in the fluid milk chain could be performed. Understanding better the behavior of these strains will allow for the

implementation of effective control measures at farm and plant level, for the benefit of the final product.

While assessing the behavior of sporeforming bacteria (i.e. heat resistance), the laboratory conditions used for the production of spore suspensions are critical (Cazemier et al., 2001). As mentioned in the literature review, the heat resistance of spores depends in part on the presence of minerals and thermal adaptation (Beaman and Gerhardt, 1986). A study reported that spores of different *Bacillus* strains were more heat resistant when prepared on nutrient agar with different metal ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, K^{2+} and Mn^{2+}) than when prepared on nutrient agar with only Mn^{2+} (Cazemier et al., 2001). In the dairy industry, the milk provides Ca^{2+} and Mg^{2+}, thus spores formed in a dairy environment might be more resistant to heat (Burgess et al., 2010). Furthermore, the temperature at which the spores are formed is also important, as studies have reported an enhanced heat resistance of spores formed at sporulation temperatures higher than the optimum growth temperature (Beaman and Gerhardt, 1986; Palop et al., 1999).

In the evaluation of biofilm formation by sporeforming bacteria many factors should be taken into account. For example, this process is dependent on the particular sporeforming strain (Faille et al., 2010b) and their ability to form biofilms on surfaces at air-liquid interfaces or on submerged surfaces (Ryu and Beuchat, 2005; Wijman et al., 2007; Zhao et al., 2013). Moreover, biofilm formation depends on the enrichment temperature (Zhao et al., 2013) and can be enhanced due to synergistic interactions between multiple species of bacteria (Zhao et al., 2013; Ren et al., 2013). The proper

formation of biofilms in the laboratory will contribute to the evaluation of the efficiency of cleaning and sanitization procedures.

10.2. Evaluate the spoilage potential of sporeforming bacteria: Redefining spoilage of pasteurized milk

As mentioned before, the mere presence of sporeforming bacteria at high numbers in milk does not specifically mean the spoilage of the products. As there might be sporeformers that cannot grow in milk; while others may grow well in milk, but don't produce enzymes or other compounds that would render the milk unacceptable for consumption. For this reason, it is important to evaluate the spoilage potential of PPSB, and especially to reassess and propose new quality standards that actually represent the end of shelf life of pasteurized milk.

Moreover, before evaluating the spoilage potential of PPSB it is important to define a more accurate description of what spoilage would look liked based on the different factors that can limit the shelf life of pasteurized milk (i.e. sporeforming bacteria, PPC, chemical oxidation). To achieve this it would be recommended to survey pasteurized fluid milk available in grocery stores in Nebraska. This survey could include the enumeration of bacterial counts in pasteurized milk throughout shelf life and the identification of the organisms responsible for the increase in the counts (i.e. PPSB or PPC) in a similar way to what was done in the current study. In addition, sensory evaluations and in vitro detection of enzymes (i.e. proteinases, β-galactosidases and

lipases) produced by PPSB could be performed for these samples to determine the end of the shelf life.

In particular, this research would be important to assure that the efforts to eliminate sporeforming bacteria from the milk chain are actually needed, and that these potential strategies to eliminate this group of bacteria would not create other issues in the fluid milk industry. It is important to remember that the elimination of a particular group of organisms from the environment most probably will lead to the selection of another group of organisms, which might cause a greater problem than the original population.

10.3. "Keep out" approach: Farm level

In general, control strategies need to be applied along the milk chain, to achieve a reduction or elimination of sporeformers in the final product (Huck et al., 2007a; Huck et al., 2007b; Huck et al., 2008). Especially, at the farm level many improvements could be implemented to prevent sporeforming bacteria of contaminating the raw milk at the beginning of the milk chain. For example, a survey performed in dairy farms from the Netherlands suggested that feeding cows silage low in spores might lead to a reduction in the spore counts in raw milk (Vissers et al., 2007). The following contamination route of sporeformers from silage to milk has been suggested (Figure 23): silage is mixed with other ingredients to produced mixed feed, cows eat the feed, sporeformers survive the gastrointestinal tract of the cows and are shed in the manure, which contaminates the bedding material. Then, the cows lay on the bedding and dirt containing sporeformers is attached to the teats (Driehuis, 2013). Research has shown that even the best cleaning

method cannot completely eliminate all sporeformers from the teats, thus these organisms are rinsed into the milk during the milking process (Magnusson et al., 2006).

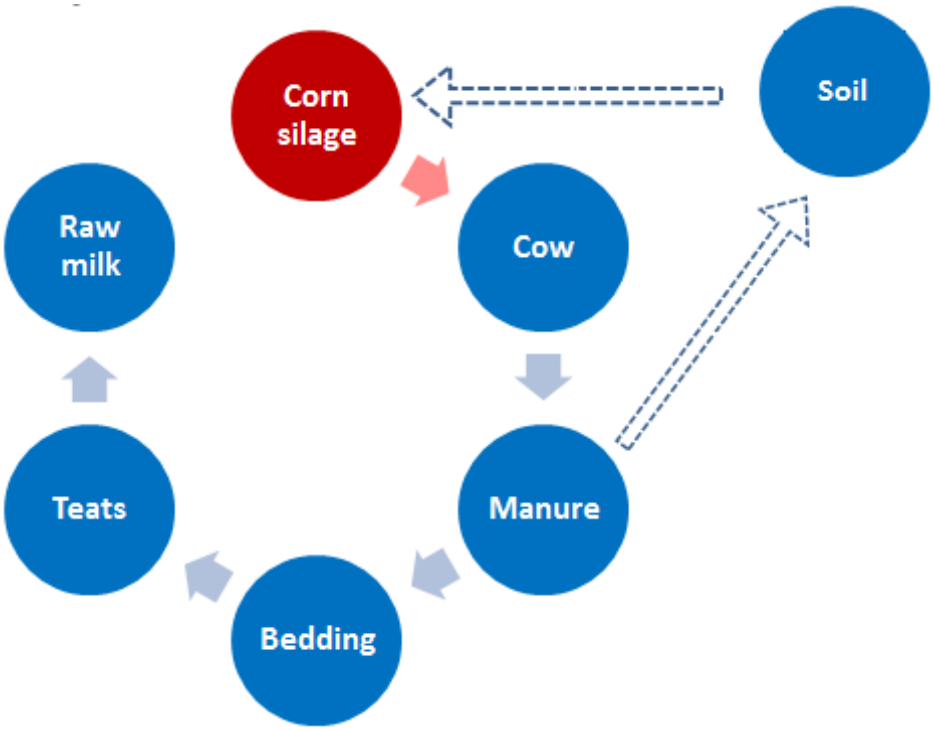

Figure 23. Contamination route of sporeforming bacteria at the farm level (Driehuis, 2013).

In our study, two PPSB identified as *Paenibacillus* spp. SAT030 and *B. licheniformis* s.l. AT001 were found in corn silage. Consequently, the first control strategy would be to control the fermentation process and reduce the aerobic deterioration of silage (Te Giffel et al., 2002). To achieve this, it would be necessary to ensile crops with low spore count. Nonetheless, this is also the first challenge, as high numbers of spores are initially in the soil where the crops are produced, probably due to the use of manure (high in sporeformers) as fertilizer in many farms (Rammer et al., 1994). This leads to a cycle that perhaps could be overcome by the use of inorganic fertilization methods or could be reduced by fertilizing the crops with farm slurry (liquid manure)

instead of farmyard manure. It was demonstrated that in crops where manure (especially farmyard manure) was used as fertilizer, sporeformers were a major issue in silage in comparison with crops where inorganic fertilization was applied (Rammer et al., 1994).

The fermentation process can be also controlled with the application of lactic acid bacteria or chemical additives (i.e. propionic acid and benzoic acid) to rapidly achieve a low pH and to inhibit aerobic deterioration (Driehuis and Oude Elferink, 2000). In addition, aerobic deterioration can be reduced by maintaining anaerobic conditions with the use of oxygen barrier films to cover the silage. Aerobic sporeformers (i.e. *Paenibacillus*) increase in numbers a few days after the silage is opened, but the use of these special films has been demonstrated to slow down the growth of sporeformers. Nonetheless, this only extends the quality of the silage for a few extra days, thus silage should be produced in smaller portions, to assure that cows are always fed good quality silage (Borreani et al., 2013).

Furthermore, four PPSB identified as *Bacillus* spp. SAT011, *P. odorifer* AT049, *B. safensis* AT012 and *B. licheniformis* s.l. AT001 were detected in manure. Thus, it is recommended that the pens should be kept as clean as possible. Manure removal from the floor and more frequent changes in the bedding material could potentially help in controlling sporeformers at this point. However, high counts of sporeformers were obtained in new bedding material [22-S12, 23-S12, 22-F12 and 23-F12] in this study, indicating the need for controlling sporeformers in fresh bedding as well. Lactation pens

that are cleaned at least three times a day have been correlated with a lower likelihood of farms producing milk with high spore counts (Miller et al., 2013).

At the milking parlor one suggestion would be improving the cleaning and sanitization of the milking line, especially by using a method that would remove biofilms formed by sporeformers as described above. PPSB (*B. licheniformis* s.l. AT001 and AT025) were identified in the rinse water of the milking line, thus this is an important entry point of sporeformers. In Farm A, milk is usually moved from each milking station through stainless steel pipelines to a balance tank, then it passes through filters and two heat exchangers before it gets to the sampler where samples of raw milk from the farm [28-S12, 28-F12, A1-S13]. This particular farm cleans and sanitizes the milking line and equipment every 7 hours (based on personal communications with the farm manager), which is more often than what the PMO establishes (every 12 hours; (FDA, 2011). Consequently, it would be necessary to improve the cleaning and sanitization method to be more effective against sporeformers, rather than increasing the frequency at which these procedures are performed.

Improvements could be also implemented during the cleaning of the teats as PPSB were found in these samples, including *P. odorifer* AT049, *Bacillus* spp. SAT011 and *B. licheniformis* s.l. AT001 and AT025. In Farm A, the cleaning of the teats is usually performed by dipping the teats in an iodine solution and wiping the teats with a side of a clean cloth towel for a few seconds (based on personal communications with the farm manager). A study demonstrated that by using a moist washable towel followed by

drying with a dry paper towel for 20 seconds, 96% of the sporeformers were removed from the teats (Magnusson et al., 2006). As with every other strategy, it would be necessary to find an optimum method that would remove as many sporeformers, but still be practical and economical.

Furthermore, PPSB (*Bacillus* spp. SAT011, *B. safensis* AT012, *B. licheniformis* s.l. AT001 and AT025) were identified in clean towels, indicating that the method that is being used to wash the towels is not effective against sporeformers. Likewise, the teat cups also contained PPSB, including *P. odorifer* AT049, *B. licheniformis* s.l. AT001 and AT025. In Farm A, the teat cups are cleaned and sanitized every 7 hours and are rinsed in between cows (based on personal communications with the farm manager). During milking manure usually splashes and can contaminate the teat cups at any time. Therefore, it would be recommended to do the cleaning and sanitization of the teat cups at more frequently intervals and rinse them better in between cows. In addition, as with any cleaning and sanitization procedure, and as mentioned before, it would be necessary to find a method that would be effective against sporeformers.

10.4. "Keep out" approach: Raw milk and detection tools

The raw milk supply was identified as a source of seven allelic types of PPSB (*P. odorifer* AT049 and AT073; *B. safensis* AT012; *B. licheniformis* AT001 and AT025; *Bacillus* spp. SAT012; and *Paenibacillus* spp. SAT030). Based on the initial evaluation of the quality of raw milk (Objective 1) it was concluded that all samples presented a

high initial quality, nonetheless these PPSB were able to germinate and grow during refrigerated storage in four of the heat-treated samples [1-S12, 57-F12, TA3-S13, TA4-S13]. These results confirm that tests commonly used to access the quality of raw milk (i.e. SPC, PBC, CC, MSC) haven't been able to predict the shelf life of pasteurized milk, because these tests lack the sensitivity and specificity needed to detect all forms of sporeforming bacteria (Martin et al., 2011), especially all forms of PPSB. Thus, the development of tools to consistently detect PPSB in raw milk has been recommended in the literature (Martin et al., 2011). For example, a real-time PCR was developed for the detection of *Paenibacillus* spp. in raw milk. However, this protocol takes a few days and lacks the sensitivity and specificity needed to detect all PPSB (Ranieri et al., 2012). The success of molecular techniques as detection tools of sporeforming bacteria in milk will be enhanced by the identification of new targets through genomic comparison. Some of these targets could include genes in cold adapted *Paenibacillus* strains encoding for important features for cold growth in milk (Moreno-Switt et al., 2014).

Although, other studies have suggested the need of highly sensitive and specific detection methods to be used as quality control tools (Martin et al., 2011), it should be rather recommended their use for research purposes only; at least for now and for the fluid milk industry in Nebraska. In particular, because the information about PPSB is still limited and their spoilage potential in pasteurized milk has not been assessed and defined in this industry. The main concern with the use of these detection tools for quality control lies in the economic implications of rejecting trucks of raw milk that might have yielded pasteurized milk with no signs of spoilage at the end of shelf life, just because the quality

control tool was too sensitive but non-specific. However, these methods could be helpful to support the assessment of control strategies to reduce sporeformers in the milk chain.

10.5. "Keep out" approach: Plant level

Improved cleaning and sanitization procedures should be implemented along the milk chain to effectively remove biofilms formed by sporeforming bacteria. The development and implementation of nonfouling stainless steel (i.e. nanosphere modified steel) could help prevent biofilms and fouling by sporeforming bacteria, thus allowing for longer production runs that result pasteurized milk with low spore counts (DRI, 2013). In addition, it is important to evaluate the efficacy of different cleaning and sanitization compounds and procedures, such as Clean-In-Place (CIP) against biofilms formed by sporeforming bacteria. A study reported that temperatures above 60°C and concentrations over 0.5% of NaOH during CIP cleaning are needed for the reduction of spore viability and spore adhesion to stainless steel surfaces. Moreover, it is important to take into account a possible re-adhesion of spores to processing lines during CIP (Faille et al., 2010a).

Clean trucks pick up the raw milk at Farm A to transport it to Plant A. These trucks are cleaned and sanitized at the plant (based on personal communications with the farm and plant managers). In this study, the trucks did not seem to contribute much to contamination of milk with PPSB. However, the cleaning and sanitization procedures of the trucks should be optimized to be effective against sporeformers.

Also, as mentioned before, PPC with psychrotrophic Gram-negative bacteria and lactic acid bacteria was the main factor limiting the shelf life extension of pasteurized milk samples in this study (Objective 3). Therefore, the main recommendation to extend the shelf life of pasteurized milk would be to improve the sanitization of the processing plant, especially of the tanks and fillers. In addition, routine cup maintenance and the replacement of the rubber cup portion of each nozzle from the filler at regular intervals could contribute to the control of PPC (Ralyea et al., 1998). However, if the contamination with PPC persists after doing all these modifications, then aseptic packaging could be considered. None of the non-sporeforming bacteria that were identified due to PPC in pasteurized milk were found in more than one season (Tables 10-12), indicating that these organisms do not persist in the plant environment and that their elimination from this location can be accomplished.

10.6. Other approaches: Plant level

The majority of the strategies mentioned here are based on the "keep out" approach, which might represent the less expensive option for the control of sporeformers in pasteurized fluid milk. The "keep out" approach focuses mainly on preventing sporeformers from entering the raw milk supply before processing and on eliminating all sporeformers and microbes from the plant environment that could contaminate the product after processing. Other strategies could be implemented to physically remove, to inhibit the growth or to kill sporeformers in fluid milk. Nonetheless, these strategies might require a more radical change in the processing of this product, which might

represent a higher economical cost. In addition, it would be important to assure that any modification to the process would not adversely affect the sensory acceptability of the product.

Some studies have indicated that plant factors (i.e. PPC, biofilm formation and pasteurization conditions) may have a significant effect on pasteurized milk quality and shelf life (Fromm and Boor, 2004; Martin et al., 2011). For example, shorter production runs may contribute to control sporeforming bacteria and other microorganisms, as it allows for more often and thorough cleaning and sanitization between runs (Fromm and Boor, 2004). Furthermore, the use of lower pasteurization temperatures has been correlated with a reduction in SPC of pasteurized milk after 21 days of storage (Ranieri et al., 2009; Martin et al., 2012b). However, before making any recommendation about changing pasteurization conditions in the processing plants in Nebraska or in other regions across the US, it is important to consider that this strategy might not have the same effect on the allelic types isolated in the current project, as both of the aforementioned studies were done in New York State. As it was described in objective 6, in the current study many previously unreported allelic types of sporeforming bacteria were identified. Consequently, it would be recommended to evaluate if the reduction of the pasteurization conditions in other plants outside New York State would yield the same effect before suggesting the change in process parameters as an industry wide guideline.

Moreover, microfiltration (membrane filtration) could be used to physically remove sporeformers from fluid milk, which is a process currently used for the production of milk with extended shelf life (Elwell and Barbano, 2006). Moreover, the induction of spore germination before processing could reduce heat resistance of spores, thus facilitating their inactivation during pasteurization (Eijlander et al., 2011; Aouadhi et al., 2012; Aouadhi et al., 2013). As described in the literature review, germination is usually triggered by specific nutrients (Setlow, 2003). The incubation of milk (i.e. 60 minutes at 35°C) with low concentrations of specific germinants (such as L-alanine, D-glucose) before processing have been reported to give optimal germination of *B. sporothermodurans* spores (Aouadhi et al., 2013). In addition, spore germination can be induced with the use of high hydrostatic pressure (Aouadhi et al., 2012). In particular, the use of ultra-high pressure homogenization has been assessed during the last years as an alternative to conventional pasteurization methods, such as HTST pasteurization (Pereda et al., 2007; Pinho et al., 2011; DRI, 2013).

The final strategy in controlling sporeforming bacteria in pasteurized milk is the proper refrigeration (<7°C) of the product from the processing plant to the consumer's house. This essential step is critical for delaying the growth of sporeforming bacteria in milk, however some psychrotrophic sporeforming bacteria (such as *Paenibacillus* spp., *B. weihenstephanensis*, *Viridibacillus* spp.) can still grow at low temperatures, causing the spoilage of pasteurized milk (Ivy et al., 2012). Consequently, it is important to control and reduce these problematic organisms from the raw materials and final products.

CHAPTER 5: FUTURE RESEARCH AND CONCLUSIONS

Future research

1. Assessment of psychrotrophic, mesophilic and thermophilic sporeforming bacteria in other dairy industries (such as condensed milk) and in a wider variety of dairy farms in Nebraska.
2. Evaluate the behavior of PPSB, such as potential for biofilm formation, heat resistance profiles, and their cold growth profile under refrigerated conditions commonly found in the fluid milk chain.
3. Evaluation of farm practices that might lead to a reduction in sporeformers in raw milk.
4. Implementation of strategies proposed in objective 6 at the farm and plant level, followed by evaluation of their impact, feasibility and efficiency.
5. Development of a reliable, rapid and economical tool for the detection of PPSB in milk to support the assessment of control strategies to reduce sporeformers in the milk chain.
6. Evaluate the spoilage potential of PPSB and redefined spoilage of pasteurized milk by doing a survey of pasteurized milk available in grocery stores in Nebraska.

Conclusions

1. The majority of raw and pasteurized milk samples showed high initial quality based on bacterial counts (SPC and CC), which were mostly within the regulatory limits established in the PMO (FDA, 2011).
2. Packaged pasteurized milk samples showed the highest SPC after 21 days of refrigerated storage, followed by in-line pasteurized milk samples. Lastly, the heat-treated raw and pasteurized milk samples showed the lowest SPC, suggesting the presence of different microbiota (i.e. sporeforming bacteria and/or non-sporeforming bacteria due to PPC) among these three sets of samples.
3. Heat-treated environmental samples from Farm A and Plant A showed an initial and variable presence of sporeformers, and MSC of some samples from both locations increased 1-5 logs after 21 days of refrigerated storage, indicating these environments as possible sources of psychrotrophic sporeformers for pasteurized fluid milk.
4. A vast diversity of sporeforming bacteria, varying in species and proportion within sources, time points and seasons, was identified throughout the farm-to-table continuum. Moreover, a variety of non-sporeforming bacteria were also identified in most pasteurized fluid milk samples due to PPC, especially during the filling process.
5. Only a few subtypes of "potentially problematic sporeforming bacteria" (PPSB) were present in laboratory heat-treated or commercially pasteurized milk samples with high bacterial counts (MSC or SPC) after 21 days of refrigerated storage;

however, many entry points of these PPSB were identified all over the milk chain (i.e. raw milk, farm and plant environment).

6. Some subtypes identified in this study had been previously found in other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009; Ivy et al., 2012), but many more seem to be specific to Nebraska, suggesting the need of region-specific strategies to better control sporeformers in the milk chain.
7. The high diversity of sporeformers and their multiple entry points hinders any efforts towards the development of universal strategies to better control sporeformers in pasteurized fluid milk, thus a systematic and comprehensive approach, including specific solutions for specific species/strains, will have to be applied along the farm-to-table continuum.

CHAPTER 6: APPENDIX

A. Production data for Farm A and processing parameters for Plant A.

Table 14. Production data for Farm A during the first quarter of 2012 and 2013.

Processing parameter	First quarter 2012	First quarter 2013
Volume of milk produced (million kg)	4.4	4.4
Cows shipping milk	1373	1369
Dry cows	183	166
Hospital cows	18	19
Total cows	1574	1554

(Personal communication with farm manager)

Table 15. Processing parameters for Plant A in 2012 and 2013.

Processing parameter	Description
Pasteurization conditions	Minimum of 79°C for at least 27 seconds
Average days in code	Usually 18-20 days*
Frequency of processing (days/week)	4-5**
Average volume of milk processed(gallons/day)	5,000**
Types of dairy products processed	Whole, 2% fat, 1% fat, skim and flavored milk. Cream, half-and-half cream.

*Depends on time of the year and type of milk.

**Varies throughout the year, with no particular tendency.

(Personal communication with plant manager)

Table 16. Average temperature (°C) of the collection days at Plant A and Farm A.

Season	Location	Date	Average temperature (°C)
Spring 2012	Farm A	03/20/2012	13
	Plant A	03/28/2012	14
		03/29/2012	18
Fall 2012	Farm A	09/07/2012	19
	Plant A	09/24/2012	14
		09/25/2012	19
Spring 2013	Farm A	03/18/2013	1
	Plant A	05/13/2013	19
		05/14/2013	26

(Weather Underground, Inc.)

B. Protocols for DNA sequencing

1. Optimized protocol for DNA Extraction using the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, Ca)

1. Streak each isolate on Standard Methods Agar (SMA), incubate at 32°C for 24h, and check for purity.
2. Use a single colony from a plate to inoculate Triptic Soy Broth (TSB) and incubate overnight at 32°C.
3. Transfer 1mL of each culture to a sterile 1.5mL tube with 0.3g of beads and place on ice.
4. Centrifuge at 7000g for 5 min and discard 0.9mL of supernatant.
5. Add 1mL of enzymatic lysis buffer (with no enzyme), vortex, and centrifuge at 7000g for 5 min. Discard 0.9mL of supernatant.
6. Resuspend bacterial pellet in 180µL enzymatic lysis buffer with 20mg/mL of lysozyme (to break open cell and nuclear membrane) and vortex.
7. Incubate at 37°C for 30 min (optimum temperature for lysozyme).
8. Add 25µL of proteinase K (to denature the proteins and keep the DNA intact) and vortex.
9. Add 200µL of Buffer AL (lysis buffer) and vortex.
10. Incubate at 56°C for 30 min (optimum temperature for proteinase K).
11. Incubate at 95°C for 15 min (enzyme inactivation).
12. Place tubes on ice until they cool down.
13. Beat beating for 2 min at maximum speed and place tubes on ice until they cool down.
14. Transfer supernatant to 1.5mL sterile Eppendorf tubes.
15. Add 200µL of 96-100% ethanol (to precipitate the DNA) and vortex.
16. Apply mixture to the column.
17. Centrifuge at 6000g for 1 min and discard the flow.
18. Add 500µL of Buffer AW1 (wash buffer), centrifuge for 1 min at 6000g and discard the flow.
19. Add 500µL of Buffer AW2 (wash buffer), centrifuge for 3 min at 6000g, discard the flow and the tube.
20. Place spin column in new 1.5mL Eppendorf tubes.
21. Add 200µL of Buffer AE (elution buffer).
22. Incubate at room temperature for 1min.
23. Centrifuge for 1 min at 6000g and remove the column.
24. Store DNA at -20°C.

2. Optimized protocol for *rpoB* PCR (Drancourt et al., 2004; Durak et al., 2006)

Table 17. Components, concentrations and volumes for *rpoB* PCR.

Component	Concentrations	Volumes
Primer SF2012_F (20pmol/µL, Invitrogen) 5' – AARYTIGGMCCTGAAGAAAT – 3' (Strepto F; Drancourt et al., 2004)	0.5µM	2.5 µL
Primer SF2012_R (20pmol/µL, Invitrogen) 5' –TGTARTTTRTCATCAACCATGTG – 3' (Strepto R; Drancourt et al., 2004)	0.5µM	2.5 µL
dNTPs (TaKaRa)	200µM each	8.0 µL
Buffer 10X (Mg^{2+} free; TaKaRa)	1X	10.0 µL
$MgCl_2$ (25mM; TaKaRa)	1.8mmol	7.2 µL
molecular biology grade water (0.3µm filtered, DNase-, RNase- and protease-free; Fisher BioReagents)		67.3 µL
DNA Template (DNA of each bacterial isolate)		2.0 µL
rTaq polymerase (5U/µL; TaKaRa)	$2.5x10^{-2}$U/µL	0.50 µL
Volume per PCR reaction		**100.0 µL**

Procedure:

1. Primer preparation: Primers were diluted with molecular biology grade water (0.3µm filtered, DNase-, RNase- and protease-free; Fisher BioReagents) to obtain a stock solution of 40pmol/µL. Then, individual tubes containing dilutions of 20pmol/µL were prepared using the same water.
2. Place empty PCR tubes and components on ice.
3. Add DNA template of bacterial isolates and PCR controls (positive – *B. licheniformis* B. 4282 (ARS Culture Collection, USDA); negative – molecular biology grade water) directly to the corresponding 200µL PCR tubes.
4. PCR mixture (98.0 µL per PCR reaction): Mix all the other components (Primer SF_2012F, Primer SF_2012R, dNTPs, Buffer 10X, $MgCl_2$, water, rTaq) for all the different PCR reactions (total number of DNA samples, positive and negative controls) in a sterile PCR-grade tube.
5. Add the PCR mixture to each tube using a multichannel pipette.
6. Run the PCR.
7. Store the PCR products at -20°C.

PCR Conditions (Durak et al., 2006):

- 94°C for 3 min.
- 20 cycles of denaturation at 94°C for 30 s, primer annealing at TD (Touchdown PCR from 60°C to 50°C with temperature decrease of 0.5°C per cycle) for 30 s and DNA extension at 72°C for 1 min.
- 20 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 30 s and DNA extension at 72°C for 1 min.
- 72°C for 7 min.

PCR Equipment: T100™ Thermal Cycler (Bio-Rad Laboratories)

3. Optimized protocol for PCR Products Purification using the QUIAquick PCR Purification Kit (Quiagen Inc., Ca)

1. Add 5 volumes Buffer PB (binding buffer) to 1 volume of the PCR reaction and mix (i.e. 450 µL PB + 90 µL PCR for *rpoB* PCR products; 200 µL PB + 40 µL PCR for 16S DNA PCR products).
2. Bind DNA by applying the sample to the QUIAquick column, centrifuge for 1 min at 13,000 rpm and discard flow-through.
3. Wash by adding 750 µL Buffer PE (wash buffer) to the column, centrifuge for 1 min at 13,000 rpm and discard flow-through.
4. Centrifuge for 1 min at 13,000 rpm to remove residual wash buffer.
5. Elute DNA by adding 30 µL of molecular biology grade water (0.3µm filtered, DNase-, RNase- and protease-free; Fisher BioReagents) to the center of the membrane, let the column stand for 5 minutes and centrifuge the column for 1 min at 13,000 rpm.
6. Stored purified PCR products at -20°C

C.1. Description of 94 unique *rpoB* Allelic Types (ATs) identified among 313 sporeformers isolates collected from Farm A and Plant A during Spring 2012, Fall 2012 and Spring 2013.

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
***rpoB* AT**	**16S SAT**					**GenBank (*rpoB*)**	**Id.**	**GenBank (16S)**	**Id.**	**CAT***
AT001	SAT001	*Bacillus*	*licheniformis* s.l.	42C1SP	105	EF156897.1	100%	EF156868.1	100%	CAT001
AT002	SAT001	*Bacillus*	*licheniformis* s.l.	51C1SP	2			EF156868.1	100%	
AT003		*Bacillus*	*licheniformis* s.l.	13B1SP	17	EF156902.1	100%			CAT006
AT004		*Bacillus*	*licheniformis* s.l.	44B1SP	2	EF156905.1	100%			CAT009
AT005		*Bacillus*	*licheniformis* s.l.	41B1SP	14	EF203109.1	100%			CAT031
AT006	SAT006	*Bacillus*	cf. *megaterium*	16B1SP	1	EF157041.1	100%	EF156947.1	100%	CAT085
AT007	SAT002	*Bacillus*	*subtilis* s.l.	26B1SP	1	EU138851.1	99%	EF156933.1	100%	
AT008		*Bacillus*	*pumilus*	1C1SP	1	EF156916.1	100%			CAT020
AT009		*Bacillus*	*pumilus*	7B1SP	1	EF156916.1	99%			
AT010		*Bacillus*	*safensis*	5B1SP	6	EU147211.1	100%			CAT124
AT011		*Bacillus*	*aerophilus* s.l.	10A1SP	1	EU147221.1	99%			
AT012		*Bacillus*	*safensis*	1A1SP	7	EU147226.1	100%			CAT140
AT013	SAT008	*Bacillus*	*safensis*	16B14SP	6	EU147227.1	100%	EF156981.1	100%	CAT141
AT014	SAT008	*Bacillus*	*safensis*	25A1SP	2	EU147227.1	99%	EU147188.1	100%	
AT015		*Bacillus*	*safensis*	42B1SP	3	EU147227.1	99%			
AT016		*Bacillus*	*safensis*	44C1SP	2	EU147227.1	99%			
AT017	SAT008	*Bacillus*	*safensis*	8B1SP	3	EU147227.1	99%	EU147188.1	100%	
AT018		*Bacillus*	*cereus* s.l.	2B1SP	1	EU147240.1	100%			CAT129
AT019		*Bacillus*	*subtilis* s.l.	24B1SP	5	EF157022.1	99%			
AT020		*Bacillus*	*subtilis* s.l.	6B1SP	1	EF157022.1	99%			
AT021		*Bacillus*	*subtilis* s.l.	8A1SP	2	EF157023.1	99%			
AT022		*Bacillus*	*subtilis* s.l.	4A1SP	8	EF157023.1	99%			

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
***rpoB* AT**	**16S SAT**					**GenBank (*rpoB*)**	**Id.**	**GenBank (16S)**	**Id.**	**CAT***
AT023		*Bacillus*	*subtilis* s.l.	13A7SP	1	HG328254.1	100%			
AT024		*Bacillus*	*subtilis* s.l.	4A7SP	1	EU138812.1	98%			
AT025		*Bacillus*	*licheniformis* s.l.	40B7SP	15	EF203109.1	99%			
AT026		*Bacillus*	*aerophilus* s.l.	40C7SP	2	EU147221.1	100%			CAT135
AT027		*Bacillus*	*aerophilus* s.l.	42A7SP	1	EU147221.1	99%			
AT028		*Bacillus*	*safensis*	9B7SP	7	EU147227.1	99%			
AT029		*Bacillus*	*subtilis* s.l.	1A7SP	4	EF157023.1	99%			
AT030		*Bacillus*	*subtilis* s.l.	44D7SP	1	EF157023.1	99%			
AT031		*Bacillus*	*pumilus*	40A14SP	2	EF157025.1	100%			CAT137
AT032		*Bacillus*	*safensis*	1A14SP	4	EF157062.1	100%			CAT106
AT033		*Bacillus*	*aerophilus* s.l.	43A14SP	1	EU147221.1	99%			
AT034		*Bacillus*	*aerophilus* s.l.	46C14SP	1	EU147221.1	99%			
AT035		*Bacillus*	*safensis*	22C14SP	1	EU147227.1	99%			
AT036		*Bacillus*	*safensis*	42B14SP	1	EU147227.1	99%			
AT037		*Bacillus*	*safensis*	45B14SP	1	EU147227.1	99%			
AT038		*Bacillus*	*safensis*	3C14SP	1	JX680049.1	100%			
AT039		*Bacillus*	*subtilis* s.l.	5A14SP	2	EF157022.1	99%			
AT040		*Bacillus*	*subtilis* s.l.	15C14SP	2	EF157022.1	99%			
AT041		*Bacillus*	*subtilis* s.l.	21A14SP	4	EF157023.1	99%			
AT042	SAT002	*Bacillus*	*subtilis* s.l.	12B21SP	1	HF563562.1	100%	EF156933.1	100%	CAT066
AT043		*Bacillus*	*licheniformis* s.l.	15B21SP	1	EF156897.1	97%			
AT044		*Bacillus*	*aerophilus* s.l.	8A21SP	2	EF157026.1	100%			CAT069
AT045		*Bacillus*	*aerophilus* s.l.	27A21SP	1	EU147221.1	99%			
AT046		*Bacillus*	*aerophilus* s.l.	9C21SP	1	JX680033.1	100%			
AT047		*Bacillus*	*safensis*	6A21SP	1	EU147227.1	99%			

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
***rpoB* AT**	**16S SAT**					**GenBank (*rpoB*)**	**Id.**	**GenBank (16S)**	**Id.**	**CAT***
AT048		*Bacillus*	*safensis*	14A21SP	1	EU147227.1	99%			
AT049		*Paenibacillus*	*odorifer*	1A21SP	9	EF156923.1	100%			CAT027
AT050	SAT005	*Bacillus*	*cereus* s.l.	43E1SU	1	EU147240.1	99%	EU147176.1	100%	
AT051		*Bacillus*	*subtilis* s.l.	21C14SU	1	EF157022.1	99%			
AT052	SAT038	*Paenibacillus*	spp.	16C14SU	1			JF309261.1	99%	
AT053	SAT002	*Bacillus*	*subtilis* s.l.	39A21SU	1	HG514499.1	99%	EF156933.1	100%	CAT066
AT054		*Bacillus*	*safensis*	6A21SU	2	EU147209.1	100%			CAT122
AT055		*Bacillus*	*aerophilus* s.l.	42A21SU	1	EU147221.1	99%			
AT056	SAT039	*Paenibacillus*	*lautus*	15A21SU	1	EU147204.1	99%	EU147168.1	100%	CAT117
AT057		*Bacillus*	*psychrosaccharalyticus*	EA1A1SP	2	EU147212.1	100%			CAT281
AT058		*Bacillus*	*aerophilus* s.l.	TA1A1SP	1	EU147221.1	99%			
AT059		*Bacillus*	*aerophilus* s.l.	A11E1SP	1	EU147221.1	99%			
AT060		*Bacillus*	*aerophilus* s.l.	HA4B1SP	1	EU147221.1	99%			
AT061		*Bacillus*	*aerophilus* s.l.	PA2A1SP	1	JX680062.1	99%			
AT062		*Bacillus*	*safensis*	PA4D7SP	2	EU147227.1	99%			
AT063		*Bacillus*	*safensis*	TA1A21SP	1	EU147227.1	99%			
AT064		*Bacillus*	*safensis*	PA1B1SP	1	EU147227.1	99%			
AT065		*Bacillus*	*safensis*	PA1C1SP	1	EU147227.1	99%			
AT066		*Bacillus*	*safensis*	HA1A1SP	1	EU147227.1	99%			
AT067		*Bacillus*	*safensis*	EA3A1SP	1	EU147227.1	99%			
AT068		*Bacillus*	*safensis*	A8B1SP	1	EU147229.1	99%			
AT069		*Bacillus*	*pumilus*	PA1A7SP	1	KF734907.1	99%			
AT070		*Bacillus*	*pumilus*	HA2B21SP	1	EU147223.1	99%			
AT071		*Bacillus*	*cereus* s.l.	TA1D1SP	1	EU147240.1	99%			
AT072		*Bacillus*	*cereus* s.l.	HA5B21SP	1	EF157048.1	100%			CAT092

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
***rpoB* AT**	**16S SAT**					**GenBank (*rpoB*)**	**Id.**	**GenBank (16S)**	**Id.**	**CAT***
AT073		*Paenibacillus*	*odorifer*	TA3A21SP	3	EF156917.1	100%			CAT021
AT074		*Paenibacillus*	*odorifer*	HA3A21SP	1	EF157000.1	100%			CAT050
AT075		*Paenibacillus*	*odorifer*	PA2A21SP	1	EF157002.1	100%			CAT035
AT076	SAT006	*Bacillus*	cf. *megaterium*	13A1SP	1	EU147237.1	100%	EU147197.1	100%	CAT151
AT077		*Bacillus*	cf. *megaterium*	23A14SP	1	EU147237.1	99%			
AT078		*Bacillus*	*subtilis* s.l.	A11B14SP	1	EF157023.1	99%			
AT079	SAT006	*Bacillus*	cf. *megaterium*	21A21SP	1	EF157041.1	99%	EU147197.1	100%	CAT151
AT080	SAT035	*Lysinibacillus*	spp.	49C7SP	1			EU147183.1	99%	
AT081		*Solibacillus*	spp.	A6B14SP	2	EU147213.1	100%			CAT126
AT082	SAT034	*Lysinibacillus*	spp.	26A21SU	1			EU147183.1	99%	
AT083		*Bacillus*	spp.	55B21SU	2	EU147213.1	99%			
AT084	SAT036	*Solibacillus*	spp.	47A21SU	2	EU147213.1	99%	JX202553.1	100%	
AT085	SAT035	*Lysinibacillus*	spp.	A2D7SP	1	EU147222.1	99%	EU147183.1	100%	CAT136
AT086	SAT008	*Bacillus*	*safensis*	A2B14SP	1	EU147227.1	99%	EU147188.1	100%	CAT141
AT087	SAT046	*Rummeliibacillus*	*pycnus*	PA1B21SP	1			JN650277.1	99%	
AT088	SAT013	*Bacillus*	*sporothermodurans*	HA1D1SP	1			U49080.1	99%	
AT089	SAT022	*Paenibacillus*	spp.	A4A21SP	1			EF156934.1	98%	
AT090	SAT048	*Bacillus*	*nealsonii*	HA1C21SP	1			KF054865.1	100%	
AT091	SAT049	*Bacillus*	*flexus*	A8A1SP	1			GU566359.1	100%	
AT092	SAT035	*Lysinibacillus*	spp.	A5C1SP	1	EU147222.1	99%	EU147183.1	100%	CAT136
AT093	SAT035	*Lysinibacillus*	spp.	A7D1SP	1			EU147183.1	100%	CAT136
AT094	SAT035	*Lysinibacillus*	spp.	A5B7SP	1			EU147183.1	100%	CAT136

* CAT are Allelic Types identified in similar research projects done in other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009; Ivy et al., 2012).

C.2. Description of 53 unique 16S rDNA Allelic Types (SATs) identified among 195 bacterial isolates collected from Farm A and Plant A during Spring 2012, Fall 2012 and Spring 2013.

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
16S SAT	*rpoB* AT					GenBank (16S)	Id.	GenBank (*rpoB*)	Id.	CAT*
SAT001	AT001	*Bacillus*	*licheniformis* s.l.	42C1SP	9	EF156868.1	100%	EF156897.1	100%	CAT001
SAT002		*Bacillus*	*subtilis* s.l.	14A1SP	29	EF156933.1	100%			CAT066
SAT003		*Bacillus*	*clausii*	40C21SP	2	EF156928.1	99%			
SAT004		*Bacillus*	*coagulans*	49A14SP	3	CP003056.1	100%			
SAT005	AT050	*Bacillus*	*cereus* s.l.	43E1SU	1	EU147176.1	100%	EU147240.1	99%	CAT129
SAT006	AT006	*Bacillus*	cf. *megaterium*	16B1SP	3	EF156947.1	100%	EF157041.1	100%	CAT085
SAT007		*Bacillus*	*oleronius*	18B21SP	7	AY988598.1	100%			
SAT008	AT013	*Bacillus*	*safensis*	16B14SP	13	EF156981.1	100%	EU147227.1	100%	CAT141
SAT009		*Bacillus*	*badius*	7C21SU	3	AY803745.1	100%			
SAT010		*Bacillus*	*circulans*	12B14SP	2	GU726174.1	100%			
SAT011		*Bacillus*	spp.	44A14SU	8	JF799969.1	99%			
SAT012		*Bacillus*	spp.	47C14SP	11	JX173285.1	100%			
SAT013		*Bacillus*	*sporothermodurans*	16B1SU	5	U49080.1	99%			
SAT014		*Brevibacillus*	spp.	A2B1SP	2	AY319301.1	99%			
SAT015		*Brevibacillus*	*borstelensis*	24C1SU	12	AB116134.1	100%			
SAT016		*Paenibacillus*	*borealis*	A3A21SP	2	KC236524.1	99%			
SAT017		*Brevibacillus*	spp.	45A21SP	12	KF217127.1	99%			
SAT018		*Exiguobacterium*	spp.	PA4A14SP	4	KF054759.1	99%			
SAT019		*Exiguobacterium*	spp.	PA5C7SP	1	JX625993.1	99%			
SAT020		*Lysinibacillus*	spp.	22A1SU	3	DQ350820.1	99%			
SAT021		*Paenibacillus*	spp.	PA4C1SP	3	KC354448.1	98%			
SAT022		*Paenibacillus*	spp.	A9A21SP	2	EF156934.1	98%			

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
16S SAT	*rpoB* AT					GenBank (16S)	Id.	GenBank (*rpoB*)	Id.	CAT*
SAT023		*Paenibacillus*	*borealis*	PA3D21SP	1	EF156931.1	99%			
SAT024		*Paenibacillus*	*macerans*	PA3B1SP	2	EU071599.1	99%			
SAT025		*Paenibacillus*	*macerans*	19A21SU	1	EU071599.1	100%			
SAT026		*Paenibacillus*	spp.	52D1SP	2	EF156930.1	98%			
SAT027		*Paenibacillus*	*pueri*	PA2B1SP	1	EU391155.1	99%			
SAT028		*Bacillus*	*aerophilus* s.l.	41C1SU	1	AJ831842.1	100%			
SAT029		*Paenibacillus*	spp.	40C1SP	1	EF156980.1	99%			
SAT030		*Paenibacillus*	spp.	18A1SU	13	AY266990.1	99%			
SAT031		*Paenibacillus*	spp.	17B1SU	1	JX011004.1	100%			
SAT032		*Pseudomonas*	spp.	52B7SP	5	KF153216.1	100%			
SAT033		*Bacillus*	*novalis*	38E21SU	1	JN650281.1	99%			
SAT034	AT082	*Lysinibacillus*	spp.	26A21SU	1	EU147183.1	99%			
SAT035	AT085	*Lysinibacillus*	spp.	A2D7SP	5	EU147183.1	100%	EU147222.1	99%	CAT136
SAT036	AT084	*Solibacillus*	spp.	47A21SU	1	JX202553.1	100%	EU147213.1	99%	
SAT037		*Aneurinibacillus*	*aneurilyticus*	19B21SU	1	AB680012.1	100%			
SAT038	AT052	*Paenibacillus*	spp.	16C14SU	1	JF309261.1	99%			
SAT039	AT056	*Paenibacillus*	*lautus*	15A21SU	2	EU147168.1	100%	EU147204.1	99%	CAT117
SAT040		*Leuconostoc*	*mesenteroides*	43A21SU	1	HG799977.1	100%			
SAT041		*Lactococcus*	spp.	52A21SU	1	EU689105.1	100%			
SAT042		*Acinetobacter*	*baumannii*	53C1SP	2	CP003967.1	100%			
SAT043		*Paenibacillus*	spp.	14B7SU	1	JF309261.1	100%			
SAT044		*Pantoea*	spp.	53B14SU	2	JX458430.1	99%			
SAT045		*Pseudomonas*	spp.	44A14SP	3	KF465842.1	100%			
SAT046	AT087	*Rummeliibacillus*	*pycnus*	PA1B21SP	1	JN650277.1	99%			
SAT047		*Terribacillus*	*saccharophilus*	HA5C7SP	1	AB243845.1	100%			

Allelic Types		Genus	Species	Reference Isolate	Isolates	BLAST Results				
16S SAT	*rpoB* AT					GenBank (16S)	Id.	GenBank (*rpoB*)	Id.	CAT*
SAT048	AT090	*Bacillus*	*nealsonii*	HA1C21SP	1	KF054865.1	100%			
SAT049	AT091	*Bacillus*	*flexus*	A8A1SP	1	GU566359.1	100%			
SAT050		*Psychrobacter*	spp.	PA5B21SP	1	KF186667.1	100%			
SAT051		*Kurthia*	*gibsonii*	PA5A1SP	1	JN409471.1	100%			
SAT052		*Enterobacter*	spp.	PA5D1SP	1	FJ577974.1	99%			
SAT053		*Paenisporosarcina*	spp.	A7A14SP	1	AB712362.1	99%			

* CAT are Allelic Types identified in similar research projects done in other regions of the US (i.e. Northeast, Southeast, South, Midwest and West; Ranieri and Boor, 2009; Ivy et al., 2012).

D. Analysis of Variance of Standard Plate Counts (SPC) of milk samples

1. Milk samples at Day 1

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.8374	2	0.4187	F (2, 30) = 1.522	P = 0.2347
Residual (within columns)	8.255	30	0.2752		
Total	9.092	32			

Test details	Mean 1	Mean 2
HT Raw milk vs. In-line pasteurized	1.370	1.290
HT Raw milk vs. Packaged pasteurized	1.370	1.743
In-line pasteurized vs. Packaged pasteurized	1.290	1.743

2. Milk samples at Day 7

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	7.066	2	3.533	F (2, 30) = 10.20	P = 0.0004
Residual (within columns)	10.39	30	0.3464		
Total	17.46	32			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
HT Raw milk vs. In-line pasteurized	0.1605	-0.4177 to 0.7388	No	ns
HT Raw milk vs. Packaged pasteurized	-1.126	-1.815 to -0.4375	Yes	***
In-line pasteurized vs. Packaged pasteurized	-1.287	-2.036 to -0.5377	Yes	***

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
HT Raw milk vs. In-line pasteurized	1.354	1.193	0.1605	0.2346	17	10	0.9679	30
HT Raw milk vs. Packaged pasteurized	1.354	2.480	-1.126	0.2795	17	6	5.700	30
In-line pasteurized vs. Packaged pasteurized	1.193	2.480	-1.287	0.3039	10	6	5.988	30

3. Milk samples at Day 14

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	51.03	2	25.52	F (2, 30) = 14.89	P < 0.0001
Residual (within columns)	51.41	30	1.714		
Total	102.4	32			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
HT Raw milk vs. In-line pasteurized	-0.7575	-2.044 to 0.5286	No	ns
HT Raw milk vs. Packaged pasteurized	-3.389	-4.921 to -1.856	Yes	****
In-line pasteurized vs. Packaged pasteurized	-2.631	-4.298 to -0.9645	Yes	**

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
HT Raw milk vs. In-line pasteurized	1.566	2.324	-0.7575	0.5217	17	10	2.053	30
HT Raw milk vs. Packaged pasteurized	1.566	4.955	-3.389	0.6216	17	6	7.709	30
In-line pasteurized vs. Packaged pasteurized	2.324	4.955	-2.631	0.6760	10	6	5.504	30

4. Milk samples at Day 21

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	135.7	2	67.85	F (2, 30) = 18.68	P < 0.0001
Residual (within columns)	109.0	30	3.632		
Total	244.7	32			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
HT Raw milk vs. In-line pasteurized	-2.065	-3.937 to -0.1923	Yes	*
HT Raw milk vs. Packaged pasteurized	-5.474	-7.705 to -3.243	Yes	****
In-line pasteurized vs. Packaged pasteurized	-3.409	-5.835 to -0.9827	Yes	**

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
HT Raw milk vs. In-line pasteurized	2.131	4.196	-2.065	0.7595	17	10	3.845	30
HT Raw milk vs. Packaged pasteurized	2.131	7.605	-5.474	0.9050	17	6	8.554	30
In-line pasteurized vs. Packaged pasteurized	4.196	7.605	-3.409	0.9842	10	6	4.898	30

5. Heat-treated raw milk samples over shelf life

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	6.746	3	2.249	F (3, 64) = 2.652	P = 0.0561
Residual (within columns)	54.26	64	0.8478		
Total	61.01	67			

Test details	Mean 1	Mean 2
Day 1 vs. Day 7	1.370	1.354
Day 1 vs. Day 14	1.370	1.566
Day 1 vs. Day 21	1.370	2.131
Day 7 vs. Day 14	1.354	1.566
Day 7 vs. Day 21	1.354	2.131
Day 14 vs. Day 21	1.566	2.131

6. In-line pasteurized milk samples over shelf life

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	58.31	3	19.44	F (3, 36) = 6.229	P = 0.0016
Residual (within columns)	112.3	36	3.121		
Total	170.7	39			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Day 1 vs. Day 7	0.09700	-2.031 to 2.225	No	ns
Day 1 vs. Day 14	-1.034	-3.162 to 1.094	No	ns
Day 1 vs. Day 21	-2.906	-5.034 to -0.7783	Yes	**
Day 7 vs. Day 14	-1.131	-3.259 to 0.9967	No	ns
Day 7 vs. Day 21	-3.003	-5.131 to -0.8753	Yes	**
Day 14 vs. Day 21	-1.872	-4.000 to 0.2557	No	ns

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Day 1 vs. Day 7	1.290	1.193	0.09700	0.7900	10	10	0.1736	36
Day 1 vs. Day 14	1.290	2.324	-1.034	0.7900	10	10	1.851	36
Day 1 vs. Day 21	1.290	4.196	-2.906	0.7900	10	10	5.202	36
Day 7 vs. Day 14	1.193	2.324	-1.131	0.7900	10	10	2.025	36
Day 7 vs. Day 21	1.193	4.196	-3.003	0.7900	10	10	5.376	36
Day 14 vs. Day 21	2.324	4.196	-1.872	0.7900	10	10	3.351	36

7. Packaged pasteurized milk samples over shelf life

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	126.9	3	42.32	F (3, 20) = 68.13	P < 0.0001
Residual (within columns)	12.42	20	0.6211		
Total	139.4	23			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Day 1 vs. Day 7	-0.7367	-2.010 to 0.5368	No	ns
Day 1 vs. Day 14	-3.212	-4.485 to -1.938	Yes	****
Day 1 vs. Day 21	-5.862	-7.135 to -4.588	Yes	****
Day 7 vs. Day 14	-2.475	-3.748 to -1.202	Yes	***
Day 7 vs. Day 21	-5.125	-6.398 to -3.852	Yes	****
Day 14 vs. Day 21	-2.650	-3.923 to -1.377	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Day 1 vs. Day 7	1.743	2.480	-0.7367	0.4550	6	6	2.290	20
Day 1 vs. Day 14	1.743	4.955	-3.212	0.4550	6	6	9.983	20
Day 1 vs. Day 21	1.743	7.605	-5.862	0.4550	6	6	18.22	20
Day 7 vs. Day 14	2.480	4.955	-2.475	0.4550	6	6	7.693	20
Day 7 vs. Day 21	2.480	7.605	-5.125	0.4550	6	6	15.93	20
Day 14 vs. Day 21	4.955	7.605	-2.650	0.4550	6	6	8.237	20

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