Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk

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ABSTRACT

Bacterial endospores (also referred to as spores) present in raw milk are capable of surviving pasteurization and other adverse conditions encountered during dairy powder production. Therefore, requiring low spore levels in raw ingredients (e.g., raw milk) may be necessary for producing dairy powders with low spore counts. To identify potential associations between management practices and spore levels in raw milk, we sampled bulk tank raw milk from 33 farms throughout New York State every other month for 1 yr. Following spore pasteurization (80°C for 12 min), samples were incubated at 3 different temperatures to enumerate psychrotolerant (6°C for 10 d), mesophilic (32°C for 48 h), and thermophilic (55°C for 48 h) spores. An additional enrichment procedure was used to detect spores present at low levels (<10 spores/mL). Overall, psychrotolerant, mesophilic, and thermophilic spores were detected (at levels ≥ 10 spores/mL) in 1, 74, and 58% of bulk tank raw milk samples, respectively. Although thermophilic spore levels could not be quantified (due to bacterial swarming), mesophilic spore levels ranged from below detection (<10 spores/mL) to 680 spores/mL. Data collected through surveys were used to identify management practices associated with either mesophilic or thermophilic spore levels. We found that different management practices are associated with mesophilic and thermophilic spore levels. Low mesophilic spore levels in bulk tank raw milk samples were associated with (1) large herd size, (2) use of sawdust or sand bedding, and (3) not fore stripping during the premilking routine. Management practices that were associated with lower odds of having a thermophilic spore level ≥ 10 spores/mL are (1) large herd size, (2) spray-based application of the postmilking disinfectant, (3) dry massaging the udder during the premilking routine, and (4) the use of straw bedding. Collectively, these results suggest that

different management practices may influence mesophilic and thermophilic spore levels in raw milk.

Key words: spores, raw milk, management practices

INTRODUCTION

Sporeforming bacteria of the *Clostridiaceae* and *Bacillaceae* families represent important spoilage organisms for a variety of dairy foods (Scheldeman et al., 2005; De Jonghe et al., 2010; Ivy et al., 2012). In HTST pasteurized fluid milk, psychrotolerant *Bacillus* spp. and *Pseudomonas* spp. contribute off flavors and aromas which reduce product shelf life (Ranieri and Boor, 2009). In semi-hard cheeses, the anaerobic interior provides a favorable environment for *Clostridium tyrobutyricum*, which metabolizes lactate into hydrogen gas, resulting in “late blowing” defects (Klijn et al., 1995). In dairy powders, stringent customer specifications mandate low spore levels (A. Bienvenue, United States Dairy Export Council, Arlington, VA, personal communication). According to a recent survey by the US Dairy Export Council, dairy powders destined for use in baby formulas have aerobic mesophilic and thermophilic spore limits that range from <500 to 1,000 cfu/g (A. Bienvenue, United States Dairy Export Council, Arlington, VA, personal communication).

Raw milk has been implicated as an important source of endospores produced by mesophilic sporeforming bacteria (abbreviated here as mesophilic spores; **MS**), by thermophilic sporeforming bacteria (thermophilic spores; **TS**), and by psychrotolerant sporeforming bacteria (psychrotolerant spores; **PS**). However, several studies have shown that the spore populations of dairy powders are generally different from those of raw milk (Scott et al., 2007; Burgess et al., 2010; Hill and Smythe, 2012). For example, although TS of *Anoxybacillus* spp. and *Geobacillus* spp. are frequently isolated from dairy powders, they are rarely isolated from raw milk (McGuigan et al., 2002; Rückert et al., 2004; Scott et al., 2007). It has been suggested that TS of *Anoxybacillus* spp. and *Geobacillus* spp. are either (1) present at very low levels in raw milk and are able to

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germinate and produce vegetative cells capable of growing in the product during processing, or (2) that the TS are able to attach to surfaces within the processing environment where they germinate and form biofilms that contaminate the product throughout the processing run (Burgess et al., 2010; Watterson et al., 2014).

A large proportion of MS and TS found in bulk tank (BT) raw milk are thought to originate from the farm environment, as high levels of MS and TS have been isolated from silage, bedding materials, and manure (McGuiggan et al., 2002; Vissers et al., 2007; Quiberoni et al., 2008). Furthermore, Julien et al. (2008) used DNA-based analyses to show that the same spore subtypes were isolated from both BT raw milk and the corn silage fed to milking cows, implicating feed as an important source of spore contamination.

Although numerous studies have examined management practices associated with SCC and SPC levels in BT raw milk (Barkema et al., 1998; Barnouin et al., 2005; Elmoslemany et al., 2010), our understanding of the influence of management practices on the transmission of spores from the farm environment into BT raw milk remains limited despite the significant economic importance of spore counts in the context of dairy powder production. Therefore the goals of this study were to (1) enumerate psychrotolerant, mesophilic, and thermophilic spores present in BT raw milk, and (2) determine management practices that are associated with spore levels in BT raw milk.

MATERIALS AND METHODS

Farm Enrollment

A total of 33 dairy farms located throughout New York State were included in the study. Participating farms were recruited based on their being enrolled in Quality Milk Production Services (QMPS) facilitated monitoring programs (College of Veterinary Medicine, Cornell University, Ithaca, NY). Farms were selected to represent 4 general geographic areas in upstate New York from the 4 QMPS laboratory locations (see Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2015-9406>). At least 1 certified organic farm was included per region. Herds were also selected to represent a range of sizes and historical SCC levels (see Supplemental Table S1 for a detailed summary of farm characteristics).

Bulk Tank Raw Milk Sample Collection

Bulk tank raw milk samples were collected from each of the 33 dairy farms every other month between April 2012 and March 2013 (i.e., half of the farms had their first sample date in April 2012, whereas the remaining

farms were first sampled in May 2012). Bulk tank raw milk was agitated before collection of approximately 400 mL of milk with a sterile sampling receptacle. Bulk tank raw milk samples were divided between two 300-mL polyethylene terephthalate vials and were held at $\leq 6^{\circ}\text{C}$ during transport to the Milk Quality Improvement Program (MQIP) laboratory (Cornell University, Ithaca, NY). Samples were either brought directly to the laboratory by QMPS technicians, or were shipped via overnight delivery within 24 h of sampling; raw milk BT samples thus may have been tested between 24 and 72 h after milking. Because spores are unlikely to germinate at 6°C , time between milking and testing of BT milk was not presumed to affect the spore counts. Bulk tank raw milk sample temperatures were taken immediately upon receipt to the MQIP laboratory. If the sample temperature exceeded 6°C , a new sample was collected. Bulk tank raw milk samples were also submitted for SCC analysis (Dairy One Cooperative, Ithaca, NY).

Survey Administration

Surveys were administered verbally to dairy farm personnel at each sample collection by QMPS technicians. Questions focused on housing and bedding types used, premilking routine, and sanitation practices (see Table 1). Cow hygiene was assessed by technicians using a convenience sample of 10 cows per farm per sample date; a scale of 1 to 4 was used to score the cleanliness of the udder and hind legs of each cow, with lower scores indicating better hygiene (Barkema et al., 1998). Stall cleanliness was assessed using predefined terms (good, fair, and poor). All survey data were entered into an Access database (Microsoft Corporation, Seattle, WA).

Quantification of Psychrotolerant, Mesophilic, and Thermophilic Spores

Approximately 200 mL of each BT raw milk sample were aseptically transferred to a sterile glass Pyrex (Corning Inc., Corning, NY) bottle and agitated according to the *Standard Methods for the Evaluation of Dairy Products*, 17th ed. (SMEDP; Frank and Yousef, 2004). Each sample of BT raw milk was evaluated for total bacteria count on SPC agar (Difco, Franklin Lakes, NJ). Aliquots of each BT raw milk sample were spore pasteurized (SP) at 80°C for 12 min (Frank and Yousef, 2004), followed by rapid cooling to $\leq 6^{\circ}\text{C}$. Spore pasteurized samples were spiral plated (Auto-plate 4000, Spiral Biotech, Norwood, MA) onto brain heart infusion agar (BHI; Difco, Franklin Lakes, NJ) in duplicate for (1) psychrotolerant spore count (PSC;

Table 1. Summary of farm management practice data collected from surveys administered at each sampling occasion (n = 6 visits per farm) for 33 farms located throughout New York State

Factor ¹	Description ²
Seasonal factors	Sample collection date
Cow hygiene	Score assigned from 1 to 4 (1 representing cleanest and 4 representing most soiled) based on the presence of organic material and manure on hind quarters, udder, and anal region (among lactating cows)
Milking routine	Preparation of udder for attachment of milking equipment, specifically (1) whether gloves were worn during milking, (2) type of teat disinfectant application used (spray, dip, other), (3) whether problem cows segregated during milking, (4) whether problem cows were milked last
General	Number of cows milked, average production per cow
Housing	Housing type, bedding type, frequency of bedding change, ventilation in housing area (air flow), frequency of pen cleaning, use of recycled water
Sanitation	Type of bulk tank sanitizer and cleanser used, whether milking units were washed between milking groups, frequency of bulk tank cleaning

¹General categories of farm management practices used in data analysis.

²Summary of the data collected from questions asked at each sampling.

incubation at 6°C for 10 d), (2) mesophilic spore count (**MSC**; incubation at 32°C for 48 h), and (3) thermophilic spore count (**TSC**; incubation at 55°C for 48 h).

An additional 60 mL of SP milk was divided between two 2-oz (59.15 mL) sterile plastic vials, which were incubated at 32°C and 55°C for 48 h; this enrichment procedure allowed for the detection of low levels of MS and TS, respectively. Enrichments were plated on BHI (incubated at 32 or 55°C for 24 h) for SP samples that did not yield bacterial colonies upon direct plating, which indicated low levels of spores (<10 spores/mL). Due to the swarming nature of the thermophilic sporeforming bacteria isolated in this study, individual colonies could not be obtained for the majority of TSC analyses and therefore TSC data were categorized into 3 general levels: (1) present on direct plating (≥ 10 spores/mL), (2) present after enrichment (≥ 0.03 to <10 spores/mL), and (3) below detection limit after enrichment (<0.03 spores/mL). Mesophilic spore count data are reported as levels; however, it is important to note that these data represent continuous values, whereas TS levels represent categorical levels.

Bacterial Isolation

Morphologically distinct colonies observed on agar plates representing PSC, MSC, and TSC count plates from direct plating or enriched samples were streaked onto BHI agar plates (i.e., if multiple distinct colony morphologies were identified on a given plate, all colony morphologies were sub-streaked). Using this approach, the 198 milk samples tested yielded a total of 684 bacterial isolates representing spores from PSC, MSC, and TSC plates (2, 425, and 257 isolates, respectively). Pure cultures were preserved in 15% glycerol wt/vol solution, and were stored at -80°C until further characterization. Additional information on isolates collected

in this study can be found at www.foodmicrobetracker.net.

Statistical Analyses

Statistical analyses were performed using R statistical software (version 3.0.1; R-project, Vienna, Austria). For all microbiological analyses, bacteriological data were log transformed. All data were stored in Microsoft Access and Excel databases (Microsoft Corporation).

Linear Mixed Effect Model Construction. A linear mixed effects model was used to assess the associations between management practices and log MS level, using the lme4 package (Bates et al., 2013). The best fitting model was selected based on a backward stepwise elimination procedure using the Akaike information criterion (**AIC**; Akaike, 1973). The AIC for the candidate model was calculated and compared with the AIC of models with 1 explanatory variable removed. If the removal of an explanatory variable resulted in a decrease in the AIC of the candidate model (i.e., AIC decreased by >0 units), the explanatory variable was removed from the final model. Interaction terms were generated for both sample date and average cow hygiene scores and were chosen using a forward stepwise addition procedure. Farm was included as a random effect in the model. The final model was not influenced by outliers, and the residual values were normally distributed.

Logistic Regression Model Construction. Logistic regression was used to model the associations between management practices and the log odds of the presence of having a TSC ≥ 10 spores/mL. The model was constructed using the lme4 package (Bates et al., 2013).

Variables were removed from the candidate model using a backward stepwise elimination process depend-

ing on the change in AIC as described above (Akaike, 1973). Interaction terms were generated and were selected using a forward stepwise addition process. None of the interaction terms reduced the AIC, and therefore none of the terms were included in the model.

Hierarchical Cluster Analyses

Hierarchical cluster analyses were performed on log-transformed data to group farms by MS and TS level across each of the 6 sample collection dates. To perform the cluster analysis for MS level, a distance matrix was first generated by calculating the difference in MS level between farms for each of the 6 trials. For MS levels below detection for direct plating (<10 spores/mL) but detectable after enrichment, we approximated a median value using a normal distribution extrapolated from the upper 50% of the MSC data. The assumption that MS levels between 0.03 and 10 spores/mL are normally distributed was supported by preliminary results available from other research studies (MQIP, unpublished data). Therefore, we assigned samples with MS level between 0.03 and 10 spores/mL a value of 4 spores/mL, and performed the cluster analysis with these approximated values. The *hclust* script in the *stats* package was used to perform the cluster analysis (R Development Core Team, 2013). Bootstrap values and adjusted unbiased *P*-values were determined using the *pvcust* package (Suzuki and Shimodaira, 2015). Due to the categorical nature of the TSC data, the cluster analysis was generated by coding the data based on spore level (i.e., ≥ 10 spores/mL, ≥ 0.03 and <10 spores/mL, and <0.03 spores/mL). The package *pheatmap* (Kolde, 2013) was used to generate heat maps for both MS and TS levels.

RESULTS AND DISCUSSION

Psychrotolerant Spores Are Considerably Less Common in Bulk Tank Raw Milk Compared with Mesophilic and Thermophilic Spores

A total of 198 BT raw milk samples were collected and analyzed. Table 2 and Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2015-9406>) contain a summary of farm characteristics and sample SCC. The geometric mean SCC level of BT raw milk samples collected was 226,000 cells/mL (range: 46,000 to 810,000 cells/mL). This value is comparable to a study by Cicconi-Hogan et al. (2013) that found a mean BT SCC of 191,000 cells/mL (range: 41,000 to 725,000 cells/mL) among 290 farms throughout New York, Wisconsin, and Oregon. The geometric mean SPC for the samples tested here was 6,300 cfu/mL (range: 50 to 500,000 cfu/mL); by comparison the geometric mean SPC found by

Cicconi-Hogan et al. (2013) was 5,200 cfu/mL. These SPC and SCC values indicate that the milk quality of the samples evaluated here was similar to the milk quality reported in other studies.

Overall, only 1% of samples had PS levels ≥ 10 spores/mL. By comparison, 74 and 58% of samples had MS and TS levels ≥ 10 spores/mL, respectively (Figure 1). The low levels and prevalence of PS in raw milk is consistent with previous studies, which also found a considerably lower prevalence for PS than MS (Boor et al., 1998; Huck et al., 2008; Martin et al., 2012). Additionally, PS have also been found at very low levels in dairy powders (Watterson et al., 2014). Although PS were present at relatively low levels, it has been found that even very low levels (potentially just 1 spore per container of pasteurized milk) of PS are capable of outgrowth at refrigerated temperatures to levels that are sufficient to cause severe sensory defects (Martin et al., 2012).

Mesophilic Spores Were Detected in All Samples and Their Levels in Raw Milk Differed Significantly Between Farms

Mesophilic spores were detected in 74% of the samples collected at levels ≥ 10 spores/mL; the remaining 26% of samples were positive following enrichment (representing levels ≥ 0.03 but <10 spores/mL; Figure 1). Hence MS appear to be ubiquitous in BT raw milk, at least in New York. The geometric mean MS level for samples with spores detected by direct plating (≥ 10 spores/mL) was 50 spores/mL (range: 10 to 680 spores/mL). The range of MS levels obtained in this study was comparable with MS levels obtained in other studies (te Giffel et al., 2002; Coorevits et al., 2008). For example, Coorevits et al. (2008) found that the mean MS level in BT raw milk collected from 10 Belgian farms sampled on 2 different occasions was 28 spores/mL. Similar to our findings, Coorevits et al. (2008) isolated MS (≥ 2 spores/mL) from 85% of BT raw milk samples, further supporting our findings that MS are ubiquitous in BT raw milk.

Previous reports have shown that the majority of spores isolated from raw milk represent bacteria that show optimal growth between 25 and 40°C, including *Bacillus licheniformis*, *Paenibacillus* spp., *Bacillus cereus* group organisms, and *Bacillus subtilis* (Coorevits et al., 2008; De Vos et al., 2009; De Jonghe et al., 2010); therefore, it is not surprising that the samples analyzed in this study had a high prevalence of spores representing bacteria capable of growing at mesophilic temperatures (32°C). It is important to note that growth temperature minima for these organisms range from 5 to 20°C, and maxima range from 35 to 55°C (De

Table 2. Summary of sample characteristics and associated management practices collected through surveys administered at each sample collection for 198 bulk tank raw milk samples from 33 different farms

Variable ²	Quality Milk Production Services location ¹				
	Ithaca (n = 8)	Canton (n = 8)	Cobbleskill (n = 9)	Geneseo (n = 8)	Total (n = 33)
Average number of milking cows	312	286	144	264	252
Average cow hygiene score ³	1.89	1.90	1.48	1.69	1.74
Average SCC	225	264	206	210	226
Tails docked					
Yes	6	20	24	31	81
No	32	18	30	12	92
Intermediate	10	10	0	5	25
Udder hair clipped					
Yes	14	34	0	36	84
No	34	12	54	12	112
Intermediate	0	2	0	0	2
Gloves worn during milking					
Yes	42	24	42	41	149
No	6	22	12	0	40
Intermediate	0	2	0	7	9
Dry massage during premilking routine					0
Yes	12	0	6	0	18
No	36	48	48	48	180
Fore strip during premilking routine					
Yes	36	48	38	36	158
No	12	0	16	12	40
Postmilking disinfectant application method					
Dip	48	31	54	38	171
Spray	0	17	0	10	27
Teat disinfectant active ingredient					
Iodine	48	48	54	38	188
Dodecylbenzenesulfonic acid	0	0	0	4	4
Chlorhexidine	0	0	0	4	4
Salicylic acid	0	0	0	2	2
Problem cows milked last ⁴					
Yes	26	27	30	24	107
No	11	21	18	24	74
Intermediate	10	0	6	0	16
N/A	1	0	0	0	1
Type of housing ⁵					
Stanchion	5	6	0	0	11
Tiestall	8	24	29	7	68
Freestall	35	30	34	41	140
Loose	4	0	0	0	4
Pasture	11	12	13	6	42
Type of bedding material ⁵					
Shavings	1	6	6	0	13
Sawdust	13	6	6	36	61
Straw	20	0	0	0	20
Hay	4	17	18	12	51
Mat	9	17	0	6	32
Sand	10	10	30	6	56
Clay	0	1	0	0	1
Paper	0	2	5	0	7
Cellulose	0	10	0	0	10
Recycled manure	6	0	0	0	6
Mattress	6	6	7	18	37
Frequency of pen cleaning					
Daily	4	4	5	42	55
More than once per day	44	43	49	0	136
Once per week	0	1	0	0	1
N/A	0	0	0	6	6
Milking units washed between groups					
Yes	16	12	6	12	46
No	28	29	42	28	127
Intermediate	4	7	6	8	25

Continued

Table 2 (Continued). Summary of sample characteristics and associated management practices collected through surveys administered at each sample collection for 198 bulk tank raw milk samples from 33 different farms

Variable ²	Quality Milk Production Services location ¹				
	Ithaca (n = 8)	Canton (n = 8)	Cobleskill (n = 9)	Geneseo (n = 8)	Total (n = 33)
Bulk tank cleaning agent active ingredient					
Acid	12	1	17	12	42
Chlorine	24	12	6	3	45
Multiple ⁶	6	35	11	21	73
Other ⁷	6	0	12	6	24
N/A	0	0	8	6	14

¹n = refers to the number of farms at each location; total is the total number of farms included in the study.

²Intermediate = data could not be attributed to a single category (e.g., intermediate “tails docked” refers to a herd that contained cows with both tails docked and tails undocked); N/A = information not available; except for the first 3 variables listed, numbers shown represent numbers of samples in a given category.

³Average cow hygiene score = see materials and methods for description.

⁴Problem cows milked last = reported segregation of cows due to lameness, illness, and so on, as reported by milking personnel.

⁵Bedding and housing types reported, multiple bedding types were reported per farm.

⁶Multiple = multiple bulk tank sanitizing agents were reported.

⁷Sanitizer used for which the active ingredient could not be identified.

Vos et al., 2009). Therefore, a given organism that can grow under mesophilic conditions may also be capable of growing under psychrotolerant (6°C) or thermophilic (55°C) conditions (or both) as well. Hence, PSC, MSC, and TSC test procedures cannot be used to definitively classify bacterial spore isolates as psychrotolerant, mesophilic, or thermophilic, simply based on the fact that bacteria were isolated through PSC, MSC, or TSC procedures, respectively, a convention that is used in the SMEDP (Frank and Yousef, 2004). This issue has also been raised by Coorevits et al. (2008), who showed that some sporeforming genera have the potential to be isolated at 3 different growth temperatures (i.e., 20, 37, and 55°C). Further characterization of isolates obtained from each spore count method will be necessary to better understand the sporeformer diversity associated with the milk samples tested here.

To assess the variance in MS level between farms, we performed a cluster analysis of MS levels for each of the 6 BT raw milk samples collected per farm (see Materials and Methods). Overall, farms were assigned to 5 separate clusters (Figure 2). The approximated geometric mean MS level for all 198 BT raw milk samples tested was 25 spores/mL; MS spore levels ranged from below detection (<10 spores/mL; multiple farms) to 680 spores/mL (farm C9). Clusters 1 and 2 represent farms with the lowest approximated mean MS levels (based on all 6 BT raw milk samples collected per farm). The overall geometric mean MS level of samples from farms in clusters 1 and 2 (representing n = 4 and n = 8 farms, respectively) were 5 and 10 spores/mL, respectively. The majority of farms were grouped into cluster 3 (n = 13), which had a cluster geometric mean

MS level of 30 spores/mL. Farms with the highest MS levels were grouped into 2 distinct clusters (clusters 4 and 5). The cluster 4 mean MS level (n = 7 farms) was 100 spores/mL. Farm C9 represents group 5, which had a geometric mean MS level of 440 spores/mL. In contrast, farm D4 (Figure 2, cluster 1) had consistently

Figure 1. Distribution of mesophilic and thermophilic spore levels in bulk tank raw milk samples (n = 198) collected from 33 farms, sampled every other month for 1 yr. Bulk tank raw milk samples were spore pasteurized (80°C for 12 min) and plated on brain heart infusion agar incubated at 32°C and 55°C to enumerate mesophilic and thermophilic spores, respectively. MSC = mesophilic spore count; TSC = thermophilic spore count.

Figure 2. Hierarchical cluster analysis of mean mesophilic spore level of bulk tank raw milk samples collected from 33 dairy farms sampled every other month for 1 yr (sample dates 1–6). Farms were located in 4 regions in New York State [coded by location (A–D) and number (1–9)]. The cluster analysis yielded 5 distinct clusters supported by approximate unbiased (AU) values >0.85 . Individual squares represent the mesophilic spore level for one sample and are shaded based on spore level.

low BT raw milk MS levels (all 6 samples collected from this farm had a MS level ≥ 0.03 and <10 spores/mL). The observed variation in MS level between farms is consistent with the findings of other studies that examined variations in total bacteria count (TBC) between farms over time (Hayes et al., 2001; Pantoja et al., 2009). For example, Hayes et al. (2001) observed spikes in TBC (elevated TBC compared with other samples collected from the same farm) when 13 farms were sampled daily over a 2-wk period; the lowest and highest mean TBC between farms differed by a factor of 40. In our study, we found an approximate 170-fold difference between the lowest and highest MS levels for individual BT raw milk samples. A one-way ANOVA

confirmed that the variation in MS level in BT raw milk samples between farms sampled in this study was significant ($P < 0.001$).

Low Mesophilic Spore Levels Were Associated With Large Herd Size, the Use of Sawdust or Sand Bedding, and Not Including Fore Stripping As Part of the Milking Routine

The variance in MS level observed for BT raw milk samples was examined further using a linear mixed model to assess the relationships between management practices and MS levels present in BT raw milk samples. In general, management practices that were found to be associated with lower MS levels include (1) a larger herd size, (2) use of sawdust or sand bedding, and (3) not including fore stripping during the milking routine (see Tables 3 and 4).

Larger herds are predicted to have a lower MS level than smaller herds (Tables 3 and 4). The model predicts that the MS level would be 0.100 log spores/mL lower for each additional 100 cows. The observation that larger herd sizes were associated with lower MS level is consistent with the findings of van Schaik et al. (2002), who found that BT milk samples from larger herds had lower bacterial counts compared with BT milk collected from smaller herds. This observed effect has several possible explanations: (1) larger farms have more frequent milk collections, thus reducing the potential germination-outgrowth-spore formation cycle; (2) larger herds are more likely to have financial resources for improved milking hygiene; or (3) herd size might also be associated with differences in milking practices such as the use of automated milking systems, as found by Jayarao et al. (2004). In their study, Jayarao et al. (2004) also found that raw milk samples collected from farms in the largest herd size category had lower SPC than raw milk collected from farms with fewer cows.

Bedding materials are well-documented sources of environmental bacteria on dairy farms (Hogan and Smith, 1997; Zdanowicz et al., 2004; Magnusson et al., 2007). Our model predicts that milk collected from farms that use sawdust bedding is associated with MS levels that are 0.503 log spores/mL lower than milk collected from farms that do not use sawdust bedding. Sand bedding use was also significant in the model. Sand use was associated with a lower log MSC; however, this effect is lessened as the average cow hygiene score increases. For example, as the average cow hygiene score increases from 1 to 2 (representing an increase in visible soiled material), the predicted log MSC for milk collected from farms that use sand is 0.449 and 0.065 log spores/mL, respectively, lower than raw milk collected from farms that do not use sand. These results suggest that either

Table 3. Associations between farm management practices and mesophilic spore counts of bulk tank raw milk samples collected from 33 farms every other month for 1 year (n = 198 samples)

Variable ⁷	Estimate ¹	SE ²	df	T statistic ³	P-value ⁴
Intercept	2.512	0.302	144	8.329	<0.001
Sample date 2	-0.767	0.310	118	-2.481	0.015
Sample date 3	-0.445	0.369	117	-1.206	0.230
Sample date 4	-1.101	0.367	120	-3.001	0.003
Sample date 5	-0.715	0.362	117	-1.975	0.051
Sample date 6	-0.252	0.349	116	-0.721	0.472
Number of milking cows ⁸	-0.001	0.002	38	-2.820	0.008
Average cow hygiene score ⁵	-0.431	0.140	131	-3.074	0.003
Dry massage during premilking routine = yes ⁶	-1.500	1.002	145	-1.497	0.137
Fore strip during premilking routine = yes ⁶	0.455	0.155	32	2.935	0.006
Bedding material reported = sawdust	-0.503	0.113	43	-4.441	<0.001
Bedding material reported = sand	-0.833	0.335	145	-2.484	0.014
Sample date 2 × dry massage during premilking routine = yes	-0.968	0.348	121	-2.779	0.006
Sample date 3 × dry massage during premilking routine = yes	-0.865	0.364	112	-2.381	0.019
Sample date 4 × dry massage during premilking routine = yes	-1.085	0.412	116	-2.633	0.010
Sample date 5 × dry massage during premilking routine = yes	-0.904	0.358	113	-2.518	0.013
Sample date 6 × dry massage during premilking routine = yes	-0.120	0.361	115	-0.334	0.739
Sample date 2 × average cow hygiene score	0.335	0.164	117	2.045	0.043
Sample date 3 × average cow hygiene score	0.191	0.204	118	0.940	0.349
Sample date 4 × average cow hygiene score	0.558	0.196	120	2.841	0.005
Sample date 5 × average cow hygiene score	0.411	0.198	118	2.074	0.040
Sample date 6 × average cow hygiene score	-0.055	0.173	115	-0.317	0.752
Average cow hygiene score × bedding material reported = sand	0.384	0.189	142	2.031	0.044
Average cow hygiene score × dry massage during premilking routine = yes	1.132	0.610	146	1.855	0.066

¹Parameter estimate of explanatory variable in linear mixed effect model.²Standard error of explanatory variable in linear mixed effect model.³T statistic of Student's *t*-test for explanatory variable.⁴P-value of Student's *t*-test for explanatory variable.⁵Represents the average cow hygiene score of 10 individual cows (see Materials and Methods) for each sample collection for each farm.⁶Reported as "yes" or "no" or "intermediate" as observed by a Quality Milk Production Services technician at the time of sample collection.⁷x = interaction term between variable 1 and variable 2.⁸Unit size is 1 milking cow.

(1) the inherent MS level of sawdust and sand bedding types is lower than other bedding types such as straw or recycled manure solids; (2) that these bedding types do not support the outgrowth of MS, and thus result in lower MS levels; or (3) that these bedding types repre-

sent some other effect such as cow cleanliness. Magnusson et al. (2007) found that certain bedding materials such as peat or peat-straw/sand mixtures inhibit the growth of *B. cereus*, likely due to the low pH of these bedding types. Interestingly, we found that the use of

Table 4. Analysis of variance table for linear mixed effects model with log mesophilic spore count as response

Variable ¹	χ^2 statistic ²	df ³	P ($>\chi^2$) ⁴
Sample date	23.116	5	<0.001
Number of milking cows ⁵	7.952	1	0.005
Average cow hygiene score	2.742	1	0.098
Dry massage during premilking routine = yes	5.628	1	0.018
Fore strip during premilking routine = yes	8.613	1	0.003
Bedding type reported = sawdust	19.725	1	<0.001
Bedding type reported = sand	3.276	1	0.070
Sample date × average cow hygiene score	15.132	5	0.010
Sample date × dry massage during premilking routine = yes	18.739	5	0.002
Average cow hygiene score × bedding type reported = sand	4.126	1	0.042
Average cow hygiene score × dry massage during premilking = yes	3.443	1	0.064

¹x = interaction term between variable 1 and variable 2.²Statistic of linear mixed effect model.³df = degrees of freedom for the χ^2 statistic.⁴P = level of significance of the χ^2 statistic compared with the χ^2 distribution.⁵Unit size is 1 milking cow.

recycled manure solids was not significantly correlated with MS level, even though levels of *Bacillus* spp. in treated manure have been shown to persist throughout the treatment process (Bagge et al., 2010). Although our data indicate that sawdust and sand bedding types are associated with lower MS levels, further analyses are needed to assess whether this effect was due to a reduction in the transmission of spores from the bedding to the raw milk, or whether these bedding types have inherently lower levels of MS. Further studies are also needed to determine where different practices for recycling sand bedding affect raw milk MS counts.

Milk collected from farms which fore strip during the milking routine has a predicted MS level that is 0.455 log spores/mL higher than milk collected from farms that do not fore strip (Tables 3 and 4). The observed association between fore stripping and higher predicted BT raw milk MS levels was unexpected and is not necessarily easy to explain. In theory, fore stripping facilitates the removal of bacteria residing in the teat canal and on the end of the teat. It has been documented that fore stripping is associated with reduced BT SCC (Wenz et al., 2007). Our finding that fore stripping was associated with higher MSC could suggest that increased farm worker contact during the premilking routine may be associated with increased levels of spores being introduced to the udder region before milking unit attachment. Alternatively, similar to the action of dry massaging (discussed below), fore stripping might loosen soiled material on the teat end, which is then able to contaminate the milk. Further work is necessary though to determine whether this association is reproducible and to explore the mechanisms of how fore stripping may increase MS levels in BT raw milk.

The predicted effect of dry massaging (as opposed to not dry massaging) on log MSC is dependent on both the average cow hygiene scores and sample collection date (Tables 3 and 4). The predicted effect of dry massaging on the log MSC of samples collected from farms with low average cow hygiene scores is lower than the predicted log MSC of samples collected from farms with high average cow hygiene scores, for most sample collection dates. For example, for milk collected from a farm with an average cow hygiene score of 1 and which dry massaged, the predicted log MSC was, on average, 1.132 log spores/mL lower than milk collected from a farm with the same average cow hygiene score but which did not dry massage. However, given the same scenario except that the average cow hygiene score is 2, the effect of dry massaging is now associated with a log MSC that is marginally higher (0.107 log spores/mL higher). It is possible that as the average cow hygiene score increases, the amount of visible soiled material that is loosened by the action of dry

massaging increases, but the amount of soiled material that is removed at subsequent steps is not sufficient to prevent transmission into the milk. Alternatively, it is also possible that the association of dry massaging with MSC is due to other factors that were not directly examined in this study, but that also contribute to MS contamination.

Although our statistical analysis showed that some of the variance in sample MS level was attributable to the sample collection date, this association likely reflects variables not captured in our current data set (i.e., weather). Future analyses should also examine specific external factors such as precipitation, average daily temperature, humidity, and other weather-related events that may have an effect on spore contamination. The effect of sample collection date on log MSC was significantly influenced by the average cow hygiene score, and by whether or not cows were dry massaged. This suggests that the average cow hygiene score is likely to change as the weather conditions change (precipitation, temperature, and so on), as does the amount of soiled material that remains on the udder after dry massaging but before attachment of the milking unit.

The effect of average cow hygiene score was inconsistent. Overall, higher hygiene scores were associated with marginally to substantially higher log MSC, depending on sample collection date, use of sand bedding, and dry massaging. Use of bedding materials other than sand, and not dry massaging, was associated with log MSC values that were slightly lower than log MSC for samples collected from farms that used sand and dry massaged. This demonstrates the complex relationship between farm management practices and spore counts.

Taken together, the significant associations observed between BT raw milk MS level and bedding type, preparation of the udder during the premilking routine, herd size, and average cow hygiene scores suggest that the cleanliness of the farm environment as well as the premilking routine play important roles in BT raw milk MS levels. Therefore, future studies aimed at determining specific sources of spore contamination of BT raw milk should be designed to examine these associations in greater detail.

Thermophilic Spores Were Detected Less Frequently Than Mesophilic Spores in Bulk Tank Raw Milk

Overall, just 58% of the BT raw milk samples examined here had TS levels above the detection limit on direct plating (≥ 10 spores/mL; Figure 1); an additional 38% of samples had detectable levels of TS (i.e., ≥ 0.03 to <10 spores/mL) following enrichment. Only 4% of the BT raw milk samples had TS levels <0.03 spores/mL. Accurate quantification of TS level was not pos-

Figure 3. Hierarchical cluster analysis of farms based on thermophilic spore level. Six samples were collected (designated sample dates 1–6) from each farm [coded by location (A–D) and number (1–9)]. Thermophilic spore levels were treated as categorical values because bacterial isolates for thermophilic spore counts had a “swarming” growth, which made it impossible to obtain accurate counts. Therefore, data were categorized into 3 general levels: present upon direct plating (≥ 10 spores/mL), present at enrichment (0.03–10 spores/mL), or present at levels below detection following enrichment (< 0.03 spores/mL).

sible, as a large number of the samples contained bacteria that swarmed (sometimes referred to as spreaders). Results are thus reported as categorical values [i.e., (1) < 0.03 spores/mL, (2) ≥ 0.03 to < 10 spores/mL, and (3) ≥ 10 spores/mL (Figure 1)].

As with the MS level, the TS level varied considerably between farms (Figure 3). The farms were assigned to 4 general clusters. Cluster 1 consisted of 1 farm, D4, which had TS levels below detection (< 0.03 spores/mL) for the majority of the BT raw milk samples collected (4/6 samples; Figure 3). Cluster 4 represents farms that had BT raw milk TS levels ≥ 10 spores/mL for the majority of the samples collected, with 8 of these farms contributing BT raw milk with ≥ 10 spores/mL for all

6 samples (indicating high TS levels compared with other BT raw milk samples analyzed in this study). In contrast, for 4 farms (D1, D4, D6, and A7), all 6 of the farms' BT raw milk samples were negative by direct plating, indicating consistently low levels of TS (< 10 spores/mL).

Because TS is the primary category of sporeforming bacteria of concern for dairy powders (Burgess et al., 2010; Watterson et al., 2014), our data provide important benchmarking information that could help to develop raw milk specifications and potential premium payment systems for low TS levels in BT raw milk. The importance of benchmarking TS levels is illustrated by the fact that some customer specifications require a TSC < 100 cfu/g of dairy powder. For example, the production of whole milk powder (WMP) represents removal of water from milk that equates to a concentration effect of approximately 7 fold. Therefore, raw milk with a TS level of approximately 14 spores/mL would result in WMP with approximately 100 spores/mL (assuming no TS die-off or additional contamination; Watterson et al., 2014). In the study reported here, just 40% of the samples had TS levels < 10 spores/mL (due to the categorical nature of the TSC data we were unable to quantify the number of samples that had levels present between 10 and 14 spores/mL); thus, a considerable proportion of the BT raw milk samples tested in this study may not be suitable for production of WMP with a stringent specification of < 100 TS/g, unless processing interventions (e.g., removal via centrifugation) are also used.

Although limiting TS levels in raw milk is considered to be important for achieving low TS levels in dairy powders, few other studies have previously evaluated the variance in TS levels in BT raw milk. In one study, Coorevits et al. (2008) reported that among 10 farms sampled twice, the BT raw milk TS level varied between sample collections. McGuiggan et al. (2002) also noted considerable variation in PS, MS, and TS levels found in the raw milk collected from a processing plant sampled over a 1-yr period.

Low Thermophilic Spore Levels Are Associated with Large Herd Size, Use of Straw Bedding, and Select Aspects of the Premilking and Postmilking Routines

Associations between management practices and TS level (i.e., < 10 spores/mL or ≥ 10 spores/mL) in BT raw milk samples were determined using a logistic regression model. Management practices that were associated with a lower odds of a BT raw milk sample having a detectable TS level (i.e., present at ≥ 10 spores/mL) include (1) large herd size, (2) use of straw bedding, (3) dry massaging during the premilking routine, and (4)

Table 5. Associations between farm management practices and the log odds of presence (≥ 10 spores/mL) or absence of thermophilic spores (< 10 spores/mL) in spore pasteurized bulk tank raw milk samples from 33 farms

Variable	Estimate ¹	Fold change ²	SE ³	z-value ⁴	Pr(> z) ⁵
Intercept	-6.038	-418.887	1.724	-3.503	0.001
Number of milking cows ⁶	-0.003	-1.003	0.001	-3.022	0.003
Average cow hygiene score	1.975	0.139	0.654	3.018	0.003
Tails docked = yes	-0.043	-1.044	0.504	-0.085	0.933
Tails docked = intermediate ⁷	1.434	0.238	0.645	2.222	0.026
Dry massage during milking	-1.823	-6.191	0.679	-2.685	0.007
Postmilking disinfectant = spray	-1.482	-4.403	0.724	-2.048	0.041
Housing type reported = stanchion	1.285	0.277	0.861	1.493	0.136
Housing type reported = tie stall	1.990	0.137	0.532	3.739	<0.001
Bedding type reported = straw	-2.406	-11.087	0.717	-3.356	0.001
Milking units washed between milking groups = sometimes	0.696	0.499	0.632	-1.102	0.271
Milking units washed between milking groups = yes	1.010	0.364	0.505	2.176	0.030
Log standard plate count of bulk tank raw milk	1.378	0.252	0.312	4.422	<0.001

¹Parameter estimate of variable in logistic regression model.

²Calculated by taking the reciprocal of the estimate raised to the power of e. Fold change indicates the change in likelihood of a sample showing presence of thermophilic sports at ≥ 10 spores/mL.

³Standard error of explanatory variable in logistic regression model.

⁴Corresponding z-value of parameter estimate.

⁵Level of significance for z-value compared with z-distribution.

⁶Unit size is 1 milking cow.

⁷Herd contained a mix of cows with and without docked tails.

utilization of a postmilking disinfectant spray (Table 5).

Similar to the relationship between MS level and herd size, the odds of a BT raw milk TS level ≥ 10 spores/mL are predicted to be lower with larger herd sizes. For example, a 100 cow increase in herd size is associated with a 1.284 times lower odds of a BT raw milk sample with TS level ≥ 10 spores/mL (Table 5).

For farms that use straw bedding, the predicted odds of having a BT raw milk TS level ≥ 10 spores/mL is 11.087 times lower than raw milk collected from farms that do not use straw bedding (Table 5). The association between straw bedding and a lower odds of TS level ≥ 10 spores/mL implies that either (1) the TS level of straw is lower than other bedding types, or (2) that straw bedding is able to reduce the transmission of spores from the environment to the udder, and subsequently, into the milk. Magnusson et al. (2007) found a positive correlation between the spore load in bedding materials (both straw and sawdust were tested) and the spore load in raw milk samples. The authors noted that the inherent spore levels of both straw and sawdust bedding materials were similar (both below detection < 50 spores/g), but that these levels increased after the bedding became soiled with manure (Magnusson et al., 2007). Although our data suggest that straw bedding use is associated with significantly lower BT raw milk TS levels, compared with the use of other bedding types, further analyses are necessary to further discriminate this relationship.

Dry massaging during the premilking routine was associated with a predicted odds of a BT raw milk

sample TS level ≥ 10 spores/mL that was 6.191 times lower than BT raw milk collected from a farm that does not dry massage (Table 5). In the MSC linear mixed model, the predicted effect of dry massaging on MS levels was dependent on sample collection date, average cow hygiene score, and whether or not sand was used. For some sample dates, but not all, an increase in the average cow hygiene score is predicted to increase the log MSC, but for other sample dates, it is associated with a predicted decrease in log MSC. It is possible that the observed difference in the effect of dry massaging on raw milk MS and TS levels could be attributed to differences in the response modeled (i.e., MS level used as a continuous variable versus TS level reported as categorical data). Alternatively, it is possible that this discrepancy is suggestive that the thermophilic and mesophilic spores isolated in this study may be derived from different environmental sources, possibly that TS are more frequently present in manure and soil, which are dislodged during dry massaging, but MS contamination occurs at steps after attachment of the milking unit (i.e., milking equipment-derived contamination).

Application of a postmilking disinfectant in the form of a spray was also associated with a lower odds of having a TS level ≥ 10 spores/mL. For farms that use a spray instead of a dip to apply postmilking teat disinfectant, the odds of having a BT raw milk TS level ≥ 10 spores/mL are 4.403 times lower than farms that use a dip-based application. Overall, 27 and 171 samples represented raw milk from a sampling where spray- and dip-based applications of a postmilking disinfectant product were used, respectively. In contrast to our find-

ings, Jayarao et al. (2004) reported that using a dip application of the postmilking disinfectant was associated with significantly lower bacterial counts, compared with a spray application.

The model predicts that tie stall housing use is associated with a predicted odds of having a TS level ≥ 10 spores/mL that is 7.315 times higher than if an alternative housing type is used; the majority of samples were collected from BT on farms that reported using free stalls ($n = 140$; Table 2), and 68 samples were collected from farms in which cows were housed in tie stalls. Interestingly, Magnusson et al. (2007) found that *B. cereus* was isolated more frequently from tie stall bedding than from free stall bedding material, even though the hygiene scores of the cows housed in both housing types did not differ. One could hypothesize that tie stall use is also associated with other management practices that may have not been assessed here, but lead to increased environmental exposure to TS. To determine if tie stall use is associated with higher average cow hygiene scores, we performed a one-way ANOVA, which showed that the average cow hygiene score of cows housed in tie stalls was not significantly different ($P = 0.813$) from the overall average cow hygiene score for all housing types. Although our data suggest that tie stall use may be associated with higher TS level, further investigations into the mechanism of the association are needed.

We also found that higher average cow hygiene scores (indicating higher levels of soiled material present on the udder and rear legs) were associated with a higher predicted odds (7.204 times higher) of a sample TS level ≥ 10 spores/mL. This observation was expected because TS have been isolated from soil and cow manure previously (Wang et al., 2007). The association between soiled cows and TS levels ≥ 10 spores/mL suggests that cow hygiene is important for achieving low TS levels, although further studies will be necessary to confirm the observed relationship between hygiene and TS level.

The model also predicts that the odds of having a BT raw milk sample with TS levels ≥ 10 spores/mL for milk collected from herds with a mixed population of cows with tails docked and without tails docked is 4.195 times higher compared with farms that contain herds in which all cows have undocked tails, and 4.237 times higher compared with farms that dock all tails. A study by Schreiner and Ruegg (2003) did not find an association between cow hygiene and milk quality that was attributable to docking tails. It is probable that the information captured by whether or not tails were docked is explaining some of the variance in the data that was not examined in our survey, or that tail docking is contributing to cow hygiene, even though

an interaction term examining the relationship of these variables was not significant in the final model.

Washing the milking units between groups of cows is associated with a predicted odds of a BT raw milk sample having a TS level ≥ 10 spores/mL that is 2.745 times higher compared with not washing between cow groups, or 1.369 times higher than doing so intermittently. In our study, 6 out of 33 farms reported washing milking units between milking groups. This result was also unexpected because one would hypothesize that washing between milking groups would reduce the presence of biofilms, which are thought to be important sources of thermophilic bacilli (Scott et al., 2007). It is possible that the amount of time elapsed between sanitization cycles is not sufficient to allow for biofilm formation in the milking equipment. Interestingly, in dairy powder manufacturing plants, TS levels in dairy powders have been shown to increase as the processing run time increases (Scott et al., 2007; Watterson et al., 2014). Although, to our knowledge, the relationship between TS level and the frequency at which milking units are washed has not been previously studied, our data suggest that more frequent washing of milking equipment is associated with higher odds of TS levels ≥ 10 spores/mL in raw milk. It is possible that more frequent washing provides for additional moisture that facilitates spore transmission; this could be analogous to food processing facilities where mid-shift cleaning has been associated with increased microbial contamination. This hypothesis will require further investigation as we are unable to discern from the data collected, the means by which the milking units were washed, and exactly how this might have an effect on the resulting spore load of the BT raw milk.

Last, the total bacteria count was significantly associated with TS level. A 1-log increase in SPC was associated with an odds of having a TS level ≥ 10 spores/mL that is 3.966 times higher. It is noteworthy that endospores from bacteria with strictly thermophilic growth temperature requirements would not have been isolated from the SPC performed, as the assay was performed at 32°C. Therefore, this finding supports the idea that higher total bacteria counts may be indicative of less sanitary milking conditions, which could correlate with higher levels of TS in the milk.

Different Management Practices Are Associated with Mesophilic and Thermophilic Spore Levels in Bulk Tank Raw Milk

With the exception of herd size, where larger herd sizes were associated with lower MS levels and lower odds of TS level ≥ 10 spores/mL in BT raw milk, different management practices were associated with higher

MS levels and higher odds of BT raw milk samples with TS level ≥ 10 spores/mL. These differences are likely a result of (1) the ecological differences of spore-formers that grow at 32 and 55°C, or (2) their mode of transmission from the environment into raw milk. Overall, 2 management practices were associated with higher MS level, and 5 management factors were associated with higher odds of TS level ≥ 10 spores/mL. Although future work confirming these findings is needed, the differences in the direction of the effect of management practices on MS levels and the odds of TS level ≥ 10 spores/mL could be attributed to differences in environmental sources for MS and TS. For example, the finding that higher average cow hygiene scores are associated with a higher odds of TS levels ≥ 10 spores/mL, but lower MS levels, may indicate that manure and bedding materials may be important sources of TS but not MS. Because we did not collect and test environmental samples, it is difficult to assess differences in transmission between the environment and the raw milk. Mesophilic spores may also be more ubiquitously distributed than TS, and therefore might contaminate BT raw milk from multiple environmental sources. Although few studies have examined different parts of the farm environment as potential sources of spore contamination, Scheldeman et al. (2005) isolated *B. licheniformis* from several different environmental sources including the milking equipment and several feed stuffs, whereas other species such as *Brevibacillus borstelensis* were only isolated from fodder concentrate. The use of molecular subtyping methods to identify the MS and TS collected in this study will provide further information that will aid in our understanding of matching specific management practices with specific sporeforming bacteria genera isolated from raw milk.

CONCLUSIONS

Overall, our data show that MS are more abundant in BT raw milk samples compared with PS and TS, and that the management practices that are associated with MS and TS levels in raw milk are different. Increasingly stringent customer specifications for low MS and TS levels continue to present a major hurdle in dairy powder markets. It is apparent that farm management practices may play an important role in reducing the spore load of BT raw milk. Because spores are resistant to thermal and chemical treatments, preventing their transmission into the raw milk represents an important strategy in the production of dairy powders with low spore counts. Our results provide important baseline data for management practices that contribute significantly to BT raw milk spore loads, and specifically suggest that future research should focus on the roles

of bedding material types, specifically straw, sawdust, and sand, as well as the milking routine to elucidate the roles that these factors play in the transmission of spores from the environment to the milk.

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