The microbiome of bulk tank milk: Characterization and associations with somatic cell count and bacterial count

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ABSTRACT

Numerous studies have evaluated associations between bacterial groups and milk quality parameters. However, to our knowledge, no research has been published that has analyzed associations between the microbiome and quality parameters of bulk tank milk (BTM). Thus, the aims of this study were to identify the core microbiome of BTM and to examine associations between the microbiome and milk quality parameters. Four hundred seventy-two BTM samples from 19 different dairy farms located in New York State were analyzed by next-generation sequencing and quantitative PCR of the 16S rRNA gene to assess the milk microbiome and measure total bacterial load, respectively. Flow cytometry was used to determine bacterial and somatic cell counts. Heatmaps were constructed and simple linear regressions and response screening analysis were performed. To facilitate data analysis and interpretation of the results, we dichotomized the BTM samples into high (HSCC, >200,000) and low somatic cell count (LSCC, ≤200,000) and into high (HSPC, >3.6) and low log₁₀ SPC (LSPC, ≤3.6). Spoilage-causing, spore-forming, and pathogenic bacteria of importance to the dairy industry were identified in the core microbiome. In addition, the taxa *Thermoanaerobacterium* and 5–7N15 were identified in the core microbiome; to our knowledge, these genera have not been previously identified in milk samples. Several bacterial genera were encountered in significantly higher relative abundances in the HSCC group when compared with the LSCC group, including *Corynebacterium*, *Streptococcus*, *Lactobacillus*, *Coxiella*, *Arthrobacter*, and *Lactococcus*. Additionally, several bacterial taxa were found in significantly higher relative abundances in the HSPC groups versus the LSPC groups: *Acinetobacter*, *Enterobacteriaceae*, *Corynebacterium*, and *Streptococcus*. In addition, *Streptococcus* was highly correlated with HSPC, and this genus was the second most abundant bacterial taxon detected in samples classified as HSCC. Bacterial diversity (Shannon index) was negatively correlated with bacterial load, suggesting that the microbiomes of high-bacterial load BTM samples are dominated by smaller groups of bacterial taxa. In conclusion, the associations described corroborated current knowledge about pathogens and spoilage bacteria in relationship to milk quality, and also indicated that other bacterial taxa should be a focus of further investigations.

Key words: milk, microbiome, quality, next-generation sequencing, *Streptococcus*

INTRODUCTION

Ensuring the safety and quality of raw milk is a challenge worldwide (Gschwendtner et al., 2016). Since the 1990s, bulk tank milk (BTM) has been used to diagnose current and potential problems in dairy herds related to milk quality and mastitis (Jayarao et al., 2004). In addition, the food industry and cooperatives have been using BTM analysis to identify higher quality milk for which they pay premium prices (Jayarao et al., 2004; Barbano et al., 2006) based on one or more parameters (Gillespie et al., 2012). Nightingale et al. (2008) evaluated the effect of a premium program for high-quality milk in a United States cooperative focused on BTM SCC, which is a widely used criterion for milk quality premium payments (Barbano et al., 2006). Somatic cells are naturally present in milk (Li et al., 2014); commonly, SCC has been used to indicate the prevalence of IMI in dairy herds (Gillespie et al., 2012) and overall milk quality (Li et al., 2014). Associations among milk components, milk quality parameters, and presence of bacteria have been evaluated elsewhere (Park et al., 2007; Katholm et al., 2012).

Milk quality is influenced by several bacterial counts; for example, laboratory pasteurization count and SPC (Gillespie et al., 2012). A high SPC in raw milk indicates mastitis occurrence and problems in milking or general

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hygiene (Gillespie et al., 2012). Microbial contamination of BTM can occur through several sources and by distinct microorganisms (Elmoslemany et al., 2009). Among the microorganisms, spore-forming bacteria is a concern (Barbano et al., 2006); raw milk is a source of endospores produced by mesophilic, thermophilic, and psychrotolerant spore-forming bacteria (Miller et al., 2015). Standard laboratory analysis (based on traditional culturing) following pasteurization is a good technique to indicate the level of spores (Barbano et al., 2006). The majority of microbiological analyses of raw milk microbiota have been based on culture-dependent methods (Fricker et al., 2011); however, culture-dependent methods have several limitations when compared with culture-independent methods (e.g., viable but nonculturable cells cannot be identified by culturing; Weber et al., 2014). Consequently, the use of different culture-independent methods to describe the bacterial composition has emerged. The composition of the bacterial community of raw milk has been described using different culture-independent methods (Kuang et al., 2009; Oikonomou et al., 2014; Weber et al., 2014); however, it is believed that a knowledge gap still exists in our understanding of native bacterial communities in raw milk (Fricker et al., 2011). Moreover, to our knowledge, no research has been published that has analyzed associations between the microbiome and quality parameters of BTM. Therefore, we hypothesized that the bulk tank milk microbiome will be associated with milk quality parameters (e.g., SCC and SPC).

MATERIALS AND METHODS

BTM Samples Collection

Dairy farms associated with Cayuga Marketing LLC (central region, NY), which regularly send BTM samples to Dairy One Co-op Inc. (Ithaca, NY) for milk analyses, were invited to participate in this study. Letters of consent were sent to the dairy farmers requesting permission to collect BTM samples to perform milk microbiome analyses and to use data from milk analyses carried out by Dairy One. From 29 members contacted, 19 dairy farms agreed to participate in the research, from which 472 BTM samples were obtained during the study period, September to October 2015. Trained employees collected milk samples from bulk tanks aseptically into vials, which were stored under refrigeration during transport to Dairy One Milk Laboratory. The samples submitted to Dairy One were subjected to milk quality analysis, and aliquots aseptically collected from those samples (approximately 6 mL) were sent, under refrigeration, to our laboratory at Cornell University

(Ithaca, NY). The samples were received within 48 h after collection at the farms, and farm, tank, and date were recorded on a spread sheet. Samples were stored at -20°C for downstream molecular analysis.

SCC and Total Bacteria Count

The SCC and total bacterial count measurements were carried out at Dairy One Milk Laboratory. The SCC was determined by flow cytometry using a Fossomatic FC Somatic Cell Counter (Foss, Hillerød, Denmark). Briefly, a mixture of each sample and staining solution was prepared, passed through a flow cell in which somatic cells emit fluorescent light pulses, and the fluorescent light pulses were counted (cells/mL). Total bacterial count was determined using a BactoScan FC+ instrument (Foss), which is also based on flow cytometry. The results for individual bacterial count were converted to colony-forming units by BactoScan FC+ software (Foss), considering SPC as reference method (International Dairy Federation, 1991; IDF Standard 100B: 1991, the reference or anchor method applied in this technology); SPC was used to express the final results for total bacterial count.

Next-Generation Sequencing of the Bacterial 16S rRNA Gene

The DNA was extracted from all samples using a PowerFood Microbial DNA Isolation Kit (MO BIO Laboratory Inc., Carlsbad, CA) following the manufacturer's protocol. The V4 hypervariable region of the bacterial/archaeal 16S rRNA gene was amplified by PCR according to a previously described protocol and optimized for the Illumina MiSeq platform (Caporaso et al., 2012) using different 12-bp error-correcting Golay barcodes for the 16S rRNA gene PCR (Lima et al., 2015). The PCR were performed using 10 μM of each primer (515F and 806R), EconoTaq Plus Green 1 $\times$ Master Mix (Lucigen, Middleton, WI), 5 to 50 ng of individual metagenomic DNA samples, and ultrapure water to bring the final reaction volume to 25 μL . Blank controls in which no DNA was added to the reaction were also performed. All reactions were set up in triplicate, and the PCR conditions for amplification included an initial denaturing step of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 90 s, and a final elongation step of 72°C for 10 min. Replicates were pooled and the amplified DNA visualized by electrophoresis using 1.2% (wt/vol) agarose gels stained with 0.5 mg/mL of ethidium bromide. The DNA was purified using a Gel/PCR Fragments Extraction Kit (IBI Scientific, Peosta, IA). Quantifica-

tion of purified DNA was carried out using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). All samples were standardized to the same DNA concentration and pooled for sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) in 2 different runs according to individual barcode primers. Final equimolar libraries were prepared and sequenced using the MiSeq Reagent Kit V2–300 cycles (Illumina Inc.).

Quantitative PCR

For assessment of the total bacterial load by quantitative (q) PCR, a subsample of 120 samples was randomly selected. For determination of bacterial load, a plasmid containing the amplified V6 hypervariable region was cloned into TOP10 cells using a Zero Blunt TOPO PCR cloning kit (Life Technologies, Darmstadt, Germany). Plasmid was purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and quantified using Quant-iT PicoGreen and a dsDNA Broad Range Assay Kit (Life Technologies Corporation, Carlsbad, CA). Insertion of the DNA fragment was confirmed by agarose gel electrophoresis, and by sequencing at the Cornell University Life Science Core Laboratories Center. The 16S rRNA copy numbers were measured by qPCR using forward 5'-TGGAGCATGTGGTTTAATTCTGA-3' and reverse 5'-TGC GGGACTTAACCCAACA-3' primers previously described (Nonnenmacher et al., 2004). Reactions were performed in 15- μ L volumes composed of 1 $\times$ iQ Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA), 300 nM of each primer, and 5 pg to 50 ng of genomic DNA (or plasmid DNA standards). The thermal cycler conditions were denaturation at 95°C for 3 min, 40 amplification cycles (95°C for 10 s, 55°C for 30 s), and 2 final steps at 95°C for 1 min and 55°C for 1 min followed by melting curve analysis. All reactions were performed in duplicate (plasmid standards, BTM samples, and blank control) using a MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories). Quantification of 16S rRNA target DNA was achieved by 10-fold serial dilutions, ranging from 10⁰ to 10⁷ plasmid copies of the previously quantified plasmid standard. The average of the cycle threshold value was used for calculation of the bacterial load.

Bioinformatics and Statistical Analysis

The 16S rRNA sequences obtained were processed through the open-source pipeline Quantitative Insights into Microbial Ecology (QIIME; version 1.7.0-dev; Caporaso et al., 2010). Sequences were filtered for quality using established guidelines (Bokulich et al., 2013) and

were binned into operational taxonomic units (OTU) based on 97% identity using UCLUST (Edgar, 2010) against the Greengenes reference database (McDonald et al., 2012; May 2013 release). Chimeric sequences were removed, and low-abundance clusters were filtered using USEARCH (Edgar, 2010). The representative sequences for each OTU were compared against the Greengenes database for taxonomy assignment, and only full-length, high-quality reads ($r = 0$) were used for analysis. Shannon diversity index output was generated by the QIIME pipeline. Before estimating the Shannon index, all sample libraries were rarefied to an equal depth of 10,000 sequences using QIIME.

The OTU data obtained from bioinformatics analyses were used to describe the most abundant phyla of BTM samples in terms of relative abundance (%), and the data also were used to generate heatmaps to describe the BTM core microbiome and the 30 most abundant bacterial taxa. Analysis of associations between bacterial taxon and milk quality parameters were first performed using the heatmaps according to quartiles of SCC (quartile 1 = 54,000 cells/mL; quartile 2 = 120,000 cells/mL; quartile 3 = 170,000 cells/mL; and quartile 4 = 450,000 cells/mL) and log₁₀ of the SPC (quartile 1 = 3.0 log cfu/mL; quartile 2 = 3.0 log cfu/mL; quartile 3 = 3.60 log cfu/mL; and quartile 4 = 5.07 log cfu/mL). The heatmaps were plotted using Cell Plot in JMP Pro 11 (SAS Institute Inc., Cary, NC). Using JMP Pro 11, mean, standard deviation, and range of SCC and of log₁₀ SPC were calculated per dairy farm. Response screening analysis was performed in JMP Pro 11; according to the JMP Pro 11 software, this method “fit a large number of y by x combinations to get an idea of what affects what.” Therefore, this method was used to determine which bacterial taxa were most associated or most important to high SCC (HSCC; >200,000 cells/mL), low SCC (LSCC; $\leq$ 200,000 cells/mL), high log₁₀-SPC (HSPC; >3.6 log cfu/mL), and low log₁₀-SPC (LSPC; $\leq$ 3.6 log cfu/mL). The 200 most prevalent OTU were added as outcome variables and the dichotomized SCC variable was added as the dependent variable; the same procedure was performed using log₁₀-SPC. *P*-values were adjusted for false discovery rate (FDR) (Benjamini and Hochberg, 1995) and presented as FDR LogWorth (i.e., $-\log_{10}P$ -value). The mean relative abundance for each bacterial taxon observed in the BTM samples was used as a reference for calculation of fold changes. The effect size was explored because it shows the magnitude of the difference found. Operational taxonomic units statistically significant on the response screening analysis (log₁₀ of the FDR $\geq$ 1.3) are presented; bacterial taxa with higher relative abundance in HSCC compared with LSCC samples and

bacterial taxa with higher relative abundance in LSCC compared with HSCC samples were shown in graphics (mean relative abundance $\pm$ SEM); the same procedure was also applied to $\log_{10}$ SPC. Simple linear regressions between $\log_{10}$ SPC and Shannon diversity index were assessed using JMP Pro 11 software (P -value <0.001). Simple linear regressions were also fitted in JMP software to assess the association between the total bacterial load ($\log_{10}$ copy numbers of the 16S rRNA gene) and 4 factors evaluated: Shannon index, $\log_{10}$ SPC, relative abundance of *Enterobacteriaceae*, and relative abundance of *Acinetobacter* (an initial screening was performed and these 2 bacterial taxa presented the highest associations with total bacterial load).

RESULTS

Sequencing Results, Prevalence of Bacterial Taxa, and Core Microbiome

Quality filtered reads for the 16S rRNA sequences were de-multiplexed, yielding 24,509,428 raw sequences in total (median = 45,821; range = 6,977–158,311), with a median length of 301 bases per read and an average of 51,965 sequences per sample. The most prevalent bacterial phyla were *Firmicutes*, *Fusobacteria*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, and *Tenericutes*; the mean relative abundance of each phylum by farm is depicted in Figure 1. The 30 most prevalent OTU identified in the BTM samples from each farm are shown in Figure 2. The core microbiome across the 19 farms studied is composed of *Ruminococcaceae*, *Acinetobacter*,

Clostridiales, *Bacteroidales*, 5–7N15, *Pseudomonas*, *Staphylococcus*, *Lachnospiraceae*, *Corynebacterium*, *Planococcaceae*, *Bacillus*, and *Thermoanaerobacterium* (Figure 3).

Milk Quality Parameters, Bacterial Load by qPCR, and Bacterial Taxa Associations

The mean, standard deviation, and range of SCC and $\log_{10}$ SPC are shown in Table 1. Associations between SCC quartiles or $\log_{10}$ SPC quartiles and mean relative abundance of the 12 most common OTU identified in the BTM samples were assessed (Figure 4). The relative abundances of *Acinetobacter*, *Enterobacteriaceae*, and *Streptococcus* were higher in the highest quartile of $\log_{10}$ SPC in comparison to the lower $\log_{10}$ SPC groups (Figure 4A). Regarding SCC, *Enterobacteriaceae* was identified with a higher relative abundance in the fourth quartile than in the other quartiles, as was *Corynebacterium*, and the mean relative abundance of *Streptococcus* also increased with the increase in SCC (from the first quartile to the fourth quartile).

Screening analysis was performed to identify the main microbial types distinguishing the microbiomes in samples classified as HSCC and LSCC and $\log_{10}$ SPC. Figure 5 shows the OTU significantly associated with HSCC and LSCC. In HSCC, *Coxiella*, *Dermacoccus*, and *Lactococcus* had higher effect sizes, whereas *Corynebacterium* and *Streptococcus* were the most abundant (Figure 5). *Ruminococcaceae*, *Clostridiales*, and *Bacteroidales* were the bacterial taxa with the highest abundances in the HSCC category. More detailed analyses

Figure 1. Mean relative abundance of the most prevalent bacterial phyla identified in milk samples of 19 farms studied. Color version available online.

Figure 2. Heatmap illustrating the relative abundance of the 30 most prevalent bacterial taxa in bulk tank milk from the 19 dairy farms enrolled in this study. Each square in the heat map represents the abundance level of a single category. Small relative abundance values are represented by light green, progressing to higher values as black and dark red. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus). OTU = operational taxonomic units. Color version available online.

were performed using OTU classified as statistically significant ($\log_{10}$ of the FDR ≥ 1.3) in the response screening analysis for the HSCC (Figure 6) and LSCC categories (Figure 7). The effect of milk microbiome on $\log_{10}$ SPC was also evaluated using response screening analysis (Figure 8). In HSPC, the *Streptococcus* genus

had the highest effect size whereas the most abundant bacterial taxa were *Acinetobacter* and *Enterobacteriaceae* (Figure 8). In LSPC, *Thermoanaerobacterium* and *Bacillus* had the highest effect sizes, whereas *Ruminococcaceae*, *Clostridiales*, *Bacteroidales*, and *Staphylococcus* were the most abundant bacterial taxa. Detailed

Figure 3. The bulk tank milk core microbiome, defined as the bacterial taxa detected in all bulk tank milk samples across all 19 farms enrolled in this study. Each square in the heat map represents the abundance level of a single category. Small relative abundance values are represented by light green, progressing to higher values as black and dark red. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus). OTU = operational taxonomic units. Color version available online.

analysis was performed using bacterial taxa statistically significant in the response screening in the HSPC (Figure 9) and LSPC categories (Figure 10).

Log₁₀ SPC and Shannon index were linearly correlated (Figure 11); bacterial taxa diversity decreased with higher values of log₁₀ SPC. The total bacterial load (log₁₀ of the number of 16S rRNA gene copies) was positively and linearly correlated with the relative abundances of *Acinetobacter* and *Enterobacteriaceae*, as

well as with log₁₀ SPC (Figure 12A, B, and D). Additionally, a negative correlation was detected between the total bacterial load and the Shannon diversity index (Figure 12C).

DISCUSSION

To our knowledge, this is the first report to have described associations between the BTM bacterial com-

Table 1. Number of samples (N), mean, SD, and range of log₁₀ SPC and SCC per dairy farm enrolled in this study

Dairy farm	N	log ₁₀ SPC			SCC		
		Mean	SD	Range	Mean	SD	Range
1	11	4.04	0.14	0.47	73,545	8,298	34,000
2	22	3.04	0.10	0.30	134,285	12,071	50,000
3	16	3.38	0.22	0.69	147,500	19,148	70,000
4	24	3.11	0.26	0.90	140,833	23,759	100,000
5	24	3.31	0.31	1.51	167,500	19,615	90,000
6	16	4.11	0.61	1.77	120,437	16,382	53,000
7	8	3.84	0.53	1.77	162,500	16,690	40,000
8	19	3.43	0.25	0.84	240,000	19,148	70,000
9	50	3.37	0.33	1.30	157,800	14,468	60,000
10	72	3.60	0.37	1.89	203,472	68,116	330,000
11	18	3.88	0.35	1.46	176,833	37,281	167,000
12	13	3.40	0.19	0.69	121,538	23,666	760,00
13	29	3.25	0.23	0.77	151,724	16,051	600,00
14	55	3.51	0.38	1.47	162,000	27,514	140,000
15	12	3.07	0.13	0.30	120,416	15,733	45,000
16	1	3.90	—	—	240,000	—	—
17	23	3.01	0.06	0.30	80,260	8,491	32,000
18	25	3.26	0.16	0.47	123,520	16,676	62,000
19	34	3.44	0.31	1.17	77,529	11,602	52,000

Figure 4. Heat maps illustrating the mean relative abundance of the 12 most common bacterial taxa relative to log₁₀ of the SPC (A) and SCC (B) quartiles for all milk samples evaluated in this study. Each square in the heat map represents the abundance level of a single category. Small relative abundance values are represented by light green, progressing to higher values as black and dark red. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus). OTU = operational taxonomic units. Color version available online.

Figure 5. Bubble graph illustrating the operational taxonomic units (OTU) that were significantly associated with SCC. Bulk tank milk samples were divided into high ($>200,000$) and low SCC ($\leq 200,000$). The graph illustrates the $\log_{10}$ of the false discovery rate along the y-axis, and the fold change of the relative abundance of the each specific OTU comparing high- versus low-SCC samples along the x-axis. The size of the bubbles represents the overall abundance of each OTU and the colors represent the size of the detected effect (blue = small effect and red = large effect). OTU with x-axis values greater than 1 were more abundant in high-SCC milk, and OTU with x-axis values below 1 were more abundant in low-SCC milk. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus). Color version available online.

munity and milk quality parameters (SSC and SPC) based on high-throughput sequencing of the 16S rRNA gene. Previous studies focused only on identification of specific bacterial species and their relationships to milk quality parameters, or conducted a simple characterization of the raw milk microbiome. In our study, important spoilage (e.g., *Acinetobacter*), spore-forming (e.g., *Bacillus*), and pathogenic (e.g., *Staphylococcus*) bacteria were identified in the core microbiome of BTM, all of which are bacterial groups of known importance in the dairy industry. We also observed that *Streptococcus* was the second most abundant bacterial genus detected in samples classified as HSCC, and it was highly correlated with HSPC (highest size effect),

which corroborates the existing knowledge that species of *Streptococcus* are important mastitis pathogens. A negative correlation between the Shannon diversity index and total bacterial load ($\log_{10}$ of the copy number of the 16S rRNA gene) was observed, suggesting that a BTM environment with high bacterial count may be dominated by a smaller group of bacterial taxa.

The phylum-level structure of the BTM microbiome detected in the present study is similar to those of previous studies that described the microbial communities of raw milk (Quigley et al., 2013) and BTM (Raats et al., 2011). Furthermore, the common genera previously detected in raw milk, such as *Acinetobacter* (Gurung et al., 2013), *Pseudomonas* (von Neubeck et al., 2015),

Corynebacterium (Quigley et al., 2013), *Staphylococcus*, and *Streptococcus* (Park et al., 2007; Zanardi et al., 2014), were also identified in our samples. Additionally, the bacterial taxa that presented the highest abundance in BTM samples were *Ruminococcaceae*, *Acinetobacter*, and *Enterobacteriaceae*. Our results are in line with studies that previously reported *Ruminococcaceae* in the milk microbiome (Oikonomou et al., 2014; Young et al., 2015). Members of this family have been widely identified in samples across the different anatomical parts of the gastrointestinal tract of dairy cattle (Lima et al., 2015; Mao et al., 2015). Therefore, it has been suggested that microorganisms from maternal gastrointestinal microbiota reach the mammary secretions, contributing to the bacterial components in milk (Young et al., 2015). The existence of the enteromammary pathway in humans and mice has been described, where microbes from the gut lumen travel

through the mesenteric lymph nodes and then reach the mammary gland (Perez et al., 2007; Donnet-Hughes et al., 2010; Fernández et al., 2013). The mechanism by which microorganisms cross body sites is not fully understood and may involve immune cells (Perez et al., 2007; Donnet-Hughes et al., 2010). Young et al. (2015) investigated if a similar mechanism was evident in cows and found support for the presence of an endogenous enteromammary pathway during lactation in the cow when the same bacterial taxon (i.e., *Ruminococcus* genus) was found in milk, blood, and feces of healthy lactating cows. Therefore, contamination by this bacterial taxon during milking and milk storage should be also considered.

Acinetobacter adapts to a variety of environmental conditions, and emerging pathogens have been reported in this genus (Gurung et al., 2013). In the milk production process, the lipolytic and proteolytic activities of

Figure 6. Bar graph depicting the difference of relative abundances between the bulk tank milk samples that were classified as high- and low-SCC (>200,000 and ≤200,000, respectively). This graph depicts the taxa that were found in significantly higher relative abundance in high- compared with low-SCC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit for low- (light gray bars) and high-SCC samples (dark gray bars). The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus).

Figure 7. Bar graph depicting the difference of relative abundances between the bulk tank milk samples that were classified as high and low SCC ($>200,000$ and $\leq 200,000$, respectively). This graph depicts the taxa that were found in significantly higher relative abundance in low-compared with high-SCC samples. The mean relative abundances ($\pm$ SEM) are shown for each operational taxonomic unit for low- (light gray bars) and high-SCC samples (dark gray bars). The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus).

Acinetobacter contribute to spoilage of dairy products (Hantsis-Zacharov and Halpern, 2007). Another important group of bacteria related to food safety and spoilage are members of the *Enterobacteriaceae* family, which can be detected in almost all natural environments and are often associated with intestinal infections (Anand and Griffiths, 2011). Contamination of raw milk by cow feces during milking is common; thus, *Enterobacteriaceae* have been used as indicators of hygiene and microbial quality (Anand and Griffiths, 2011). Elmoslemany et al. (2009) highlighted the importance of cow and stall hygiene and equipment hygiene to bacteriological quality of bulk tank milk. Management practices have influence in quality milk (Jayarao et al., 2004; Masiello et al., 2014; Miller et al., 2015), characteristics such as use of used automatic milking detachers, bedding material, technique used for teat dipping, and practices pre- and postdipping are relevant (Jayarao et al., 2004). Management practices associated with cow cleanliness

may ensure better microbial quality, longer shelf life, of fluid milk (Masiello et al., 2014).

Additionally, in the present study, we observed by using qPCR that the relative abundances of *Acinetobacter* and *Enterobacteriaceae* increase with increasing total bacterial load in BTM samples. As mentioned above, *Acinetobacter* are psychrotolerant bacteria (Fricker et al., 2011), and within the *Enterobacteriaceae* family some members, such as *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia*, do grow very well in refrigerated milk (Anand and Griffiths, 2011).

An interesting finding from our study was the identification of 12 bacterial taxa common to all 19 dairy farms enrolled, suggesting that a core BTM microbiome may exist. Several bacterial taxa identified in the core microbiome have been previously described in raw milk (Hantsis-Zacharov and Halpern, 2007; Oikonomou et al., 2014; Weber et al., 2014; Miller et al., 2015). To our knowledge, ours is the first report describ-

Figure 8. Bubble graph illustrating the operational taxonomic units (OTU) that were significantly associated with the $\log_{10}$ of the SPC. Bulk tank milk samples were divided into high (>3.6) and low $\log_{10}$ -SPC (≤ 3.6). The graph illustrates the $\log_{10}$ of the false discovery rate along the y-axis and the fold change of the relative abundance of each specific OTU in comparison with high- versus low- $\log_{10}$ -SPC samples along the x-axis. The size of the bubbles represents the overall abundance of each OTU and the colors represent the size of the detected effect (blue = small effect and red = large effect). OTU with x-axis values greater than 1 were more abundant in high $\log_{10}$ -SPC milk, and OTU with x-axis values below 1 were more abundant in low $\log_{10}$ -SPC milk. The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus). Color version available online.

ing 5-7N15 and *Thermoanaerobacterium* in raw milk samples, both in the milk core microbiome described. 5-7N15 has been identified in fecal samples from dairy cows (Young et al., 2015); however, information regarding this genus is limited. The *Thermoanaerobacterium* genus has generated interest within the food sector in the last decade due its potential as a contaminant during manufacturing involving high temperatures, because the genus contains thermophilic spore-forming bacteria (Mtimet et al., 2016). Spores present in raw milk can survive pasteurization and other conditions during dairy-product processing (Miller et al., 2015), and the spores can germinate postprocessing to cause dairy-product spoilage (Masiello et al., 2014) or food-borne disease (Bennett et al., 2013). Raw milk has been implicated as an important source of spores produced by psychrotolerant (Masiello et al., 2014; Miller et al., 2015), mesophilic, and thermophilic spore-forming bacteria (Miller et al., 2015).

We identified a high abundance of *Ruminococcaceae* in all SCC and $\log_{10}$ SPC quartiles; therefore, further studies should be conducted to understand the origin and the role of *Ruminococcaceae* in milk samples. The observed high abundance of *Enterobacteriaceae* in the highest quartiles of $\log_{10}$ SPC and SCC can indicate poor hygiene or a high prevalence of mastitis in the herd. In addition, the abundance of *Acinetobacter* was clearly higher in the fourth quartile of $\log_{10}$ SPC, and the positive correlation between *Acinetobacter* and total bacterial load that we observed suggests that bacteria within the *Acinetobacter* genus are an important environment contaminant. This genus is known to cause spoilage (Hantsis-Zacharov and Halpern, 2007) and is also a rare primary cause of mastitis (Oliver and Murinda, 2012). The mean relative abundances of *Streptococcus* and *Corynebacterium* increased from the first to the fourth quartiles of SCC, consistent with their importance as etiological agents of mastitis.

The bacterial genera associated with samples classified as HSCC were *Macrococcus*, *Enterococcus*, *Coxiella*, *Lactococcus*, *Dermacoccus*, and *Lactobacillus*. *Enterococcus*, *Coxiella*, and *Lactococcus* are known mastitis pathogens; however, *Macrococcus*, *Dermacoccus*, and *Lactobacillus* are not associated with bovine mastitis. *Dermacoccus* was first isolated from human skin and described as *Micrococcus* (Stackebrandt et al., 1995), *Macrococcus* is phylogenetically close to the genus *Staphylococcus* (Baba et al., 2009), and *Lactobacillus* spp. are known for their beneficial properties (Bouchard et al., 2015). In addition, food-borne illness can be caused by *Enterococcus* (Giraffa, 2002). The highest relative abundances of *Corynebacterium* and *Streptococcus* were encountered in samples classified as HSCC, and both are known to be important mastitis pathogens (Smith et al., 1985; Jayarao et al., 2004; Gonçalves et al., 2016). Species within the genus *Streptococcus* have been previously associated

with increased SCC in BTM samples (Jayarao et al., 2004). Although, *Corynebacterium bovis*, a contagious pathogen, was already associated with increased SCC in milk samples from dairy cows with subclinical mastitis (Gonçalves et al., 2016), the SCC found in a previous study was lower than the threshold of HSCC used in our experiment. A precedent study, performed by Lam et al. (1997), detected that the prevalence of *Corynebacterium* spp. may be an indicator of the use of postmilking teat-disinfection; thus, this pathogen may be a potential indicator of hygiene issues in the parlor, which can have an important effect on mammary gland health and consequently on bulk tank SCC. On the other hand, in samples classified as LSCC, *Ruminococcaceae*, *Clostridiales*, and *Bacteroidales*, were the most abundant taxa. These groups, mainly *Ruminococcaceae* and *Bacteroidales*, have been previously detected during cold storage of bovine raw milk (Gschwendtner et al., 2016).

Figure 9. Bar graph depicting differences of relative abundances between the bulk tank milk samples that were classified as high and low log₁₀ of the SPC (>3.6 and ≤3.6, respectively). This graph depicts the bacteria that were found in significantly higher relative abundance in high-compared with low-log₁₀ SPC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit for low- (light gray bars) and high-log₁₀ SPC samples (dark gray bars). The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus).

In samples classified as HSPC, our microbiome analysis showed HSPC to be associated with a small group of bacterial taxa. In most cases the increase of bacterial count is correlated with unsanitary conditions (Jayarao et al., 2004), which may explain the high prevalence of *Enterobacteriaceae* in BTM samples classified as HSPC and the positive correlation between relative abundance of *Enterobacteriaceae* and total bacterial load described in the present study. Presence of coliforms, an important group within this family, is suggestive of fecal contamination in BTM samples or contamination of water used in the cleaning process (Jayarao et al., 2004). Furthermore, some *Enterobacteriaceae* are environmental mastitis pathogens that access BTM not only from IMI, but also from cow skin, teat ends and skin, bedding, manure, and water (Jayarao et al., 2004). *Streptococcus* was the bacterial taxon with the highest effect size in samples classified

as HSPC. This genus is frequently detected in BTM samples (Zadoks et al., 2004) and includes contagious and environmental mastitis pathogens (Jayarao et al., 2004, Jørgensen et al., 2016). Similarly, Gillespie et al. (2012) reported the highest correlation between SPC and *Streptococcus* spp. count. In another study, Hayes et al. (2001) found *Streptococcus uberis* to be associated with increased SPC in BTM samples (Hayes et al., 2001). In summary, *Streptococcus* abundance was highly correlated with both SCC and SPC, suggesting that this genus remains a very significant problem to the dairy industry and that more preventive actions should be implemented to reduce its incidence. In addition, an important remaining concern is the presence of spore-forming bacteria in milk (Anand and Griffiths, 2011), which may survive pasteurization; indeed, in the present study, we identified spore-forming species in LSPC groups of BTM.

Figure 10. Bar graph depicting differences of relative abundances between the bulk tank milk samples that were classified as high and low log₁₀ of the SPC (>3.6 and ≤3.6, respectively). This graph depicts the bacteria that were found in significantly higher relative abundance in low- compared with high-log₁₀ SPC samples. The mean relative abundances (±SEM) are shown for each operational taxonomic unit for low- (light gray bars) and high-log₁₀ SPC samples (dark gray bars). The letters in front of the bacterial names identify the lowest level of classification (k = kingdom, p = phylum, c = class, o = order, f = family, and g = genus).

CONCLUSIONS

Our description of the microbiome of BTM samples and its association with milk quality parameters corroborate the current knowledge regarding the bacterial community of raw milk and the effects on milk quality parameters. Bacterial taxa widely studied in milk were among the most abundant identified in our BTM samples, with *Ruminococcaceae* being the most abundant. Spoilage bacteria, such as *Acinetobacter* and *Pseudomonas*, were ubiquitous in BTM for all studied dairy farms. In samples classified as HSPC, *Acinetobacter* and *Enterobacteriaceae* presented higher relative abundances, and both increased as bacterial load increased. Spore-forming bacteria (*Thermoanaerobacterium* and *Bacillus*) were identified in the core microbiome of BTM, which is a concern to the dairy industry. Among the pathogens associated with SCC and SPC, *Streptococcus* was highlighted. Other pathogens

(e.g., *Corynebacterium*) were observed at significantly higher relative abundances in the HSCC group when compared with the LSCC group; a similar result was obtained in the analysis of SPC. We also found that the BTM environment is dominated by a smaller group of bacterial taxa in cases with higher bacterial load. For further investigation, we suggest studying the bacterial taxa that were detected at significantly higher relative abundance in BTM samples classified as HSCC (e.g., *Lactobacillus*, *Arthrobacter*, and *Lactococcus*) to verify the influence of these genera on general milk quality and herd health.

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Figure 11. Simple linear regression illustrating the relationship between the Shannon index and $\log_{10}$ of the SPC. The shaded area is the 95% confidence interval. Color version available online.

Figure 12. Simple linear regression illustrating the relationship between the $\log_{10}$ copy number (16S rRNA) and relative abundance of genus (*g*) *Acinetobacter* (A), relative abundance of family (*f*) *Enterobacteriaceae* (B), Shannon index (C), and $\log_{10}$ of the SPC (D). The shaded area is the 95% confidence interval. Color version available online.

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