Bulk tank raw milk microbiota differs within and between farms: A moving goalpost challenging quality control

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ABSTRACT

Microbial contamination of bovine raw milk often occurs at the farm. To acquire a deeper knowledge of the microbiota of farm tank milk, we studied milk from 45 farms situated in 2 geographical areas in Norway. Each farm was visited on 3 different occasions, with at least 2 wk between visits. We combined both bacterial cell counts and a sequence variant inference method of amplicon-based high-throughput sequencing to achieve a high-resolution overview of the microbiota in each sample. Compositional variation of the farm milk microbiota was shown in relation to the 2 areas, between the farms and between the sampling times. Despite the near constant level of bacteria enumerated in milk from each individual farm, the dominant microbiota differed significantly between the samplings. The predominant microbiota was dominated by spoilage genera, such as *Pseudomonas* and *Bacillus*, as well as the dairy fermentation genus *Lactococcus* and mastitis-causing organisms (*Streptococcus*). Analysis of the identified sequence variants within these genera showed that the populations of *Pseudomonas* and *Lactococcus* in milk had similar composition between the farms, but that *Bacillus* and, in particular, *Streptococcus* populations changed between collection days from the same farm and between farms and geographical areas. Furthermore, the levels and composition of *Bacillus* and *Paenibacillus* were different between the 2 geographical areas. The results presented here provide new insight into the farm milk microbiota and show that this microbiota is a dynamic community highly subject to variation.

Key words: raw milk microbiota, farm, sequence variant inference, bacterial cell count

INTRODUCTION

Bovine raw milk has one of the most diverse microbial communities detected in food matrixes, and this diversity is one of the factors causing shifts in milk pricing, safety, and quality of both raw milk and dairy products (Quigley et al., 2013b). Although the dairy industry attempts to strictly control microbial quality throughout the milk processing lines, a large number of bacteria that can cause quality problems are constantly transported to the dairy plant with the raw milk. These bacteria include spoilage bacteria such as psychrotrophs, thermotolerant and thermo-resistant bacteria, and pathogens, and these are regularly detected in milk during storage in silo tanks and in the dairy environment (Cleto et al., 2012; von Neubeck et al., 2015; Porcellato et al., 2018). Spore-forming microorganisms and thermotolerant enzymes produced by psychrotrophs are not destroyed by pasteurization and represent common quality problems in milk and dairy products. Therefore, greater control of the initial microbial composition of the raw milk is of fundamental importance for the dairy industry in order to reduce or limit economical losses caused by quality problems.

Microbiological contamination of raw milk takes place mainly at the farm level, and bacteria may originate from a variety of sources, ranging from inside the udder to the milking and storage equipment. Milk is not sterile within the udder of healthy cows, as previously thought, and contains a wide range of microorganisms, including *Ruminococcus*, *Bifidobacterium*, and *Peptostreptococcaceae*, which are thought to enter the milk via the entero-mammary pathway (Young et al., 2015). In addition, contamination occurs from the teat surface microbiota and from the milking and milk handling equipment (Elmoslemany et al., 2010). The mastitic udder sheds large numbers of pathogenic microorganisms (including *Streptococcus*, *Staphylococcus*, and *Escherichia*) into the milk. Considering all this, the milk microbiota is understandably a complex community where many different species from diverse sources are present, and this becomes a challenge for the dairy

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industry, which is required to produce high-quality milk and milk products.

Many bacterial taxa have been detected in raw milk by both culture-dependent and culture-independent methods (reviewed by Quigley et al. 2013a). In recent studies performed with amplicon-based high-throughput sequencing (HTS), the number of different taxa detected in bovine raw milk samples was shown to be in the order of thousands (Hou et al., 2015; Kable et al., 2016; Doyle et al., 2017a). Despite this overall diversity, some bacteria groups were frequently found in studies using both culture-dependent and culture-independent approaches. Of these, the most abundant were lactic acid bacteria, psychrotrophic bacteria, and spore-forming bacteria (Quigley et al. 2013b).

New methods have recently been applied to describe the composition of the raw milk microbiota (Kable et al., 2016; Doyle et al., 2017b; Rodrigues et al., 2017). These methods, based on culture-independent approaches and amplicon-based HTS, are able to successfully identify changes in microbiota associated with seasonal housing, farm management, and the transfer of milk from truck to milk-processing facilities. These techniques allow an in-depth analysis of the milk microbiota and are able to detect taxa not found using traditional cultivation methods (Quigley et al., 2011). However, the use of amplicon-based HTS suffers from bias because the values obtained are transformed to relative abundance. Therefore, the abundance of taxa found cannot be converted to real values and, as they are dependent on each other, results for different samples cannot be readily quantitatively compared (Xia and Sun, 2017). Methods for absolute quantification have been implemented in some of these studies (by quantitative PCR), but these results were not directly linked to the abundance of several different taxa (Doyle et al., 2017a). New bioinformatics tools, with greater resolution than before, are being continuously developed to study microbial community data (Callahan et al., 2016). These new methods can be used to provide a better description of the diversity of the milk microbiota so that the dairy industry can act appropriately to improve microbiological quality of their milk and dairy products.

The objective of our study was to obtain an overview of the microbial composition and diversity of raw milk (1) between different collection days at the same farm, (2) between farms, and (3) between geographical regions. The microbiota was studied by applying a novel approach of combining the culture-independent information from the bacterial counts with amplicon-based HTS-sequencing. Further, we examined the application of sequence variant (SV) inference as an alternative

method to OTU clustering to obtain a better resolution of the microbiota determination.

MATERIALS AND METHODS

Study Design, Milk Samples, and Sampling

Forty-five farms, distributed within 2 geographical areas in Norway (20 farms in area A and 25 farms in area B), were selected for this experiment. The farms from each area deliver bovine raw milk to 2 regional dairy plants, which are situated 110 km apart in the southern part of Norway, where climate conditions and elevation are similar. Each farm was visited on 3 occasions between January and March 2017, with 2 wk between each sampling for farms in area A and 3 wk between each sampling in area B. In Norway, farm milk is usually collected after 2 or 3 d of storage at the farm. In this study, the milk was collected from farms where collection of the milk is performed only every 3 d. The milk was collected on the same weekday and was a mix of 6 consecutive milking (evening on d 1, morning and evening on d 2 and 3, and morning of the collection day). The farms were categorized based on the type of milking system (free roaming with parlor milking system, free roaming with automatic milking system, and stall with pipeline milking system; Supplemental Table S1, <https://doi.org/10.3168/jds.2017-14083>). At each sampling, raw milk was collected during the transfer from the bulk farm tank to the tanker truck. The milk was collected in a sterile 50-mL tube using the sampling outlet of the truck and stored at 4°C during transportation. Immediately after arrival at the dairy plant, milk from each sample was removed for Bactocount analysis (Bentley Instrument Inc., Chaska, MN), and the remainder was transported at 4°C (within 2 h) to the Faculty of Chemistry, Biotechnology and Food Science laboratory, Norwegian University of Life Sciences, for DNA extraction on the same afternoon, evening, or early the following morning.

Bacterial Level in Milk Samples

Flow cytometric counting of the bacteria in the milk samples was performed using the Bactocount IBCm instrument (Bentley Instrument Inc.). Milk was analyzed for total bacterial count (TBC) immediately after arrival of the truck at the dairy plant. One milliliter of milk was taken from the collection tube and the analysis was performed according to manufacturer's instructions (Bentley Instrument Inc.; <ftp://ftp.bentleyinstruments.com/Bactocount-Manual.pdf>).

DNA Extraction from Bacteria in Milk Samples

A bacterial pellet was prepared from 40 mL of each milk sample. The milk was centrifuged for 10 min at $8,000 \times g$ at 4°C and the pellet was resuspended and washed twice with 1 mL of 2% sodium citrate water (wt/vol). The pellet was then resuspended in 200 µL of lysis buffer (20 mM Tris-Cl pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, Sigma-Aldrich, St. Louis, MO) and transferred to a Fastprep tube (MP Biomedicals, Santa Ana, CA) containing 0.5 g of acid-washed glass beads (Sigma-Aldrich). The tubes were subjected to 3 rounds of bead-beating in a Fastprep-96 Instrument (MP Biomedicals) for 50 s at 1600 rpm and cooled on ice for 1 min between bead-beating rounds. The tubes were then centrifuged for 1 min at $16,000 \times g$ at room temperature and 50 µL of supernatant was used for DNA extraction. The DNA extraction was performed using the Mag Midi kit (LGC, Luckenwalde, Germany) according to manufacturer's instructions (<https://www.lgcgroup.com/LGCGroup/media/PDFs/Products/Extraction/Kits%20inserts/mag-midi.pdf>); DNA was stored at -20°C.

16S rRNA Marker Gene Sequencing and Bioinformatics

The DNA extracted from the milk samples was amplified using the method described by Porcellato and Skeie (2016) with minor changes. Briefly, 2 µL of DNA was added to the PCR reactions mix containing 1× HF Buffer (Biorad, Hercules, CA), 0.5 mg/mL of BSA, 0.2 mM of each primer, 200 mM of each deoxynucleotide triphosphate, and 0.02 U/mL of iProofTM *taq* polymerase (Biorad). The PCR amplification conditions were similar to those previously reported (Porcellato and Skeie, 2016). The PCR product was purified using 0.6× Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA) according to the manufacturer's instructions (<https://www.beckmancoulter.com/wsrportal/techdocs?docname=B37419>). Five microliters of the purified PCR product was used as a template for the second PCR using primers with unique 8-bp barcodes. The PCR reaction was similar to the previous PCR and the amplification was performed as follows. Initial denaturation at 98°C for 30 s and 10 cycles of denaturation for 15 s, annealing for 30 s at 55°C, and elongation for 20 s at 72°C. Final elongation was performed for 5 min at 72°C. The PCR product was then purified and normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, MA). The purified library was quantified using the Kapa library quantification kit (Kapa Biosystems, Wilmington, MA) and sequenced on an Illumina Miseq platform (Il-

lumina Inc., San Diego, CA) using the Miseq Reagent Kit v3 (Illumina Inc.). Sequences were deposited at the European Nucleotide Archive with accession number PRJEB24669. Raw files were merged and quality-filtered using Usearch 8.0 with Max E-value of 0.5 (Edgar, 2010). Reads were assigned to the different SV using the R package "dada2" (Callahan et al., 2016). Assignment of taxon for each SV was performed after training the classifier using the RDP training set 14 and the function "assignTaxonomy" from the R package "dada2" (Callahan et al., 2016).

Statistical Analysis

All the statistical analysis was performed using R version 3.3.3 (R Core Team, 2017). The TBC was calculated per milliliter of milk and then transformed to log TBC per milliliter. The TBC in milk samples collected from different farm types and milking systems were compared using the Kruskal-Wallis test. The type of farm and the milking system were used as a single independent variable with 3 levels: free roaming with parlor milking system, free roaming with automatic milking system, and stall with pipeline milking system. The SV table was normalized using the cumulative-sum scaling method using the R package "metagenomeSeq" (Paulson et al., 2013). Permutational analysis using the same dissimilarity matrixes was used to test differences in the composition of the community between groups of samples (number of permutations = 999). Principal coordinate analysis was chosen as ordination method using 3 distance matrixes: weighted and unweighted UniFrac dissimilarities (Lozupone and Knight, 2005) and Bray-Curtis. Samples with less than 2,000 sequences ($n = 7$) were not considered for statistical analysis, but were included in the relative and absolute abundance analysis. The relative abundance of each taxon was transformed to absolute value (log TBC/mL) after computing the percentage of that taxon according to the total levels obtained from the Bactocount analysis for each sample. This was calculated by considering q copy of 16S rRNA per TBC. Statistical analysis of the genus-associated SV was performed using the Bray-Curtis distance matrix and permutational multivariate ANOVA between groups of samples; a P -value < 0.05 was considered significant. The nonparametric Mann-Whitney U test was used to compare the abundance of the most abundant genera (expressed as log TBC/mL) between the 2 geographical areas. One consideration during the analysis of the SV results was that the number of SV is correlated with the phylogenetic variation of the amplified 16S fragment (in this study the V3 and V4 region of the 16S rRNA) of the genus considered;

thus, the results do not take into consideration the number of 16S copies present in the bacterial genome.

RESULTS

The TBC in the 135 milk samples ranged from log 3.6 to log 5.28 TBC/mL, with a mean value of 4.27 TBC/mL and median of 4.25 TBC/mL. Only 2 samples out of 135 showed a log TBC per milliliter greater than 5. Overall, the TBC was similar between the 3 samplings from each single farm with few exceptions, such as farm 34, where the bacterial count of sample 2 was 1 log TBC/mL greater than the other 2 samples (Figure 1). Milk from parlor farms with automatic milking systems showed a greater bacterial count than the other 2 types of farm with different milking system. The overall TBC was significantly different ($P < 0.05$) between the 3 types of farms, and distribution of the TBC grouped by type of farm is shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2017-14083>).

From the 16S rRNA sequencing, an average of 11,667 (median = 8,515) sequences per sample was obtained. The sequences were distributed in 5,490 SV, representing 105 taxonomical families and 334 genera. Microbial communities of the raw milk samples were grouped according to the geographical area of collection and

the sampling times using principal coordinate analysis (Figure 2). Furthermore, permutational ANOVA showed these grouping to be diverse (Adonis $P < 0.05$) with respect to geographical area and sampling time (Table 1). The microbiota in the milk samples from individual farms showed poor homogeneity between the collections days (Figure 1). The large distribution of the homogeneity between collection days at the same farm indicates that the microbiota composition differs between sampling, for example on farm 1, 8, and 28.

The most abundant genera we detected were *Pseudomonas*, *Lactococcus*, *Bacillus*, and *Streptococcus*, accounting for 26.6, 12.0, 11.0 and 6.4% of the total sequences, respectively; these values were converted to give an average abundance per sample of 3.6, 3.2, 2.6, and 2.7 log TBC/mL, respectively. Whereas *Lactococcus* and *Streptococcus* levels did not differ between the 2 different areas ($P > 0.05$), the levels of *Pseudomonas* and *Bacillus* were significantly different between the 2 geographical areas (Figure 3). The levels of other genera, such as *Staphylococcus*, *Paenibacillus*, *Psychrobacter*, *Chryseobacterium*, *Aerococcus*, and *Rhizobium*, also differed significantly between the 2 areas (Figure 3).

The milk microbiota from the same farms was not similar at the 3 sampling times. The dominant genera in milk samples collected from the same farms varied

Figure 1. Average individual bacterial counts (IBC) from bulk tank milk collected from each farm (blue) and the homogeneity of group dispersion (beta diversity: orange) at each farm obtained from the weighted Unifrac dissimilarity matrix (Lozupone and Knight, 2005). Milk samples from farm 1 to 20 were collected from area A, and from farms 21 to 45 from area B. The top and bottom of the box indicate the third and first quartile, whereas the middle line indicates the median. The upper and lower whiskers indicate the greatest and least value excluding outliers, respectively.

Figure 2. Principal coordinate (PC) analysis plot obtained from the weighted Unifrac distance matrix (Lozupone and Knight, 2005) for the microbiota of 135 milk samples. A1 = area A, sampling 1; A2 = area A, sampling 2; A3 = area A, sampling 3; B1: area B, sampling 1; B2 = area B, sampling 2; B3 = area B, sampling 3.

between the collection days (Figure 4). For example, the genera *Bacillus* (in farm 1, 2, 9), *Acinetobacter* (in farm 19, 21), *Paenibacillus* (in farm 23), *Staphylococcus* (in farm 44), and *Chryseobacterium* (in farm 28) varied in relative abundance between the 3 sampling days. In addition, the genus *Bacillus* was present in greater abundance from most of the farms in collection area A compared with *Paenibacillus*, which showed greater abundance from farms in area B (Figure 3). On one of

the collection days at farm 34, a marked increase in the total bacteria level was found to be due to a specific increase of *Streptococcus* (Figure 4).

Of the 5,490 SV, *Corynebacterium* was the genus with the highest number of SV detected ($n = 278$), although it only represented 2.38% of the total number of reads. Of the 4 most abundant taxa, the genus *Pseudomonas* was divided into 132 SV and *Lactococcus* into 206. In addition, *Pseudomonas* showed the highest number of SV per sample (mean = 10.4, median = 10). The distribution of the SV in each sample showed that some of the dominant *Pseudomonas* and *Lactococcus* SV could be detected in all the samples with few exceptions, such as farms 7, 8, 9, 15, 18, and 20 for *Pseudomonas* and farms 3, 4, 11, 14, 16, 17, and 21 for *Lactococcus* (Figure 5). Permutational ANOVA indicated that the *Pseudomonas* and *Lactococcus* populations were significantly different according to collection area, sampling time, and the type of housing or milking systems.

Bacillus was detected in all the samples from area A and also from 75% of the samples from area B (Figure 6), but in lower numbers (Figure 3). On the other hand, *Paenibacillus* was detected only in 32 and 68% of the samples from area A and B, respectively. Analysis of the SV for these 2 genera clearly showed the difference in the presence and abundance of these genera in the milk samples. The genus *Bacillus* was divided in 56 SV, whereas 50 SV were detected within the genus *Paenibacillus*. The genus *Bacillus* detected in milk from

Table 1. Sequence variants description of the 15 most abundant bacterial genera found in the raw milk from 45 farms

Item	Percentage number of reads over total	Number of Sequence variants	Average sequence variants per sample	Median sequence variant per sample	P-value ¹			
					Between areas	Between farms	Between sampling	Between housing/milking system ²
Microbiota weighted Unifrac ³					0.001	0.003	0.001	0.001
<i>Pseudomonas</i>	26.58	132	10.43	10	0.001	0.362	0.001	0.037
<i>Lactococcus</i>	12.03	206	6.30	6	0.001	0.678	0.001	0.001
<i>Bacillus</i>	11.30	56	3.43	2	0.001	0.116	0.001	0.610
<i>Streptococcus</i>	6.42	184	4.86	3	0.005	0.001	0.035	0.393
<i>Clostridium XI</i>	5.54	64	3.59	2	0.001	0.888	0.243	0.625
<i>Acinetobacter</i>	3.44	240	3.40	2	0.234	0.001	0.001	0.001
<i>Staphylococcus</i>	2.77	163	2.16	1	0.057	0.057	0.567	0.252
<i>Facklamia</i>	2.68	230	3.77	2	0.708	0.001	0.038	0.009
<i>Corynebacterium</i>	2.38	278	3.62	2	0.044	0.285	0.213	0.123
<i>Paenibacillus</i>	2.23	50	0.86	1	0.201	0.973	0.341	0.384
<i>Psychrobacter</i>	1.74	88	1.62	1	0.451	0.001	0.050	0.021
<i>Aerococcus</i>	1.47	110	2.06	1	0.163	0.111	0.423	0.616
<i>Sphingomonas</i>	1.08	22	1.14	1	0.731	0.599	0.208	0.048
<i>Turicibacter</i>	0.97	73	1.22	1	0.002	0.710	0.804	0.400
<i>Chryseobacterium</i>	0.85	79	0.93	0	0.273	0.029	0.370	0.030
Other	18.5	3515	0.11	0				

¹P-value obtained from the permutational multivariate ANOVA (R function Adonis from R package “Vegan”; R Core Team, 2017).

²Type of housing/milking system: free roaming with parlor milking system, free roaming with automatic milking system, and stall with pipeline milking system.

³Unifrac distance matrix (Lozupone and Knight, 2005).

Figure 3. Distribution [log individual bacterial counts (IBC)/mL] of the 15 most represented genera of bacteria detected in 135 samples of raw milk from farm bulk tanks divided in the 2 geographical areas in Norway. Orange = area A. Blue = area B. The top and bottom of the box indicate the third and first quartile, whereas the middle line indicates the median. The upper and lower whiskers indicate the greatest and least value excluding outliers, respectively. *Significant differences ($P < 0.05$) between the 2 areas.

area A (farms 1 to 20) showed similar SV distribution (with few exceptions) between the farms, whereas in milk from farms in area B (farms from 21 to 45) the SV types were diverse both between farms and with respect to sampling days. For *Paenibacillus*, the SV distribution was also different between milk collected in the 2 areas, between farms, and between collection days.

The composition of the *Streptococcus* SV varied with respect to farm, sampling day, and area (Figure 7). The sample with extremely large numbers of *Streptococcus* (collection d 2 from farm 34) contained a mix of 13

different *Streptococcus* SV (Figure 7). Interestingly, 9 of the SV detected in the second day of collection from that farm were also detected in the last day of collection, whereas none of the SV from the first day of collection were detected in the second day of collection.

The type of housing or milking system had a significant effect on the total microbial composition at genus level. Both *Lactococcus* and *Pseudomonas* SV compositions were significantly influenced by the housing types, as were *Acinetobacter*, *Facklamia*, and *Psychrobacter* ($P < 0.05$).

DISCUSSION

We demonstrated that the microbial composition of raw milk in farm milk tanks differs (1) between collection days for the same farm, (2) between farms,

and (3) between regions. This was investigated using a novel combination of culture-independent bacterial count and next-generation sequencing, which allowed the quantitative evaluation of the composition of the milk microbiota.

Figure 4. Distribution of the 15 most abundant sequence variants of bacterial genera detected in raw milk samples. Each bar represents a sample of milk and each farm was sampled 3 times. The height of each bar indicates the absolute level of bacteria (log copy number) obtained by normalizing genera relative abundances (in %) against the absolute bacterial level. (A) Farm 1 to 20 collected in area A; (B) farm 21 to 45 collected in area B.

Figure 5. Distribution of the sequence variants for *Pseudomonas* and *Lactococcus* detected in the milk samples from 45 farms. Each color indicates a different sequence variant expressed as relative abundances of the height of the bar. The height of the bar indicates the absolute level of the genus indicated calculated over the total bacterial level [log individual bacterial counts (IBC)/mL].

Figure 6. Distribution of the sequence variants for *Bacillus* and *Paenibacillus* detected in the milk samples from 45 farms. Each color indicates a different sequence variant expressed as relative abundances of the height of the bar. The height of the bar indicates the absolute level of the genus indicated calculated over the total bacterial level [log individual bacterial counts (IBC)/mL].

Tank Milk Microbiota Between Collection Days

Although the TBC was found to be similar in the 3 milk samples from individual farms, the microbial composition changed significantly between the collection days. This was detected for several farms, where an increased abundance of 1 or more genera were detected in 1 sample out of 3 from the same farm. This indicates that the composition varied randomly among samples from the same farms and, thus, removed the possibility of bias caused by sampling procedure. The TBC was performed the moment the milking tanker arrived at the dairy plant, whereas DNA extraction was performed the same day or the next morning after transportation to the university facility. Moreover, the milk was stored at 4°C and the same procedure was used for all samples from the same farms.

A shift in microbial composition between collection days at the same farm occurred over a period

of 2 to 3 wk. Several factors, such as changes in feed quality, farm environment, weather conditions, and animal health, might have contributed to these changes (Hernandez-Sanabria et al., 2010; Vacheyrou et al., 2011; Quigley et al., 2013b; Oikonomou et al., 2014). However, we did not record such data from the farms, and the correlation between the microbial changes and these factors could not be directly identified. In farm 34, for example, the second collection day showed an increase of the taxa *Streptococcus* and an increase in TBC, which might denote the presence of a significant mastitis problem in the herd at that time (Zadoks et al., 2004). Further, SV analysis of samples from farm 34 showed that the second collection day did not contain the same SV found in the first day of collection. The various streptococci might have been present but not detected, due to the detection limit of the method, on the first day, or new streptococci might have been introduced in the milk between collection days (e.g., new

Figure 7. Distribution of the *Streptococcus* sequence variants detected in milk samples from 45 farms. Each color indicates a different sequence variant expressed as relative abundances of the height of the bar. The height of the bar indicates the absolute level of the genus indicated calculated over the total bacterial level [log individual bacterial counts (IBC)/mL].

cows might have been added to the herd). This suggests that the *Streptococcus* population is dynamic and continuously varied in composition between samplings at the same farm. We confirmed this by SV analysis of this genus, where the composition was highly diverse with respect to collection day, individual farm, and geographical location. In a previous study performed in Norway, mastitis-causing *Streptococcus* were identified in 4% of cows, independent of their udder health status (Reksen et al., 2006). In this present study, all the milk samples contained at least 1 *Streptococcus* SV, but identification at species level could not be inferred due to the short fragments sequenced. *Streptococcus* is a heterogeneous genus and includes several species found in the farm environment, such as on the teat skin (Braem et al., 2012) and dairy equipment (Marchand et al., 2012; Delgado et al., 2013). Some species are responsible for bovine mastitis (Waage et al., 1999; Osterås et al., 2006; Tenhagen et al., 2006).

Changes in milk microbiota between samplings have also been attributed to seasonal changes or weather conditions, such as temperature and humidity (Li et al., 2018). We collected the milk samples during the same season (winter), which might have affected the level of some psychrotrophic bacteria. In particular, *Pseudomonas*, which was the most abundant genus we detected, have been previously correlated with winter milk (Vithanage et al., 2016; Porcellato et al., 2018). The level of *Pseudomonas* could also be explained by the 3-d storage period of the milk at the farm before collection. The storage of milk for up to 3 d is a usual practice in the Norwegian dairy industry, and milk is kept at refrigerated temperature before being collected and processed. The low storage temperature encourages the growth of psychrotrophic bacteria, such as *Pseudomonas* and *Acinetobacter* (Vithanage et al., 2016).

Tank Milk Microbiota Between Farms

Both TBC and microbial composition were significantly different between milk samples collected at different farms, which is in accordance with findings in previous studies (Bonizzi et al., 2009; Feligini et al., 2015). This indicates that each farm can be considered as a particular niche containing a dynamic microbiota. On-farm management practices and milking systems were shown to have a great effect on the TBC and bacterial composition of milk (Elmoslemany et al., 2010; Doyle et al., 2017b). Overall, the TBC levels we detected were comparable to previous studies performed in farm milk samples from Norway and from other countries (Elmoslemany et al., 2010; De Jonghe et al., 2011; Fricker et al., 2011). The type of housing and milking system can also explain some of the differences in TBC

in milk from different farms. Milk from farms with automatic milking systems showed higher TBC compared with the other 2 types of milking systems. This might be attributable to inferior washing of the teat, the lack of a drying step before milking, and challenges with equipment cleaning in the automatic milking system (Jago et al., 2006; Jacobs and Siegford, 2012).

Although significant differences exist between the farms, the 2 most abundant genera (*Pseudomonas* and *Lactococcus*) were detected in all milk samples (with few exceptions). Previously, *Pseudomonas* and *Lactococcus* were positively linked to milk from cows on pasture (Doyle et al., 2017b). However, our samples were collected from cows housed indoor, but these 2 genera represented over one-third of the total microbiota present in the milk. The great abundance of *Pseudomonas* in our samples might reflect the seasonal and storage conditions (as described above), whereas great abundance of *Lactococcus* was not surprising, as this genus has been described by other authors as one of the most abundant in raw milk samples (Quigley et al., 2013a; von Neubeck et al., 2015; Porcellato et al., 2018). In addition, the *Lactococcus* population isolated from raw milk samples has been previously shown to be dominated by psychrotolerant strains (von Neubeck et al., 2015). The composition of psychrotrophic and psychrotolerant microbiota affects the milk quality, in particular when low-temperature storage for several days is used before processing. In our study, both *Lactococcus* and *Pseudomonas* showed a great intragenus diversity (number of SV detected per sample), which might indicate multiple source of bacterial contamination in milk; this affects the complexity of the raw milk microbiota.

Another aspect that might have influenced the diversity between milk samples collected from different farms is the individual cow microbiome and the farm size. The average farm herd size in Norway is 27 cow/farms (Statistics Norway, 2017), and in our study the milk was collected from large and small farms (amount of milk delivered after 3 d >4,000 L and <1,000 L, respectively). The first source of bacterial contamination in milk is the cow (udder and teat microbiome), and the number of cows in each farm might affect the diversity of taxa and SV detected in our study. For example, the diversity of *Streptococcus* could reflect the herd size, where farms with more cows could have a greater microbiota diversity. Here, the *Streptococcus* SV distribution was influenced by the farm and collection day, which indicates that the source of *Streptococcus* could be linked to the size of the herd. However, sampling from each individual cow is needed to evaluate the importance of each cow and farm size on the milk microbiota.

Tank Milk Microbiota in the 2 Sampling Areas

We showed significant variation in milk microbiota related to the 2 geographical areas. Whereas the 2 areas were not different with regard to climate conditions and elevation, difference in farming practices, feed suppliers, and other sources of contamination could explain this variation (Mallet et al., 2012). Interestingly, 7 of the 15 most abundant genera were significantly different in level between the 2 areas, including *Bacillus* and *Paenibacillus*. *Bacillus* was more abundant in milk from area A and *Paenibacillus* was more abundant in milk samples from area B. These 2 bacterial genera are the predominant aerobic endospore-forming taxa detected in milk samples and are of concern to the dairy industry for their ability to survive pasteurization and cause subsequent spoilage in stored pasteurized milk (Coorevits et al., 2008; Ivy et al., 2012; Masiello et al., 2014). Several factors have been identified that influence the levels of aerobic endospore-forming bacteria in milk, such as bedding materials and feed (Vaerewijck et al., 2001; Magnusson et al., 2007), season variation (Bartoszewicz et al., 2008), and contamination from equipment (Salustiano et al., 2009). These factors could explain some of the differences between the 2 geographical areas of collection and can be assumed from the composition of the SV. *Bacillus* SV clearly showed a consistent presence of some dominant SV between collection days and farms in most milk samples from area A, whereas in milk samples from area B the *Bacillus* SV were different both within and between farms. Similar to *Bacillus* SV from area B, the *Paenibacillus* SV identified in all milk samples from area B were different between collection days and farms. *Paenibacillus* was detected in only 32% of the milk samples from area A as compared with 68% in milk from farms in area B.

Conclusions and Future Perspectives

This present study showed that the microbiota of the raw milk delivered to the dairy industry continuously shifts in composition, possibly even on a daily basis. We detected a complex milk microbiota with great intra-genus and intraspecies diversity, thus implying highly variable sources of contamination at the farm level. The between-farm variation was expected due to differences in the farm environment, but the variation connected to sampling at the same farm and geographical location was unexpected and cannot be explained by available data. Seemingly random variation in milk microbiota is a challenge for the dairy industry, as the quality of dairy products is related to the quality of the raw milk received for processing. The study of the milk microbiota at farm level can also help the dairy industry to

identify farms with undesirable microbiota and to work with farmers to identify the contamination sources and reduce or remove unwanted microorganisms from the milk.

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