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Rumen fluid, feces, milk, water, feed, airborne dust, and bedding microbiota in dairy farms managed by automatic milking systems

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Abstract

Microbiota of the gut, milk, and cowshed environment were examined at two dairy farms managed by automatic milking systems (AMS). Feed, rumen fluid, feces, milk, bedding, water, and airborne dust were collected and the microbiota on each was assessed by Illumina MiSeq sequencing. The most abundant taxa in feed, rumen fluid, feces, bedding, and water were *Lactobacillaceae*, *Prevotellaceae*, *Ruminococcaceae*, *Ruminococcaceae*, and *Lactobacillaceae*, respectively, at both farms. *Aerococcaceae* was the most abundant taxon in milk and airborne dust microbiota at farm 1, and *Staphylococcaceae* and *Lactobacillaceae* were the most abundant taxa in milk and airborne dust microbiota at farm 2. The three most prevalent taxa (*Aerococcaceae*, *Staphylococcaceae*, and *Ruminococcaceae* at farm 1 and *Staphylococcaceae*, *Lactobacillaceae*, and *Ruminococcaceae* at farm 2) were shared between milk and airborne dust microbiota. Indeed, SourceTracker indicated that milk microbiota was related with airborne dust microbiota. Meanwhile, hierarchical clustering and canonical analysis of principal coordinates demonstrated that the milk microbiota was associated with the bedding microbiota but clearly separated from feed, rumen fluid, feces, and water microbiota. Although our findings were derived from only two case studies, the importance of cowshed management for milk quality control and mastitis prevention was emphasized at farms managed by AMS.

KEYWORDS

automatic milking system, cowshed environment, gut, microbiota, milk

1 | INTRODUCTION

Mastitis, an inflammation of the mammary gland regarded as the most important disease affecting dairy herds, is triggered by pathogens derived from infectious and environmental bacteria. Assessment of milk microbiota is thus important for preventing mastitis and maintaining herd health (Jayarao, Pillai, Sawant, Wolfgang, & Hegde, 2004; Olde Riekerink, Barkema, & Stryhn, 2007). Typical infectious bacteria include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, and *Mycoplasma* spp. Therefore, if mastitis is caused by these pathogens the farmer should revise their milking procedure and sequence. If environmental bacteria, such as coliforms, coagulase negative *Staphylococci*, and *Streptococci* other than *S. agalactiae*, are pathogenic agents, the farmer should improve the hygiene of their cowshed.

Implementation of automatic milking systems (AMS) is one of the most important technological advancements in the dairy industry in the past 20 years. The use of AMS enables cows to be milked whenever they want. Therefore, milk production may increase as the milking frequency of individual cow increases as compared with regular twice per day milking (Kruip, Morice, Robert, & Ouweltjes, 2002). Moreover, milk quality, cow behavior, cow welfare, and herd management have been shown to be affected by AMS (Jacobs & Siegford, 2012) and the risk of udder contamination associated with farmer contact is expected to be minimized. However, the teat orifice could be damaged more when using an AMS than when employing conventional milking techniques, because the curtailed milking interval may retard the recovery of the mammary gland. Furthermore, infectious mastitis, if present, could spread throughout the herd via the AMS, because the sequence of milking is not controlled. Although, studies have shown inconsistent results regarding the risk of mastitis during the transition from conventional milking to AMS (Berglund, Pettersson, & Svennersten-Sjaunja, 2002; Hovinen & Pyorala, 2011; Jacobs & Siegford, 2012).

A lot of research has been conducted, by both plate-culture and culture-independent methods, to analyze milk microbiota in association with milking practices and the farm management. Milk microbiota is shown to be influenced by the microbiota present on teat skin, bedding, feed (hay), and in the surrounding air (Doyle, Gleeson, O'Toole, & Cotter, 2016; Quigley et al., 2013; Vacheyrou et al., 2011). Likewise, region, season, cowshed environment, and hygiene of the milking practices are known environmental factors that influence the microbiota (Elmoslemany et al., 2010; Kable et al., 2016; Kim et al., 2017).

Although increasing data for milk microbiota, determined by next-generation sequencing (NGS) analyses, have become available, the results were highly variable between experiments and the understanding of high-throughput data is largely complicated by factors such as milk quality control and mastitis prevention (Bhatt et al., 2012; Bonsaglia et al., 2017; Doyle et al., 2016; Falentin et al., 2016; Kuehn et al., 2013; Oikonomou et al., 2014; Young, Hine, Wallace, Callaghan, & Bibiloni, 2015). Furthermore, information on the milk microbiota of cows managed by AMS is lacking.

In this study, microbiota analysis by NGS (MiSeq) was performed for rumen fluid, feces, milk, water, feed (total mixed ration silage), bedding, and airborne dust collected at two dairy farms managed by AMS. The aim of this study was to characterize the microbiota of the gut, milk, and cowshed environment. Diet and nutrition were shown to affect the composition of milk microbiota and the risk of mastitis (Zhang, Huo, Zhu, & Mao, 2015). Therefore, feed, rumen fluid, and feces were examined to see how diet and gut microbiota associate with each other and raw milk.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Samples were collected at two farms located in Okayama (farm 1) and Hiroshima (farm 2) prefectures, which operated AMS (Lely Astronout A4, Cornes AG. Ltd., Eniwa, Japan) for management of lactating dairy cows. The sites were >100 km away from each other. At both farms the cows were housed in a free stall barn and fed total mixed ration (TMR) silage, which was formulated to have 500–600 g/kg of dry matter (DM), 160–180 g/kg DM of crude protein ($N \times 6.25$), and 720–740 g/kg DM of total digestible nutrients. Samples were collected between 10:00 and 12:00 hr in April at farm 1 and between 13:00 and 15:00 hr in September at farm 2. Daily minimum and maximum temperatures on the day of sampling were 9 and 19°C for farm 1 and 16 and 24°C for farm 2, respectively. Rumen fluid was obtained using a flexible stainless spring tube (Lumenar stomach evacuator outfit, Fujihira Industry Co. Ltd., Tokyo, Japan), and fecal samples were collected from the rectum. Milk samples were collected manually from four udders and then mixed as a composite sample. Following surface cleaning, several streams of foremilk were discarded prior to sample collection. Airborne dust samples were collected by placing three petri dishes for 5 min approximately 1.0 m above the ground and then gathered into a tube using sterile physiological saline. Bedding samples were collected from three separate places in a cowshed and water was collected from three different water cups. Feed was sampled by taking three piles of total mixed ration silage. In the free stall system, cows could move and rest freely and determining their resting place was difficult. Thus, a composite sample prepared from three separate samples was thus regarded as a representative means of assessing airborne dust, water, bedding, and feed microbiota at a farm. All samples were immediately refrigerated on ice and transported to Okayama University. Procedures and protocols for the animal experiments were approved by the Animal Care and Use Committee, Okayama University, Japan.

2.2 | Determination of N-acetyl -β-D-glucosaminidase (NAGase) activity in milk

NAGase activity was determined by a fluorometric method using micro templates (Hovinen et al., 2016). Eight milk samples with known somatic cell count (1.4×10^4–1.3×10^6 cells/ml) were used to define the association between NAGase and somatic cell count. The fluorescence of 4-MU released from the substrate 4-MUAG was measured with a Grating Based Multimode Reader SH-9000 (CORONA Electric Co., Ltd., Ibaraki, Japan) using a 355 nm excitation filter and a 460 nm emission filter.

2.3 | Preparation of bacterial DNA

Bacterial DNA from feed, milk, water, and airborne dust samples was extracted and purified using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA). Bacterial pellets were obtained by centrifugation at 16,000×*g* for 2 min. For feed sample, a 10 g of silage was vigorously mixed with 90 ml of sterilized saline and the gauze-filtered extract was centrifuged to obtain the pellet (Ni et al., 2016). For milk, water, and airborne dust samples, a 250 μL sample was centrifuged to collect the pellet. All pellet samples were lysed with 180 μL of lysozyme solution (20 g/L lysozyme, 0.02 M Tris-HCl [pH

8.0]), 0.002 M sodium EDTA [pH 8.0], 1.2 g/L Triton X-100) at 37°C for 1 hr. Subsequent bacterial DNA purification was performed following the manufacturer's recommendations. For rumen fluid, feces, and bedding samples, 0.1 g of the sample was used to prepare bacterial pellets and bacterial DNA was purified using the DNeasy Stool Mini Kit (Qiagen, Germantown, MD, USA).

2.4 | Quantitative real-time PCR for total bacteria

The total bacterial count was determined by quantitative PCR (qPCR). Each sample DNA solution (2 μL) was added to 23 μL of a PCR mixture containing 12.5 μL of KAPA SYBR FAST Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) and 8 μM primers targeting the V3 region of the 16S rRNA genes (forward: 5′-ACGGGGGGCCTACGGGAGGCAGCAG-3′; reverse: 5′-ATTACCGCGGCTGCTGG-3′). The qPCR was performed with a Mini Opticon real-time PCR system (Bio-Rad Laboratories Inc., Tokyo, Japan) with initiation at 95°C for 30 s followed by 35 cycles of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. A standard curve was prepared from plasmid DNA employing 16S rRNA genes from *Escherichia coli* (JCM 1649). The copy number of the standard plasmid was calculated using the molecular weight of the nucleic acid and the length (base pairs) of the cloned plasmid.

2.5 | Illumina MiSeq sequencing

The PCR amplification using primers targeting the V4 region of the 16S rRNA genes (forward: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3′; reverse: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3′) was employed (Tang, Han, Yu, Tsuruta, & Nishino, 2017). The PCR protocol was as follows: initiation at 94°C for 2 min and followed by 25 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and a final elongation of 72°C for 5 min. The products were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and moved to a second round of PCR with adapter-attached primers. The second PCR protocol was as follows: initiation at 94°C for 2 min followed by 10 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s and a final elongation of 72°C for 5 min. The PCR products were again purified as described above. The purified DNA was then ligated to the 16S rRNA amplicons prior to 250 bp paired-end sequencing performed on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan).

2.6 | Bioinformatics and microbiota characterization

Raw sequences were processed using QIIME (version 1.9.0) running the virtual box microbial ecology pipeline. Before pair-end joining, raw sequence data were sheared using "sickle pe" to obtain a phred quality score above 30 and ensure that sequences were longer than 135 bp. Paired-end sequences were joined using fastq-join with more than 20 bp overlap required between all paired sequences. Chimeric sequences were identified with USEARCH and removed. The remaining DNA sequences were grouped into an OTU with 97% matched with the closed-reference OTU picking method in QIIME, when assessed with default settings. Both chimera checking and OTU picking used Greengenes 13.8 as the reference database and sequences were aligned using PyNAST. The results of the sequence analysis are available in the DDBJ Sequence Read Archive under project identification number PRJDB7427.

2.7 | Statistical analyses

Comparison of total population and bacterial composition between the two farms was examined by analysis of variance.

Microbiota data were also subjected to canonical analysis of principal coordinates to define assignment and clustering that explained variations in the microbiota. Discriminant vectors with a Pearson correlation >0.7 were considered significant. Likewise, hierarchical clustering and heat map construction were done. These analyses were performed using Primer version 7 with Permanova+ add-on software (Primer-E, Plymouth Marine Laboratory, Plymouth, UK).

Sources of environmental contamination of milk were assessed using the SourceTracker algorithm (Knights et al., 2011). During this source tracking, airborne dust, bedding, water, and feed microbiota were regarded to be a common source of contamination on a farm, that is, the source of milk contamination could vary between cows even if they were kept in a same housing.

3 | RESULTS

The MiSeq sequencing resulted in non-chimeric sequence reads with an average of 38,647, 36,635, 41,551, 35,122, 38,497, 35,981, and 46,666 for rumen fluid, feces, milk, bedding, airborne dust, water, and feed samples, respectively. The somatic cell counts of the milk, estimated by the NAGase activity, were 1.9–2.0 × 10^5 cells/ml for farm 1 and 1.0–2.3 × 10^5 cells/ml for farm 2, respectively. The cows examined in this study did not show any systemic signs of mastitis.

Regardless of the farms, *Prevotellaceae* (31.9% and 25.5%, respectively, at farm 1 and 2) was the most abundant taxa in rumen fluid microbiota (Figure 1, Figure S1, and Table S1). Other bacteria identified included *Ruminococcaceae* (11.2%), *Lachnospiraceae* (9.3%), *Paraprevotellaceae* (2.9%), and *Veillonellaceae* (1.7%) at farm 1, and *Succinivibrionaceae* (13.3%), *Ruminococcaceae* (10.8%), *Lachnospiraceae* (5.2%), and *Veillonellaceae* (4.4%) at farm 2.

The most abundant taxa in the feces microbiota were different from those in rumen fluid microbiota. *Ruminococcaceae* was found at 38.5% and 39.2% at farm 1 and 2, respectively. Additionally, *Lachnospiraceae* (7.8%), *Clostridiaceae* (6.6%), *Bacteroidaceae* (6.1%), and *Peptostreptcoccaceae* (3.0%) were found at farm 1, and *Bacteroidaceae* (11.5%), *Lachnospiraceae* (5.1%), *Clostridiaceae* (4.5%), and *Rikenellaceae* (3.5%) were detected at farm 2.

The five most abundant taxa in the milk microbiota were *Aerococcaceae* (24.3%), *Staphylococcaceae* (12.3%), *Ruminococcaceae* (11.4%), *Corynebacteriaceae* (5.9%), and *Lachnospiraceae* (5.1%)

at farm 1, and *Staphylococcaceae* (21.0%), *Lactobacillaceae* (10.8%), *Ruminococcaceae* (6.3%), *Corynebacteriaceae* (6.1%), and *Enterobacteriaceae* (5.6%) at farm 2. Although *Aerococcaceae* and *Staphylococcaceae* were present in the two highest proportions in the milk microbiota at farm 1 and 2, one out of three cows had *Ruminococcaceae* as the most abundant taxa at both farms.

Although only one composite sample of bedding was examined for each farm, *Ruminococcaceae* (19.5% and 10.8% at farm 1 and 2, respectively) was found as the predominant taxa at the two farms. Some taxa varied greatly between farms with *Aerococcaceae* (15.0%), *Staphylococcaceae* (9.7%), *Corynebacteriaceae* (8.8%), and *Lachnospiraceae* (6.4%) observed at farm 1, and *Moraxellaceae* (10.4%), *Idiomarinaceae* (8.5%), *Halomonadaceae* (8.2%), and *Corynebacteriaceae* (7.0%) observed at farm 2.

In airborne dust microbiota, *Aerococcaceae* (25.2%) was the most abundant at farm 1 followed by *Ruminococcaceae* (12.0%), *Staphylococcaceae* (10.3%), *Lachnospiraceae* (5.8%), and *Corynebacteriaceae* (5.7%). At farm 2, *Lactobacillaceae* (64.5%) were found at far greater proportions than *Staphylococcaceae* (5.6%), *Ruminococcaceae* (3.1%), *Pseudomonadaceae* (2.2%), and *Aerococcaceae* (1.8%).

Regardless of the farms, *Lactobacillaceae* (38.8% and 55.7% at farm 1 and 2, respectively) was the most abundant taxa in the water microbiota. Other taxa were seen at proportions <10% including *Comamonadaceae* (6.8%), *Moraxellaceae* (5.7%), *Pseudomonadaceae* (5.1%), and *Staphylococcaceae* (3.6%) at farm 1, and *Moraxellaceae* (9.5%), *Aeromonadaceae* (4.3%), *Neisseriaceae* (4.2%), and *Weeksellaceae* (3.8%) at farm 2.

At both farms, total mixed ration silage was exclusively fed to dairy cows and the proportions of *Lactobacillaceae* exceeded 95% in the feed microbiota. The second most prominent taxon was *Leuconostocaceae*, but the proportions were as low as 1.0%–2.6%.

According to heatmap, the rumen fluid and feces microbiota were clearly separated with the bedding microbiota (Figure 2). Few differences were seen in the rumen fluid and feces microbiota between individual cows across the two farms. Farm-to-farm and cow-to-cow differences in milk microbiota appeared to be greater compared with the feed, rumen fluid, and feces microbiota. The milk microbiota at farm 1 were grouped with the airborne dust and bedding microbiota. Although the milk microbiota for two samples at farm 2 was grouped with the bedding microbiota, that for one sample was related with the airborne dust and water microbiota.

Canonical analysis of principal coordinates further clarified the taxa associated with rumen fluid, feces, milk, water, feed, airborne dust, and bedding samples (Figure 3). Rumen fluid and feces, which were characterized by *Prevotellaecae* and *Ruminococcaceae*, respectively, were regarded as separate groups at a 60% similarity level. As depicted in the heatmap, the milk, airborne dust, and bedding at farm 1 were considered to be in a same group, which was characterized with a high abundance of *Aerococcaceae*. Feed, airborne dust, and water at farm 2 formed one group and was characterized

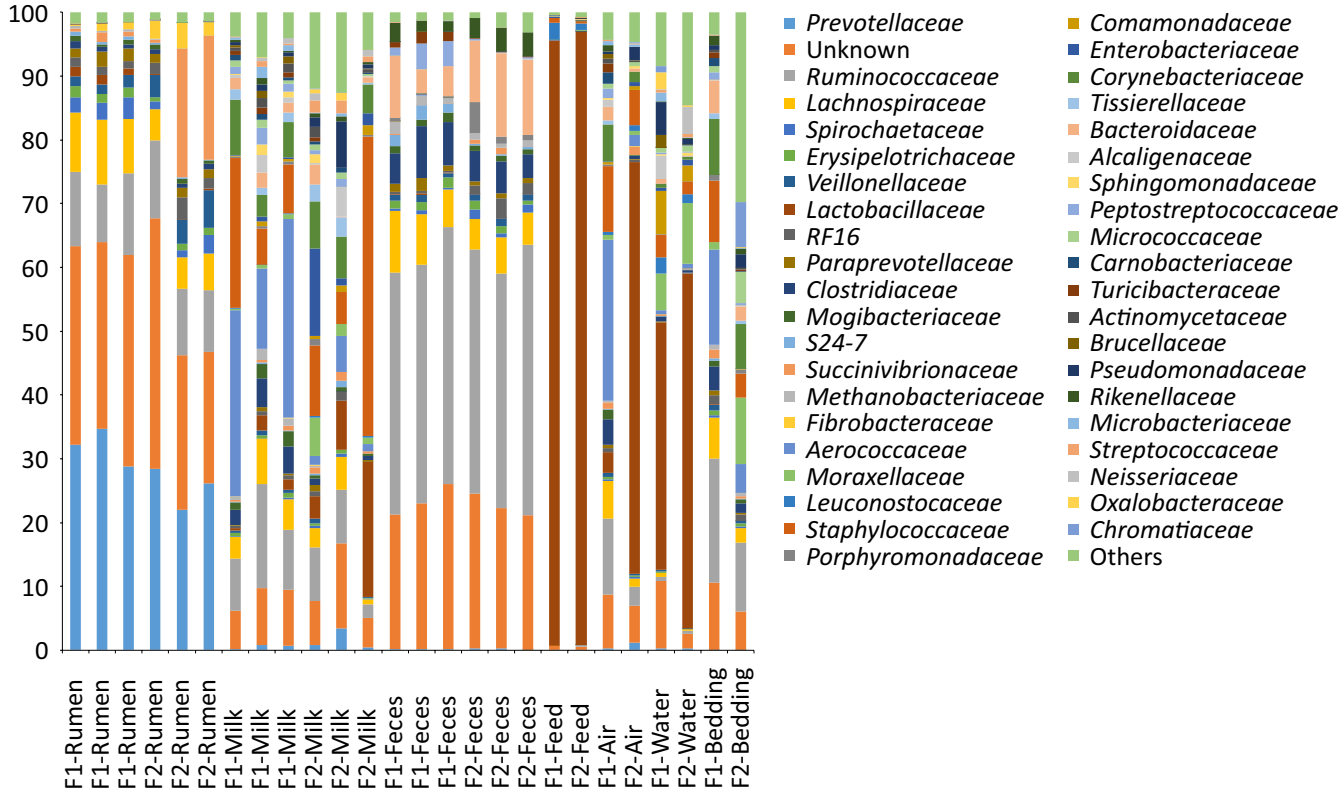


FIGURE 1 Family level proportions of feed, rumen fluid, feces, milk, bedding, water, and airborne dust microbiota in two dairy farms managed by an automatic milking system. F1, F2, rumen, and air indicate farm 1, farm 2, rumen fluid, and airborne dust, respectively

by the high abundance of *Lactobacillaceae*. Differences with respect to airborne dust and bedding between farm 1 and 2 were apparent.

The SourceTracker algorithm was used to identify the likely source of milk microbiota in the dairy farm environment (Figure 4). Regardless of the farms, airborne dust was identified as the greatest contributor (53.0% and 37.9% at farm 1 and 2, respectively) to the milk microbiota, followed by feces (13.8%), bedding (13.7%), and water (4.3%) at farm 1, and bedding (9.7%), feces (8.0%), and rumen fluid (6.4%) at farm 2. Farm-to-farm differences were not evident ($p > 0.05$) for any source of contamination. When SourceTracker analysis was performed by combining the data from the two farms; the contributions of airborne dust, feces, and bedding were calculated to be 45.5%, 10.9%, and 10.1%, respectively.

4 | DISCUSSION

Although *Prevotellaceae* was the most abundant taxa (>25%) in the rumen fluid, the proportion was <0.3% in feces. *Ruminococcaceae* was the second (farm 1) and the third (farm 2) most abundant taxa in the rumen fluid, but the relative abundance was the highest in feces for any cows regardless of the farm. *Prevotellaceae* is regarded as a major soluble carbohydrate degrader and the proportion correlated with grain feeding (Khafipour et al., 2016). Therefore, differences in the taxon proportions between rumen fluid and feces indicated that availability of the soluble carbohydrates was greatly lowered over digestion from the rumen to the large intestine. *Lachnospiraceae* was found at similar proportions in rumen fluid and feces, whereas *Veillonellaceae* was higher in rumen fluid and *Bacteroidaceae* and *Clostridiaceae* were higher in feces. *Ruminococcaceae*, *Bacteroidaceae*, *Lachnospiraceae*, and *Clostridiaceae* were identified as the four most abundant taxa in feces, which was similar to that seen in our previous study (Tang et al., 2017). The observation that *Prevotellaceae* is predominant in rumen fluid whereas the abundance was greatly different between the rumen fluid and feces was also similar to that reported by Mao, Zhang, Liu, and Zhu (2015).

Well-preserved TMR silage with *Lactobacillaceae* at >95% was exclusively fed to the cows at both farms. Therefore, the observation that the mean proportion of *Succinivibrionaceae* in rumen microbiota was numerically higher at farm 2 (13.3%) than farm 1 (0.9%)

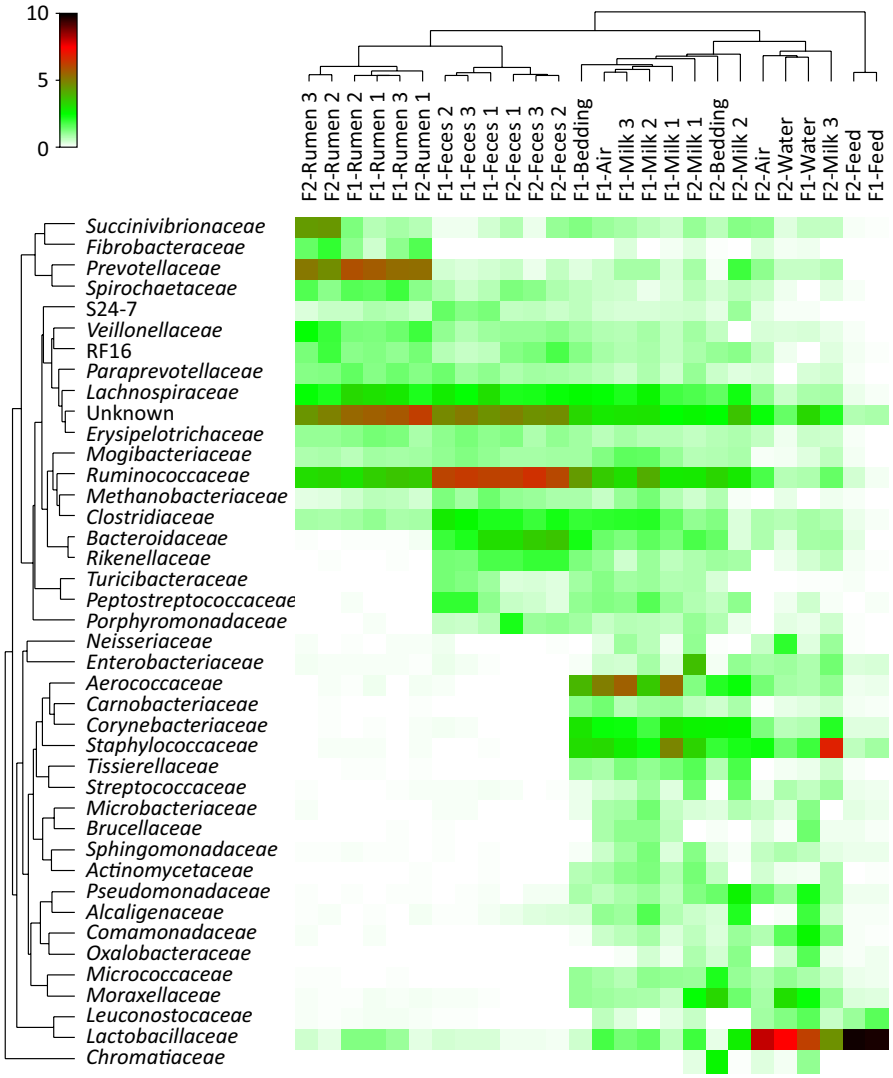
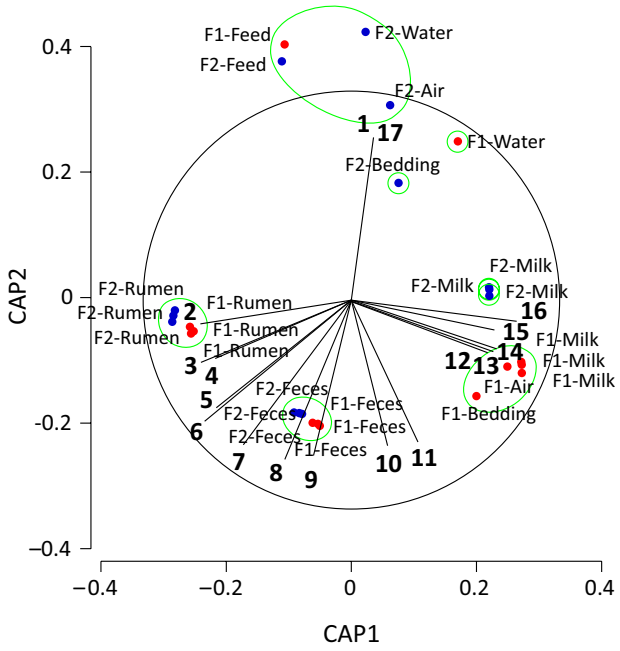


FIGURE 2 Heatmap showing the relative abundance of major taxa (detected at >1.0% at least two different samples) in feed, rumen fluid, feces, milk, bedding, water, and airborne dust microbiota in two dairy farms managed by an automatic milking system. Clustering was performed using the Euclidean distance as a similarity metric. F1, F2, rumen, and air indicate farm 1, farm 2, rumen fluid, and airborne dust, respectively



1. *Lactobacillaceae*
2. *Prevotellaceae*
3. *Veillonellaceae*
4. *Spirochaetaceae*
5. *Paraprevotellaceae*
6. Others
7. *Erysipelotrichaceae*
8. *Lachnospiraceae*
9. *Ruminococcaceae*
10. *Clostridiaceae*
11. *Mogibacteriaceae*
12. *Carnobacteriaceae*
13. *Yaniellaceae*
14. *Aerococcaceae*
15. *Actinomycetaceae*
16. *Corynebacteriaceae*
17. *Leuconostocaceae*

FIGURE 3 Canonical analysis of principal coordinates plot characterizing the microbiota of feed, rumen fluid, feces, milk, bedding, water, and airborne dust in the two dairy farms managed by an automatic milking system. The operational taxonomy unit with Pearson's correlation >0.7 is overlaid on the plot as vectors. Samples for farm 1 and 2 are presented as red and blue plots, respectively. Samples enclosed in a green circle are regarded to be in the same group at a 60% similarity level. F1, F2, rumen, and air indicate farm 1, farm 2, rumen fluid, and airborne dust, respectively

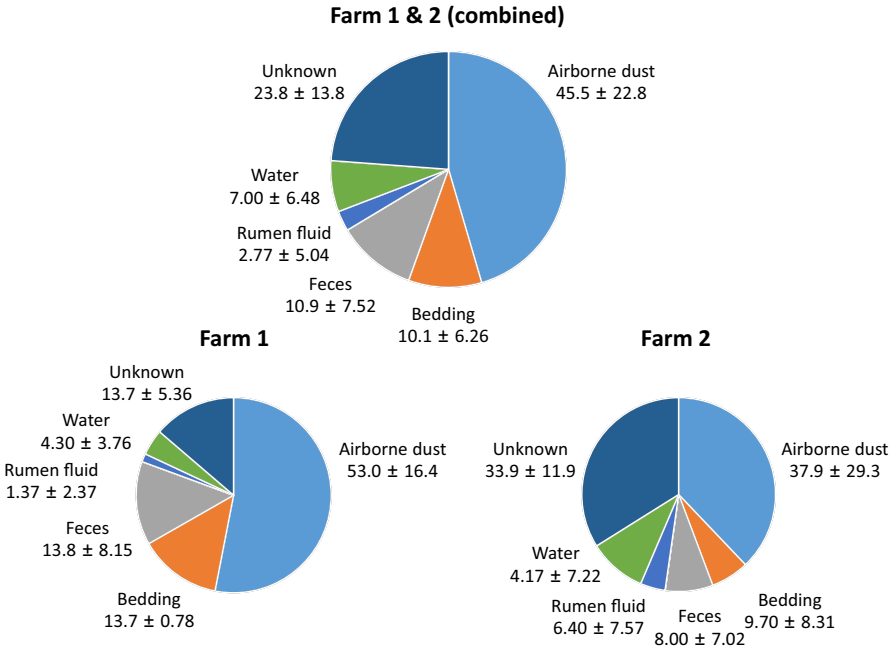


FIGURE 4 Pie charts of the percentages of inferred sources of milk microbiota in two dairy farms managed by an automatic milking system. Cows moved freely and determining their resting place was difficult in the free stall system. A composite sample prepared from three separate samples was thus regarded as a representative means of assessing airborne dust, water, bedding, and feed microbiota at a farm. The values are the means and standard deviations for three cows

was difficult to explain. However, one cow among three cows at farm 2 had *Succinivibrionaceae* at <1.0%, which was similar to the mean proportion of *Succinivibrionaceae* in the rumen fluid at farm 1. Thus, although *Succinivibrionaceae* has been known to correlate with grain feeding (Khafipour et al., 2016), the taxa may show a large cow-to-cow variation in rumen fluid.

Staphylococcaceae, *Ruminococcaceae*, and *Corynebacteriaceae* were identified as the most abundant taxa in the milk microbiota at both farms. *S. aureus* is the representative agent of infectious mastitis and the proportion of *Staphylococcus* spp. was shown to increase to >50% in clinical mastitis (Bhatt et al., 2012). Relative abundances of *Staphylococcaceae* detected in this study (12.3% and 21.0% at farm 1 and 2, respectively) should be regarded as high levels, but the cows from which milk was collected did not show any symptoms of clinical or subclinical mastitis in this study.

Although a lot of studies have examined the milk microbiota of cows with and without mastitis, no typical microbiota has been defined as the microbiota for healthy cow's milk. Bonsaglia et al. (2017) reported that *Corynebacterium* spp. (*Corynebacteriaceae*) and *Psychrobacter* spp. (*Moraxellaceae*) were the two most abundant taxa in milk samples and *Acinetobacter* spp. (*Moraxellaceae*), *Staphylococcus* spp. (*Staphylococcaceae*), and *Micrococcus* spp. (*Micrococcaceae*) were found at >5.0% abundance. Falentin et al. (2016) found that *Oscillospira* spp. (*Ruminococcaceae*) and *Staphylococcus* spp. (*Staphylococcaceae*)

were prevalent taxa and *Bifidobacterium* spp. (*Bifidobacteriaceae*) was found as the next most abundant taxon in the milk microbiota. Because milk microbiota can vary between seasons (Li et al., 2018), to define the microbiota of healthy cow's milk may be difficult.

In the water microbiota, *Lactobacillaceae* and *Moraxellaceae* were found in both farm 1 and 2. A high proportion of *Lactobacillaceae* may indicate a transfer from mouth to cows fed *Lactobacillaceae*-rich TMR silage, and that of *Moraxellaceae* could indicate the presence of the psychrophilic *Acinetobacter* spp. The relative abundance of *Lactobacillaceae* was high in airborne dust at farm 2. Therefore, a high proportion of *Lactobacillaceae* in water may also indicate a transfer from the surrounding air.

At both farm 1 and 2, *Ruminococcaceae* and *Corynebacteriaceae* were found as the major taxa in the bedding microbiota, which agrees with the findings of Doyle et al. (2016). *Ruminococcaceae* is regarded as a gut inhabitant, whereas *Corynebacteriaceae* is known to inhabit diverse environments. Our results indicating that milk and feces microbiota are separately grouped from bedding microbiota were similar to those of Doyle et al. (2016). *Ruminococcaceae* and *Lachnospiraceae* were identified as the major taxa in feces, bedding, and milk microbiota at farm 1, indicating that milk microbiota could be contaminated by gut-associated groups (Oikonomou et al., 2014). However, neither *Ruminococcaceae* nor *Lachnospiraceae* are considered to be mastitis pathogens. Rather, *Aerococcaceae*, *Staphylococcaceae*, and *Corynebacteriaceae* were found at high relative abundances. Therefore, non-gut groups like *Aerococcus viridans*, *S. aureus*, and *C. bovis* might provoke mastitis at farm 1. Regardless, at two farms examined in this study, and apparently well managed by AMS, gut-associated microbiota was not a primary risk factor for mastitis.

Aerococcaceae, *Ruminococcaceae*, and *Staphylococcaceae* were major taxa in the airborne dust microbiota at farm 1 and 2. de Evgrafov et al. (2013) reported that the major taxa of microbiota of airborne dust collected at a milking parlor were *Lachnospiraceae* (26%), *Aerococcaceae* (12.9%), *Peptostreptococcaceae* (11.0%), and *Moraxellaceae* (6.2%) and *Staphylococcaceae* (2.0%), *Corynebacteriaceae* (2.5%), *Lactobacillaceae* (1.1%), and *Pseudomonadaceae* (1.7%) were low in relative abundance. Although they reported that these bacteria were detectable in non-farm outdoor samples collected 8 km away from the dairy farm, they did not detect *Ruminococcaceae* in either the cowshed or non-farm outdoor environment. In this study, three airborne dust samples collected at the free-barn were composited. Therefore, *Ruminococcaceae* have a greater likelihood of detection than those samples collected near milking parlor. The observation that *Lactobacillaceae* is a major taxon in airborne dust at farm 2 was difficult to explain, but *Lactobacillaceae* was found at a high relative abundance (10.8%) in water at this farm. Thus, although the route and source were unclear, *Lactobacillaceae* could become a major taxon of the airborne dust microbiota in the cow shed environment. Luongo et al. (2017) reported that *Lactobacillus* spp. (*Lactobacillaceae*), *Streptococcus* spp. (*Streptococcaceae*), *Micrococcus* spp. (*Micrococcaceae*), *Corynebacterium* spp. (*Corynebacteriaceae*), *Haemophilus* spp. (*Pasteurellaceae*), and *Finegoldia* spp. (*Peptoniphilaceae*) were found as the major taxa in the airborne dust microbiota of non-farm indoor samples (university dormitory rooms).

Although relative abundances were different between milk and airborne dust, *Ruminococcaceae*, *Aerococcaceae*, and *Staphylococcaceae* were the three most abundant taxa found in common between milk and airborne dust at farm 1. Likewise, *Staphylococcaceae*, *Lactobacillaceae*, and *Ruminococcaceae* were the three most abundant taxa found in common between milk and airborne dust at farm 2. Indeed, SourceTracker indicated that the milk microbiota may be related to the airborne dust microbiota in non-mastitis healthy cows. Furthermore, *Corynebacteriaceae* was found at both farm 1 and 2 at stable relative abundances (5.8%–8.8%) in milk and bedding microbiota. *Corynebacteriaceae* in milk may have been derived from that in the bedding. Therefore, management of the bedding should be a primary consideration as a measure to prevent mastitis.

Based on hierarchical clustering and canonical analysis of principal coordinates, milk microbiota, particularly at farm 1, was associated with the bedding microbiota. Although Doyle et al. (2016) clarified that the teat microbiota showed the greatest similarity with the milk microbiota, we did not examine teat or udder skin microbiota in this study. The relationship between airborne dust, bedding, and the teat microbiota should be determined in the forthcoming studies.

4.1 | Conclusion

This study examining the microbiota of the gut, milk, and cowshed environment in dairy farms managed by AMS displayed greater farm-to-farm and cow-to-cow differences in milk microbiota compared with the feed, rumen fluid, and feces microbiota. Milk microbiota appeared to be influenced by airborne dust based on the source tracking. Hierarchical clustering and canonical analysis of principal coordinates demonstrated that the milk microbiota was associated with the bedding microbiota but clearly separated from feed, rumen fluid, feces, and water microbiota. Our findings were derived from only two case studies. Therefore, it is unclear if these findings should be limited to the farms managed by AMS. Regardless, the importance of cowshed management should be emphasized to maintain cow's health and prevent mastitis.

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SUPPORTING INFORMATION

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