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Research Article

Fecal, Milk, Uterine, Airborne Dust, and Water Microbiota in Dairy Farms in Southern Vietnam: A Pilot Study

Tu Thi Minh Tran[1]*, Diep Hoang Tran[1], Thuong Thi Nguyen[2], Tomas J. Acosta[3], Takeshi Tsuruta[4], Naoki Nishino[4], Hai Thanh Duong[5]

[1]Faculty of Agriculture and Food Technology, Tien Giang University, My Tho, Vietnam; [2]Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh, Vietnam; [3]Graduate School of Life and Environmental Science, Okayama University, Okayama, Japan; [4]Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan; [5]Faculty of Animal Sciences and Veterinary Medicine, University of Agriculture and Forestry, Hue University, Vietnam.

Abstract | Six sets of fecal, milk, and uterine samples from dairy cows and two sets of airborne dust and water samples from the cowshed were collected from two farms in Ho Chi Minh City at 20–40 days postpartum. The microbiota was characterized using Illumina sequencing of the V4 hypervariable region of the 16S rRNA genes. The predominant species in the fecal microbiota was not as predominant in the milk microbiota, but occasionally appeared in the uterine microbiota. The microbiota from the airborne dust and water samples did not show a relation with either the milk or the uterine microbiota, whereas Moraxellaceae, the most abundant family in the airborne dust and water microbiota, was detected at low proportions in the milk microbiota. The farm-to-farm difference was more apparent for the milk microbiota than for the fecal and uterine microbiota. It is noteworthy that Dermacoccaceae, Bacillaceae, and Methylobacteriaceae were the prevalent families in the microbiota of several milk and uterine samples; however, these findings did not help understand the role of the microbiota in fertility and subsequent conception. Despite the small number of dairy cows examined in this study, this report is the first to characterize the fecal, milk, uterine, airborne dust, and water microbiota in dairy farms in southern Vietnam.

Keywords | Dairy cows, Environment, Microbiota, Next generation sequencing, Tropics

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***Correspondence** | Tu Thi Minh Tran, Faculty of Agriculture and Food Technology, Tien Giang University, My Tho, Vietnam; **Email:** tranminhtu@tgu.edu.vn
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INTRODUCTION

Hot and humid climates have a negative impact on the reproductive performance and milk production of dairy cows, especially for the high-yielding and improved breeds (Mayer et al., 1999; Morton et al., 2007). Nevertheless, the population of dairy cows in Vietnam has increased at an annual rate of 10% in the last decade, and this trend is predicted to continue for the next 30 years. However, dairy production is not a traditional practice in Vietnam; hence, farmers and industries have suffered from production-related diseases such as mastitis and infertility. Ostensson et al. (2013) reported that the prevalence of subclinical mastitis (>200,000 somatic cell count per milliliter of milk) was 63.2% at quarter-level and 88.6% at cow-level in southern Vietnam. Likewise, Nguyen-Kien et al. (2017) demonstrated poor reproductive performance (postpartum anestrus and infertility) in cows at dairy smallholders in Ho Chi Minh City; 49.9% of cows were not detected in estrus on day 60 postpartum. Although these

diseases are considered multifactorial, infectious agents are shown to be involved and the traits can be exacerbated by nutrition and management.

During parturition, physical barriers of the cervix, vagina, and vulva are compromised, allowing microorganisms from the environment and fecal material to enter the uterus. After calving, dairy cows have high nutritional requirements to ensure milk production, and thus face the risk of negative energy balance, inflammation, and immunosuppression. These disorders may result in bacterial infection and disruption of the normal microbiota, thus encouraging the development of metritis, endometritis, and mastitis during the postpartum period (Esposito et al., 2014). Although different bacteria might contaminate different organs/tissues in cows, pathogens such as *Escherichia coli*, *Trueperella pyogenes*, *Streptococcus* spp., *Fusobacterium necrophorum*, and *Staphylococcus* spp. have been occasionally detected in both milk and uterine microbiota (Santos and Bicalho, 2012; Bicalho et al., 2012; Oikonomou et al., 2014), which raises the question of whether these bacteria originate from the same source.

In this study, we determined the fecal, milk, and uterine microbiota of dairy cows and the airborne dust and water microbiota in farms in southern Vietnam. Our aim was to examine if the milk and uterus microbiota interact with the fecal and environmental microbiota in hot and humid dairy farms. Uterus samples were collected from visibly healthy cows with translucent vaginal discharge because an understanding of the variability and stability of the microbiota was necessary before considering the diseases and their prevention.

MATERIALS AND METHODS

Animals and sample collection

Two industry-scale dairy farms in Ho Chi Minh City were visited in the rainy season. Farm 1 and 2 both had >200 heads of Holstein × Lai Sind crossbred dairy cows and offered the diets described in Table 1.

Milk samples were manually collected from four udders and then mixed as composite samples. Fecal samples were collected from the rectum, and uterine mucus samples were collected using a cytobrush (Fujihira Industry Co. Ltd., Tokyo). To avoid vaginal contamination, the instrument was covered with a sanitary plastic sheath and then inserted into the cervix. Inside the cervix, the plastic sheath was ruptured and the instrument was then advanced through the cervix toward the base of the larger horn, at which point the stainless-steel tube was retracted to expose the cytobrush. Uterine mucus was collected by rotating the cytobrush while in contact with the uterine wall (Jeon et al., 2015), and the cytobrush was collected by cutting and placing into an Eppendorf tube. Airborne dust was collected by placing several Petri dishes in the cowshed for five minutes and then gathered into a tube using sterile physiological saline. Water samples were collected from water troughs. All the samples were kept on ice during transportation to the laboratory and stored at -20°C until further analyses. Procedures and protocols for the animal experiments were approved by the Animal Care and Use Committee, Tien Giang University, Vietnam.

Table 1: Composition of diet offered for the herd, bacterial numbers in the cowshed, and records of sampling time, reproduction traits, milk yield, and bacterial numbers in fecal, milk, and uterine samples collected from two dairy farms.

	Farm 1			Farm 2			Farm to farm difference (t-test)
	a	b	c	d	e	f	
Diet composition							
Elephant grass (DM kg/day)	2.0			8.0			---
Rice straw (DM kg/day)	4.5			0.0			---
Concentrate (DM kg/day)	10			5.4			---
Bacterial numbers in the cowshed							
Airborne dust (log10 copies/petri dish)	6.56			6.57			---
Water (log10 copies/mL)	6.21			6.43			---
Records for individual cows							
Sampling time (days after postpartum)	28	36	37	27	39	28	---
Times of calving	3	2	1	5	2	2	---
Calving to conception interval (days)	158	157	98	>180	155	>180	---
Milk yield (kg/day)	27.6	22.0	21.3	17.2	13.5	13.9	0.020
Fecal bacteria (log10 copies/g)	9.86	9.93	9.96	9.66	9.64	9.55	0.002
Milk bacteria (log10 copies/mL)	6.67	6.70	6.57	6.70	6.21	6.83	0.746
Uterine bacteria (log10 copies/brush)	9.43	7.68	6.66	7.90	8.35	6.95	0.840

a–f, individual dairy cows.

On the day of visitation, four and five cows were at 3–6 weeks postpartum at farm 1 and 2 respectively. Fecal, milk, and uterine samples were collected from these cows. One sample set from farm 1 was excluded because of bloody milk, and one set from farm 2 was excluded owing to a history of treatment for retained fetal membrane. The remaining seven sample sets were derived from cows that received no medical treatment for at least one month before the sampling. Based on a preliminary evaluation by denaturing gradient gel electrophoresis, one set of farm 2 samples that showed a similar band profile for uterine microbiota as other samples were excluded. Thus, three sets of fecal, milk, and uterine samples and one set of airborne dust and water samples for farm 1 and 2 were subjected to high-throughput Illumina sequencing as described below.

DNA extraction, PCR amplification, and MiSeq sequencing

Bacterial DNA from milk, uterine, water, and airborne dust samples was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) (Han et al., 2014). A 250 µL composite milk sample was transferred to an Eppendorf tube, and the pellets were obtained by centrifugation at 16,000 × *g* for 2 min. For uterine samples, the thawed cytobrush was soaked in 1 mL of sterile phosphate buffered saline (PBS; pH 7.4) for 1 h to release the uterine microbiota. The PBS suspension was centrifuged at 16,000 × *g* for 2 min to collect the pellet. For airborne and water samples, a 1 mL sample was transferred to an Eppendorf tube, and then centrifuged to collect the pellet. All of the 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 h. Subsequent bacterial DNA purification was performed following the manufacturer's recommendations. For fecal samples, 0.1 g of the feces was used for bacterial DNA purification using the DNeasy Stool Mini Kit (Qiagen, Germantown, MD, USA).

The DNA samples were subjected to two-step polymerase chain reaction (PCR) procedures to generate amplicon libraries for the MiSeq sequencing. In the first round of PCR, primers targeting the V4 region of 16S rRNA genes (forward: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3′; reverse: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3′; tail sequences are underlined) were used (Tang et al., 2017). The PCR program was as follows: an initial denaturation step at 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 at 72°C for 10 min (Jeon et al., 2015). The PCR products were then purified with a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) after electrophoretic separation on a 20 g/L agarose gel. The second round of PCR was performed with adapter-attached primers, with the following protocol: Initial denaturation at 94°C for 2 min; followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 30 s; and a final elongation step at 72°C for 5 min. The PCR products were purified by the same method as used above for the first-round products.

The purified amplicons were pair-end sequenced (2 × 250) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan). Raw sequences were processed using the QIIME (version 1.9.0) microbial ecology pipeline. The 250-bp reads were truncated at any site receiving an average quality score <20 over a 20-bp sliding window, and truncated reads that were shorter than 50 bp were discarded. Likewise, two nucleotide mismatches in primer matching were removed, sequences that overlapped by more than 50 bp were assembled, and chimeric sequences were identified and removed. Only sequences at least 130-bp long after quality filtering were grouped into operational taxonomic units (OTUs) using a 97% similarity threshold. Sequences were classified from the phylum to the genus level using the default settings of the Ribosomal Database Project classifier. Results from sequence analysis are available at the DDBJ Sequence Read Archive under project identification number PRJDB4975.

Quantitative PCR (qPCR) assessment of total bacterial numbers

Amplification and detection of bacterial DNA were performed by using the MiniOpticon Real-Time PCR System (Bio-Rad Laboratories Inc., Tokyo, Japan), with primers (forward: 5′-CCTACGGGAGGCAGCAG-3′; reverse: 5′-ATTACCGCGGCTGCTGG-3′) targeting the V3 region of the 16S rRNA genes (Bai et al., 2016). Plasmid DNA prepared with 16S rRNA genes of *E. coli* (JCM 1649^T) was used as the standard for qPCR assessment.

Data analysis

Data for qPCR and dominant families determined by MiSeq were subjected to one-way analysis of variance to examine the differences between the two farms by using the JMP software (version 11; SAS Institute, Tokyo, Japan). Data for the bacterial families were also subjected to canonical analysis of principal coordinates (CAP) 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).

RESULTS AND DISCUSSION

In the present study, fecal, milk, and uterine samples were collected from dairy cows at 3–6 weeks postpartum because damaged endometrial tissues normally regenerate during week 2–4 postpartum and evidence of previous pregnancy may disappear from the genital tract by week 6 after calving (Sheldon et al. 2009). Likewise, incidence of bacterial infection is known to be quite high before 3 weeks postpartum; hence, it is rather difficult to discriminate healthy cows. The six cows examined did not show any systemic signs of disease and their uterine mucus samples were translucent. However, it was unclear whether subclinical endometritis might have persisted in several cows, as detailed diagnosis, e.g., polymorphonuclear cell counting, was not performed in this study.

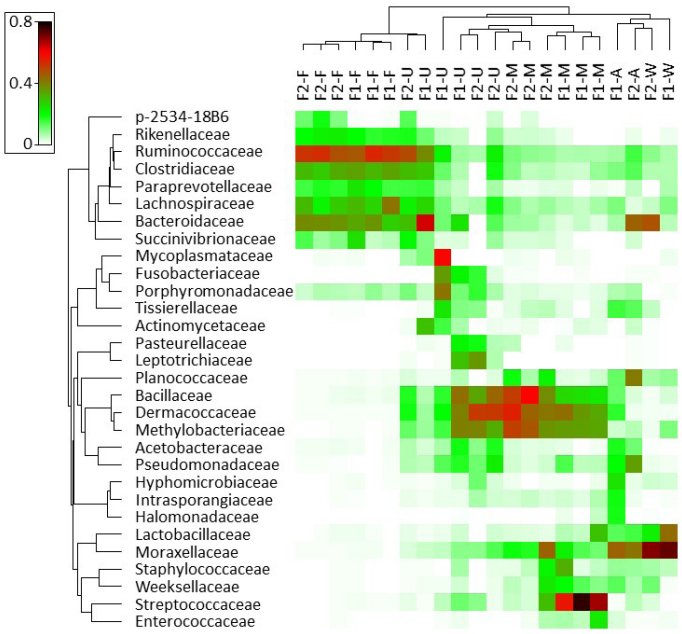

Figure 1: Heatmap analysis showing hierarchical clustering of the relative abundance of the 30 most occurring OTUs in the fecal, milk, uterine, airborne dust, and water microbiota in dairy farms.F, fecal samples; M, milk samples; U, uterine samples; A, airborne dust samples; W, water samples; 1–2, the farm number.The color of each cell indicates the relative abundance of the bacterial family.

Total bacterial numbers for the fecal (log10 copies/g), milk (log10 copies/mL), uterine (log10 copies/brush), airborne dust (log10 copies/petri dish), and water (log10 copies/mL) samples were 9.75, 6.61, 7.82, 6.39 and 6.50, respectively. Although the differences were small, the total numbers of fecal bacteria were greater in farm 1 than in farm 2. No farm-to-farm differences were seen in the total bacterial numbers for the milk and uterine samples. Variation between cows appeared to be greater in the uterine samples than in the fecal and milk samples. The uterine samples from two cows clustered into the same group as the fecal samples (Figure 1) had numerically higher total bacterial numbers than the other uterine samples.

The milk yield of the dairy herd at farm 1 was greater than that at farm 2 (Table 1). Veterinary records indicated that although one pregnant cow reported acceptable calving to conception interval (98 days), other cows required a longer time (155–158 days) or did not show a distinctive heat signal even after 5 months from calving. Although diet is considered the primary factor influencing gut microbiota, fecal microbiota was regarded as one group for the two farms (Figures 1 and 2). The ratios of forage concentrates were 40:60 and 60:40 for farm 1 and 2, respectively, and both farms used fresh harvest of elephant grass as the main forage in the diet. Therefore, although the difference in diet formulation did cause varied milk production between the two farms, this difference could be insufficient to alter fecal microbiota of dairy cows.

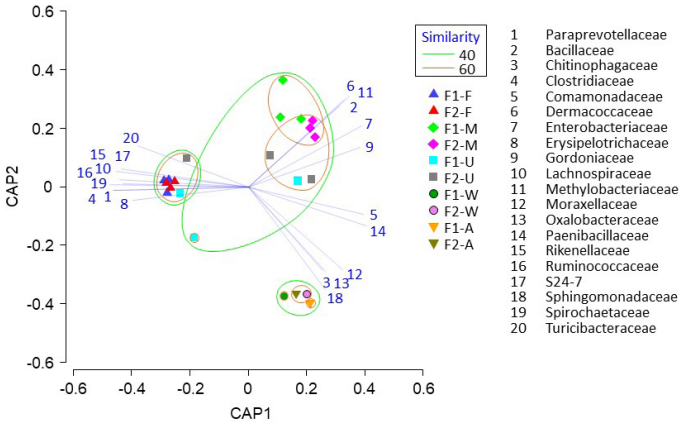

Figure 2: Principal coordinate analysis exploring the differences betweenfecal, milk, uterine, airborne dust, and water microbiota in dairy farms. The operational taxonomy unit with Pearson's correlation >0.7 is overlaid on the plot as vectors. F, fecal samples; M, milk samples; U, uterine samples; A, airborne dust samples; W, water samples; 1–2, the farm number.

Illumina Miseq sequencing of the 22 samples resulted in a total of 1,568,908 non-chimeric sequence reads with an average of 75,140, 70,034, 52,353, 101,530, and 90,340 sequence reads for fecal, milk, uterine, airborne dust, and water samples, respectively. Based on the rarefaction curves, the microbiota diversity was the greatest in fecal samples, followed by uterine, airborne dust, milk, and water samples (Supplementary Figure S1). The CAP indicated that the fecal microbiota from two farms were classified into the same group with clear separation from the airborne dust, water, and milk microbiota (Figure 2). The milk and uterine microbiota from the two farms were grouped together at 40% similarity level, whereas the milk microbiota from farm 1 was distinct from the uterine microbiota at 60% similarity level. Uterine microbiota was dispersed

irrespective of the farm; three cow samples showed the same group of milk microbiota, two cow samples showed microbiota with close similarity to the fecal microbiota, and one cow sample was distinct from other cow samples with a 60% similarity level.

Predominant families in the fecal microbiota included Ruminococcaceae (21.0–29.1%), Bacteroidaceae (7.37–14.3%), Clostridiaceae (7.72–11.6%), Lachnospiraceae (4.51–17.3%), Paraprevotellaceae (2.12–5.66%), and Rikenellaceae (2.69–4.89%) (Supplementary Table S1). All fecal samples were grouped into the same cluster in the heatmap (Figure 1). In fecal microbiota, Ruminococcaceae was found as the most dominant family ranging from 21.0–29.1%. Similar results were reported by Young et al. (2015) and Liu et al. (2015), who identified Ruminococcaceae at 35.1 and 19.6%, respectively, in fecal microbiota of dairy cows. Bacteroidaceae was the second dominant family ranging from 7.37–14.3%, with large variation in the relative abundance, observed between the two farms (Supplementary Table S1). Bacteroidaceae is known to associate with hydrolyzing polysaccharides of the plant cell wall (Power et al., 2014).

The uterine microbiota from the three cow samples showed the presence of Dermacoccaceae (25.7–39.4%), Bacillaceae (11.4–21.0%), and Methylobacteriaceae (10.6–22.2%) as the three most abundant families (Supplementary Table S3), and these families were also prevalent in milk microbiota. In the other two cow samples, gut-associated bacterial families, namely Ruminococcaceae, Bacteroidaceae, Clostridiaceae, and Lachnospiraceae appeared as highly abundant taxa; hence, the uterine microbiota of these two samples was clustered into the same group as the fecal microbiota (Figure 1). For one cow sample from farm 1, Mycoplasmataceae, Porphyromonadaceae, Tissierellaceae, Fusobacteriaceae, and Actinomycetaceae accounted for 72% of the uterine microbiota. Therefore the cow was clustered into a separate group on the heatmap (Figure 1). Fecal microbiota, namely Ruminococcaceae, Bacteroidaceae, Clostridiaceae, and Lachnospiraceae were detected as prevalent families in the uterine microbiota of two cows, one each from farm 1 and 2 (Supplementary Table S3; Figure 1). It is generally thought that the fecal associated families are likely to transmit from feces via bedding to the postpartum uterine environment; however, Knudsen et al. (2016) reported that, regardless of normal and endometritis cows, Ruminococcaceae and Bacteroidaceae were found at high abundance in the endometrial biopsies at week 7 after calving. Gilbert and Santos (2015) indicated that *Clostridium* spp., particularly *C. perfringens,* were isolated at approximately 20% from the uterus of postpartum dairy cows, but their prevalence was limited to the very-early lactation period (<7 days postpartum). Although the uterine microbiota of the two cow samples suggested occasional infection with feces, this fact did not necessarily define the time for conception after calving in this study (Table 1).

Large variation in the relative abundance of milk microbiota was seen between the two farms. The proportion of Streptococcaceae was greater in farm 1 than in farm 2, whereas those of Bacillaceae and Ruminococcaceae were greater in farm 2 than in farm 1 (Supplementary Table S2). Doyle et al. (2016) reported that *Lactococcus* spp., *Pseudomonas* spp. (Pseudomonadaceae), and *Lactobacillus* spp. (Lactobacillaceae) were higher in milk when no teat preparation was done prior to milking. The difference in the proportions of Streptococcaceae could thus be attributed to differences in milking management, whereas the proportions of Pseudomonadaceae and Lactobacillaceae were similar between the two farms.

Dermacoccaceae, Bacillaceae, and Methylobacteriaceae were found as prevalent families for three cows in both uterine and milk microbiota. La Duc et al. (2007) reported a substantial resistance of *Methylobacterium* spp. to chlorine; hence, Methylobacteriaceae may have persisted in milk despite the use of cleaning agents in milking parlors. Wagener et al. (2015) reported that *Bacillus* spp. (Bacillaceae) could be isolated from cow uterus within 4 weeks postpartum, and their detection was more frequent in cows with clear vaginal discharge than in those with vaginal discharge containing >50% pus. In this study, however, only one out of the three cows recorded an acceptable time interval (98 days) between calving and conception. Because the prevalence of Dermacoccaceae, Bacillaceae, and Methylobacteriaceae in milk and uterus was unexpected observation, further survey of many healthy and diseased cows is needed.

The uterine microbiota of one cow was largely different from that of other cows at farm 1. Mycoplasmataceae (34.5%) was found as the most prevalent, whereas the family was detected at <1.0% in other uterine samples. The proportions of Porphyromonadaceae (17.4%) and Fusobacteriaceae (12.4%), which were found at <2.0% in other cows, were also high in the uterine microbiota of the cow (Supplementary Table S3). Jeon et al. (2015) and Knudsen et al. (2016) reported that Mycoplasmataceae, Porphyromonadaceae, and Fusobacteriaceae were associated with metritis and endometritis in dairy cows at 4 weeks postpartum. Machado et al. (2012) found that *Ureaplasma* spp. and *Fusobacterium* spp., which are members of the Mycoplasmataceae and Fusobacteriaceae, respectively, were higher in uterine lavage samples derived from cows that were not pregnant for 200 days after calving. Therefore, subclinical endometritis may have occurred in

the cow, whereas the time to conception (157 days) was not the worst among the six cows.

In the airborne dust microbiota, the total proportion of gut-associated families such as Bacteroidaceae, Ruminococcaceae, Clostridiaceae, and Lachnospiraceae was 2.52% in farm 1 but 23.7% in farm 2. Similarly, this proportion in the water microbiota was 1.71% in farm 1 and 23.4% in farm 2 (Supplementary Table S4). In addition, the airborne dust and water microbiota were classified in the same group by CAP (Figure 2). This finding indicated that the airborne dust microbiota could be correlated with the water microbiota in dairy farms.

Moraxellaceae, the family *Acinetobacter* spp. belong to, accounted for 17.2–19.6% of the airborne dust microbiota and 48.3–51.8% of the water microbiota. This family showed a large cow to cow variation in the milk microbiota (1.20–4.76% for farm 1 and 2.99–19.6% for farm 2), but its proportions in the fecal (<1.0%) and uterine (<1.82%) microbiota were low. Braem et al. (2011) detected a 52% abundance of *Acinetobacter* spp. in teat apices from the herd of dairy cows and identified it as the predominant genus in the sub-clinically infected quarter. Although *Acinetobacter* spp. are categorized as potential pathogens causing mastitis and endometritis in dairy cows (Carneiro et al., 2016), their presence in airborne dust and water did not necessarily indicate that they constituted a higher proportion in the milk and uterus.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, fecal microbiota was not seen as predominant in milk microbiota, but occasionally appeared in uterine microbiota; hence, feces might be more likely to contaminate the uterus than the milk. The fecal microbiota from two farms were classified into the same group with clear separation from the airborne dust, water and milk microbiota. The microbiota from the airborne dust and water samples were not related with the milk or uterine microbiota, although the most abundant family (Moraxellaceae) in the airborne dust and water microbiota was detectable at low proportions in the milk microbiota. Farm-to-farm difference was more apparent for milk microbiota than for fecal, uterine, airborne dust, and water microbiota, suggesting a significant influence of milking management on the microbiological quality of milk. The findings that Dermacoccaceae, Bacillaceae, and Methylobacteriaceae were the prevalent microbiota families in several milk and uterine samples should be noteworthy. Further studies are required to understand the origin of these bacteria and their involvement in the milk quality, health, and fertility of dairy cows.

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NOVELTY STATEMENT

In this paper, we concluded that fecal microbiota was not seen as predominant in milk microbiota, but occasionally appeared in uterine microbiota; hence, feces might be more likely to contaminate the uterus than milk. Farm-to-farm difference was more apparent for milk microbiota than for fecal, uterine, airborne dust and water microbiota, suggesting a significant influence of milking management on the microbiological quality of milk.

AUTHOR'S CONTRIBUTION

TU Thi Minh TRAN created the idea, designed the experiments, experimented, analyzed and wrote the primary manuscript. Naoki NISHINO edited and finalized the manuscript.

SUPPLEMENTARY MATERIAL

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.aavs/2022/10.7.1525.1531

Conflict of interest

The authors have declared no conflict of interest.

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