The Journal of Veterinary Medical Science

FULL PAPER

Internal Medicine

Examination of the microbiota of normal cow milk using MinION™ nanopore sequencing

Yasunori SHINOZUKA[1,2], Kazuhiro KAWAI[1,2], Tomomi KURUMISAWA[1,2], Yuko SHIMIZU[1], Tadashi IMANISHI[3], Ayumu OHNO[3], Mano TAKAHASHI[3], Sohei KANEKO[4] and Naoki SUZUKI[4,5]*

[1]School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 252-5201, Japan
[2]Azabu University Mastitis Research Center, Sagamihara, Kanagawa 252-5201, Japan
[3]Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan
[4]Higashi-Hiroshima Veterinary Clinical Center, Higashi-Hiroshima, Hiroshima 739-2106, Japan
[5]Present address: Graduate School of Integrated Science for Life, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8528, Japan

ABSTRACT. The aim of this study was to evaluate the microbiota of normal milk in dairy cows and their relationship with host factors, such as the age of the cow (Age), somatic cell counts in milk (SCCs), and days in milk (DIM). We investigated 48 milk samples from 22 cows with no systemic or local clinical signs using MinION™ nanopore sequencing for a *16S rRNA* gene amplicon. Bacterial richness was positively correlated with the DIM (P=0.043), and both the Shannon-Wiener Index and Simpson's Index, which are metrics of alpha-diversity, were also significantly positively correlated with the SCC (P<0.001). The composition ratios of both Actinobacteria at the phylum level and *Kocuria* spp. at the genus level in the milk microbiota were significantly correlated with the SCC (P<0.001 and P<0.001, respectively). In the beta-diversity test, the one-way analysis of similarities test showed a significant difference (P=0.0051) between the low- and high-SCC groups. This study clarified that the composition of the normal milk microbiota in this herd was related to the SCC. It also raised the possibility of variations in bacterial genera in the normal milk microbiota between the low- and high-SCC groups. However, to clarify the actual condition of the milk microbiota and to elucidate the relationship with the SCC, it is necessary to perform further analyses taking into account not only the relative abundance, but also the absolute abundance of microbes.

KEY WORDS: cow, milk microbiota, 16S rRNA

J. Vet. Med. Sci.
83(11): 1620–1627, 2021
doi: 10.1292/jvms.21-0353

Received: 21 June 2021
Accepted: 29 August 2021
Advanced Epub:
15 September 2021

The presence of intramammary bacterial flora has been reported in humans, cattle, and other animals [26]. The first study on the microbiota in bovine milk was a pyrosequencing study that was published in 2012 [3]. More recent studies have reported that the composition of the intramammary flora differs between mastitis and non-mastitis cows [27], and that the intramammary flora may be associated with mastitis and mastitis sensitivity [10]. Attempts to prevent mastitis by controlling the intramammary flora have been reported [17]. Oikonomou *et al*. [26] reported several factors that influence milk and the milk-associated microbiota, including host factors (number of lactation, stage of lactation, delivery mode, body mass index, genetics, health status, mastitis, breast cancer, and antibiotic use) and environmental factors (geography, housing, farm environment, diet and milk micronutrient composition, and milking hygiene). To apply the intramammary flora for the prevention or treatment of mastitis, the accumulation of basic knowledge on the composition of the intramammary flora and the factors that influence it is needed.

Previous studies on the intramammary flora by second-generation sequencing have targeted and amplified partial (approximately 100 to 500 bp) sequences of the *16S rRNA* gene, such as V1-V3 or V2-V3, and evaluated the sequences at the genus level. Researchers have to select the most effective target regions for identifying taxa from full-length *16S rRNA* gene sequences that contain nine hypervariable regions (V1-V9) as phylogenetically informative markers. Recently, the first commercial sequencer using nanopore technology [4] was released by Oxford Nanopore Technologies (Oxford, UK); it is called MinION™, and it has significant advantages, such as a long-read output, low cost, and capability for rapid real-time analysis, as compared to other DNA sequencing technologies. In 2019, full-length *16S rRNA* gene amplicon analysis of the human gut microbiota using MinION™ nanopore sequencing was reported, providing species-level resolution [25]. To the best of our knowledge, there has been no study evaluating cow milk microbiota at the species level using a comprehensive bacterial flora analysis technique for the entire length of the *16S rRNA* gene. Therefore, the purpose of this study was to evaluate the indigenous bacterial flora in the milk of cows raised in the same environment and with the same feed, and the factors that affect the bacterial flora at the species level.

*Correspondence to: Suzuki, N.: naosuzuki@hiroshima-u.ac.jp

MATERIALS AND METHODS

The protocol for milk sample collection from lactating dairy cows adhered to the guidelines of Azabu University Animal Experimentation Committee, and was approved by the committee (No. 200803-1).

Study animals and milk sample collection

The present study was performed on a commercial dairy farm (Tie-stall housing, rubber mat with raw wood chips, separate feeding system) with 70 Holstein cows in Hiroshima Prefecture, Japan. Milk samples were collected from 32 cows on this dairy farm in July 2020. An interview survey for dairy farmer was conducted on host factors (number of lactation, stage of lactation, delivery mode, body condition score (instead of the body mass index), health status, mastitis, and antibiotic use) and environmental factors (geography, housing, farm environment, diet and milk micronutrient compositions, and milking hygiene) that have been reported to influence milk and the milk-associated microbiota. However, among the factors reported by Oikonomou *et al*. [26], genetics and breast cancer were not investigated in this study. The farm owner provided permission for the sampling and for the use of the data obtained in this study. Since the composition of the milk microbiota does not differ between milk samples obtained by different sampling methods (collected by hand-squeezing, a teat canal cannula, or a trans-teat wall needle aspirate) [9], samples were collected by hand-squeezing, which is a non-invasive procedure, from the viewpoint of animal welfare. Milk samples were collected from each quarter without any signs of mastitis or any other diseases, and the cows were not under any antimicrobial treatment. After stripping and before applying the milking unit to the cow, milk samples were aseptically collected into tubes by a trained veterinarian. A total of 111 samples were successfully collected during the experiment. The sample tubes were immediately stored on ice, and transported to the laboratory within 2 hr from the last sampling.

Culturing and identification of isolates

Ten microliters of each milk sample was directly cultured on a sheep blood agar plate (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). After 24 hr of aerobic incubation at 37°C, the obtained colonies were subcultured to obtain pure cultures, and identified using BD BBL CRYSTAL GP (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) or BD BBL CRYSTAL GN (Becton, Dickinson and Co.) based on the results of Gram staining.

Milk test

The somatic cell count (SCC) and the activity of lysosomal N-acetyl-β-D-glucosaminidase (NAGase) in each milk sample were determined. The SCC was determined using an electronic cell counter DCC (DeLaval International AB, Tumba, Sweden) based on the method of Kawai *et al*. [19]. NAGase activity was determined using the β-N-Acetylglucosaminidase Assay Kit (Sigma-Aldrich Co., LLC, St. Louis, MO, USA). Briefly, milk samples were centrifuged at 3,000 rpm for 10 min at 20°C, and the resultant whey was used to determine the NAGase activity, which was calculated after the absorbance of the unreacted substrate of the whey sample (background control) was subtracted from the absorbance of the same whey sample to avoid the effect of the color of the whey.

DNA extraction, 16S rRNA gene amplicon sequencing, and preprocessing of sequence reads

DNA from each milk sample was extracted using ISOSPIN Fecal DNA (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Milk samples were subjected to mechanical disruption by bead-beating, and DNA was isolated using silica membrane spin columns. The quantity and purity of the extracted DNA were determined with NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) by measuring both the 260/280 and 260/230 absorbance ratios. For amplification of the V1-V9 region of the *16S rRNA* gene, the previously described universal primers S-D-bact-0008-c-S20 (5′-AGRGTTYGATYMTGGCTCAG-3′) and S-D-bact-1391-a-A-17 (5′-GACGGGCGGTGWGTRCA-3′) [20] were used for polymerase chain reaction (PCR). PCR amplification of the *16S rRNA* gene was conducted using KAPA^{TM} HiFi HotStart ReadyMix (Nippon Genetics, Tokyo, Japan) in a total volume of 1 µl containing inner primer pairs (1.25 nM each). The conditions for PCR amplification were as follows: 5 min at 95°C, 35 cycles of 20 sec at 98°C, 15 sec at 69°C, and 60 sec at 72°C for annealing, followed by a final extension at 72°C for 5 min. Next, the barcoded outer primer mixture (25%) from the PCR Barcording Kit (SQK-RBK004; Oxford Nanopore Technologies) was added and amplified under the following conditions: 1 min at 30°C, and 1 min at 80°C. All PCR products were purified using AMPure® XP (Beckman Coulter, Brea, CA, USA) and quantified according to the absorbance as described above with NanoDrop, and fluorescence was detected with a Quantus^{TM} Fluorometer (Promega, Madison, WI, USA). A total of 100 ng of DNA was incubated with 1 µl of Rapid Adapter at room temperature for 5 min. The prepared DNA library (11 µl) was mixed with 34 µl of Sequencing Buffer, 25.5 µl of Loading Beads, and 4.5 µl of water, then loaded onto the Spot-on Flow Cell R9 version (FLO-MIN106D; Oxford Nanopore Technologies), and sequenced on the MinION™ Mk1C. MINKNOW software ver. 20.6.17 (Oxford Nanopore Technologies) was used for data acquisition and the production of FASTQ files.

Taxonomic mapping, classification, diversity, and community analyses

The resulting FASTQ files were trimmed and/or filtered by Nanofilt [8] software with filtering set to a minimum average read quality score less than 10, remove all sequences shorter than 500 nucleotides, and trim the first 50 nucleotides of all reads. After trimming and size selection, on average, 52,683 reads per sample (maximum=110,546; minimum=17,859) passed and were retained for bacterial identification. For each read, a minimap2 search with 5,850 representative bacterial genome sequences stored in the GenomeSync database [21] was performed. The taxa were determined based on the National Center for Biotechnology

Information taxonomy database [12]. Low-abundance taxa (less than 0.01% of total reads) were discarded from the analysis. Among the factors that were reported to affect the composition of the milk microbiota [26], statistical analyses were performed for background conditions that differed between the cows in this study such as the number of lactation, stage of lactation, and mastitis; these were determined to correlate to age of the host (Age), days in milk (DIM), and SCC in milk, respectively.

The correlations with cow factors, such as Age, SCC, and DIM, were examined for each of the richness and alpha-diversity metrics (Shannon-Wiener Index and Simpson's Index) by the Spearman's rank correlation coefficient, which is a non-parametric test. The correlations between SCC and the milk microbiota composition at the phylum and genus levels were also examined by the Spearman's rank correlation coefficient. A P-value <0.05 was considered to be indicative of a statistically significant difference. These analyses were performed using EZR (ver. 1.53; Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [18].

To investigate the fluctuations in the milk microbiota according to the Age, DIM, and SCC, the samples were divided into two groups for each factor as follows: Age (L-Age: less than 36 months; H-Age: 36 months or more), DIM (L-DIM: less than 80 days; H-DIM: 80 days or more), SCC (L-SCC: less than 200,000 cells/ml; H-SCC: 200,000 cells/ml or more). The Age classification standard of 36 months was used to distinguish between primiparous and multiparous cows. The DIM classification standard of 80 days was based on the day that dry matter intake was maximal on this farm. The SCC classification standard of 200,000 cells/ml was based on the report by PYÖRÄLÄ [28] as the threshold for healthy milk. Analysis of similarities (ANOSIM), a non-parametric test, was used as a beta diversity metric to evaluate differences between the two groups of Age, SCC, and DIM based on the Bray-Curtis distance measure [7]. These data were analyzed with PAST 4.03 [13]. The data for genus clusters were obtained, and bacterial taxa that were significantly enriched in a certain sample group were extracted by linear discriminant analysis (LDA) effect size (LEfSe) analysis using the online interface Galaxy [30]. For LEfSe analysis, the alpha value for the factorial Kruskal-Wallis test was set to <0.05, and the threshold on the logarithmic LDA score for discriminative features was set to <2.0.

RESULTS

As the result of selecting samples that had 1) a sufficient concentration of purified DNA (10 ng/µl or more) for sequencing analysis, and 2) NAGase activity less than 10 nmol/min/ml for excluding milk with an infectious episode [16] to avoid any effect from the administration of intramammary antibiotics on the milk microbiota, from among the 111 milk samples, a total of 48 milk samples from 22 dairy cows were included in this study. Although multiple samples were sometimes obtained from a single cow, all samples were included in the analysis, because the bacterial flora of each quarter was considered to be independent [24]. The mean (median) age and DIM of cows were 46.4 (39.0) months and 114.6 (97.0) days, respectively. The mean (median) SCC of quarter milk samples was 359,000 (205,000) cells/ml. For analyzing the bacterial culture results, we used the definition of "A" of Dohoo *et al*. [11] as valid for our study, and we identified quarters as having possible intramammary infection when the number of colonies was >1 per 0.01 ml. The total percentage of infected quarters was 100%. Fifteen of the pure bacterial isolates were unidentifiable. Among the identified bacteria, the most frequently isolated species was *Aerococcus viridans* (10.4%); however, at the genus level, *Staphylococcus* was the most common (27.1%) with six identified species. *Staphylococcus xylosus* (30.1% of the total isolates) and *Staphylococcus haemolyticus* (23.1%) were the most abundant *Staphylococcus* identified. *Corynebacterium propinquum* was the most abundant species within the *Corynebacterium* genus, accounting for 25.0% of the total number of isolates. The rest were *Streptococcus uberis*, *Escherichia coli*, and *Klebsiella pneumoniae* (one each).

Amplicon sequencing of the *16S rRNA* gene was performed for all samples. After trimming and filtering the sequence data to remove sequences that would adversely affect the analysis, the mean (median) quality, length, and number of reads were 11.4 (11.45), 989.6 (1016.9) bp, and 52,683 (50,355) reads, respectively.

A total of 330 bacterial species were identified from the analysis. At the phylum level, Actinobacteria, Firmicutes, and Proteobacteria accounted for more than 99.9% of the milk microbiota. At the class level, Actinobacteria, Erysipelotrichia, and Alphaproteobacteria were the most common, followed by Clostridia and Bacilli (Table 1). At the species level, the mean (median) bacterial richness was 29.4 (21.5), and the alpha-diversity metrics, i.e., Shannon-Wiener Index and Simpson's Index, were 1.57 (1.64) and 0.68 (0.75), respectively.

Although the DIM was significantly positively correlated with the bacterial richness of normal milk (P=0.043, rs=0.2931), the Age and SCC were not. The SCC was significantly correlated with the alpha-diversity metrics, Shannon-Wiener Index and Simpson's Index (P=0.000268, rs=0.5089, and P=0.00591, rs=0.3940, respectively), while the Age and DIM were not (Table 2). The composition of the milk microbiota and the SCC were significantly negatively correlated with Actinobacteria at the phylum level (Fig. 1A), and *Kocuria* at the genus level (Fig. 1B; P<0.001 and P<0.001, respectively). In contrast, the composition of the milk microbiota and the SCC were significantly positively correlated with Euryarchaeota, Firmicutes, and Proteobacteria (P=0.0497, P<0.001, and P<0.001, respectively) at the phylum level, and *Mycolicibacterium*, *Acetobacter*, *Catenibacterium*, *Corynebacterium*, *Acetanaerobacterium*, *Bacillus*, and *Anaerobutyricum* (P<0.001, P<0.001, P=0.015, P=0.0288, P<0.001, P<0.001, and P<0.001, respectively) at the genus level.

After the samples were divided into two groups for each factor, i.e., for the Age (L-Age: n=20; H-Age: n=28), DIM (L-DIM: n=17; H-DIM: n=31), and SCC (L-SCC: n=23; H-SCC: n=25), the composition of the milk microbiota in each group was compared. There were no differences in the composition of the milk microbiota between the L-Age and H-Age groups (P=0.1794) nor between the L-DIM and H-DIM groups (P=0.7495), but there was a significant difference between the L-SCC and H-SCC groups (P=0.0051; Fig. 2). Because the ANOSIM test detected a significant difference in microbiota diversity between the L-SCC and H-SCC groups,

Table 1. Taxonomic abundance of the microbiota of normal bovine milk examined in this study (n=48)

Taxonomy level			Relative abundance (%)	
Kingdom	Phylum	Class	Mean	SD
Bacteria	*Actinobacteria*		64.08	14.54
		Actinobacteria	(64.08)	(14.54)
Bacteria	*Firmicutes*		20.88	8.87
		Erysipelotrichia	(12.38)	(6.81)
		Clostridia	(4.50)	(2.17)
		Bacilli	(4.00)	(3.02)
Bacteria	*Proteobacteria*		14.50	6.88
		Alphaproteobacteria	(13.97)	(6.86)
		Gammaproteobacteria	(0.41)	(1.51)
		Betaproteobacteria	(0.10)	(0.28)
		Epsilonproteobacteria	(0.01)	(0.01)
Archaea	*Euryarchaeota*		0.33	1.35
		Halobacteria	(0.33)	(1.35)
Bacteria	Others*		0.05	0.14

*Others included Bacteroidetes, Cyanobacteria, Tenericutes and unclassified phyla. SD, standard deviation.

Table 2. Correlation of microbiota diversity in normal milk with age, days in milk (DIM) and somatic cell counts (SCC)

	Richness		Alpha-diversity metrics			
	Number of detected species		Shannon H'		Simpson D	
	Correlation *rs**	*P* value	Correlation *rs*	*P* value	Correlation *rs*	*P* value
Age	0.0189	0.899	0.0257	0.862	0.0150	0.920
DIM	0.2931	0.043	0.1158	0.433	−0.1315	0.373
SCC	0.2122	0.148	0.5089	0.000268	0.3940	0.00591

*The correlation was analyzed with Spearman's Rank Correlation Coefficient.

the bacterial taxa that differed between the two groups were examined. Results indicated that *Kocuria* spp. belonging to the phylum Actinobacteria were enriched in the L-SCC group, while Corynebacteriales were enriched in the H-SCC group (Fig. 3A, 3B).

DISCUSSION

In this study, there was a large proportion of *Kocuria* spp., which has rarely been reported in previous studies of the milk microbiota. *Kocuria* spp. are Gram-positive coagulase-negative coccoid Actinobacteria belonging to the family Micrococcaceae, suborder Micrococcineae, order Actinomycetales, and they are found in the environment and on human skin [29]. The presence of *Kocuria* spp. has been reported in the milk of water deer and reindeer [23], and the most frequently detected species in this study, *Kocuria carniphila* (previously classified as *Micrococcus lactis*), has been isolated from dairy industry waste [6]. The milk samples used in this study were collected by hand-squeezing with conventional aseptic techniques after pre-squeezing. Although it is possible that this classical milk sampling approach may be affected by the bacteria present in the areas near the teat skin and/or apex that were exposed to the environment [14], the microbiota does not differ between milk collected by different sampling methods, including hand-squeezing [9]. Therefore, it is considered that the milk sampling method used in this study was appropriate for the analysis of the microbiota. In this study, many environmental bacteria were detected in the milk microbiota, and it is possible that the composition of the microbiota is strongly influenced by the environment. Since the bacterial flora has been reported to be different in the teat skin, teat apex, teat canal, and milk cistern [10], to evaluate the effect of the bacteria derived from the environment, it is necessary to perform not only a DNA survey of the bedding materials and the environment of the cow, but also the bacterial flora of the teat apex, teat canal, and milk cistern.

There was a correlation between the SCC and the composition of the microbiota in the milk at the phylum/class level. To extract the normal milk samples from among the collected samples, the criterion for NAGase activity, which is a marker of mammary gland tissue damage, was set to 10 nmol/min/ml or below to eliminate mastitic milk [16]. However, there were some samples with a SCC over 500,000/ml. NAGase activity increases following increases in the SCC at the early stages of mastitis [31]; as such, this study may have included samples from cows with mastitis at the early stages of infection showing a high SCC, but before the NAGase activity increased.

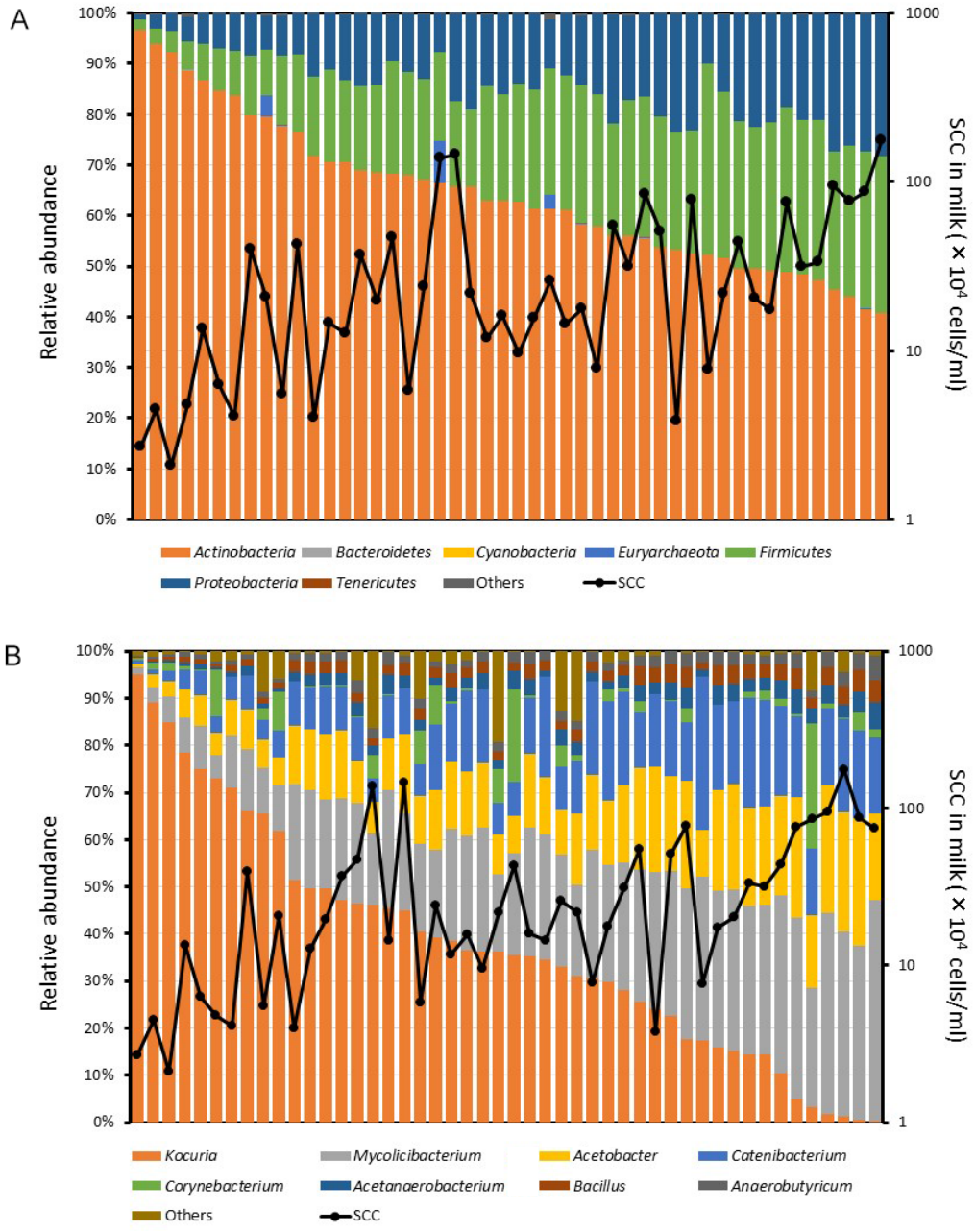

Fig. 1. Relationship between the composition of the milk microbiota and the somatic cell counts (SCC). The stacked bar graphs show the compositions of the bacterial flora in milk sorted in the order of abundance according to the phylum **(A)** or genus **(B)** (scale bars on the left y axes). The line graphs show the SCCs on a logarithmic scale (scale bars on the right y axes). Statistical analysis was performed using Spearman's rank correlation coefficient, a non-parametric test, for the relationship between the SCC and Actinobacteria (A) or *Kocuria* spp. (B).

Analysis of the diversity of microbial communities at the species level for each of the factors of Age, SCC, and DIM revealed that the species richness was significantly positively correlated with the DIM. Of the metrics used for alpha-diversity, the Shannon-Wiener Index is strongly influenced by species richness and by rare species, while Simpson's Index gives more weight to evenness and common species. Since there were no differences in either of the alpha-diversity metrics for the DIM, the number of bacterial species that make up the milk microbiota may increase with increasing DIM without altering the alpha-diversity of the milk microbiota. The DIM represents the accumulation of milking exposure opportunities, and a higher DIM may indicate a higher chance of bacterial invasion from the environment through the stressed teat apex. In addition, the alpha-diversity was strongly and positively correlated with the SCC. Although the alpha-diversity increased at a high SCC, it was not accompanied by an increase in species richness, suggesting that specific bacterial species may account for a large proportion of the flora of milk with a low SCC. The above results suggest that it was the diversity of the bacterial flora, and not the richness of the bacterial species, that affected the SCC. However, this analysis was performed based on relative species abundance, and it is necessary to consider the absolute

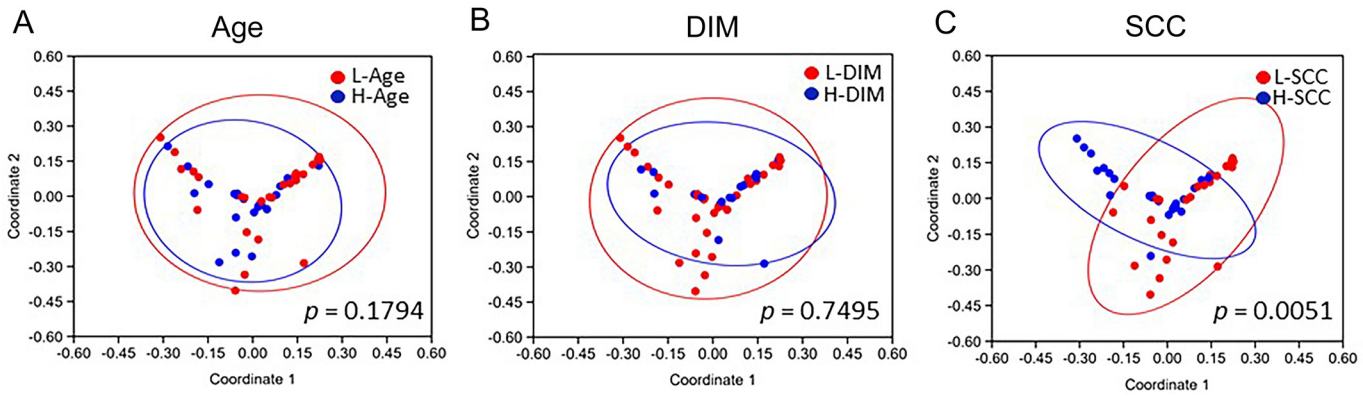

Fig. 2. Principal coordinate analysis plots and differences between groups in the milk microbiota for each of the factors of Age, days in milk (DIM), and somatic cell counts (SCC). Principal coordinate analysis plots obtained from the Bray-Curtis dissimilarity for the microbiota for each of the factors of **(A)** Age, **(B)** DIM, and **(C)** SCC. The samples were divided into two groups for each factor as follows: Age (L-Age: less than 36 months; H-Age: 36 months or more), SCC (L-SCC: less than 200,000 cells/ml; H-SCC: 200,000 cells/ml or more), and DIM (L-DIM: less than 80 days; H-DIM: 80 days or more), and the 95% confidence intervals are indicated by the circles. Statistical analysis was performed using the analysis of similarities (ANOSIM) test to identify significant differences between the two groups of each of the factors of Age (L-Age vs. H-Age), DIM (L-DIM vs. H-DIM), and SCC (L-SCC vs. H-SCC).

abundance for further verification.

Analysis of the beta-diversity (differences in microbial abundances between two groups) within each of the factors of Age, SCC, and DIM at the species level revealed that there was no difference in the milk microbiota between the H-Age and L-Age groups. The composition of human milk microbiota changes with time [5], but in the case of cattle, the milk-producing period is short, and milk-producing cows are generally young, so the effects of aging may not have been properly evaluated. Since the age distribution of herds varies from farm to farm, the results obtained in this study may not reflect the situation on other farms. Between the L-DIM and H-DIM groups, there was no difference in diversity. It has been reported that concentrate feeding, which is correlated with the DIM, affects the intramammary flora through the effects of the rumen microbial status [32]; however, no effect was observed between the L-DIM and H-DIM groups. The reason for this was thought to be that the difference in feed between the groups was not as large as in the previous study. Since feed design and supply vary from farm to farm, it is unclear whether similar results would be obtained on other farms. There were significant differences between the L-SCC and H-SCC groups; since there were significantly more *Kocuria* spp. in the L-SCC group, this bacterium may have a lower ability to stimulate the migration polymorphonuclear leukocytes, which account for the majority of somatic cells in the SCC, than other bacterial species. On the other hand, the H-SCC group tended to have more *Corynebacterium* spp. Although there are conflicting opinions on the relevance of the commensal pathogen *Corynebacterium bovis* as an etiological agent of mastitis [1], the general consensus is that it is an important causative agent of mastitis [22]. Coryneforms do not reach udder tissues, but seem to colonize only the teat canal [2]. In recent years, it has been reported that *C. bovis* is easily reduced by innate immune factors in milk with subclinical mastitis [15]. As such, one of the possible reasons for an increase in the SCC with no growth of a causative organism and no clinical symptoms is the stimulation from *Corynebacterium* spp. that promotes the migration of polymorphonuclear leukocytes. Oikonomou *et al*. [27] reported a relationship between the SCC and the milk microbiota composition, and this relationship was found in samples obtained from various farms. Although the bacterial species reported by Oikonomou *et al*. differ from those reported in the present study, it is possible that similar trends may be found on other farms.

This study is the first report of a 16S full-length metagenomic analysis of DNA extracted from normal milk of dairy cows in Japan. This study clarified that the composition of the microbiota of normal milk was not affected by the Age or DIM, but was related to the SCC. Furthermore, it was shown that there may be variations in the bacterial species in normal milk with a high SCC. This was a cross-sectional study, and unfortunately, it was not possible to determine causal relationships. For the analysis of the microbiota, we examined the relative ratio of bacterial species, and absolute quantification was not performed. We believe that further research, such as longitudinal studies or cross-sectional studies combining absolute quantification methods, is needed to clarify the actual condition of the milk microbiota and to elucidate the relationship with the SCC. As a specific method for estimating the absolute quantity of various bacteria, when sample bacterial DNA is amplified by PCR, DNA sequences with known *16S rRNA* gene copy numbers can be spiked into the sample and sequenced as external standards for estimating the quantity of the sample DNA, and thus the quantity of the bacteria.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

ACKNOWLEDGMENT. This work was funded by Grants-in-Aid for the Promotion of the Livestock Industry from the Japan Racing Association.

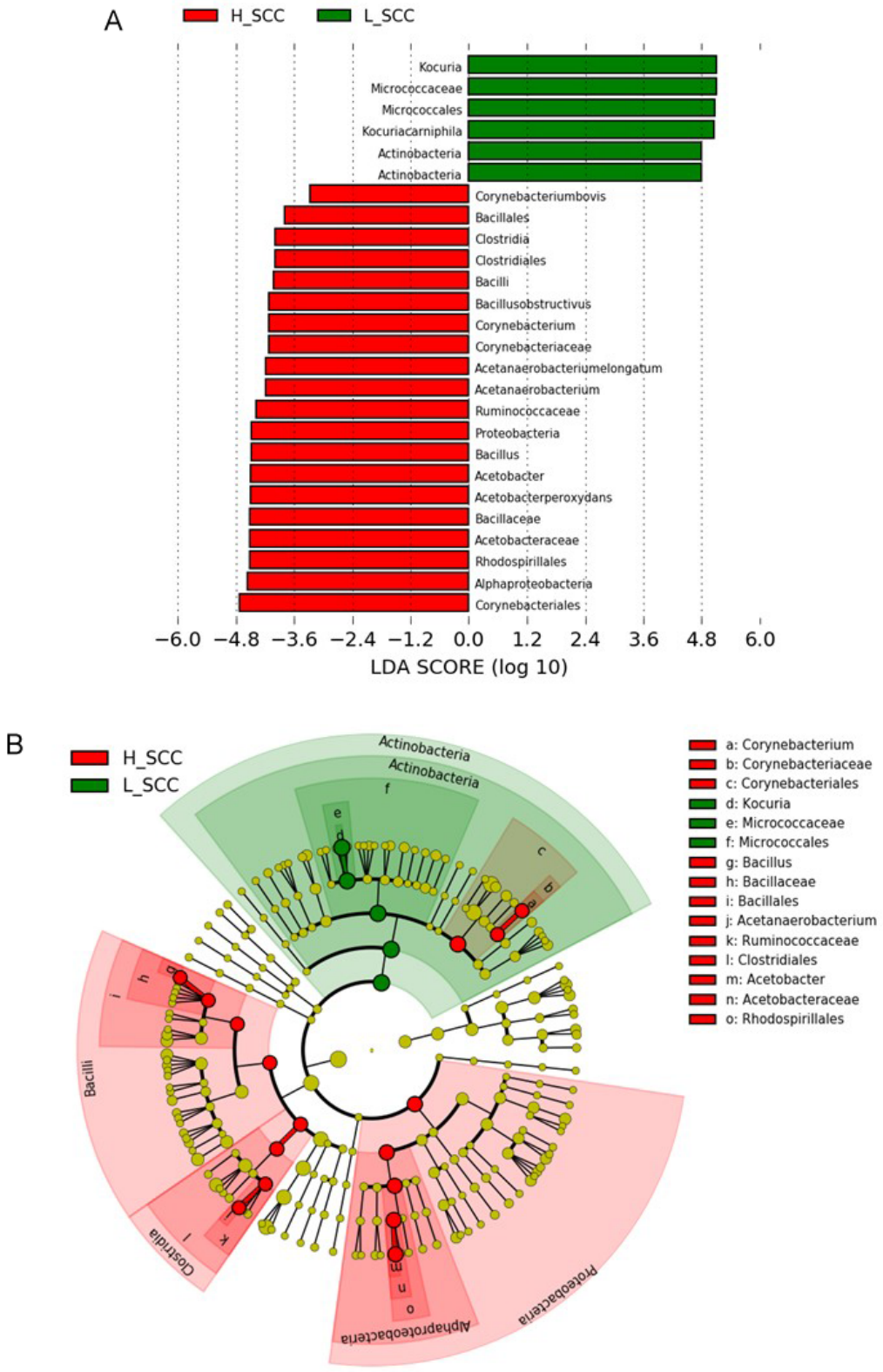

Fig. 3. Differences in the abundance of bacterial taxa among the milk microbiota in the L-somatic cell count (SCC) and H-SCC groups. The bacterial taxa of the L-SCC and H-SCC groups (L-SCC: less than 200,000 cells/ml; H-SCC: 200,000 cells/ml or more) are shown in green and red, respectively. **(A)** List of bacterial taxa detected by LEfSe analysis. For LEfSe analysis, the Kruskal-Wallis test alpha value of 0.05, and the linear discriminant analysis (LDA) score of <2.0 (P<0.01) were used as thresholds. The bacterial taxa in the L-SCC and H-SCC groups are indicated as positive and negative LDA scores, respectively. **(B)** Taxonomic cladogram generated from the LEfSe analysis.

REFERENCES

1. Bexiga, R., Koskinen, M. T., Holopainen, J., Carneiro, C., Pereira, H., Ellis, K. A. and Vilela, C. L. 2011. Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing. *J. Dairy Res.* **78**: 49–55. [Medline] [CrossRef]
2. Bexiga, R., Pereira, H., Pereira, O., Leitão, A., Carneiro, C., Ellis, K. A. and Vilela, C. L. 2011. Observed reduction in recovery of Corynebacterium spp. from bovine milk samples by use of a teat cannula. *J. Dairy Res.* **78**: 9–14. [Medline] [CrossRef]
3. Bhatt, V. D., Ahir, V. B., Koringa, P. G., Jakhesara, S. J., Rank, D. N., Nauriyal, D. S., Kunjadia, A. P. and Joshi, C. G. 2012. Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. *J. Appl. Microbiol.* **112**: 639–650. [Medline] [CrossRef]
4. Branton, D., Deamer, D. W., Marziali, A., Bayley, H., Benner, S. A., Butler, T., Di Ventra, M., Garaj, S., Hibbs, A., Huang, X., Jovanovich, S. B., Krstic, P. S., Lindsay, S., Ling, X. S., Mastrangelo, C. H., Meller, A., Oliver, J. S., Pershin, Y. V., Ramsey, J. M., Riehn, R., Soni, G. V., Tabard-Cossa, V., Wanunu, M., Wiggin, M. and Schloss, J. A. 2008. The potential and challenges of nanopore sequencing. *Nat. Biotechnol.* **26**: 1146–1153. [Medline] [CrossRef]
5. Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E. and Mira, A. 2012. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am. J. Clin. Nutr.* **96**: 544–551. [Medline] [CrossRef]
6. Chittpurna, S. P. K., Singh, P. K., Verma, D., Pinnaka, A. K., Mayilraj, S. and Korpole, S. 2011. Micrococcus lactis sp. nov., isolated from dairy industry waste. *Int. J. Syst. Evol. Microbiol.* **61**: 2832–2836. [Medline] [CrossRef]
7. Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **18**: 117–143. [CrossRef]
8. De Coster, W., D'Hert, S., Schultz, D. T., Cruts, M. and Van Broeckhoven, C. 2018. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics* **34**: 2666–2669. [Medline] [CrossRef]
9. Dahlberg, J., Williams, J. E., McGuire, M. A., Peterson, H. K., Östensson, K., Agenäs, S., Dicksved, J. and Waller, K. P. 2020. Microbiota of bovine milk, teat skin, and teat canal: Similarity and variation due to sampling technique and milk fraction. *J. Dairy Sci.* **103**: 7322–7330. [Medline] [CrossRef]
10. Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema, H. W., Plaizier, J. C. and Khafipour, E. 2018. Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. *J. Dairy Sci.* **101**: 10605–10625. [Medline] [CrossRef]
11. Dohoo, I. R., Smith, J., Andersen, S., Kelton, D. F., Godden S., Mastitis Research Workers' Conference. 2011. Diagnosing intramammary infections: evaluation of definitions based on a single milk sample. *J. Dairy Sci.* **94**: 250–261. [Medline] [CrossRef]
12. Federhen, S. 2012. The NCBI Taxonomy database. *Nucleic Acids Res.* **40**: D136–D143. [Medline] [CrossRef]
13. Hammer, Ø., Harper, D. A. T. and Ryan, P. D. 2001. PAST : Paleontological statistics software package for education and data analysis. *Palaeontol. Electronica* **4**: 1–9.
14. Hiitiö, H., Simojoki, H., Kalmus, P., Holopainen, J., Pyörälä, S. and Taponen, S. 2016. The effect of sampling technique on PCR-based bacteriological results of bovine milk samples. *J. Dairy Sci.* **99**: 6532–6541. [Medline] [CrossRef]
15. Hisaeda, K., Koshiishi, T., Watanabe, M., Miyake, H., Yoshimura, Y. and Isobe, N. 2016. Change in viable bacterial count during preservation of milk derived from dairy cows with subclinical mastitis and its relationship with antimicrobial components in milk. *J. Vet. Med. Sci.* **78**: 1245–1250. [Medline] [CrossRef]
16. Hovinen, M., Simojoki, H., Pösö, R., Suolaniemi, J., Kalmus, P., Suojala, L. and Pyörälä, S. 2016. N-acetyl -β-D-glucosaminidase activity in cow milk as an indicator of mastitis. *J. Dairy Res.* **83**: 219–227. [Medline] [CrossRef]
17. Hu, X., Li, S., Fu, Y. and Zhang, N. 2019. Targeting gut microbiota as a possible therapy for mastitis. *Eur. J. Clin. Microbiol. Infect. Dis.* **38**: 1409–1423. [Medline] [CrossRef]
18. Kanda, Y. 2013. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.* **48**: 452–458. [Medline] [CrossRef]
19. Kawai, K., Hayashi, T., Kiku, Y., Chiba, T., Nagahata, H., Higuchi, H., Obayashi, T., Itoh, S., Onda, K., Arai, S., Sato, R. and Oshida, T. 2013. Reliability in somatic cell count measurement of clinical mastitis milk using DeLaval cell counter. *Anim. Sci. J.* **84**: 805–807. [Medline] [CrossRef]
20. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **41**: e1. [Medline] [CrossRef]
21. Kryukov, K. 2020. GenomeSync. http://genomesync.org [accessed on June 10, 2021].
22. Langoni, H., Guimarães, F. F., Salina, A., Ribeiro, M. G., Baio, P. V. P., Ramos, J. N., Mota, H. F., Vieira, V. V. and Mattos-Guaraldi, A. L. 2016. Molecular characterization of Corynebacterium bovis causing clinical mastitis and increasing somatic-cell count. *Int. J. Adv. Vet. Sci. Technol.* **5**: 248–255. [CrossRef]
23. Li, Z., Wright, A. G., Yang, Y., Si, H. and Li, G. 2017. Unique bacteria community composition and co-occurrence in the milk of different ruminants. *Sci. Rep.* **7**: 40950. [Medline] [CrossRef]
24. Metzger, S. A., Hernandez, L. L., Skarlupka, J. H., Suen, G., Walker, T. M. and Ruegg, P. L. 2018. Influence of sampling technique and bedding type on the milk microbiota: results of a pilot study. *J. Dairy Sci.* **101**: 6346–6356. [Medline] [CrossRef]
25. Mitsuhashi, S., Kryukov, K., Nakagawa, S., Takeuchi, J. S., Shiraishi, Y., Asano, K. and Imanishi, T. 2017. A portable system for rapid bacterial composition analysis using a nanopore-based sequencer and laptop computer. *Sci. Rep.* **7**: 5657. [Medline] [CrossRef]
26. Oikonomou, G., Addis, M. F., Chassard, C., Nader-Macias, M. E. F., Grant, I., Delbès, C., Bogni, C. I., Le Loir, Y. and Even, S. 2020. Milk Microbiota: What Are We Exactly Talking About? *Front. Microbiol.* **11**: 60 [CrossRef]. [Medline]
27. Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., Teixeira, A. G., Santisteban, C., Schukken, Y. H. and Bicalho, R. C. 2014. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PLoS One* **9**: e85904 [CrossRef]. [Medline]
28. Pyörälä, S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Vet. Res.* **34**: 565–578. [Medline] [CrossRef]
29. Savini, V., Catavitello, C., Masciarelli, G., Astolfi, D., Balbinot, A., Bianco, A., Febbo, F., D'Amario, C. and D'Antonio, D. 2010. Drug sensitivity and clinical impact of members of the genus Kocuria. *J. Med. Microbiol.* **59**: 1395–1402. [Medline] [CrossRef]
30. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. *Genome Biol.* **12**: R60 [CrossRef]. [Medline]
31. Shinozuka, Y., Kawai, K., Sato, R., Higashitani, A., Hamamoto, Y., Okita, M. and Isobe, N. 2018. Blood ionized calcium levels and acute-phase blood glucose kinetics in goats after intramammary infusion of lipopolysaccharide. *J. Vet. Med. Sci.* **80**: 242–246. [Medline] [CrossRef]
32. Young, W., Hine, B. C., Wallace, O. A. M., Callaghan, M. and Bibiloni, R. 2015. Transfer of intestinal bacterial components to mammary secretions in the cow. *PeerJ* **3**: e888. [Medline] [CrossRef]