The Journal of Veterinary Medical Science

FULL PAPER

Internal Medicine

Regression tree analysis of the relationship between the concentrations of antimicrobial components and the microbiota of normal milk from dairy cows

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

[1)]School of Veterinary Medicine, Azabu University, Kanagawa, Japan
[2)]Azabu University Mastitis Research Center, Kanagawa, Japan
[3)]Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, Japan
[4)]Higashi-Hiroshima Veterinary Clinical Center, Hiroshima, Japan
[5)]Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa, Japan

ABSTRACT. The purpose of this study was to determine the concentrations of antimicrobial components (immunoglobulin A (IgA), lactoferrin (LF), lingual antimicrobial peptide (LAP), and S100A7) in normal milk and their relation to host factors (Age, somatic cell count (SCC), days in milk, richness, and alpha diversity of the milk microbiota) in dairy cows using multivariate regression tree analyses, and to clarify how the milk microbiota is related to the obtained results. Thirty normal milk samples were collected from a commercial dairy farm in June 2020. The thresholds that predicted the concentration of each antimicrobial component in milk were obtained by regression tree analysis, and the beta-diversity of the milk microbiota composition between groups divided according to each threshold was compared by an analysis of similarities test. The IgA and LF concentrations were mainly predicted by the SCC (177,500 and 70,000 cells/ml, respectively), and the LAP and S100A7 concentrations were predicted by Age (29.667 and 40.3 months, respectively). No relationship was observed between the concentration of IgA, LAP, or S100A7 and the milk microbiota composition between the groups divided by the threshold for prediction, but the milk microbiota composition was significantly different between the groups divided by the threshold for predicting the LF concentration. Our results indicated that the LF concentration in normal milk may be associated with the milk microbiota composition.

KEY WORDS: antimicrobial component, dairy cattle, milk microbiota

J. Vet. Med. Sci.
84(3): 310–318, 2022
doi: 10.1292/jvms.21-0541

Received: 10 October 2021
Accepted: 23 December 2021
Advanced Epub:
18 January 2022

Bovine mastitis is not only the most costly disease for dairy farmers and the industry [11], but also the cause of animal suffering. Mastitis is mainly treated by the administration of antibacterial agents, but the emergence of drug-resistant bacteria associated with the use of antibacterial agents has become a public health concern, and prudent use of antibacterial agents is required [33]. Preventing mastitis is important not only to reduce the use of antimicrobial agents as much as possible, but also from the viewpoint of animal welfare.

In recent years, a shift in the etiologies of mastitis from contagious or gram-positive organisms, such as *Staphylococcus aureus* and *Streptococcus agalactiae,* to gram-negative organisms or cases yielding no bacterial growth (NG) has become a problem [37]. NG mastitis is now the most common mastitis, and its prevalence has been increasing in recent years [42]. One of the reasons for an NG status of mastitic milk is the immune system of the host that is successful in clearing the pathogens, resulting in too little pathogen to be detected [27, 45]. In fact, NG mastitis does not require antimicrobial treatment in the clinical setting [50]. Other reasons for an NG status include the selection of improper test(s) for the organism(s) of interest, failure of the cow to shed the pathogen, and the absence of an infection [27]. Nonetheless, the precise reasons for an NG status remain unknown at present [30].

The immune system, comprising innate immunity and adaptive immunity, protects against microorganisms that invade through

*Correspondence to: Shinozuka, Y.: shinozuka@azabu-u.ac.jp, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
#These authors contributed equally to this work.

the teat canal into mammary tissue. Innate immunity is activated early and non-specifically, whereas pathogen-specific adaptive immunity takes several days to activate [40]. In addition, there are innate immune factors in milk that have antimicrobial activity, including cellular factors, such as macrophages and neutrophils, as well as non-cellular factors, such as peptides, proteins, and glycoproteins. Immunoglobulin A (IgA), lactoferrin (LF), lingual antimicrobial peptide (LAP), and S100A7 have been reported as antimicrobial components in bovine milk. IgA is secreted in bovine milk, and plays an important role in protecting the mammary gland from pathogens [12, 38]. Immunoglobulins, including IgA, are known as factors of acquired immunity, but recent studies have suggested that natural antibodies derived from B1 cells can bind to pathogens and toxins, such as bacteria, lipopolysaccharide, and lipoteichoic acid, with low specificity [4, 38]. LF is an iron-binding glycoprotein that is synthesized by mammary epithelial cells [17]. Although LF is secreted in normal milk even in the absence of inflammation of the mammary gland [48], its concentration is increased in mastitic milk [16]. LAP is a β-defensin that was first isolated from inflamed bovine tongue epithelium [43]. Bovine mammary epithelial cells also express LAP mRNA, and secrete LAP into milk [19, 20]. The β-defensins are cationic peptides, and their electrostatic potential can attract the membrane of pathogens [51]. S100 proteins are calcium-binding proteins with two helix-loop-helix calcium-binding domains, which are called EF-hands [28]. S100A7 is an S100 calcium-binding protein that was first identified in epithelial cells. It has antimicrobial activity, especially against *Escherichia coli*, and thereby provides innate immune protection in the epidermis. S100A7 is expressed by epithelial cells in the bovine mammary gland, and its expression level is especially high in teat epithelium [47, 52]. Recent studies have revealed that the expression sites of these antimicrobial components in the mammary gland differ, and that the response times after microbial invasion stimuli also differ between the components [18]. In addition, the fluctuations in the concentrations of these antimicrobial components have also been clarified [48].

Since a reason for NG mastitis is thought to be that the animal is in the process of developing a spontaneous cure or it has a mild infection that may be spontaneously cured, to prevent NG mastitis, it is important to utilize the innate immune system that can respond early and non-specifically to invading microorganisms. In particular, since the value of antimicrobial peptides in milk is considered to be extremely high [29], for the prevention of NG mastitis, it is important to not reduce the concentrations of antimicrobial components in milk or to maintain them at a high level.

Advances in metagenomic pyrosequencing technology have revealed the presence of microbiota in bovine milk [35], which had previously been considered to be a sterile environment. Research on mastitis and the milk microbiota has also progressed [36], and the role of the microbiota in immunity and inflammation has been elucidated [1]. In recent years, the applicability of the gut microbiota as a possible therapeutic approach for mastitis has also been discussed [15]. However, to the best of our knowledge, there has been no study evaluating the relationship between the bovine milk microbiota and the concentrations of antimicrobial factors in normal milk.

Multiple regression analysis, which is a type of multivariate analysis, is a method for quantifying the degree of influence of a plurality of related factors (explanatory variables) when explaining an objective variable. However, since the results obtained by this method are continuous and quantified in the form of a function, it is not possible to obtain a threshold value, which is necessary for comparing the milk microbiota composition in the variables with a high degree of influence. In contrast, regression tree analysis, which is a method of subdividing the cases sequentially into branches according to the values of the explanatory variables and finally dividing them into several groups, can be used to determine thresholds, because the intergroup thresholds can be clearly calculated. This makes it possible to compare the milk microbiota composition between groups for variables with a high degree of influence [6].

The purpose of this study was to create a decision tree model of cow factors that affect the concentrations of antimicrobial components in normal milk using regression tree analyses, and to clarify how the milk microbiota is related to the obtained results.

MATERIALS AND METHODS

Study animals and data collection

In July 2020, 32 clinically healthy Holstein dairy cows at different lactation stages were enrolled from one commercial herd located in Hiroshima Prefecture, Japan. The farm owner provided permission for the sampling and for the use of the data obtained in this study. Milk samples were collected from cows diagnosed as clinically healthy by a veterinarian. The cows had not been treated with antibiotics within the last 2 weeks. Milk sampling was performed 2 hr after the morning milking without the use of invasive procedures. Briefly, after pre-milking by stripping four times, the teat end was disinfected with an alcohol-soaked cotton ball, and milk was aseptically collected by a gloved veterinarian. A total of 111 milk samples was successfully collected, immediately stored on ice, and transported to the laboratory within 2 hr from the last sampling. The protocol for milk sample collection from lactating dairy cows adhered to the guidelines of the Azabu University Animal Experimentation Committee, and was approved by the committee (No. 200803-1).

Milk screening tests

The somatic cell count (SCC) was determined using an electronic cell counter DCC (DeLaval International AB, Tumba, Sweden) based on the method of Kawai *et al*. [23]. The activity of lysosomal N-acetyl-β-D-glucosaminidase (NAGase) in each milk sample 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 from the difference between the absorbance value of the whey sample and the absorbance value of the unreacted substrate of the whey sample (background control) to avoid the effect of color.

Concentrations of antimicrobial components in milk

The milk IgA and LF concentrations were measured by enzyme-linked immunosorbent assay (ELISA) quantification following a commercial protocol (Bethyl Laboratories, Inc., Montgomery, TX, USA) [48]. Milk LAP and S100A7 concentrations were also measured by ELISA, as described previously [19, 52]. Briefly, a 96-well microtiter plate was coated with affinity-purified rabbit antibodies against LAP and S100A7 peptides (1 μg/ml). Milk samples for LAP and S100A7 analysis were diluted 10 and 5,000 times, respectively, and added to the microtiter plate. For the competition biding assay, the horseradish peroxidase-labeled LAP [19] and S100A7 [52] were added together with the samples. Then, 3,3′,5,5′-tetramethylbenzidine was added. After the reaction was stopped by the addition of sulfuric acid (1 mol/l), the optical density was measured at a wavelength of 450 nm using a spectrophotometer (Multiscan FC; Thermo Fisher Scientific, Waltham, MA, USA).

DNA extraction and 16S rRNA gene amplicon sequencing

A comprehensive analysis of the microbiota in the milk samples was performed based on previously reported methods [46] as follows. DNA was previously isolated from each milk sample using ISOSPIN Fecal DNA (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions that included an initial bead-beating step. Prior to *16S rRNA* gene sequencing, 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′) [25] 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) under the following conditions: 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 PCR Barcoding Kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, UK) was used, and amplification was performed under the following conditions: 1 min at 30°C, and 1 min at 80°C. Fifty nanograms of PCR products per sample were placed in a tube and purified using AMPure® XP (Beckman Coulter, Brea, CA, USA), then quantified by the absorbance using NanoDrop as described above, and by fluorescence using a Quantus^{TM} Fluorometer (Promega, Madison, WI, USA). The DNA library was created by incubating the purified DNA and Rapid Adapter F at room temperature for 5 min. The prepared DNA library was mixed with Sequencing Buffer, Loading Beads, and water, then loaded onto the Spot-on Flow Cell R9 version (FLO-MIN106D, Oxford Nanopore Technologies), and sequenced on the MinION™ Mk1C (Oxford Nanopore Technologies). The FASTQ files obtained from the sequencing analysis with MinKNOW software ver. 20.6.17 (Oxford Nanopore Technologies) were trimmed and filtered by Nanofilt software [3]. Briefly, we processed the data 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 was performed using 5,850 representative bacterial genome sequences stored in the GenomeSync database [26]. The taxa were determined based on the National Center for Biotechnology Information taxonomy database [5]. Low-abundance taxa (less than 0.01% of the total reads) were discarded from the analysis. From these results, the number of taxonomic groups (Richness) and the Simpson's diversity index (Simpson) were calculated as alpha-diversity metrics.

Statistical analyses

First, we examined the correlation between each objective variable (the concentration of IgA, LF, LAP, or S100A7) and each explanatory variable (Age, SCC, DIM, Simpson, or Richness). Since there were variables for which the normality of the data could not be confirmed, the Spearman's rank correlation coefficient, which is a nonparametric test, was used. Next, the concentrations of antimicrobial components in milk were estimated by regression tree analysis, which is a non-parametric method that does not assume the data distribution of the analysis target. The classification and regression trees (CART) method was used as the algorithm for the tree model, and Gini's diversity index and information gain were used as the evaluation criteria for branching. Pruning was not performed, because the number of samples were small. Both analyses were carried out using R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria).

To evaluate the differences in the milk microbiota between the two groups divided by the regression tree analysis, analysis of similarities (ANOSIM), a nonparametric test, was used as a beta-diversity metric based on the Bray-Curtis distance measure [2]. These data were analyzed with the PAST 4.03 software package [9].

To investigate fluctuations in the milk microbiota according to the results of the ANOSIM test, linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed using the Galaxy online interface [44]. For LEfSe analysis, the alpha value for the factorial Kruskal-Wallis test was set to <0.05, and the threshold of the logarithmic LDA score for discriminative features was set to <2.0.

RESULTS

After excluding samples with a SCC less than 300,000 cells/ml [39] and NAG activity less than 10 nmol/min/ml [14] to exclude abnormal milk by the screening test, a total of 30 milk samples from 18 cows were included in this study. Since the bacterial flora of each quarter is considered to be independent [32], multiple samples were sometimes obtained from a single cow, and they were all included in the analysis.

The IgA and LAP concentrations in milk were significantly positively correlated with Age (P=0.00000978 and P=0.00122, respectively), and the IgA concentration was significantly negatively correlated with Simpson (P=0.0226). The LF concentration was significantly positively correlated with the SCC (P=0.022; Table 1). Figure 1 shows a model tree diagram of the concentrations of the antimicrobial components in each milk sample obtained by regression tree analysis. The IgA concentration prediction model first diverged at a SCC of 177,500 cells/ml, then the group with a SCC less than 177,500 cells/ml diverged at an Age of 30.817 months (Fig. 1A). The LF concentration prediction model first diverged at a SCC of 70,000 cells/ml, then the group with a SCC of 70,000 cells/ml or more diverged at a Richness of 14.5 (Fig. 1B). In the LAP concentration prediction model, it branched at an Age of 29.667 months (Fig. 1C). In the S100A7 concentration prediction model, it branched at an Age of 40.3 months (Fig. 1D).

The concentrations of each antimicrobial component were divided into groups according to each threshold (IgA: SCC of 177,500 cells/ml; LF: SCC of 70,000 cells/ml; LAP: Age of 29.667 months; S100A7: Age of 40.3 months), and the milk microbiota composition was compared between the groups using the ANOSIM test (Fig. 2). Although there was no difference in the milk

Table 1. Spearman rank correlation coefficients measuring the statistical dependence between the main specified variables and the antimicrobial factors in milk

Antimicrobial components	Host factors*					*P* value				
	Age	SCC	DIM	Simpson	Richness	Age	SCC	DIM	Simpson	Richness
IgA	0.713	0.184	−0.005	−0.415	0.120	<0.001	0.330	0.979	0.0226	0.527
LF	0.287	0.419	0.134	0.105	0.110	0.124	0.022	0.479	0.579	0.563
LAP	0.562	0.316	−0.153	0.134	0.088	0.001	0.089	0.418	0.479	0.643
S100A7	0.275	0.161	−0.170	0.228	−0.098	0.141	0.396	0.368	0.225	0.606

*Age: age of the host; SCC: somatic cell counts in milk; DIM: days in milk; Simpson: Simpson's diversity index; Richness: number of taxonomic groups.

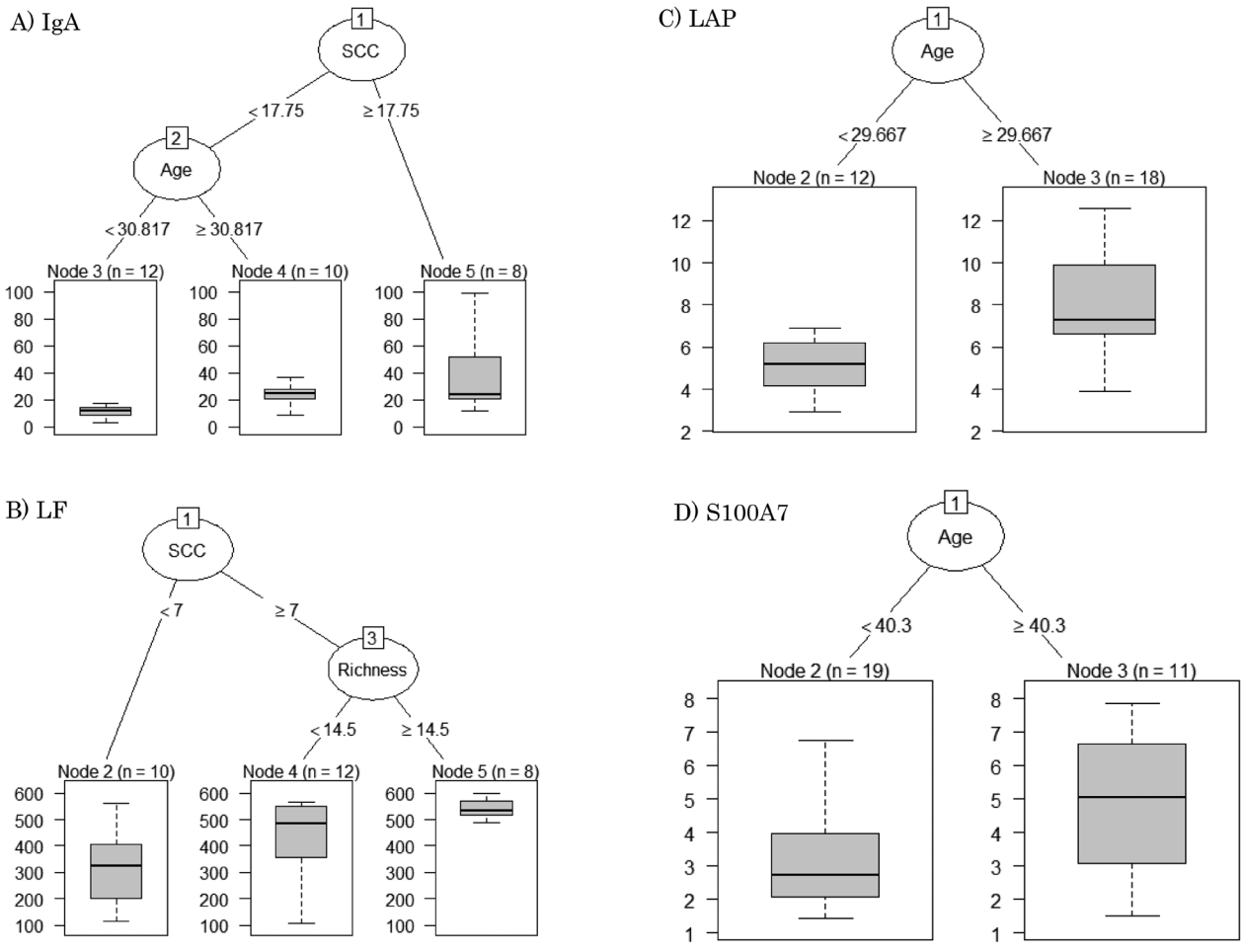

Fig. 1. Decision tree to predict the concentration of antibacterial factors in milk by regression tree analysis. The nodes in the graph represent an event or choice, and the edges of the graph represent the decision rules or conditions.

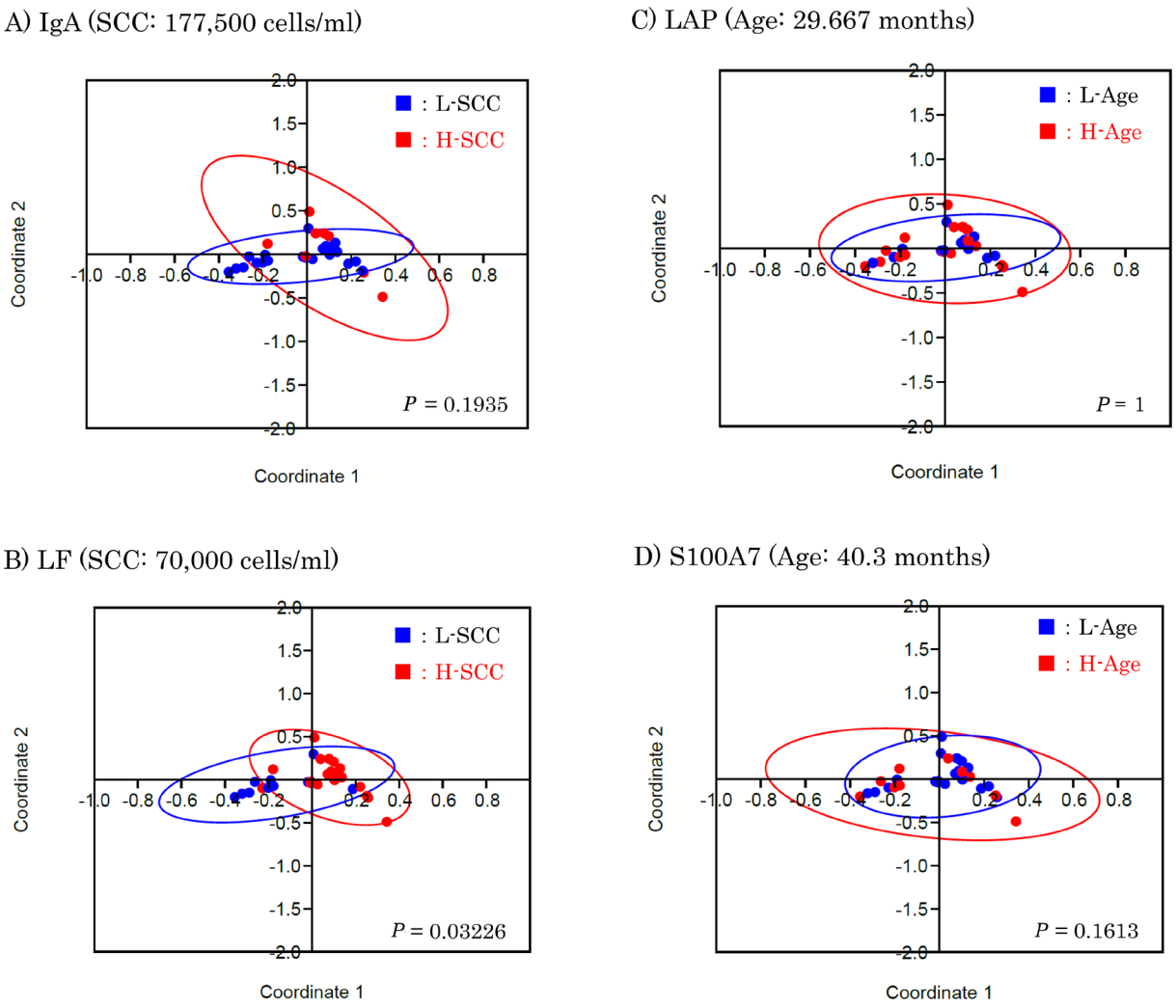

Fig. 2. Principal coordinate analysis plots and differences between groups divided according to the thresholds in the milk microbiota for the IgA, LF, LAP, and S100A7 concentrations in milk. Principal coordinate analysis plots obtained from the Bray-Curtis dissimilarity of the microbiota for the concentrations of A) immunoglobulin A (IgA), B) lactoferrin (LF), C) lingual antimicrobial peptide (LAP), and D) S100A7. The samples were divided into two groups for each factor as follows: (**A**) IgA (L-SCC: less than 175,500 cells/ml; H-SCC: 175,500 cells/ml or more), (**B**) LF (L-SCC: less than 70,000 cells/ml; H-SCC: 70,000 cells/ml or more), (**C**) LAP (L-Age: less than 29.667 months; H-Age: 29.667 months or more), and (**D**) S100A7 (L-Age: less than 40.3 months; H-Age: 40.3 months or more). The 95% confidence intervals are indicated by circles. Statistical analysis was performed using the analysis of similarities (ANOSIM) test to identify significant differences between the two groups of each factor. The obtained statistical *P* values are shown at the bottom right of each graph.

microbiota composition between the groups divided by a SCC of 177,500 cells/ml (*P*=0.1935; Fig. 2A), there was a significant difference (*P*=0.03226) when divided by a SCC of 70,000 cells/ml (Fig. 2B). There was no difference in the bacterial flora composition between the groups divided by an Age of 29.667 months (Fig. 2C) or by an Age of 40.3 months (Fig. 2D; *P*=1 and *P*=0.1613, respectively).

As a result of the LEfSe analysis, *Kocuria* spp. were found to be significantly more numerous in the group with a SCC of less than 70,000 cells/ml, and *Mycolicibacterium* spp., *Bacillus* spp., *Anaerobutyricum* spp., *Acetanaerobacterium* spp., *Catenibacterium* spp., and *Acetobacter* spp. were significantly more numerous in the group with a SCC of 70,000 cells/ml or more (Fig. 3A, 3B).

DISCUSSION

After evaluating the relationship between the biological factors and the concentrations of antimicrobial components in normal milk by correlation analysis, a prediction model of antimicrobial components was constructed by decision tree analysis. In the decision tree model, the prediction model is given in a tree-like structure, and by looking at it, the explanatory variables when the objective variable becomes larger or smaller depending on some combination of the explanatory variables and their thresholds can

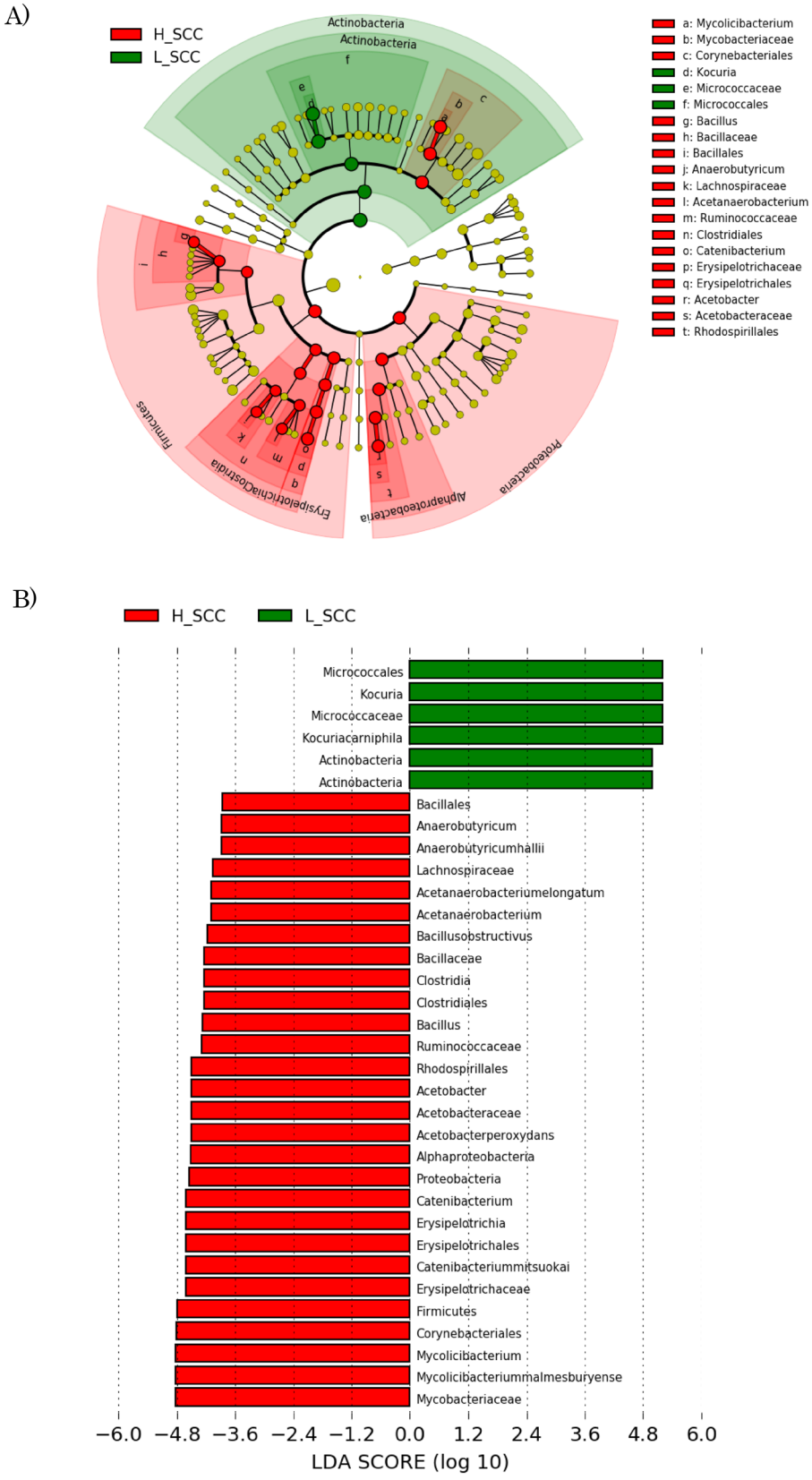

Fig. 3. Differences in the abundance of bacterial taxa among the milk microbiota in the L-SCC and H-SCC groups for lactoferrin. The bacterial taxa of the group with a somatic cell count (SCC) of less than 70,000 cells/ml (L-SCC) and the group with a SCC of 70,000 cells/ml or more (H-SCC) groups are shown in green and red, respectively. **A)** List of bacterial taxa detected by linear discriminant analysis (LDA) effect size (LEfSe) analysis. For LEfSe analysis, the Kruskal-Wallis test alpha value of 0.05 and 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.

be determined.

The IgA concentration was positively correlated with Age, and this result was consistent with previous reports that the IgA concentration increases with parity [7]. In addition, the IgA concentration was predicted from the SCC and Age, but the bacterial flora composition did not differ between the groups divided by the SCC threshold of 177,500 cells/ml and the Age threshold of 30.817 months obtained by the regression tree analysis. Gut microbiota has been shown to induce IgA-positive cells [13], while milk microbiota has been reported not to be involved in IgA production in mice [34]. From these data, it was considered that the milk microbiota may not be related to the IgA concentration in normal milk of cattle.

No correlation was found between the milk LF concentration and Age, which is consistent with the results of previous studies [10]. In addition, the milk LF concentration and SCC were positively correlated, which is also consistent with previous reports [8]. Interestingly, the milk microbiota composition was significantly different between the groups divided by the SCC threshold of 70,000 cells/ml), which could predict the LF concentration in the decision tree analysis. It has been reported that LF promotes the growth of specific bacteria, such as *Lactobacillus* and *Bifidobacteria,* in the human intestine [49]. It is possible that the milk microbiota changed as a result of the increase in the LF concentration, but such changes in bacterial species were not seen in the LEfSe analysis. LF is a glycoprotein of approximately 80 kDa in size that is synthesized in the mammary epithelium, and it binds to iron molecules with high affinity [18]. Its synthesis is increased after inflammatory stimulation of mammary epithelial cells [16]. An increased SCC indicates an immune reaction of the body against pathogens, and thus a possible disease event. Taken together, it is possible that the LF concentration increased as a result of a mild infection that can change the milk microbiota composition and stimulate inflammation in mammary epithelial cells. However, since this study is a cross-sectional study, a causal relationship between the milk LF concentration and milk microbiota could not be determined.

LAP is a β-defensin that is synthesized by mammary epithelial cells and secreted into milk. There was a negative correlation between the LAP concentration and Simpson, which is an index of alpha-diversity, but there were no differences in Richness, suggesting that there may be bacterial species that promote LAP synthesis. A significant negative correlation between Age and Simpson was observed in this study (data not shown), and the positive correlation between the LAP concentration and Age could have been caused by an aging-related shift in the microbiota that promotes LAP synthesis. However, since there was no difference in the milk microbiota composition between the groups divided by the Age threshold of 29.667 months obtained by the regression tree analysis, it is possible that the LAP concentration of normal milk and the bacterial flora composition are not related. The LAP concentration was positively correlated with Age, but not with the SCC. Previous report showed that there was no difference in the LAP concentration of bovine colostrum when the parities were divided into <2, 3 and 4 <, but the LAP concentration of third parity cows tended to be high than that of <2 parity cows [21]. In this study, of all 18 cows included in the analysis, 14 cows were under 3 parities (mean of parities was 2.3, data not shown), suggesting that the positive correlation between the LAP concentration and Age may be observed only in under third parity cows. On the other hand, it has been reported that the LAP concentration and SCC are positively correlated [22], but we obtained a different result in our study. However, this difference may be attributable to the facts that the milk inspected in this study had only a SCC of 300,000 cells/ml or less and NAG activity of 10 nmol/min/ml or less, and milk from inflamed mammary glands was inspected in the previous report [22], but not included in the present study. In fact, previous study suggested that there was no correlation between the LAP concentration and SCC in the milk with SCC of 300,000 cells/ml or less [48]. Thus, it can be inferred that an increase of LAP concentration in bovine milk was triggered by inflammation, resulting in a positive correlation between LAP concentration and SCC in milk obtained from both inflamed and non-inflamed mammary glands.

The S100A7 concentration was not correlated with the Age, SCC, DIM, Simpson, or Richness. It has been reported that inflammation-related factors, such as intramammary lipopolysaccharide administration [52] and intramammary infection of *E. coli* [41], increase the S100A7 concentration. It has also been pointed out that even in healthy milk, the S100A7 concentration is lowest in the first pre-squeezed milk, then it gradually increases [24], and it is also affected by the season [48]. From these studies, it was considered that the milk samples investigated in this study were not from mammary glands in an inflammatory state, and that known factors that influence the S100A7 concentration were sufficiently excluded. In addition, there was no difference in the milk microbiota composition between the groups divided by the Age threshold of 40.3 month, which could predict the S100A7 concentration in the decision tree analysis. As a result, it is considered that the milk microbiota is not related to the S100A7 concentration of healthy milk.

Of the four antimicrobial components in milk investigated in this study, the IgA and LF concentrations of normal milk were predictable from the SCC, although the boundary thresholds estimated by regression tree analysis were different (70,000 cells/ml and 177,500 cells/ml, respectively). It was considered that the SCC at which it begins to affect the concentration of the antimicrobial components in milk may differ depending on the type of the antimicrobial component. In addition, since the composition of the microbiota between groups was different when a SCC of 70,000 cells/ml was used as the threshold, which was the threshold for predicting the LF concentration, a SCC of 70,000 cells/ml or less may be indicative of the indigenous milk microbiota due to the biological reactions of innate immune factors.

The present study has several potential limitations that should be acknowledged. Firstly, the number of samples in this study was small (small data), and it was not sufficient for performing a decision tree analysis, which uses machine learning. However, the purpose of the decision tree analysis conducted in this study was not to construct a predictive model of antimicrobial components in milk, but to obtain a threshold for elucidating the relationship with the milk microbiota. In addition, we have a policy of reducing the number of animals used in research as much as possible from the viewpoint of animal welfare. Secondly, many antimicrobial components have been shown to be present in milk [31]. However, in this study, only four of them were

examined, and the overall picture of the relationship between innate immunity in milk and the milk microbiota was not clarified. Finally, although the effects of antimicrobial proteins have been evaluated against known pathogens, the effects remain unclear for unknown symbiotic species, and their role in the regulation of mammary microbial homeostasis is also unknown [31]. We believe that further research is needed to elucidate the relationship between innate immune factors and the microbiota of normal milk.

Although the IgA, LAP, and S100A7 concentrations in normal milk were not related to the milk microbiota composition, the LF concentration was related to it with the SCC as a common factor. Based on the present results, the threshold of the SCC at which changes in the LF concentration and milk microbiota composition could be detected was 70,000 cells/ml, and it was suggested that the normal milk microbiota composition might affect the milk LF concentration. Since LF has antimicrobial activity and is part of the innate defense [48], it may be possible to develop a new method of preventing intramammary infections by controlling the milk microbiota composition.

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

ACKNOWLEDGMENTS. This work was funded by Grants-in-Aid for the Promotion of the Livestock Industry from the Japan Racing Association. The authors would like to thank the farmers and personnel of the dairy farm for providing their cows and for their support.

REFERENCES

1. Belkaid, Y. and Hand, T. W. 2014. Role of the microbiota in immunity and inflammation. *Cell* **157**: 121–141. [Medline] [CrossRef]
2. Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **18**: 117–143. [CrossRef]
3. 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]
4. de Klerk, B., Ducro, B. J., Heuven, H. C., den Uyl, I., van Arendonk, J. A., Parmentier, H. K. and van der Poel, J. J. 2015. Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk. *J. Dairy Sci.* **98**: 2746–2752. [Medline] [CrossRef]
5. Federhen, S. 2012. The NCBI Taxonomy database. *Nucleic Acids Res.* **40**: D136–D143. [Medline] [CrossRef]
6. Gacar, B. K. and Kocakoç, I. D. 2020. Regression analyses or decision trees? *Manisa Celal Bayar University Journal of Social Sciences* **18**: 251–260.
7. Guidry, A. J. and Miller, R. H. 1986. Immunoglobulin isotype concentrations in milk as affected by stage of lactation and parity. *J. Dairy Sci.* **69**: 1799–1805. [Medline] [CrossRef]
8. Hagiwara, S., Kawai, K., Anri, A. and Nagahata, H. 2003. Lactoferrin concentrations in milk from normal and subclinical mastitic cows. *J. Vet. Med. Sci.* **65**: 319–323. [Medline] [CrossRef]
9. 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.
10. Harmon, R. J., Schanbacher, F. L., Ferguson, L. C. and Smith, K. L. 1975. Concentration of lactoferrin in milk of normal lactating cows and changes occurring during mastitis. *Am. J. Vet. Res.* **36**: 1001–1007. [Medline]
11. Hertl, J. A., Gröhn, Y. T., Leach, J. D. G., Bar, D., Bennett, G. J., González, R. N., Rauch, B. J., Welcome, F. L., Tauer, L. W. and Schukken, Y. H. 2010. Effects of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. *J. Dairy Sci.* **93**: 1551–1560. [Medline] [CrossRef]
12. Hodgkinson, A. J., Cakebread, J., Callaghan, M., Harris, P., Brunt, R., Anderson, R. C., Armstrong, K. M. and Haigh, B. 2017. Comparative innate immune interactions of human and bovine secretory IgA with pathogenic and non-pathogenic bacteria. *Dev. Comp. Immunol.* **68**: 21–25. [Medline] [CrossRef]
13. Honda, K. and Littman, D. R. 2016. The microbiota in adaptive immune homeostasis and disease. *Nature* **535**: 75–84. [Medline] [CrossRef]
14. 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]
15. 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]
16. Huang, Y. Q., Morimoto, K., Hosoda, K., Yoshimura, Y. and Isobe, N. 2012. Differential immunolocalization between lingual antimicrobial peptide and lactoferrin in mammary gland of dairy cows. *Vet. Immunol. Immunopathol.* **145**: 499–504. [Medline] [CrossRef]
17. Hurley, W. L. and Rejman, J. J. 1993. Bovine lactoferrin in involuting mammary tissue. *Cell Biol. Int.* **17**: 283–289. [Medline] [CrossRef]
18. Isobe, N. 2017. Control mechanisms for producing antimicrobial factors in ruminant mammary gland. *Anim. Sci. J.* **88**: 937–943. [Medline] [CrossRef]
19. Isobe, N., Hosoda, K. and Yoshimura, Y. 2009. Immunolocalization of lingual antimicrobial peptide (LAP) in the bovine mammary gland. *Anim. Sci. J.* **80**: 446–450. [Medline] [CrossRef]
20. Isobe, N., Nakamura, J., Nakano, H. and Yoshimura, Y. 2009. Existence of functional lingual antimicrobial peptide in bovine milk. *J. Dairy Sci.* **92**: 2691–2695. [Medline] [CrossRef]
21. Isobe, N., Shibata, A., Kubota, H. and Yoshimura, Y. 2013. Lingual antimicrobial peptide and lactoferrin concentrations and lactoperoxidase activity in bovine colostrum are associated with subsequent somatic cell count. *Anim. Sci. J.* **84**: 751–756. [Medline] [CrossRef]
22. Kawai, K., Akamatsu, H., Obayashi, T., Nagahata, H., Higuchi, H., Iwano, H., Oshida, T., Yoshimura, Y. and Isobe, N. 2013. Relationship between concentration of lingual antimicrobial peptide and somatic cell count in milk of dairy cows. *Vet. Immunol. Immunopathol.* **153**: 298–301. [Medline] [CrossRef]
23. 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]
24. Kitano, N., Isobe, N., Noda, J. and Takahashi, T. 2020. Concentration patterns of antibacterial factors and immunoglobulin A antibody in foremilk fractions of healthy cows. *Anim. Sci. J.* **91**: e13372. [Medline] [CrossRef]

25. 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]
26. Kryukov, K. 2020. GenomeSync. http://genomesync.org [accessed on June 10, 2021].
27. Kuehn, J. S., Gorden, P. J., Munro, D., Rong, R., Dong, Q., Plummer, P. J., Wang, C. and Phillips, G. J. 2013. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. *PLoS One* **8**: e61959. [Medline] [CrossRef]
28. Lee, K. C. and Eckert, R. L. 2007. S100A7 (Psoriasin)—mechanism of antibacterial action in wounds. *J. Invest. Dermatol.* **127**: 945–957. [Medline] [CrossRef]
29. Magana, M., Pushpanathan, M., Santos, A. L., Leanse, L., Fernandez, M., Ioannidis, A., Giulianotti, M. A., Apidianakis, Y., Bradfute, S., Ferguson, A. L., Cherkasov, A., Seleem, M. N., Pinilla, C., de la Fuente-Nunez, C., Lazaridis, T., Dai, T., Houghten, R. A., Hancock, R. E. W. and Tegos, G. P. 2020. The value of antimicrobial peptides in the age of resistance. *Lancet Infect. Dis.* **20**: e216–e230. [Medline] [CrossRef]
30. Makovec, J. A. and Ruegg, P. L. 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J. Dairy Sci.* **86**: 3466–3472. [Medline] [CrossRef]
31. Meade, K. G. and O'Farrelly, C. 2019. β-defensins: farming the microbiome for homeostasis and health. *Front. Immunol.* **9**: 3072. [Medline] [CrossRef]
32. 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]
33. Morley, P. S., Apley, M. D., Besser, T. E., Burney, D. P., Fedorka-Cray, P. J., Papich, M. G., Traub-Dargatz, J. L., Weese J. S., American College of Veterinary Internal Medicine. 2005. Antimicrobial drug use in veterinary medicine. *J. Vet. Intern. Med.* **19**: 617–629. [Medline] [CrossRef]
34. Niimi, K., Usami, K., Fujita, Y., Abe, M., Furukawa, M., Suyama, Y., Sakai, Y., Kamioka, M., Shibata, N., Park, E. J., Sato, S., Kiyono, H., Yoneyama, H., Kitazawa, H., Watanabe, K., Nochi, T. and Aso, H. 2018. Development of immune and microbial environments is independently regulated in the mammary gland. *Mucosal Immunol.* **11**: 643–653. [Medline] [CrossRef]
35. Oikonomou, G., Machado, V. S., Santisteban, C., Schukken, Y. H. and Bicalho, R. C. 2012. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. *PLoS One* **7**: e47671. [Medline] [CrossRef]
36. 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. [Medline] [CrossRef]
37. Oliveira, L., Hulland, C. and Ruegg, P. L. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. *J. Dairy Sci.* **96**: 7538–7549. [Medline] [CrossRef]
38. Ploegaert, T. C., Tijhaar, E., Lam, T. J., Taverne-Thiele, A., van der Poel, J. J., van Arendonk, J. A., Savelkoul, H. F. and Parmentier, H. K. 2011. Natural antibodies in bovine milk and blood plasma: variability among cows, repeatability within cows, and relation between milk and plasma titers. *Vet. Immunol. Immunopathol.* **144**: 88–94. [Medline] [CrossRef]
39. Pyörälä, S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Vet. Res.* **34**: 565–578. [Medline] [CrossRef]
40. Rainard, P. and Riollet, C. 2006. Innate immunity of the bovine mammary gland. *Vet. Res.* **37**: 369–400. [Medline] [CrossRef]
41. Regenhard, P., Petzl, W., Zerbe, H. and Sauerwein, H. 2010. The antibacterial psoriasin is induced by E. coli infection in the bovine udder. *Vet. Microbiol.* **143**: 293–298. [Medline] [CrossRef]
42. Ruegg, P. L. 2017. A 100-year review: mastitis detection, management, and prevention. *J. Dairy Sci.* **100**: 10381–10397. [Medline] [CrossRef]
43. Schonwetter, B. S., Stolzenberg, E. D. and Zasloff, M. A. 1995. Epithelial antibiotics induced at sites of inflammation. *Science* **267**: 1645–1648. [Medline] [CrossRef]
44. 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. [Medline] [CrossRef]
45. Shinozuka, Y., Morita, T., Watanabe, A. and Kawai, K. 2018. Live bacteria in clots from bovine clinical mastitis milk with no growth in conventional culturing. *Asian J. Anim. Vet. Adv.* **13**: 197–200. [CrossRef]
46. Shinozuka, Y., Kawai, K., Kurumisawa, T., Shimizu, Y., Imanishi, T., Ohno, A., Takahashi, M., Kaneko, S. and Suzuki, N. 2021. Examination of the microbiota of normal cow milk using MinION™ nanopore sequencing. *J. Vet. Med. Sci.* **83**: 1620–1627. [Medline] [CrossRef]
47. Smolenski, G. A., Cursons, R. T., Hine, B. C. and Wheeler, T. T. 2015. Keratin and S100 calcium-binding proteins are major constituents of the bovine teat canal lining. *Vet. Res. (Faisalabad)* **46**: 113. [Medline] [CrossRef]
48. Suzuki, N., Yuliza Purba, F., Hayashi, Y., Nii, T., Yoshimura, Y. and Isobe, N. 2020. Seasonal variations in the concentration of antimicrobial components in milk of dairy cows. *Anim. Sci. J.* **91**: e13427 [Medline] [CrossRef].
49. Telang, S. 2018. Lactoferrin: A critical player in neonatal host defense. *Nutrients* **10**: 1228. [Medline] [CrossRef]
50. Vasquez, A. K., Nydam, D. V., Capel, M. B., Eicker, S. and Virkler, P. D. 2017. Clinical outcome comparison of immediate blanket treatment versus a delayed pathogen-based treatment protocol for clinical mastitis in a New York dairy herd. *J. Dairy Sci.* **100**: 2992–3003. [Medline] [CrossRef]
51. Yeaman, M. R. and Yount, N. Y. 2003. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **55**: 27–55. [Medline] [CrossRef]
52. Zhang, G. W., Lai, S. J., Yoshimura, Y. and Isobe, N. 2014. Messenger RNA expression and immunolocalization of psoriasin in the goat mammary gland and its milk concentration after an intramammary infusion of lipopolysaccharide. *Vet. J.* **202**: 89–93. [Medline] [CrossRef]