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Molecular characterization of the bacterial communities present in sheep's milk and cheese produced in South Brazilian Region via *16S rRNA* gene metabarcoding sequencing

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ABSTRACT

Sheep's milk and cheese represent unexplored reservoirs of microorganisms' genetic and metabolic diversity. This study aimed to characterize the microbial communities of milk and cheese from sheep of Lacaune breed, produced in the South Region of Brazil. Milk samples (n = 15) from three dairy farms and four types of cheese (fresh, colonial, feta-type, and pecorino-type; n = 20) were subjected to partial *16S rRNA* gene sequencing. *Streptococcus* spp. and *Lactobacillus* spp. were the dominant taxa (core microbiota) of cheeses, while *Phyllobacterium* spp. and *Staphylococcus* spp. were the most prevalent genera in milk. Regarding milk samples, no differences in alpha diversity were observed between the analyzed farms. However, beta diversity analysis revealed that milk collected on Farm 1 differed from the others. These differences may be associated with sheep feeding, mammary gland diseases, and the milking practices used. Upon analyzing cheese samples, significant differences in both alpha and beta diversities were observed between the different cheese types, suggesting that processing and maturation conditions are important for shaping cheese microbiota. Notably, other bacterial groups—including decomposers and potentially pathogenic microorganisms to humans—were observed in some of the analyzed cheeses. This study expands our knowledge of the bacterial composition of sheep's milk and cheese found in different geographic regions.

1. Introduction

Dairy sheep production has recently become a profitable activity in Brazil, especially when compared to the extensive production of sheep's milk in certain European countries such as Greece, Spain, Italy, and France. The annual production of sheep's milk in Brazil corresponds to approximately 840,000 L (Bianchi, 2017), which is the sum of small- and medium-sized farm producers. Most dairy sheep activity is applied to cheese production, which is increasing globally and represents a major economic driver in many countries. Feta and pecorino cheeses have a protected designation of origin since they originate from Greece and Italy, respectively (Silva et al., 2013). Therefore, Brazilian legislation requires that locally produced versions be called "feta-type" or "pecorino-type" cheeses. The main sheep's cheeses produced in Brazil are colonial, fresh, pecorino-type, and feta-type. The soft cheeses (fresh and feta-type) have higher moisture content (44.6–45.9%) with pronounced acidity, while hard cheeses (colonial and pecorino-type) have a solid texture and lower moisture content (35.9–38.3%).

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The organoleptic characteristics of these cheeses are influenced by several factors, including the raw materials used, geographic region of the property, seasonality, sheep feed, sheep breed, manufacturing process, and maturation period (Calasso et al., 2016; Kamimura et al., 2019; Van Hoorde et al., 2010). Thus, unraveling the bacterial diversity of the milk produced in different geographical regions and different cheese types is relevant. Moreover, there is still no specific legislation for sheep's milk products in Brazil. In this sense, knowledge of raw sheep's milk and cheese microbiota will help to define the requirements for new legislation and thus promote the expansion of the sheep's cheese market. Culture-based microbiological techniques have been used to identify lactic acid bacteria as well as deteriorating and pathogenic microorganisms in milk and cheese. However, when a bacterium is not cultivable, which is true for most species, it cannot be identified via these methods. Therefore, culture independent methods such as high-throughput sequencing based on the *16S rRNA* gene are more adequate for microbiota evaluation (Sant'Anna et al., 2019). Thus, the present study aimed to characterize the microbial communities of raw sheep's milk and different types of sheep's cheeses produced in South Brazilian Region.

2. Materials and methods

2.1. Experimental design and sampling

A total of 15 sheep's milk and 20 sheep's cheese samples were evaluated in the present study: milk samples from 3 different farms (n = 5 each) and four types of cheese: feta-type (n = 5), pecorino-type (n = 5), colonial (n = 5) and fresh (n = 5). The samples were collected from Lacaune breed sheeps in dairy farms located in South Brazilian Region (Supplementary Fig. S1 and Supplementary Table S1). The milk and cheeses' samples were stored at 4 °C for maximum 24 h. The cheese was then processed and the milk stored at −18 °C until analysis.

The milk samples were collected directly on the dairy farm on different days, in sterile packaging between August 2018 and September 2018. The cheeses were purchased at the farms where milk samples were collected between April 2018 and September 2018. The manufacturing process of each type of cheese is described in the Supplementary Table S2. The cheeses were analyzed from different batches of milk collected during this study.

2.2. Sample processing

Milk samples (35 mL) were centrifuged at 10,000 rpm for 40 min at 4 °C, followed by removal of the fat from the top with a spatula. Approximately 30 mL of the supernatant was discarded. The pellet was homogenized by addition of 3 mL of sterile distilled water and vortexing. Afterwards, 1 mL of the resulting solution was centrifuged at 14,000 rpm for 5 min at 4 °C for pellet formation.

For cheese samples, 100 g were individually ground in a processor. From that, 25 g were withdrawn, diluted in 225 mL of sterile distilled water and homogenized in a shaker incubator (110 rpm for 2 h at room temperature). The samples were filtered with the aid of a sieve and gauze on top to separate the sediments. A total of 35 mL of the filtrate was centrifuged at 10,000 rpm for 40 min at 4 °C. The supernatant (30 mL) was discarded and 3 mL of sterile distilled water were added to the pellet, which was homogenized by vortexing. Then, 1 mL of this solution was added to a microtube and centrifuged at 14,000 rpm for 5 min at 4 °C for pellet formation.

2.3. DNA extraction

The pellets were resuspended in 180 μL of lysis buffer (22.5 mM Tris HCl, 2.5 mM EDTA, 1% Triton X-100, 20 mg mL-1 lysozyme), followed by homogenization in vortex and incubation for 1 h at 37 °C (homogenized every 15 min in the vortex). Subsequently, 20 μL of proteinase K (20 mg mL-1) and 200 μL of Purelink Genomic Lysis/Binding Buffer were added to the samples and incubated at 55 °C for 30 min. Then 200 μL of ethanol 100% were added and the samples were homogenized in vortex for 5 s. The sample suspension (approximately 640 μL) was used for DNA extraction with Purelink Genomic DNA kit following manufacturers' instructions. DNA was eluted in 25 μL of plain deionized water (Milli-Q) and stored at −20 °C until the time of analysis.

2.4. Library preparation and 16S rRNA sequencing

The libraries were generated amplifying the V4 domain of bacterial *16S rRNA* gene using F515 and R806 primers, both modified to contain an Illumina adapter region (Caporaso et al., 2011). Amplification was performed in a 25 μL mixture, consisting of 12.5 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 200 μM of each dNTP, 2 U Platinum Taq DNA Polymerase Platinum (Invitrogen™), and 1 X reaction buffer. Amplification was carried out in a BioRad MyCycler Thermocycler (BioRad, USA) according to the following program: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final cycle at 72 °C for 5 min.

Amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, Indiana, USA) and indexes were added to DNA libraries following the manufacturer instructions (Illumina Inc., San Diego, California, USA). Sequencing was conducted on an Illumina MiSeq System with a paired-end v2 500-cycles kit.

2.5. Bioinformatics data analysis

Bioinformatics data analysis of *16S rRNA* amplicons were performed using QIIME 2 version 2019.7 (Caporaso et al., 2012). Basically, raw sequence data were quality filtered, denoised and chimera filtered using the q2-dada2- plugin with DADA2 pipeline (Callahan et al., 2016). Reads with expected errors higher than 2 were discarded. Read length filtering was applied and the reads were discarded at the first instance of a phred score lower than or equal to 11. The remaining reads were truncated at 240 bp length. Chimera removal was performed using the consensus method. The amplicon sequence variants (ASV's) obtained by DADA2 pipeline were merged into a single feature table using the q2-feature-table plugin.

The ASV's were aligned with MAFFT (via q2-alignment) (Katoh et al., 2002) and used to construct a phylogeny with fasttree2 (via q2-phylogeny) (Price et al., 2010). Taxonomy was assigned to ASV's using the q2-feature-classifier Naïve Bayes Taxonomy Classifier (Bokulich et al., 2018). The classifier was trained using extracted Greengenes 13_8 reference sequences with 99% similarity from *16S rRNA* variable region 4 (V4). The resulting feature table, rooted tree from reconstructed phylogeny, and taxonomy classification were imported from QIIME2 to R v3.6.1 environment for further data analysis using Microbiome v1.6.0 (Lahti et al., 2017) and Phyloseq v1.28.0 R packages (McMurdie & Holmes, 2013). For Taxonomic analysis, feature table was normalized to relative abundance using transform function from Microbiome R package.

2.6. Differential abundance analysis

The feature table was filtered to remove singletons using the q2-feature-table plugin, the ASV's that was observed less than two samples and less than 10 abundance frequency were removed from the feature table. The resulting filtered features were collapsed at the genus level using q2-taxa plugin for differential abundance analysis. Differential abundance analysis was performed with ANCOM (Martino et al., 2019) using q2-composition plugin, with mean difference as fold difference in feature abundances across groups and clr as transform-function for volcano plot. ANCOM is done by calculating pairwise log ratios between all features and performing a significance test to determine if there is a significant difference in feature ratios with

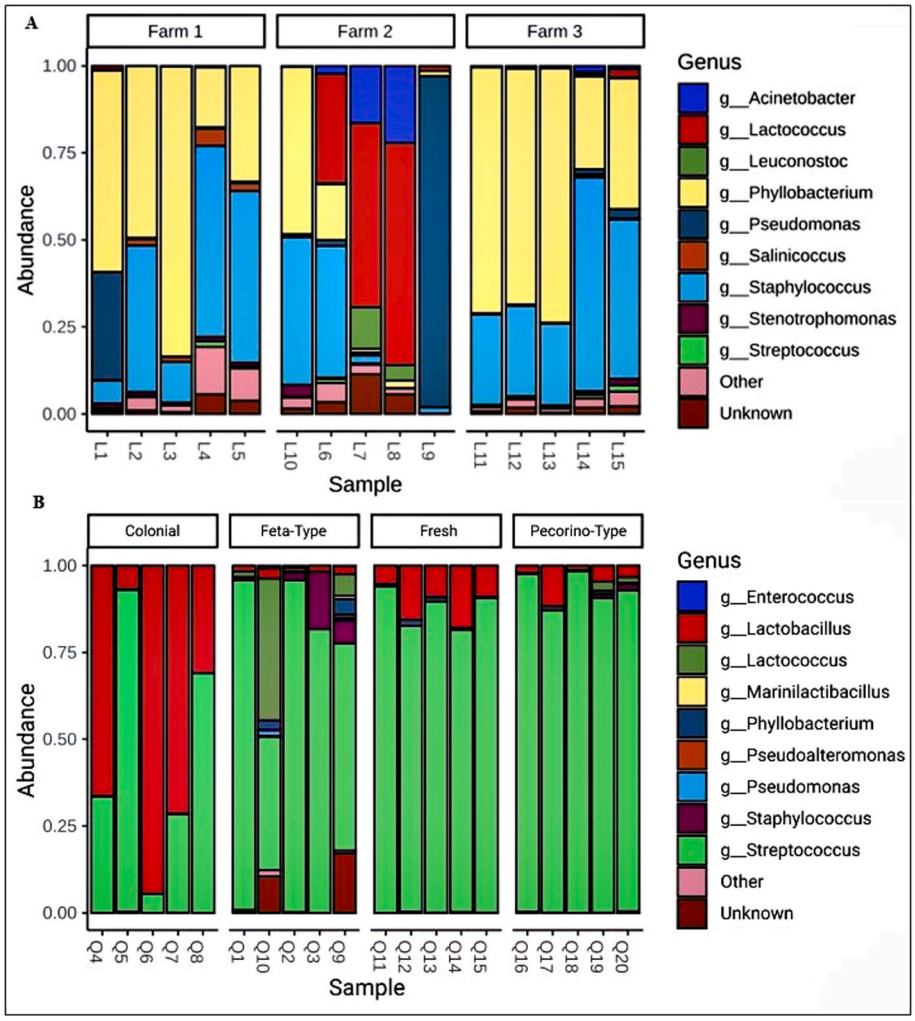

Fig. 1. Representation of the relative abundance of bacterial taxa based on the sequencing of the *16S rRNA* gene at the genus level in milk and cheese samples. Vertical bars indicate the average relative abundance of bacterial sequences present in milk (A) and cheese (B). Strings that could not be assigned are colored in brown and labeled "unknown". (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

respect to the variable of interest. "W" is the W-statistic, or number of features that a single feature is tested to be significantly different against.

2.7. Microbial community analysis

To evaluate bacterial communities' structure, alpha (Shannon, Chao1) and beta diversity (Weighted UniFrac (Lozupone & Knight, 2005) and Bray-Curtis) were estimated using Microbiome and Phyloseq packages in R. Principal Coordinate Analysis (PCoA) ordination was applied to beta diversity metrics and visualized using plot ordination function from Phyloseq. Alpha diversity significance was estimated with a pairwise comparison using a non-parametric test Wilcoxon (Wilcoxon, 1945), using Vegan R package (Oksanen et al., 2007). Beta diversity significance were estimated with a permutational multivariate analysis of variance (Anderson, 2001) using distance matrices obtained by PCoA ordination with Permutational Multivariate Analysis of variance test (PERMANOVA), implemented as Adonis function in Vegan R package (Oksanen et al., 2007).

2.8. Nucleotide sequence accession numbers

All raw sequence reads have been deposited at Sequence Read Archive (SRA) database from the National Biotechnology Information Center (NCBI) and available in BioProject PRJNA631574 (sequences of milk samples) and PRJNA555359 (sequences of cheese samples).

3. Results and discussion

3.1. Characterization of microbial communities in sheep's milk and cheese

In the present study, the microbial compositions of sheep's milk from healthy animals and four types of sheep's cheese were investigated by sequencing the V4 hypervariable region of the *16S rRNA* gene. A total of 1,292,178 reads were obtained for all samples (Supplementary Table S3), which were assigned to 501 ASV's: 429 for milk samples and 72 for cheese samples (Supplementary Table S4). The rarefaction curves showed saturation for both sample types, indicating sufficient sequencing coverage to estimate microbial composition and diversity (Supplementary Fig. S2).

A total of 14 phyla were identified in milk samples (with a relative abundance of >0.1%) that correspond to Proteobacteria (58.03%), Firmicutes (39.93%), Actinobacteria (1.79%) and Bacteroidetes (0.17%). Two phyla were identified in cheese, with a predominance of Firmicutes (97.50%) and Proteobacteria (2.48%) (Supplementary Fig. S3 A). This finding is consistent with other cheese-related *16S rDNA* metagenomics studies (Dalmasso et al., 2016; Kamilari, Anagnostopoulos, Papademas,

Kamilaris, & Tsaltas, 2020; Kamimura et al., 2020). Regarding the classes present in samples, Bacilli, Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, and Clostridia were the most abundant in both milk and cheese samples. At the order level, Rhizobiales, Bacillales, Pseudomonadales, and Lactobacillales were observed in milk, while Lactobacillales, Bacillales, Enterobacteriales, and Rhizobiales were observed in cheeses. The most abundant families in the milk samples were Phyllobacteriaceae, Staphylococcaceae, Pseudomonadaceae, and Streptococcaceae, while Streptococcaceae and Lactobacillaceae were most abundant in cheese samples (Supplementary Fig. S3 B).

At the genus level, the diversity and abundance of milk microbiota were wide-ranging (Fig. 1 A). Eighty-seven bacterial genera were identified in milk samples, of which *Phyllobacterium* spp. (42.59%), *Staphylococcus* spp. (29.20%), *Pseudomonas* spp. (10.96%), *Lactococcus* spp. (5.10%), and *Acinetobacter* spp. (1.38%) were the most abundant. Additionally, the differential abundance analysis (ANCOM) for milk samples indicated differences in *Staphylococcus* spp. abundance (w = 130) (Supplementary Fig. S4 A). *Phyllobacterium* spp. was the most prevalent genus in milk samples. This result was observed in raw goat's milk which was stored at 4 °C before freezing (Kamilari, Anagnostopoulos, Papademas, Efthymiou, et al., 2020); here the samples were also preserved at 4 °C until freezing, which could have influenced microbiota, since storage conditions are critical to microbial communities shaping in raw milk and its products (Kamilari, Anagnostopoulos, Papademas, Efthymiou, et al., 2020). Future studies may be performed to evaluate the storage influence on sheep milk microbiota. Notably, the presence of this genus can likewise be influenced by diet, environment, animal health, and manufacturing processes (McInnis et al., 2015). It is generally accepted that lactic acid bacteria predominate in the microbiota of raw sheep, goat, cow, and buffalo milk (Esteban-Blanco et al., 2019; Kamilari, Anagnostopoulos, Papademas, Efthymiou, et al., 2020; Li et al., 2016). Among the most common genera *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Streptococcus* spp., *Enterococcus* spp., *Pseudomonas* spp. and *Acinetobacter* spp. are also common in the raw milk microbiota, since they are psychrotrophic bacteria and milk is stored at low temperatures (Kamilari, Anagnostopoulos, Papademas, Efthymiou, et al., 2020; Quigley et al., 2013; Tilocca et al., 2020).

At the moment the milk is excreted, it is immediately colonized by a complex microbiota, including microorganisms that naturally inhabit the teat skin and the epithelial lining of the teat canal, which are mainly represented by the genera *Streptococcus* spp., *Staphylococcus* spp., and *Micrococcus* spp. (Bortoluzzi & Menezes, 2014). Additionally, milking equipment, animal location, feeding place, bedding material, and lactation stage also influence the raw milk microbiota. Furthermore, the raw milk microbiota is influenced by the biochemical composition of milk as well as pH and water activity, which can affect microbial growth.

In addition to the aforementioned genera, *Staphylococcus* spp. was also observed in milk samples. The presence of this genus could indicate udder inflammation in sheep since it is considered the main etiologic agent of intramammary infections. Since some *Staphylococcus* species are part of the natural microbiota of many mammals, this microorganism has frequently been observed in raw milk from healthy sheep (Esteban-Blanco et al., 2019; Gonzales-Barron et al., 2017). However, it is important to note that the presence of *Staphylococcus* spp. can also be the result of poor hygiene of the manipulator and/or facilities; however, this factor was not assessed in the present work. Since some *Staphylococcus* spp. species can be opportunistic pathogens to humans, its deeper characterization is necessary to better understand the pathogenic potential in evaluated samples.

The following genera were identified in the cheese samples: *Streptococcus* spp. (73.15%), *Lactobacillus* spp. (19.91%), *Lactococcus* spp. (2.46%), and *Staphylococcus* spp. (1.74%) (Fig. 1 B). *Lactobacillus* spp. (w = 10) and *Staphylococcus* spp. (w = 8) were differentially abundant (ANCOM) in these samples, corroborating with the taxonomic description (Supplementary Fig. S4 B). *Streptococcus* spp. and/or *Lactobacillus* spp. have been widely reported as the dominant bacteria in cheeses (Aldrete-Tapia et al., 2014; Castellanos-Rozo et al., 2020; Kamilari, Anagnostopoulos, Papademas, Kamilaris, & Tsaltas, 2020) and are associated with their flavors and textures. Bacteria of these genera produce bacteriocins that act as bioprotectors to cheese by inhibiting the

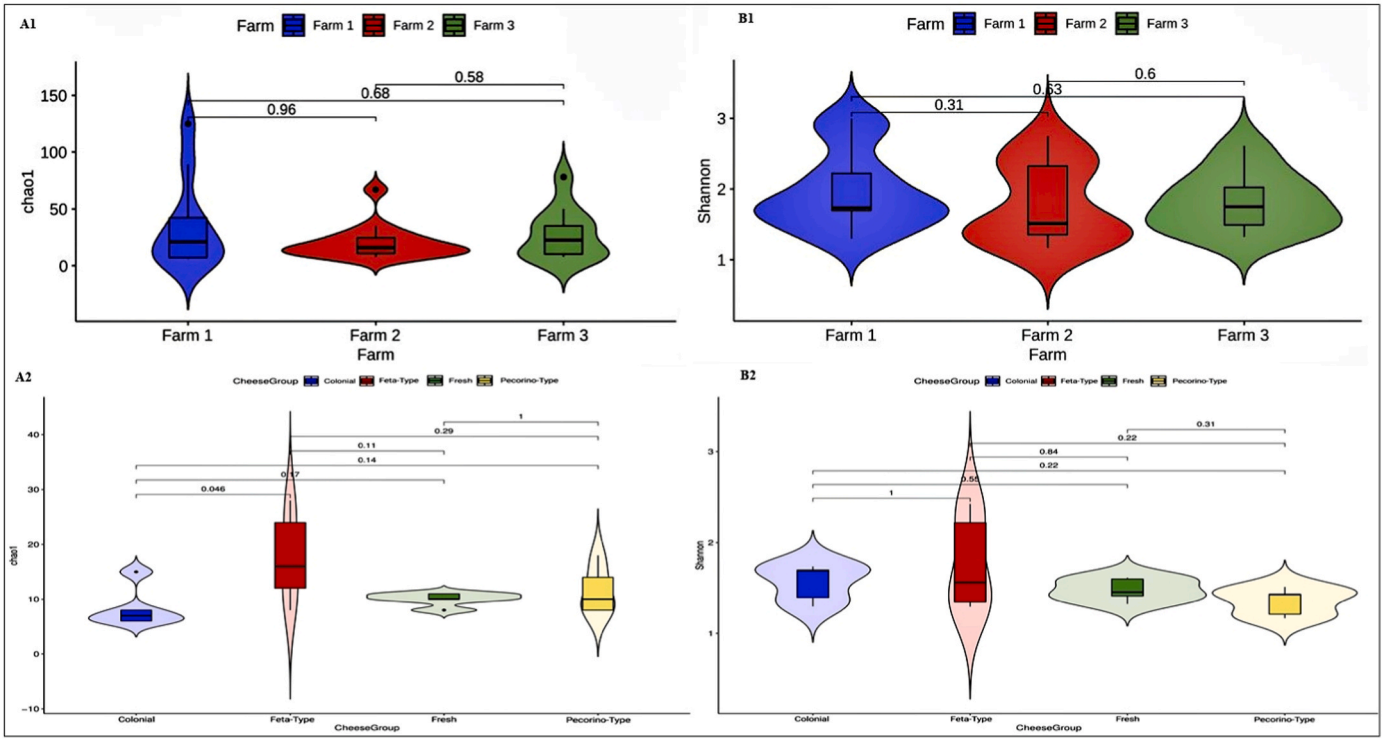

Fig. 2. Alpha diversity metrics featuring milk and cheese samples. Wealth measured using the Chao1 index (A) and Shannon index (B). (1) Milk samples collected from different farms. (2) Cheese samples from different groups.

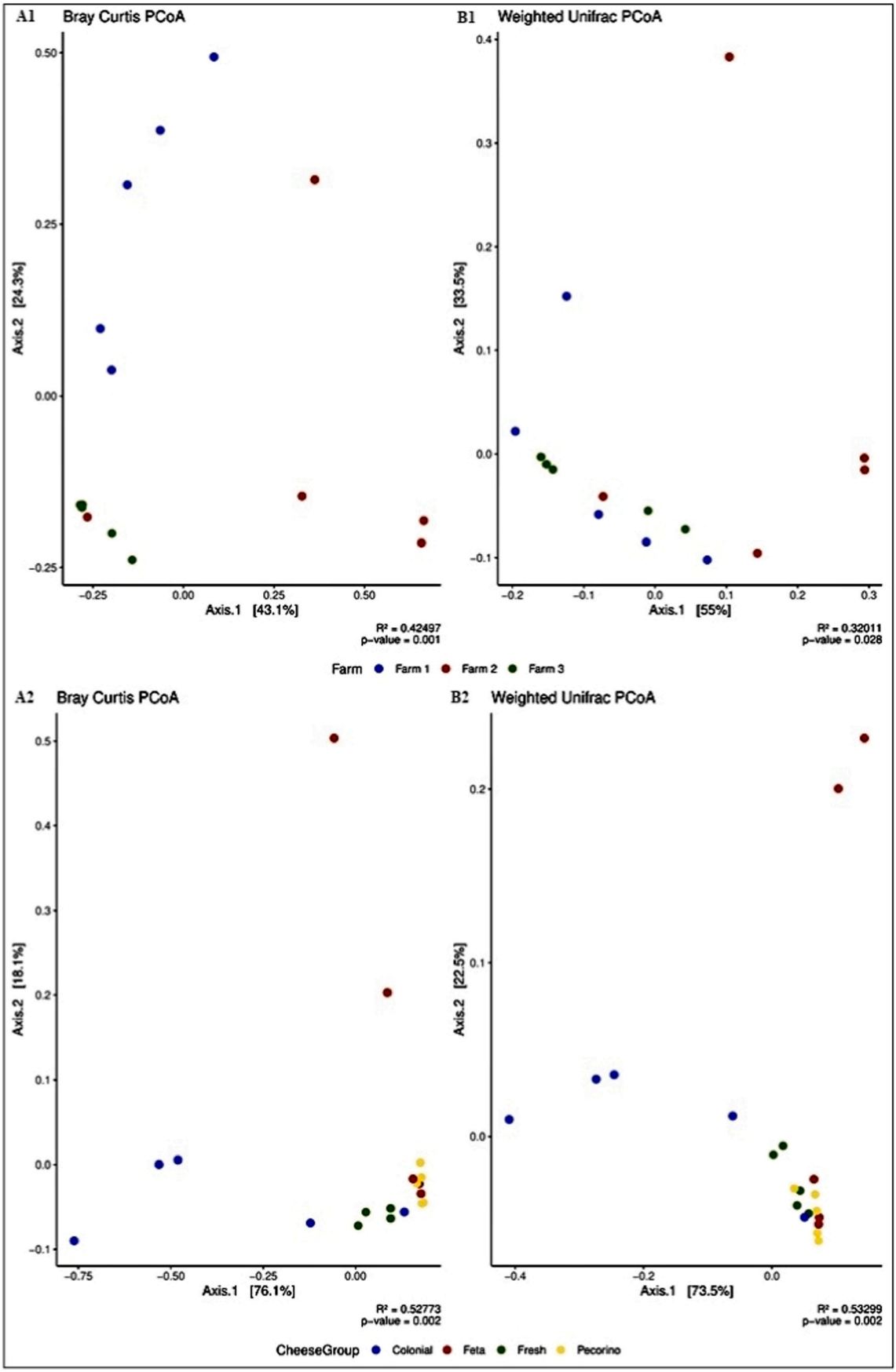

Fig. 3. Principal coordinate analysis (PCoA) based on the bacterial communities of milk and cheese samples. The PCoA analysis for these samples was based on the distance metrics of Bray-Curtis (A) and weighted Unifrac (B). (1) Milk samples collected from different farms. (2) Cheese samples from different groups.

growth of pathogenic and deteriorating microorganisms (Gontijo et al., 2020; Kaya & Simsek, 2019). The abundance of *Streptococcus* spp. and *Lactobacillus* spp. was similar in feta-type and pecorino-type cheeses, which is an expected result since the starter cultures used in the production of these cheeses are generally composed of *S. thermophilus*, *L. bulgaricus*, *L. helveticus*, and *L. casei* (Supplementary Table S2). It is interesting to note that colonial and fresh cheeses are manufactured by the addition of *L. helveticus* only; however, high levels of *Streptococcus* spp. could be observed in the analyzed samples (Fig. 1B).

While *Lactobacillus* spp. grows slowly during the initial weeks of cheese maturation, they dominate the microbiota at the end of the maturation process. These bacteria are important for the ripening process due to the release of bioactive peptides, vitamins, and oligosaccharides (Santiago-López et al., 2018). These properties explain the predominance of *Lactobacillus* spp. in hard cheeses (e.g., colonial) and their lower abundance in soft cheeses (e.g., fresh and feta-type). Notably, in cheeses with longer maturation periods, these microorganisms can die due to low pH, high salt concentrations, and decreased water content (Rantsiou et al., 2008). This justifies the low abundance of these microorganisms in pecorino-type cheese since it is matured for a longer period.

Besides genera related to cheese microbiota, the presence of *Staphylococcus* spp. may because for concern since some species are potential pathogens. This genus was mainly observed in feta-type cheese and at lower frequencies in pecorino-type cheese. Its presence may be associated with the health status of animals, handling practices, and contamination during the cheese-making process (Aldrete-Tapia et al., 2014). Thus, as for milk, further studies to determine the *Staphylococcus* spp. species in cheese samples, and their potential pathogenicity must be addressed.

Regarding species, only a few were identified in the present study. This could be related to the lower number of reads obtained by v2 Illumina kit, although the rarefaction curves show that the ASV's number was sufficient (Supplementary Fig. S2). Moreover, as published in previous studies, *16S rRNA* partial gene sequencing has not enough resolution to identify a great number of species, due to its conservation degree (Johnson et al., 2019). From milk samples, it was possible to identify *Pseudomonas fragi*, *Staphylococcus sciuri*, *Stenotrophomonas geniculate*, *Staphylococcus succinus*, and *Leuconostoc mesenteroides*. *Pseudomonas fragi* is often associated with milk and dairy products and can produce volatile esters (Filippis et al., 2014). *Staphylococcus sciuri* has been described in goat cheese and produces enterotoxin (Cunha-Neto et al., 2002). Moreover, this species and *Staphylococcus succinus* are present in the udders of animals (Derakhshani et al., 2018). Furthermore, microorganisms from the genus *Leuconostoc* spp. have been associated with milk, while *L. mesenteroides* is related to product degradation (Frantzen et al., 2017).

Lactobacillus delbrueckii was identified in cheeses, especially in colonial cheese. Additionally, *Staphylococcus equorum* and *Lactobacillus helveticus* were present in feta-type and pecorino-type cheeses. These microorganisms are often used in the production of fermented products, and their presence in cheeses is expected since they contribute to the formation of aromatic compounds during cheese maturation (Irlinger et al., 2012; Vermote et al., 2018). Their presence can be associated with sensory changes that can influence a product and its shelf life.

3.2. Sample richness and bacterial diversity

Alpha diversity data were estimated using the Chao1 and Shannon indexes (Fig. 2; Supplementary Table S5). Regarding milk diversity, no differences were observed among the three evaluated farms ($p > 0.05$). For cheese samples, the feta-type cheese had higher microbial diversity than colonial cheese (Chao1; Fig. 2; Supplementary Table S5), which may be related to the moisture content of feta-type cheese, which allows the greater proliferation of microorganisms (Lima & Leal, 2017).

Raw milk showed greater microbial diversity, which is expected since this product is rich in nutrients and has a neutral pH, which makes it capable of supporting a rich microbiota (Porcellato et al., 2018). This result has also been demonstrated for other types of milk (Falardeau et al., 2019; Kamimura et al., 2020). In contrast, the lower microbial diversity of cheeses may be related to being prepared with pasteurized sheep's milk. A recent study comparing traditional and industrial methods of cheese production shows that microbial diversity is lower when more severe heat treatments are made (Kamilari, Anagnostopoulos, Papademas, Kamilaris, & Tsaltas, 2020). Therefore, cheeses made with raw milk have greater species richness in their microbiota (Van Hoorde et al., 2010). Additionally, starter cultures are used for cheese production and to inhibit the growth of undesirable microorganisms and/or pathogens. Thus, these bacteria represent the core microbiota of the evaluated cheeses.

Table 1
Permutation test for homogeneity of multivariate dispersions (PERMANOVA) using a pseudo-F test with 9999 permutations for Beta Diversity analysis by Bray-Curtis and weighted UniFrac. Bold numbers show statistically significant differences.

Milk		Bray curtis		Weighted UniFrac	
		Adjusted p value	R[a]	Adjusted p value	R[a]
Farm 1	Farm 2	0.050	0.3070	0.177	0.2637
	Farm 3	0.021	0.4594	1.000	0.0577
Farm 2	Farm 3	0.141	0.3578	0.092	0.3304
Cheese					
Colonial	Feta-type	0.099	0.4149	0.042	0.4616
	Fresh	0.293	0.4653	0.255	0.4736
	Pecorino-type	0.061	0.5761	0.107	0.5433
Feta-type	Fresh	0.096	0.2092	0.958	0.2178
	Pecorino-type	0.706	0.1843	0.725	0.2281
Fresh	Pecorino-type	0.044	0.5053	0.406	0.3377

[a] R - Pearson correlation coefficient.

3.3. Comparison of microbial communities in milk and cheese samples

Calculating beta diversity is an important criterion used to assess the diversity of ASV's between different samples (Pastore, 2016). Based on the relative abundance of sequenced ASV's, PCoA was used to analyze differences in the composition of bacterial communities in milk samples from different farms as well as different types of cheese samples using the Bray-Curtis method (Fig. 3 A) and weighted UniFrac distance metrics (Fig. 3 B).

Significant differences in beta diversity were observed when comparing the milk samples obtained from different farms based on Bray-Curtis (Fig. 3 A1; Table 1), which showed that the milk samples collected at Farm 1 differed from those of Farms 2 and 3. Weighted UniFrac distance showed no significant differences between the milk samples ($p > 0.05$; Table 1). Differences in the milk microbiota between farms may be associated with sheep feeding, mammary gland diseases, and the milking practices employed (Esteban-Blanco et al., 2019; Kamimura et al., 2020) and were also observed for goat milk.

The composition of bacterial communities among the cheese types was also compared (Fig. 3 A2 and B2; Table 1), showing significant differences between some groups. Samples of fresh cheeses differed from the pecorino-type cheese group, which indicates that the bacterial communities in these types of cheeses have greater dissimilarity and share fewer taxa compared to others. Additionally, the weighted UniFrac distance showed a significant difference between the colonial and feta-type cheeses. The other cheese groups had very similar microbial communities, with no significant differences (Table 1). These results may be related to the effect of starter cultures, which are the same for

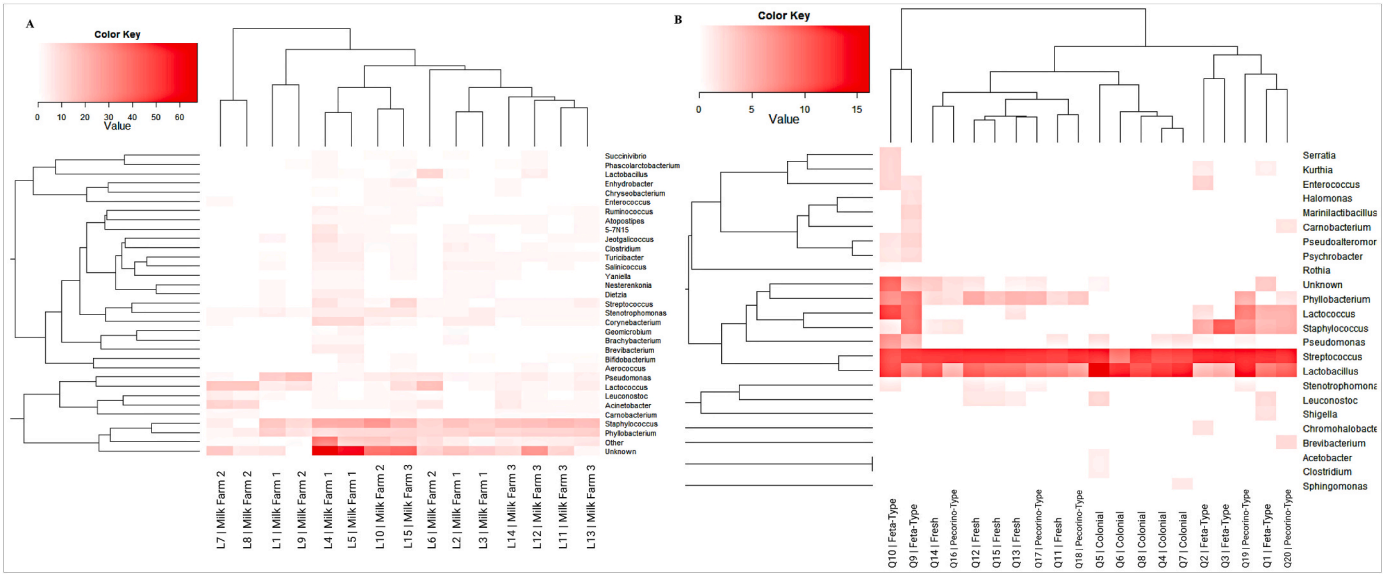

Fig. 4. Heat map and dendrogram cluster showing microbial diversity at the genus level. Hierarchical grouping in milk (A) and cheese (B) samples are presented. The color represents the relative abundance in the scale of each variable. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

feta-type and pecorino-type cheeses (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *L. helveticus*, and *Lacticaseibacillus casei*) as well as fresh and colonial cheeses (*L. helveticus*) (Supplementary Table S2). In addition, pecorino-type and feta-type are produced in the same factory, which was already showed to influence microbiota composition, probably related to the standardization of the processes along the production chain (de Paula et al., 2021).

The relationships between evaluated samples considered taxonomic classification at genus the level and were visualized using an exploratory heat map associated with hierarchical clustering based on the weighted UniFrac distances between samples (Fig. 4 A and 4 B). Since it was not possible to separate the different farms when analyzing milk samples, this suggests that even though the samples were from different lots and farms, they had similar microbiota despite originating from different lots and farms. Moreover, it was possible to confirm that *Staphylococcus* spp. and *Phyllobacterium* spp. were the dominant genera in milk samples.

Regarding cheese samples, a cluster was observed for colonial cheese and similar diversity was noted among the different types of cheese, indicating that their microbiota was preserved. Previous studies showed that the origin of cheese influences the richness, uniformity, and relative abundance of bacterial species. In the present study, geographical localization does not seem to have this influence. This result is somewhat expected since the regions included in this study present very similar characteristics. Additionally, the heat map confirms that *Streptococcus* spp. and *Lactobacillus* spp. made up the core microbiota of the analyzed cheeses.

4. Conclusions

This is the first study performed to characterize microbial communities of Lacaune breed sheep's raw milk and different types of cheese in the Southern Brazilian Region. The results demonstrated that sheep's raw milk have great microbial diversity, which could be associated with environmental conditions, diet and genetics of the animals. In addition, *Phyllobacterium* spp., *Staphylococcus* spp. and *Pseudomonas* spp. were the main genera found in milk, which could be associated with its cooling process or contamination. There was variation when analyzing beta diversity of milk samples from different farms, suggesting that animal feeding, mammary gland diseases or the milking practices may influence the milk microbiota.

The sheep's cheese microbiota showed lower diversity, with a core microbiota composed by *Streptococcus* spp. and *Lactobacillus* spp. This could be associated to the starter cultures used for cheese manufacturation and to pasteurized milk used in the process. Regarding the taxonomic diversity, the feta-type cheese had higher diversity when compared to colonial chesses. This may be related to the higher moisture content of the feta-type cheese which may facilitate microbial growth. The beta-diversity analysis also showed difference between fresh and pecorino-type cheeses. These results show that the starter cultures and the processes along the production chain may shape cheese microbiota.

The results of this study provide greater knowledge about the microbial structure on sheep's milk and cheeses and may be a useful tool to characterize the particularities of each product. Moreover, these data may be helpful to structure strategies that guarantee the quality and safety of products, serving as a basis for the creation of specific legislation for sheep's milk and cheeses in Brazil.

CRediT authorship contribution statement

Creciana M. Endres: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Ícaro Maia S. Castro:** Data curation, Formal analysis, Methodology, Software, Writing – review & editing. **Laura D. Trevisol:** Methodology, Writing – review & editing. **Juliana M. Severo:** Methodology, Software, Writing – review & editing. **Michele B. Mann:** Formal analysis, Methodology, Writing – review & editing. **Ana Paula M. Varela:** Formal analysis, Methodology, Writing – review & editing. **Ana Paula G. Frazzon:** Conceptualization, Formal analysis, Resources, Writing – review & editing. **Fabiana Q. Mayer:** Formal analysis, Methodology, Supervision, Writing – review & editing. **Jeverson Frazzon:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111579.

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