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Milk microbiota from dairy factories in the Central Region of Rio Grande do Sul, Brazil

Microbiota do leite de laticínios na Região Central do Rio Grande do Sul, Brasil

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ABSTRACT

Milk is a rich food, source of several indispensable nutrients, much consumed by the population. Its quality and microbiota are influenced by several factors. The objective of the present study was to determine the microbiota of refrigerated raw milk and processed milk in dairy products in Vale do Taquari - RS, through genetic sequencing. Three types of milk were analyzed in two dairies in the region: raw refrigerated milk, which arrives at the dairies by means of tank trucks, pasteurized milk and milk treated by Ultra High Temperature (UHT). The determination of the milk microbiota was performed through sequencing of the 16S rRNA gene. The results showed that refrigerated raw milk has the highest number of microorganisms in the two dairy products, followed by pasteurized milk and milk sterilized by the UHT process, successively. Dairy processing 2 proved to be more efficient, especially for UHT treated milk, considerably reducing the microbiota. Eighty-seven species of the Bacteria Kingdom were identified and the samples showed considerable microbiological diversity. Psychrotrophic microorganisms such as *Kurthia gibsonii* were expressive in the samples. Lactic acid bacteria such as *Streptococcus macedonicus* were found in refrigerated raw milk and pasteurized milk and *Streptococcus thermophilus* in sterilized milk. Harmful species such as *Bacillus cereus group*, *Aeromonas dhakensis* and *Acinetobacter haemolyticus* were found in UHT treated milk from both dairy products, in addition to *Aeromonas caviae*, *Enterobacter mori* and *Viridibacillus arenosi.*

Index terms: Microorganisms; metagenomics; genetic sequencing; milk quality; milk production.

RESUMO

O leite é um alimento rico, fonte de diversos nutrientes indispensáveis, muito consumido pela população. A sua qualidade e a sua microbiota são influenciadas por diversos fatores. O objetivo do presente estudo foi determinar a microbiota do leite cru refrigerado e de leites processados em laticínios do Vale do Taquari – RS, por meio do sequenciamento genético. Foram analisados três tipos de leite em dois laticínios da região: leite cru refrigerado, que chega aos laticínios por meio dos caminhões-tanques, leite pasteurizado e leite esterilizado por *Ultra High Temperature* (UHT). A determinação da microbiota do leite foi realizada por meio do sequenciamento do gene 16S rRNA. Os resultados mostraram que o leite cru refrigerado possui a maior quantidade de microrganismos nos dois laticínios, seguido do leite pasteurizado e pelo leite tratado pelo processo UHT, sucessivamente. O processamento do Laticínio 2 mostrou-se mais eficiente, principalmente para o leite UHT, reduzindo consideravelmente a microbiota. Foram identificadas 87 espécies do Reino *Bacteria* e as amostras mostraram considerável diversidade microbiológica. Microrganismos psicrotróficos, como *Kurthia gibsonii* foram expressivos nas amostras. Bactérias ácido-láticas como *Streptococcus macedonicus* foram encontradas no leite cru refrigerado e no leite pasteurizado e *Streptococcus termophilus*, no leite esterilizado. Espécies nocivas como *Bacillus cereus group*, *Aeromonas dhakensis* e *Acinetobacter haemolyticus* foram encontrados no leite UHT de ambos os laticínios, além de *Aeromonas caviae*, *Enterobacter mori* and *Viridibacillus arenosi.*

Termos para indexação: Microrganismos; metagenômica; sequenciamento genético; qualidade do leite; produção de leite.

INTRODUCTION

Milk is an essential food, with nutritional relevance, which makes it one of the main agricultural products (Carvalho et al., 2022). Milk production makes a relevant contribution to the economy and social development, with around 150 million families working in milk production worldwide. Most producers are smallholder farmers in developing countries, and this is the main activity for their livelihood (Food and Agriculture Organization of The United Nations FAO, 2021).

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Brazil is considered the fifth largest milk producer in the world and produced 5,815,050 thousand liters in the second quarter of 2021, being Rio Grande do Sul responsible for 885,000 liters (Instituto Brasileiro de Geografia e Estatística - IBGE, 2022). The Taquari Valley region, located in the central region of the state, is responsible for a large part of the state production, with an average of 4,406,428 thousand liters per year and more than one million liters of milk per day, being the third largest milk-producing region (Secretaria de Planejamento, Governança e Gestão - SPGG, 2020). Milk production is one of the core businesses of the economy of the small towns that make up Taquari Valley - RS.

The quality of milk can be influenced by a number of factors, from processing, on milk-producing properties, to processing carried out by dairy factories. In order to avoid contamination, Good Agricultural Practices (GAPs) must be adopted, which consist of a set of activities developed in the rural properties with the goal of ensuring health, well-being and safety of animals, human beings and environment (Ströher et al., 2021). Normative Instruction Number 77, from Ministry of Agriculture, Livestock and Supply (MAPA), regulates the implementation of GAPs in the stages of bovine milk production (Brasil, 2018).

MAPA's Normative Instruction Number 76 provides information on the identity and quality characteristics that refrigerated raw milk, pasteurized milk and type A pasteurized milk must present. Refrigerated raw milk is the one produced on rural properties, refrigerated and destined to milk and dairy establishments. Pasteurized milk is the fluid milk subjected to one of the pasteurization processes stated in current legislation, automatically packaged in a closed circuit and intended for direct human consumption (Brasil, 2018). UHT milk, on the other hand, is understood as homogenized milk, which has been subjected, for two to four seconds, to a temperature of 130 ºC (Brasil, 1997).

In order to be consumed, the refrigerated raw milk needs to go through the beneficiation processes carried out in the dairy factories. The dairy industry is responsible for ensuring the safety and quality of milk for consumers. The core processes for obtaining a quality product are the cooling and the beneficiation of milk (Machado et al., 2017). The cooling of raw milk must occur at temperatures up to 5 °C and remain below that until it arrives at the dairy factory, where it will be used in the production of different types of milk or dairy products (Brasil, 2018). The type of milk produced depends on the heating process that was applied: pasteurization or UHT. These heating processes eliminate pathogens and increase shelf life in closed packages. Pasteurized milk must be stored at refrigeration temperature (4° C to 7 °C) and has a shelf life of about two weeks, whereas UHT milk can be stored at room temperature and lasts up to twelve months in a closed bottle (Machado et al., 2017).

Milk microbiota can be influenced by several factors, whether endogenous or environmental, and may be composed of microorganisms that are beneficial to human health, or yet deteriorating or pathogenic. The typical composition of bovine milk microbiota was shown to be heterogeneous and characterized by an abundance of lactic acid-producing bacteria (LAB), of the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Enterococcus*, besides psychotrophic bacteria such as *Pseudomonas*, *Acinetobacter* and *Aeromonas*. More recent and more sensitive methods than traditional microbiological methods have revealed the presence of anaerobic bacteria, such as *Bacteroides*, *Faecalibacterium*, *Prevotella* and *Catenibacterium*, whose origin may be related to fecal contamination events (Tilocca et al., 2020).

Milk microbiota studies have been used to assess and improve animal health and ensure product quality, as well as safety in consumption (Yap et al., 2020). Metatranscriptome and metagenome sequencing are becoming the most used procedures to decipher the genomic potential of the entire microbiota in foods (Ferrocino; Ranstsiou; Cocolin, 2021). This field of research comprehend molecular genetics and microbial ecology, with the goal of verifying the total content of microorganisms present in the analyzed samples. By detecting the total amount of the microbiota, metagenomics provides the opportunity to reveal microbiological richness that could not be previously observed (Sudarikov; Tyakht; Alexeev, 2017). Metagenomics is a useful tool for the dairy factories as it promotes a thorough diagnosis of the quality of the milk produced, and can be used to improve milk beneficiation processes.

The goal of the present study was to determine the microbiota of refrigerated raw milk and processed milks in dairy factories in the central region of Rio Grande do Sul, in municipalities belonging to Taquari Valley.

MATERIAL AND METHODS

The study was carried out in the Taquari Valley region, Rio Grande do Sul, Brazil. A total of six samples were collected in two dairy factories, in two tows, being one sample of refrigerated raw milk from the tanker truck, one of pasteurized milk and one of UHT treated milk in each of the industries. Samples were collected at the end of March, beginning of autumn.

The dairy factories received the denomination D1 (Dairy 1) and D2 (Dairy 2) and the types of milk received their initial, being "R" for raw milk, "P" for pasteurized milk and "UHT" for UHT treated milk. At the time of collection, the sample temperature was measured using an Incoterm thermometer (model 5135). The collections were carried out with 100 mL sterilized plastic bottles, all hygiene precautions were followed, and the samples were placed in a styrofoam box with ice, which kept the temperature of the samples below 12 °C.

The high-performance sequencing of the V3/V4 regions of the 16S ribosomal gene was used to identify the bacteria present in the samples. Amplification was performed with primers for region V3-V4 of the rRNA gene 16S, 341F (5'-CCTACGGGRSGCAGCAG-3'), and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR reactions were performed in triplicates, with the conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45s, 55 °C for 30s and 72 °C for 45s and a final extension of 72 °C for 2 minutes.

The libraries were sequenced using the Illumina MiSeq platform. For single-end sequencing, the V2 kit with 300 cycles was used. The sequences were analyzed using the Sentinel pipeline. In the Sentinel pipeline, fastq files are evaluated for Phred quality (PQ) using the FastQC v.0.11.8 program. Fastq files are subjected sequence trimming (Phred < 20). The software used for this purpose was built in Python v.3.6, which is inspired by the features of the BioPython project. For paired-end data, before the trimming step, two pairs of files (R1 and R2) are merged into a single file using pandaseq v.2.11. The cluster used was the OTU (Operational Taxonomic Units). Clusters with an abundance less than two were removed from the analysis, as such structures are usually related to chimera sequences. Taxonomic identifications are performed with BLASTn v.2.6.0 (Altschul et al., 1990), using a public database as reference. Regarding the definition of the species, a Python instruction evaluated whether one of the three requirements would be met by the hits: 1) higher bit-score; 2) lower evalue; and 3) taxonomies with greater representation. Data were tabulated in Excel. The mean and standard error of the number of species were calculated using Excel. The Bioestat was used for the Q-square test in order to verify statistical differences in the species found in the six samples analyzed and the Student's *t* test was used to verify the difference in the amount of microorganisms in raw refrigerated milk for processed milk (pasteurized and UHT).

RESULTS AND DISCUSSION

The analysis of samples of refrigerated raw milk, pasteurized milk and UHT treated milk from the dairy factories in Taquari Valley (RS) showed the presence of 51,401 sequences of Bacteria Kingdom, divided into three phyla: *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, nine classes, 15 orders, 21 families, 41 genera and 87 species.

In relation to the total number of microorganisms, it is possible to observe that the samples with the highest number of microorganisms were those of refrigerated raw milk, followed by samples of pasteurized milk and UHT treated milk. The sample with the highest number of microorganisms was the refrigerated raw milk sample from Dairy 1, with 15,384 microorganisms, followed by the refrigerated raw milk sample from Dairy 2, with 13,950 microorganisms. Dairy 1 also showed the highest number of microorganisms compared to Dairy 2 for processed milks (pasteurized and UHT treated). Pasteurized milk from Dairy 1 had 9,052 microorganisms and pasteurized milk from Dairy 2 had 5,622 microorganisms. The UHT treated milk showed an even greater difference, and in Dairy 1 the total number found in the sample was 7,090 sequences, and in Dairy 2 it was only 303 sequences. In addition, the UHT treated milk from Dairy 1 has a higher number of microorganisms than the pasteurized milk from Dairy 2.

The number of microorganisms in the samples showed a reduction from the refrigerated raw milk to the pasteurized milk and to the UHT treated milk, successively, in the samples of the two dairy factories. In Dairy 1, there was a reduction of 41.15% in the total amount of microorganisms from refrigerated raw milk to pasteurized milk and 53.91% from refrigerated raw milk to UHT treated milk. In Dairy 2 it is possible to observe an even greater reduction, from 59.69% of refrigerated raw milk to pasteurized milk and from 97.83% of refrigerated raw milk to UHT treated milk. Using Student's *t* test, it is possible to verify that this reduction is significant ($p<0.05$), with the exception of the reduction in raw refrigerated milk for pasteurized milk from Dairy 2 ($p>0.05$). This shows that the beneficiation processes have been efficient in reducing the microbial load of raw milk in the two dairy factories analyzed, but Dairy 2 shows a much higher efficiency, especially in its UHT processing.

Using the Chi-square test, it is possible to observe that there is a significant variation in the species of microorganisms found in the samples of dairy factories from Taquari Valley. This statistical difference occurs when comparing the three types of milk analyzed: raw refrigerated, pasteurized and UHT treated milk, from the same dairy factory, and also when the two dairy factories are compared in relation to the three types of milk. Moreover, when comparing the same type of milk from the two dairy factories, there is also a statistical difference ($p < 0.001$).

The total average of microorganisms found in the analyzed samples was 590.8 microorganisms and the standard error was 1,836.2, due to the significant differences in the number of each species of microorganism found in the samples (Figure 1). These differences range from 8,696 sequences in one sample (pasteurized milk from Dairy 1) to zero in the other samples. The average number of microorganisms in the samples ranged from 176.8 in the refrigerated raw milk sample from Dairy 1 to 3.5 microorganisms in the UHT treated milk sample from Dairy 2, and the standard error ranged from 99.95 in the pasteurized milk sample from Dairy 1 to 1.85 in the sample of UHT treated milk from Dairy 2. The biggest difference between the averages, as well as between the total of microorganisms found, is observed for the UHT treated milk, which has 81.5 sequences of microorganisms in Dairy 1 and 3.5 sequences of microorganisms in the Dairy 2.

The reduction of the microbiota occurs mainly through thermal processes, which have the function of eliminating all pathogenic bacteria present in the food, besides inactivating the enzymes produced (Xing et al., 2020). This reduction in the number of microorganisms is necessary so that the milk can be ingested without causing harm to the consumer's health. However, in this reduction process, there is also a decrease or elimination of beneficial microbiota from the food, such as probiotic microorganisms, e.g. LAB. According to Markowiak and Śliżewska (2017), probiotics have numerous advantageous functions for human health. The main advantage is the effect on the development of the organism's own microbiota, in order to ensure the proper balance between pathogens and beneficial bacteria, necessary for its functioning. Human probiotic microorganisms mainly belong to the genera: *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus* and *Enterococcus*. For Viscard et al. (2020), the ingestion of food with present living microorganisms will promote a direct gain to health, known as the probiotic effect, or indirect (intake of microbial metabolites), known as the prebiotic effect.

According to current legislation, the total bacterial count (TBC) in refrigerated raw milk must not exceed 900,000 CFU/mL in the tanker truck that arrives at the dairy factory (Brasil, 2018). For UHT milk, the maximum amount allowed is 100 CFU/mL of mesophilic aerobes (Brasil, 1997). Pasteurized milk does not have established TBC or mesophilic levels. The levels observed in this study are above the maximum limit allowed for UHT treated milk in both dairy factories, and even the sample of UHT treated milk from Dairy 2, with 303 sequences, has three times more microorganisms than the maximum allowed. Metagenomics is a different method from conventional microbiological methods, which allows a more specific analysis of the microbiota existing in milk. There is still no legislation for the data obtained by these methods, which delimit the maximum amount admitted for each species of microorganism.

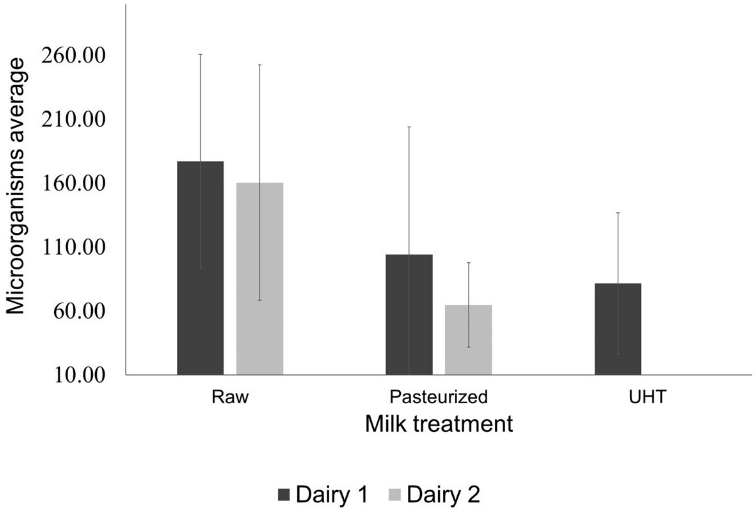

Figure 1: Average number of microorganisms found in samples of refrigerated raw milk, pasteurized milk and UHT treated milk in the dairy factories of Taquari Valley – RS. On the X axis are the types of milk analyzed in this study in the two dairy products (1 and 2) and on the Y axis is the average amount of microorganisms found in each type of milk.

According to Mariotto et al. (2020), the quality and shelf life of processed milk are directly related to the initial microbial load present in refrigerated raw milk that arrives at the dairy factory. According to Rosenberg (2020), the thermal processing of milk allows reaching commercial sterility. The quality and stability of UHT treated milk are affected by the storage and cooling conditions of the milk. In order to guarantee a quality product, it is necessary that the milk arrives at the dairy factory with a low somatic cell count (SCC) and a low count of psychrotrophic microorganisms.

The five main genera found in milk samples from the Taquari Valley dairy factories were: *Bacillus* (14,146), *Kurthia* (9,569), *Streptococcus* (9,222), *Enterobacter* (5,747), *Lysinibacillus* (3,530) and *Aeromonas* (1,776). Genera such as *Streptococcus*, *Staphylococcus* and *Aerococcus* may be related to milk from animals with mastitis. The genus *Bacillus* is composed of gram-positive psychrotrophic microorganisms, associated with spoilage, frequently reported as thermoduric and thermophilic sporulated microorganisms, which directly influence the shelf life of pasteurized milk (Ribeiro Junior et al., 2018).

The main genera in the refrigerated raw milk sample from Dairy 1 (D1-R) were: *Kurthia*, *Streptococcus*, *Lactococcus*, *Acinetobacter*, *Aeromonas*, *Bacillus*, *Providencia*, *Enterobacter*, *Escherichia*, *Enterococcus* and *Hafnia*. In pasteurized milk (D1-P) they were: *Bacillus* and *Acinetobacter* and in UHT treated milk (D2- UHT) they were: *Bacillus*, *Aeromonas*, *Kurthia*, *Streptococcus*, *Enterobacter* and *Viridibacillus*.

The main genera in the refrigerated raw milk sample from Dairy 2 (D2-R) were: *Enterobacter*, *Streptococcus* and *Lysinibacillus*. In pasteurized (D2-P) they were: *Streptococcus*, *Kurthia*, *Enterococcus*, *Aeromonas*, *Acinetobacter*, *Enterobacter* and *Lysinibacillus* and in UHT treated milk (D2- UHT) they were: *Acinetobacter*, *Lactococcus* and *Pseudomonas*.

Taponen et al. (2019) found great diversity in samples analyzed in their study. In addition, studies on the milk microbiota do not provide a minimum limit of sequences that must be observed for each species found in the analyses. Parente, Ricciardi and Zotta (2020) reported 25 genera found in milk samples, which include psychrotrophs such as *Acinetobacter*, *Chryseobacterium*, *Pseudomonas* and *Psychrobacter*, bacteria common to the intestinal microbiota of the genera *Atopostipes*, *Bacteroides*, *Romboutsia* and *Clostridium*, bacteria common to udders of animals such as *Staphylococcus*, *Aerococcus*, *Turicibacter*, *Streptococcus*, *Facklamia*, *Corynebacterium* and *Bacillus*, in addition to beneficial microorganisms of the genera *Lactobacillus*, *Streptococcus* and *Lactococcus*. Comparing data from different studies is difficult due to the lack of standard operating procedures, in addition to the absence of a well-structured database.

According to Ferrocino, Ranstsiou and Cocolin (2021), the central microbiota of dairy products is composed of *Lactococcus, Leuconostoc*, *Enterococcus* and *Streptococcis*. In smaller numbers, populations of pathogenic microorganisms occur, and there is a relationship with the state of animal health (e.g. mastitis) and the environment where the animals live (season, farm and temperature).

Considering the total number of species found in the samples, 50 species had a total incidence of less than 1% (51 sequences) of the total sequences (51,401), remaining 37 main species (Figure 2).

The main species of microorganisms found in raw milk from Dairy 1 (D1-R) were: *Kurthia gibsonii* (6, 907 sequences), *Lactococcus garvieae* (1, 298), *Streptococcus macedonicus* (1, 067), *Acinetobacter baumannii* (1,033), *Aeromonas hydrophila* (839), *Bacillus cereus group* (701) and *Streptococcus uberis* (164). These seven microorganisms add up to 12,000 sequences (78.06%), of the 15,384 sequences present in the sample. Another six microorganisms had sequence numbers between 100 and 150.

K. gibsonii represents 44.89% of the total sequences in the D1-R sample. This microorganism belongs to the phylum *Firmicutes*, being commonly found in decomposing organic matter. Ribeiro Junior et al. (2018) found *K. gibsonii* as one of the main spoilage psychrotrophic species in refrigerated raw milk samples. Gram-positive spoilage microorganisms are often related to the initial and desirable microbiota of milk and their presence is influenced by the environment, animal feed and possible infections of the mammary gland.

L. garvieae is a fish pathogen responsible for lactococcosis, a hemorrhagic septicemia with a high mortality rate and economic impact on fish farms around the world. This microorganism is also considered an emerging human pathogen, being found in the oral cavity, tonsils, rumen, intestines and feces of healthy and sick warm-blooded animals (Thiry et al., 2021). *L. garvieae* utilizes carbohydrates to produce lactic acid often found in refrigerated raw milk (Tariq et al., 2020).

According to Tarrah et al. (2018), *S. macedonicus*, the third main microorganism found in the D1-R sample, along with *Streptococcus thermophilus*, are two known species of the genus *Streptococcus* widely used as starter cultures to rapidly reduce the pH of foods, thus inhibiting the development of pathogenic microorganisms. In their study, *S. macedonicus* showed better growth rates under all pH conditions, indicating this species as a possible substitute for *S. thermophilus* in the production of certain foods. LABs of the *Streptococcus* genus, such as *S. macedonicus*, have the potential to be used as biofortified crops for human nutrition (Viscard et al., 2020).

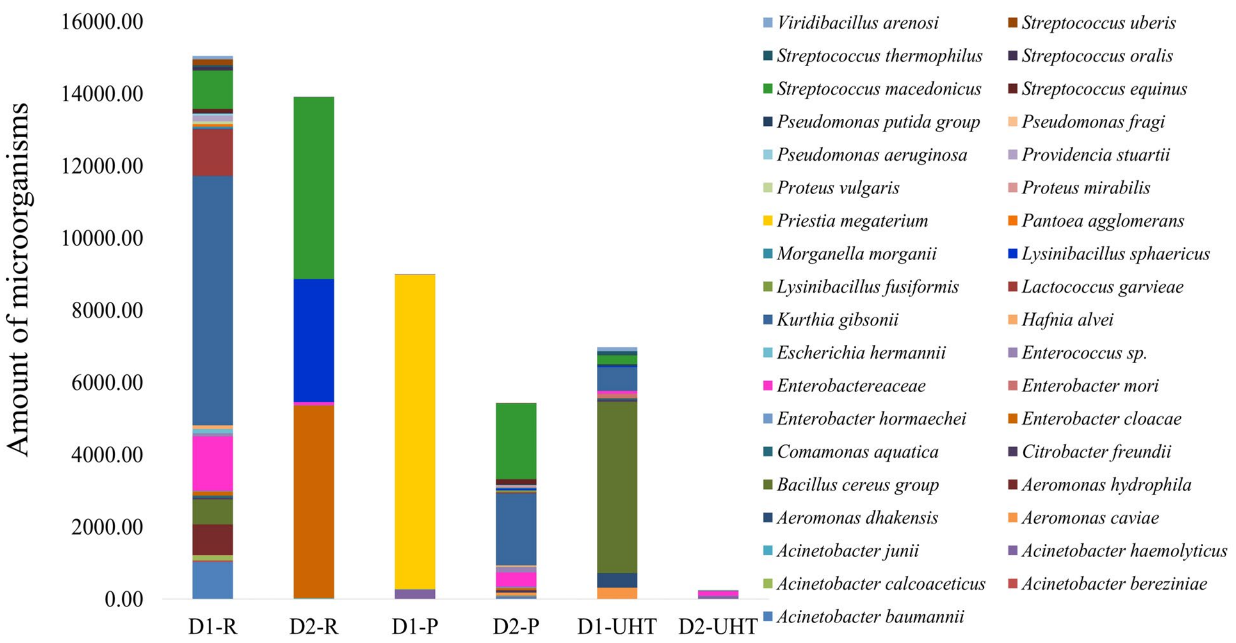

Figure 2: Main species and family *Enterobactereaceae* found in milk samples from dairy products in the Taquari Valley. D1-R: refrigerated raw milk sample from Dairy 1; D2-R: refrigerated raw milk sample from Dairy 2; D1-P: pasteurized milk sample from Dairy 1; D2-P: pasteurized milk sample from Dairy 2; D1-UHT: UHT treated milk sample from Dairy 1; D2-UHT: UHT treated milk sample from the Dairy 2.

A. baumannii is a species of the genus *Acinetobacter*, commonly found in the microbiota of skin and mouth. This bacterium is a gram-negative coccobacillus, being one of the most emerging species in the world. *A. baumanni* can be found in several foods such as fruit, vegetables and raw milk, as well as dairy products (Elbehiry et al., 2021). For Wareth et al. (2020), this microorganism causes a series of severe infections in the skin and soft tissues. *A. baumannii* was recently detected as one of the pathogenic bacteria of some fish species such as *Ictalurus punctatus* and *Channa striatus* (Malick et al., 2020). Numerous properties have ponds for fish farming, which may explain the presence of this microorganism in refrigerated raw milk.

B. cereus group is a psychrotrophic, gram-positive, facultative anaerobic microorganism, pathogenic in nature and frequently isolated from food. *B. cereus* is efficient in spore formation and these spores may have high heat resistance. This microorganism is commonly isolated in soil and food samples, and thermal processes, such as pasteurization, are not efficient enough to inactivate its spores. *B. cereus* can cause food poisoning that causes vomiting and diarrhea (Kwon et al., 2022). According to Rodrigues et al. (2017), raw milk is a source of endospores produced by mesophilic and thermophilic microorganisms and spore-forming psychrotrophic bacteria.

A. hydrophila is a gram-negative bacterium, also found in many aquatic environments, that can cause septicemia in humans. This microorganism is one of the main pathogens that can infect most farmed fish (Li et al., 2021). *S. uberis* is a gram-positive, mastitic, environmental pathogen commonly found in manure. This microorganism is cocci-shaped and occurs in pairs or chains. This bacterium has a membrane-bound special protein, which plays a central role in adherence to bovine mammary epithelial cells (Mihklepp et al., 2019).

K. gibsonii and *B. cereus group*, both psychrotrophic species, along with *S. macedonicus*, a probiotic species, represent 56.37% of the total microorganisms in the sample. According to Tilocca et al. (2020), raw milk has a microbiota with a high diversity of LAB and active microorganisms in the prevention of human pathogens. Raw milk also contains bacteriocins and antimicrobial compounds of bacterial origin, besides several antagonistic biomolecules active in preventing the growth of pathogenic microorganisms. However, pasteurization inactivates or reduces the available concentration of these compounds.

The main microorganisms found in pasteurized milk from Dairy 1 (D1-P) were *Priestia megaterium (8,696), Acinetobacter haemolyticus* (271) and *Pseudomonas fragi* (21). Another 14 microorganisms had an incidence lower than ten sequences. *P. megaterium* represents 96.06% of the microorganisms present in the sample (9,052 sequences in total), and this microorganism was restricted to the D1-P sample.

P. megaterium, formerly called *Bacillus megaterium*, is a gram-positive, endospore-forming microorganism found in seawater, soil, and dry foods. *Bacillus* species such as *P. megaterium* are commonly found in soils and are members of the microbiota of many plant hosts around the world. One of the most important characteristics of *Bacillus* strains is the ability to form spores, thus increasing their ability to resist to a wide range of stress conditions and enabling their application as plant growth-promoting bacteria (PGPB) (Nascimento et al., 2020). *P. megaterium* is restricted to the D1-P sample, and this is the second most abundant microorganism when observing the grand total of the six samples. Its presence in milk can be explained by the fact that it is a microorganism found in soil and food. The fact that it forms spores can explain its presence in pasteurized milk, resisting the thermal process. However, the first microorganism*, K. gibsonii*, with 9,569 sequences in total, was found in five of the six samples analyzed, being abundant in three. *P. megaterium* is restricted to the D1-P sample, and this is the second most abundant microorganism when observing the grand total of the six samples. However, the first microorganism, *K. gibsonii*, with 9,569 sequences in total, was found in five of the six samples analyzed, being abundant in three.

A. *haemolyticus*, present in smaller numbers, belongs to the genus *Acinetobacter*, which are emerging species as clinically relevant pathogens that can cause a wide range of infections. *Acinetobacter spp*. were previously known as environmental species, which have recently proved their potential to cause infection in humans (Malick et al., 2020).

P. fragi has an insignificant incidence in the sample, representing only 0.23% of the total sequences. This microorganism is a spoilage often found in kinds of meat, including beef, chicken, pork, lamb and fish. *P. fragi* forms biofilms under refrigerated temperature conditions used in food industries. Biofilm formation makes *P. fragi* able to survive to stressful environmental conditions (Wickramasinghe et al., 2017). Pinto Junior et al. (2017) reported in their study that thermostable peptidases produced by *P. fragi* resist to the UHT process, accelerating the milk deterioration and affecting the quality of cheese processed in dairy factories.

Pasteurized milk from Dairy 1 (D1-P), despite having a large number of microorganisms, does not represent a potential risk to consumer health, since almost all microbiological samples are composed of *P. megaterium*, a species not considered harmful.

The main microorganism found in the UHT treated milk of Dairy 1 (D1- UHT) was *B. cereus group* (4,743). Another seven microorganisms had sequence numbers above one hundred: *K. gibsonii* (661), *Aeromonas dhakensis* (400), *Aeromonas caviae* (321), *S. macedonicus* (253), *Enterobacter mori* (120) and *Viridibacillus arenosi* (113) and *S. thermophilus* (105). Considering the total number of microorganisms in the D1- UHT sample (7,090 sequences), *B. cereus group* represents 66.89%. The presence of *B. cereus group* and other spore-forming microorganisms in UHT milk is well known by the dairy industry. It is not clear whether these microorganisms are heat resistant, whether post-sterilization contamination occurs, or whether their presence is due to the formation of biofilms on equipment (Alonso; Oliveira; Kabuk, 2021).

A. dhakensis and *A. caviae* are *Aeromonas* species frequently associated with fish diseases (Azzam-Sayuti et al., 2021). According to Liang et al. (2021), A. *dhakensis* is a gram-negative waterborne pathogen that can cause gastroenteritis, being considered more virulent than other *Aeromonas* species. According to Sekizuka et al. (2019), *A. caviae* is one of the *Aeromonas* species most adapted to saline water environments, predominant in estuaries and which has often been isolated from environmental sources, such as sewage treatment plants.

E. mori is a microorganism of the *Enterobacter* genus, gram-negative, rod-shaped, non-spore-forming bacteria belonging to the *Enterobacteriaceae* family. This genus is widely distributed in nature and some species function as plant growth-promoting bacteria, while others are recognized as opportunistic pathogens causing several types of infections in humans. *E. mori* is a microorganism responsible for causing diseases in mulberry trees (Zhang et al., 2021).

V. arenosi is a spoilage spore-forming bacterium prevalent in milk (Sun; Atkinson; Zhu, 2021). According to Buehler et al. (2018), the genera *Bacillus* and *Paenibacillus* are the most common psychotolerants linked to the deterioration of dairy products, decreasing the shelf life of these foods. Members of the genera *Bacillus*, *Paenibacillus* and *Viridibacillus* have been isolated throughout the dairy production chain, including soil, silage, feed concentrate and milking equipment, in addition to raw milk and pasteurized milk. *V. arenosi* have the potential to produce enzymes that cause off-flavors and coagulate the final dairy product and therefore decrease the quality of the product.

S. thermophilus, as well as *S. macedonicus,* is used as a starter culture for the production of dairy foods. This microorganism is associated with other lactic bacteria of the genera *Streptococcus*, *Lactococcus* and *Lactobacillus* and is used for the production of fermented dairy products around the world. The proteolytic system of *S. thermophilus* is involved in the release peptide sequences of caseins and whey during lactic acid fermentation (Rodríguez-Serrano et al., 2018).

P. megaterium, found in pasteurized milk, and *B. cereus group* and *V. arenosi*, in UHT treated milk from Dairy 1, may have their presence explained because they are spore-forming species. According to Ryu et al. (2020), spores are common contaminants of food products, and their presence can cause food spoilage and even consumer illness. In addition, spores survive heat sterilization, used in dairy processing, and later form biofilms on dairy equipment, contaminating the final products. Microorganisms such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter jejuni* and *B. cereus group*, not only cause food poisoning, but also have a major influence on the acidification and spoilage of milk through the production of lipases and proteases. Milk producing properties are complex environments with a great diversity of microbial ecosystems and this has an overall impact on the microbial ecosystem found in milk.

The UHT treated milk sample from Dairy 1 (D1- UHT) showed a high number of microorganisms when compared to the total found in Dairy 2. Species considered beneficial, psychrotolerant or probiotic such as *S. thermophilus*, S. *macedonicus*, *K. gibsonii*, *E. mori* and *V. arenosi* together represent 17.65% of the total number of microorganisms. However, microorganisms considered harmful, such as *B. cereus group*, *Aeromonas dhakensis* and *Aeromonas caviae*, represent 77.06% of the sample. The presence of microorganisms that are not resistant to the thermal process in UHT milk may come from post-treatment contamination, or even with the laboratory material itself. This has already been reported in the existing literature (Elegbeleye; Buys, 2022). In addition, contamination of samples with bacterial DNA from laboratory utensils or sample collection is a well-known problem that has affected the results of studies with bovine milk samples (Ruegg, 2022).

The refrigerated raw milk from Dairy 2 (D2-R) basically presents three main microorganisms: *Enterobacter cloacae* (5,339), *S. macedonicus* (5,040), *Lysinibacillus sphaericus* (3,402), in fourth place comes *Acinetobacter junii* with only 29 sequences. The other microorganisms showed a number of sequences close to ten. From the total of sequences of microorganisms found in the sample, which was 13,950, the three main ones, *E. cloacae*, *S. macedonicus* and *L. sphaericus,* represent 38.27%, 36.12% and 24.38%, respectively, and 98.78% in total. There are two microorganisms in common with the raw milk sample from Dairy 1: *S. macedonicus* and *E. cloacae*, with 110 sequences in the D1-R sample.

E. cloacae is a member of the *Enterobacteriaceae* family. *Enterobacter* species are members of the ESKAPE group, initially described in 1960. There are 18 *Enterobacter* species. They are gram-negative, rod-shaped, generally motile, non-spore forming, oxidase-negative and facultative anaerobes. Foodborne *Enterobacter* contaminations have been found in pasteurized milk and dehydrated dairy products (Skinnader et al., 2022). Ibrahim, Saad and Hafiz (2021) reported finding several species of this family in powdered milk analyzed in their study, including *E. cloacae*. According to Odenthal, Akineden and Usleber (2016), who also found *E. cloacae* in their study of refrigerated raw milk in Germany, this is not necessarily a public health concern, as UHT heat treatment of milk safely inactivates members of the *Enterobacteriaceae* family.

Milk contamination by gram-negative microorganisms such as *A. baumannii* (D1-R), *A. haemolyticus* (L1-P) and *E. cloacae* (D2-R) is usually associated with a humid environment and with equipment and water used to clean the milk collection and storage systems (Ribeiro Junior et al., 2018).

L. sphaericus is a gram-positive, mesophilic microorganism, generally found in soil. This microorganism has been studied as an alternative for the control of the *Culex quinquefasciatus* mosquito (Guo et al., 2021). A. *junii* along with *Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter pittii* were recorded as nosocomial pathogens. *A. junii* is a microorganism found in different natural aquatic environments and is responsible for causing septicemia in humans, being also found in studies with sick fish (Malick et al., 2020). However, the incidence of this microorganism in the sample is not relevant, representing only 0.20%.

The refrigerated raw milk sample from Dairy 1 (D1-R) has the highest species diversity, with sixty-two different species, and the refrigerated raw milk sample from Dairy 2 (D2-R) has the lowest species diversity, with only seventeen different species. According to Metzger et al. (2018), the microbiota of healthy milk usually has greater richness and diversity than the microbiota of milk from glands with mastitis, even in mammary glands from the same cow. A study of teat microbiota, in which researchers collected the first milk samples and teat canal swabs, revealed that diversity was greater also in samples from healthy quarters, who had never had clinical experience of mastitis, compared to quarters that had any case of clinical mastitis. These studies cannot yet determine whether changes in the microbiota precede the development of mastitis, or whether the influx of leukocytes into the mammary gland causes changes in the microbiota.

Pasteurized milk from Dairy 2 (D2-P) showed the microorganisms *S. macedonicus* (2091), *K. gibsonii* (1992), members of the *Enterobacteriaceae* family (392), *Streptococcus equinus* (151) and *Enterococcus sp*. (150) in their main microbiota, and another twenty-five microorganisms had sequence numbers lower than 80 and higher than ten. *S. macedonicus* represents 37.19% of the microorganisms present in the sample (5,622) and *K. gibsonni* represents 35.43%.

The microorganisms belonging to the *Enterobacteriaceae* family are gram-negative, non-spore-forming bacilli and are related to aspects such as the deterioration of various foods. Members of this family most commonly found in dairy products include *Escherichia*, *Salmonella*, *Shigella*, *Yersinia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Edwardsiella*, *Erwinia*, *Morganella*, and *Providencia* (Singh; Anand, 2022).

S. equinus, synonymous of *Streptococcus bovis* and currently recognized as *S. bovis/S.equine* complex (SBSEC), is commonly found in the digestive tract of humans and ruminants. SBSEC produce lactic acid when growing rapidly with amounts of non-fibrous carbohydrates and are considered important agents of rumen acidosis (Park et al., 2021). For Metzger et al. (2018), the presence of bacterial DNA in feces and milk cannot confirm that bacterial DNA is entering the mammary gland from the intestine. DNA can also enter the mammary gland through the teat canal, coming from the animal's environment. The udder can be exposed to fecal bacteria when the cow is lying down, whereas the teat skin bacteria count is higher in cows with dirty legs and udders.

Ding et al. (2020), in their study with pasteurized milk, found *Omithimimicrobium*, *Pseudomonas*, *Cyanobacteria* and *Enterococcus* as the main genera. These genera positively modified the sensorial characteristics of the milk. *Streptococcus* and *Paeniclostridium*, also present in milk, have been shown to negatively modify these characteristics.

The UHT treated milk from Dairy 2 (D2- UHT) presented as main: the *Enterobacteraceae* family with 140 sequences and *Acinetobacter haemolyticus* with 83 sequences, respectively 46.20% and 27.39% of the total sequences in the sample (303). *S. macedonicus* and *Pseudomonas azotoformans* appear in third place with only 8 sequences. Another twenty microorganisms appear with sequence numbers lower than eight. In the literature there is a predominance of analysis of the microbiota in refrigerated raw milk. Few studies address the microbiota of pasteurized or treated UHT milk , mainly at the species level. Cremonesi et al. (2021), report a natural microbiota in UHT milk composed of the genera *Acinetobacter*, *Chryseobacterium, Enterobacter*, *Macrococcus*, *Staphylococcus*, *Pseudomonas* and *Klebsiella*.

By analyzing harmful microorganisms found in refrigerated raw milk from Dairy 1 and their prevalence in processed milks, it is possible to verify that UHT processing is inefficient for the elimination of species of the *Enterobacteriaceae* family and microorganisms such as *B. cereus group* and *A. dhakensis*. Regarding Dairy 2, processing proved to be inefficient in eliminating *Enterobacteriaceae*, *A. haemolyticus* and *S. equinus*.

Understanding the bovine milk microbiota lacks parameters established by current legislation. Thus, the data observed in the studies become difficult to interpret. Another important aspect is that the microorganisms present in the samples may come from bacterial contamination of collection utensils or laboratory materials. In addition, the DNA present in the samples may originate from inactive microorganisms, wholly or partially.

CONCLUSIONS

Milk from Dairy 2 demonstrated better quality than milk from Dairy 1. Lactic acid bacteria such as *S. macedonicus* were found in refrigerated and pasteurized raw milk and *S. thermophilus* was found in UHT-treated milk. Psychrotrophic species such as *K. gibsonni* and the *B. cereus group* form a large part of the microbiota of the three types of milk. However, harmful species such as *B. cereus*, *A. dhakensis* and *A. haemolyticus* group have been found in processed milk.

AUTHOR CONTRIBUTION

Conceptual Idea: Müller, T.; Rempel, C.; Maciel, M.J.; Methodology design: Müller, T.; Maciel, M.J.; Data collection: Müller, T., Data analysis and interpretation: Müller, T.; Rempel, C.; Maciel, M.J. and Writing and editing: Müller, T.; Rempel, C.; Maciel, M.J.

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