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MICROBIOLOGY

Bacterial communities in artisanal raw bovine milk cheeses from the southern region of Brazil

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ABSTRACT: The production of artisanal cheeses made with raw bovine milk has grown in the southern region of Brazil. It is important to obtain information about the risks of this practice, especially concerning food safety. In this study, next-generation sequencing was used to identify and characterize the bacterial communities of artisanal raw milk cheeses. We analyzed one pool of five raw milk samples (control group M1) from different dairy farms and nine pools (M2-M10) of 45 artisanal raw milk cheeses.The characterization of the bacterial communities included 199 species distributed across 59 different genera dispersed among the samples. Among the genera observed, 11 were classified as beneficial to the aroma, flavour, colour, and texture of the cheese. Thirty-one genera were classified as harmful to these characteristics. Another 17 were classified as potential pathogens for animals and humans, including *Aeromonas*, *Bacillus*, *Cronobacter*, *Salmonella*, *Staphylococcus*, and bacteria of the coliform group, including *E. coli* and *Klebsiella*. There was a significant difference ($P < 0.05$) in the number of bacterial communities identified between the control group (M1) and the two pools of artisanal raw milk cheeses (M2 and M8). This study demonstrated that next-generation sequencing provides in-depth information on the composition of the microbiota in artisanal raw milk cheeses, characterizing bacterial communities, identifying the wide microbial diversity, and identifying microbial benefits and risks.
Key words: bacterial communities, raw milk, artisanal cheese, dairy products, NGS.

Comunidades bacterianas em queijos artesanais de leite bovino cru da região sul do Brasil

RESUMO: Devido ao aumento da produção de queijos artesanais com leite bovino cru na região sul do Brasil, é importante obter informações sobre os riscos desta prática, principalmente no que se refere à segurança do alimento. Neste estudo foi utilizada a técnica de *Next Generation Sequencing* (NGS) para identificar e caracterizar comunidades bacterianas de queijos artesanais de leite cru. Foram analisados um pool de cinco amostras de leite cru como grupo controle (M1) de diferentes propriedades leiteiras localizadas na região norte do estado do Rio Grande do Sul, e nove pools (M2-M10) de 45 queijos artesanais de leite cru. A caracterização das comunidades bacterianas incluiu 199 espécies distribuídas em 59 gêneros diferentes dispersos entre as amostras. Dentre os gêneros observados, 11 foram classificados como benéficos ao aroma, sabor, cor e textura do queijo, enquanto 31 gêneros foram classificados como prejudiciais a essas características. Outros 17 foram classificados como potenciais patogênicos para animais e humanos, incluindo *Aeromonas, Bacillus, Cronobacter, Salmonella, Staphylococcus*, bactérias do grupo coliforme como *Escherichia coli e Klebsiella*. Houve diferença significativa ($P < 0,05$) entre o número de comunidades bacterianas identificadas no grupo controle (M1) e dois *pools* de queijos artesanais de leite cru (M2 e M8). Este estudo demonstra que o NGS fornece informações detalhadas sobre a composição da microbiota em queijos artesanais de leite cru, caracterizando comunidades bacterianas, identificando a ampla diversidade microbiana, os benefícios e riscos microbianos.
Palavras-chave: comunidades bacterianas, leite cru, queijo artesanal, laticínios, NGS.

INTRODUCTION

The consumption of dairy products, such as cheese, is an old tradition linked to livestock farming, since dairy products are made using ancient artisanal processes. Artisanal cheese is made from raw milk and is the main cheese produced by rural families on dairy farms in the southern region of Brazil, especially in Rio Grande do Sul (BRAZIL, 2013). The specific composition of the milk microbiota and diversity of bacterial communities directly affect the subsequent development of dairy products. In fermented and mature dairy products, such as artisanal cheese, the presence of beneficial bacteria, such as the genera *Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus*, is necessary for cheese manufacture

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and technological and organoleptic characteristics (KAMIMURA et al., 2019). The bacterial community present in raw milk is responsible for the fermentation and characteristics of consistency, texture, aroma, and flavour of artisanal cheeses. However, when milk cows do not have proper sanitary conditions, there is no guarantee of quality and safety of the raw milk. Thus, artisanal cheeses produced may pose a risk to consumer health (CRUZ & MENASCHE, 2014).

Cheese contamination can occur at numerous stages in the manufacturing process, including the use of improperly pasteurized or raw milk. Good hygiene practices when obtaining and storing raw milk, as well as the manufacture of dairy products, are crucial for the contamination, survival, persistence, and multiplication of bacteria and toxins (VELÁZQUEZ-ORDOÑEZ et al., 2019). *Bacillus cereus*, *Clostridium botulinum*, and *Staphylococcus aureus* are examples of bacteria that produce toxins that threaten human health when consumed and are resistant to pasteurization (ROSENGREN et al., 2010). In addition, pathogenic bacteria, such as *Salmonella* spp., *Listeria* spp., *Brucella* spp., and *Mycobacterium* spp., may also be present in raw milk. Consumption of contaminated raw milk and artisanal cheeses with these pathogens may lead to severe diseases (ROCHA et al., 2014).

Recently, several studies have characterized the microbial populations and properties of dairy products, such as artisanal raw milk cheeses, using next-generation sequencing (NGS) methods. In a study conducted in Mexico, researchers used NGS of 16S rRNA amplicons to investigate the bacterial communities of 26 samples of the artisanal Adobera cheese from Los Altos de Jalisco. The authors reported 15 phyla, 30 classes, 72 orders, 180 families, and approximately 400 bacterial genera (RUVALCABA-GÓMEZ et al., 2021). Other researchers evaluated microbial communities in 137 different cheeses collected in 10 countries and identified 24 genera of bacteria and fungi that were dominant in these communities (WOLFE et al., 2014).

This study was to identified and characterized bacterial communities in artisanal raw milk cheeses from a region of southern Brazil using NGS.

MATERIALS AND METHODS

Sample collection

Raw bovine milk samples were collected randomly from different dairy farms located within a 100 km radius of the city of Passo Fundo, in the northern region of the state of Rio Grande do Sul, southern Brazil, in November 2018. All dairy farms included in this study met the legal requirements for sanitary control of the herd and observed good practices for obtaining and storing milk.

A pool of five raw bovine milk samples collected directly from the cooling tanks after milking all the cows formed the control group (M1). The others nine pools of 45 purchased artisanal cheeses based on raw milk (M2, M3, M4, M5, M6, M7, M8, M9, and M10). Cheeses were traded illegally at informal fairs, busy highways, and unsupervised urban markets. The cheeses were purchased in packaging provided by the seller. They were transported to the microbiology laboratory of the UPF Institute of Biological Sciences, where they were unpacked in a laminar flow chamber for evaluation by the researchers. Samples were collected from each unit. Samples were placed in sterile plastic bags, kept refrigerated (4-8 ºC), and then sent under refrigeration (7-10 ºC) for metagenomic analysis.

Visual inspection of cheeses and sample preparation

The cheeses were evaluated visually for external and internal aspects, such as shape pattern, rind type, ripeness, internal mass, and number and size of eyes. Then, the samples were combined into one pool of five raw milk samples with 10 mL of each raw milk sample, and nine pools of 45 artisanal raw milk cheese samples with 20 g of each cheese. All samples were placed in sterile plastic bags and refrigerated (4-8 ºC) and then sent under refrigeration (7-10 ºC) for metagenomic analysis.

DNA extraction and NGS of 16S rRNA amplicons

A strategy of pooling DNA samples was used to reduce the sample numbers for the characterization of bacterial communities. DNA was obtained using a magnetic bead methodology from Neoprospecta Microbiome Technologies, Inc. (Florianopolis, Brazil). The dsDNA BR assay kit (Invitrogen, Waltham, MA, USA) was used to quantify DNA within each pool, in accordance with the manufacturer's instructions. To prepare the pools, 2–5 µL of stock DNA or 1:5 diluted (vol/vol) DNA was added to 195–198 µL of Qubit® working solution to produce a final volume of 200 µL. This volume and a working solution were then incubated at room temperature for at least 2 min before quantitation was performed using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

After quantification, the DNA was diluted to 0.5 ng/µL and stored at -20 ºC for molecular analysis. The bacteria were identified by high-

throughput sequencing of the 16S rRNA V3/V4 region using a proprietary protocol by Neoprospecta Microbiome Technologies, Inc. Amplification of the 16S rRNA V3/V4 region was performed using 341F (CCTACGGGRSGCAGCAG) (WANG & QIAN, 2009) and 806R (GGACTACHVGGGTWTCTAAT) (CAPORASO et al., 2012) primers.

PCR was performed in triplicate using Platinum Taq DNA (Invitrogen). The product of the final PCR was purified using AMPureXP beads (Beckman Coulter, Brea, CA, USA). The library was then generated by pooling 200 ng of each sample and was quantified using Picogreen dsDNA (Invitrogen). The assembled libraries were diluted for accurate quantification by qPCR using a platform quantization kit and a library quantification kits for sequencing on Illumina platforms (KAPA Biosystems, Wilmington, MA, USA). The 16S rRNA libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., San Diego, CA, USA) with the V2 kit using 300 cycles and paired-end sequencing for fragments of at least 283 bp, with a minimum coverage of 50.000 reads.

Bioinformatics analysis

The sequences were analyzed using a proprietary pipeline by Neoprospecta Microbiome Technologies, Inc. All initial sequences were checked and submitted toquality filtering and trimming adaptorsequencesusing *FastQC* (ANDREWS,2010) and Trimmomatic (BOLGERet al., 2014), based on the sum of the DNA base probability errors, allowing a maximum of 1% of accumulated errors. Subsequently, the DNA sequences corresponding to Illumina adapters were removed. The resulting sequences that presented 99% identity were clustered and used for taxonomic identification using an accurate 16S rRNA sequence database (Neobiome, Neoprospecta Microbiome Technologies, Inc.).

Statistical analyses

The Excel 2010 program was used for tabulation, analysis, and graph-making, while the GraphPad InStat 3 program was used for statistical analysis. Normally distributed data were analyzedusing one-way ANOVA (F value), Dunnett's test for multiple comparisons, and the Kruskal-Wallis test (H value) to address the significance of differences in the dispersion of bacterial communities between the control group mean values (raw milk) and artisanal cheese mean values with significance set at $P \leq 0.05$. In the presentation of the results, the bacterial communities were characterized and divided into three groups of interest: bacteria beneficial to milk production, bacteria harmful to milk production, and bacteria with pathogenic potential.

RESULTS AND DISCUSSION

Of the total samples, 32 (71.1%) of the cheeses had a circular shape and 13 (28.9%) had a rectangular shape. Concerning maturation, 25 artisanal raw milk cheese samples (55.5%) had a cured visual aspect, while 20 samples (44.5%) had a half-cure or freshness aspect. Internally, all samples differed from one another, presenting a soft to extremely compact and brittle mass, with eyes varying from few and small to numerous and medium. All cheeses based on raw milk were being marketed at informal fairs, busy highways and unsupervised urban markets without any protective packaging and at room temperature.

In addition, it is common for informal artisanal raw milk cheeses made from low-quality raw milk to present various visual and sensory defects in a rudimentary way. According to SOBRAL et al. (2017), most cheese defects may be related to the origin and quality of the milk used in manufacturing, the quality and quantity of the ingredients used, and the manufacturing and maturation techniques. Wood is one of the materials used in the manufacture of artisanal foods, such as casks for fermentation of alcoholic beverages, vats, moulds, shelves, and benches for cheese production. These surfaces have a porous structure that allows the development of microbial communities known as biofilms, which are composed of filamentous fungi, yeasts, and bacteria. Biofilms are probably responsible for the peculiar characteristics of artisanal cheeses, such as their characteristic flavour and smell, as well as cheese defects or possible foodborne diseases (PEREIRA et al., 2014).

NGS data revealed the presence of 331,280 bacterial DNA sequences spread among the pools of raw milk and cheese samples.The characterization of bacterial communities included 199 species distributed in 59 different genera dispersed in ten pools (M1-M10). M1 is the control group and M2-M10 are each artisanal raw milk cheese analyzed (Figure 1).

In terms of the relative abundanceof bacterial communities (Figure 2), lactic acid bacteria, such as *Streptococcus* and *Lactococcus*, predominated in all pools. These bacteria are beneficial for production technology (Figure 2a). However, 31 genera of bacteria that are harmful to the production technology were also identified (Figure 2b). Another 17 genera of bacteria with pathogenic potential for animals and humans were identified (Figure 2c), including *Salmonella*, *Staphylococcus*, *Aeromonas*,

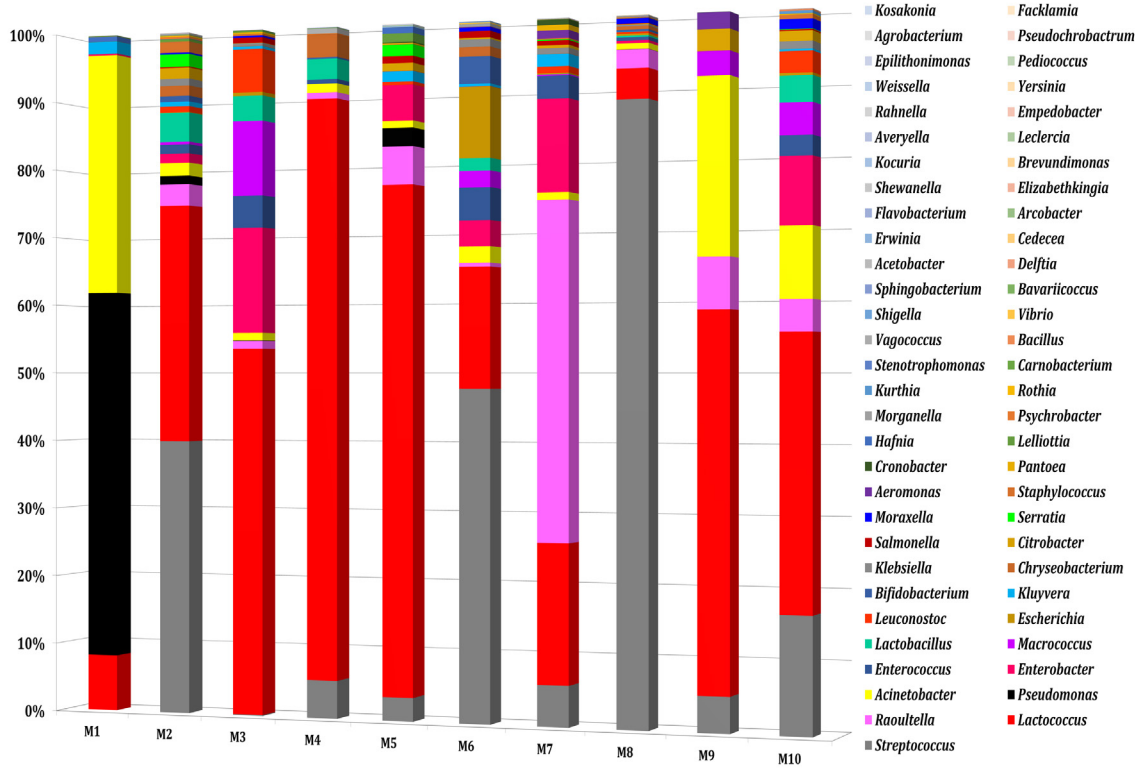

Figure 1 - According to *Next Generation Sequencing* (NGS) data, 199 species were identified distributed in 59 different genera dispersed in ten pools (M1-M10) by Neobiome Neoprospecta Microbiome Technologies, Inc. (Florianópolis, Brazil).

Cronobacter, and *Bacillus*, as well as the coliforms *Escherichia* and *Klebsiella*.

Among the genera of bacteria identified, *Acinetobacter*, *Lactococcus*, *Raoultella,* and *Streptococcus* occurred in 100% of the artisanal raw milk cheeses. *Chryseobacterium*, *Citrobacter*, *Enterococcus*, *Enterobacter*, *Lactobacillus, Leuconostoc,* and *Staphylococcus* were present in at least 80% of the evaluated cheeses.

CRUVINEL et al. (2017), JONNALA et al. (2018), and KAMIMURA et al. (2019) examined microbial populations of artisanal cheeses based on raw milk. The studies were performed in several countries, especially on the European continent, the birthplace of many famous cheese recipes. Moreover, to understand and describe the microbiome present in dairy products, studies have sought to investigate whether there is a relationship between microbial diversity and the processes of milk and cheese production, as well as seasonality or geographical and climatic aspects.

In the present study, the number of bacterial communities identified in the control group (M1) was compared to those identified in each pool of cheese evaluated (M2-M10). Dunnett's and Kruskal-Wallis tests revealed a significant difference ($P < 0.05$) only between the number of bacterial communitiesreported in the control group (M1- raw milk) and two pools of cheeses analyzed (M2 and M8) (Figure 3). Thus, biologically, cheeses featured populations rich in bacteria comprising the microbiome.

Regarding the number of bacterial communities considered beneficial for milk production technology, 11genera of bacteriawere identified. These included *Bifidobacterium*, *Carnobacterium*, *Chryseobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Psychrobacter*, *Streptococcus*, *Vagococcus*, and *Weissella*. Some microbial communities beneficial to cheese production, such as *Lactococcus* and *Lactobacillus*, have been developed to produce the preferred flavour, texture, aroma, and colour in the products in which they are used (CHEN et al., 2017). *Streptococcus thermophilus* represents the only species among the streptococci that has "Generally Regarded as Safe" status and has

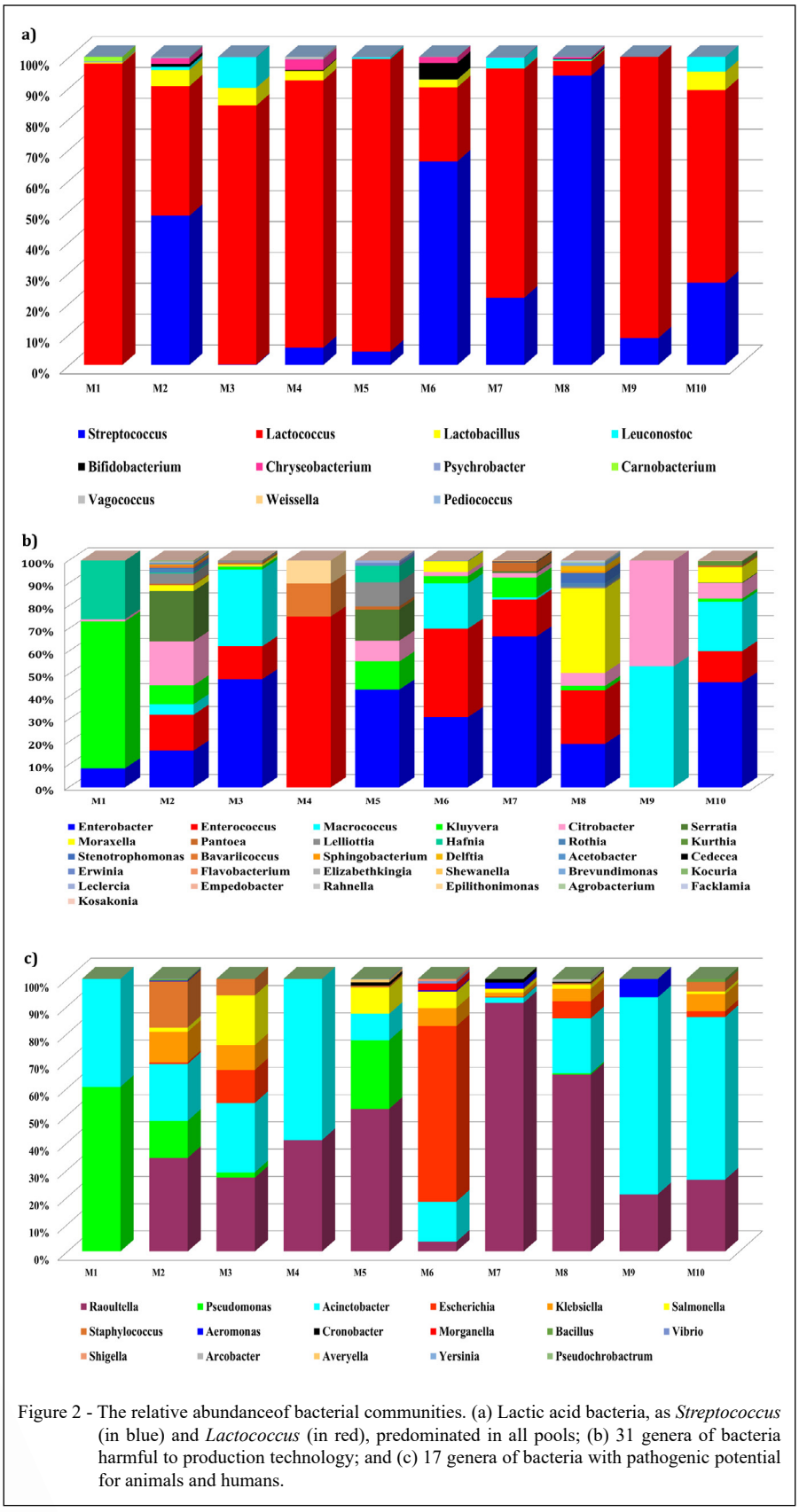

Figure 2 - The relative abundanceof bacterial communities. (a) Lactic acid bacteria, as *Streptococcus* (in blue) and *Lactococcus* (in red), predominated in all pools; (b) 31 genera of bacteria harmful to production technology; and (c) 17 genera of bacteria with pathogenic potential for animals and humans.

an economically important role in the fermentation of yogurt and cheeses with the use of commercial starter cultures, an integral part of a successful production of any fermented product (GOH et al., 2011). The term starter, or initiator, is used in dairy technology because these bacteria start the rapid production of acids in the environment in which they are inserted.

Lactobacillus delbrueckii ssp. *bulgaricus* and *Bifidobacterium animalis* ssp. *lactis* are lactic acid-producing bacteria that are widely used in the dairy industry, notably in cheese and yoghurt production. These bacteria grow slowly in milk because they lack essential proteolytic activity; therefore, they are usually combined with *Streptococcus thermophilus* (CASAROTTI et al., 2014).

Among the group of bacteria considered harmful to milk production technology, we observed that the genera with the greatest dispersion were *Raoultella*, *Pseudomonas*, *Enterobacter*, *Enterococcus*, *Macrococcus*, *Kluyvera*, *Citrobacter,* and *Serratia*, and 23 other genera.Although, metagenomic analysis has been performed with different fermented products, cheese provides new and substantial technological insights into the microbiome, which can be applied to further improve cheese production. A recent report from DERAKHSHANI et al. (2020) demonstrated that the diversity of bacteria found in raw milk and cheese comes from the skin and mammary glands of cows, including some of the genera found in the present study, and that some are is harmful for cheese making. However, bacteria can also contaminate milk because of the lack of good practices in obtaining and cooling the milk, as well as the lack of health of the animals and poor hygiene of the equipment and utensils. Arguably, this is one of the most diverse metagenomes among the microbial communities related to fermented products.

REMOR et al. (2021) analyzed the microbial genome sequences of 12 brands of colonial-type cheese in the state of Rio Grande do Sul and showed that *Pseudomonas* occurred in 100% of the evaluated samples, with the highest median among all identified microorganisms. When present in milk, harmful bacteria, such as *Pseudomonas*, can deleteriously affect the appearance, smell, and taste of milk and cheese, depending on their proportion in the microbial community. As a result, dairy products containing harmful microorganisms have reduced shelf life. Poor hygiene practices and poor cleanliness of procedure equipment, surrounding air in the milk parlour, as well as other environmental factors, including housing conditions and water supply, have an important effects on milk contamination with harmful bacteria (SAMARŽIJA et al., 2012; VELÁZQUEZ-ORDOÑEZ et al., 2019).

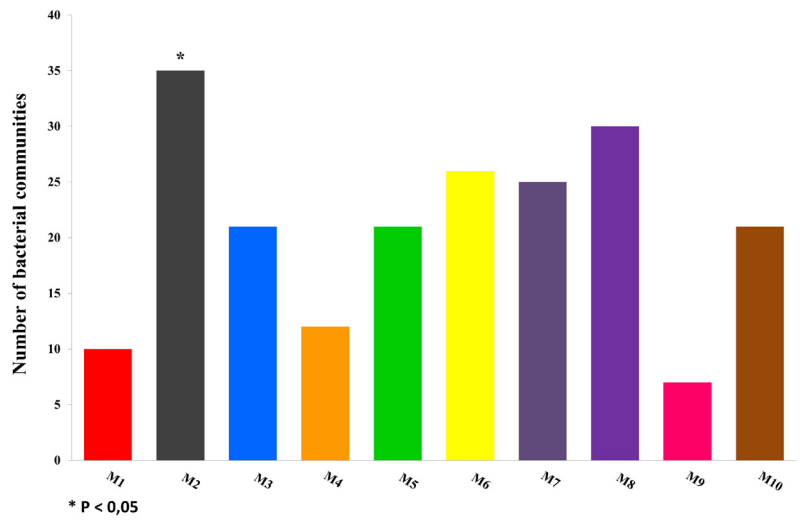

Figure 3 - Number of bacterial communities identified in the control group (M1) compared with each pool of cheese (M2-M10). Only the number of bacterial communities found in M2 (in black) and M8 (in purple) showed a significant difference (P <0.05) between the number of bacterial communities found in the control group (M1). All data were analyzed using the one-way ANOVA, Dunnett's test and the Kruskal-Wallis test.

Among the group of bacteria with pathogenic potential for human and animal health, the genera identified in the samples examined were *Acinetobacter*, *Aeromonas*, *Bacillus*, *Cronobacter*, *Escherichia*, *Klebsiella*, *Moraxella*, *Pantoea*, *Salmonella*, *Staphylococcus*, and *Vibrio*. However, to infect an animal, pathogenic bacteria must overcome both nonspecific and specific defense mechanisms of the host. Thus, bacteria penetrate more frequently through the teat canal and rarely enter the lymphatic or circulatory systems (BENIĆ et al., 2018). In Brazil, *Escherichia coli* is considered the most common bacterium of environmental origin, described in clinical mastitis in dairy cows (RIBEIRO et al., 2008), and *Klebsiella*, a common cause of clinical mastitis, can cause severe clinical symptoms.

Regardless of how often pathogenic bacteria are present in milk or cheese, they can severely affect human health. The genus *Klebsiella* includes coliforms originating from faecal contamination and other environmental sources (MARTIN et al., 2016). Moreover, *Klebsiella* spp. represent also a significant public health problem worldwide, being among the most common causes of both hospital and community-associated infections due to their virulence factors and/or antibiotic multiresistance (GELBÍČOVÁ et al., 2021).

Bacterial toxins, such as those produced by *Staphylococcus*, *Bacillus*, *Escherichia coli*, and *Clostridium*, are food safety hazards and represent a major threat to consumer health (RAJKOVIC et al., 2020). Annual reports of the European Food Safety Authority (EFSA) show that "bacterial toxins other than *Clostridium botulinum*", including *Bacillus cereus*, generally account for 16-20% of food poisoning outbreaks, behind *Salmonella* and viruses (EFSA & ECDC, 2016).

Consumption of cheese has historically been implicated in outbreaks. BIANCHI et al. (2013) concluded that unpasteurized milk can be a vehicle for a variety of microorganisms, and that outbreaks related to cheeses made with unpasteurized milk are also common. *Staphylococcus aureus*, *Salmonella* spp., and *L. monocytogenes* are the most frequent potential pathogens associated with milk or dairy products in several countries (JAKOBSEN et al., 2011). In previous studies with dairy products, plaited cheese was positive for *Cronobacter* spp. This bacterium may cause serious health problems and even death in newborns, children, and the elderly (AKSU et al., 2019). Consistent with the results of our study, the *Salmonella* and *Cronobacter* genera have also been identified in artisanal raw milk cheese samples, which demonstrated the potential health risks associated with the consumption of these products.

Although, lactic acid bacteria predominated in all pools analyzed and were beneficial to the cheesemaking process,advances are necessary to reduce microbial hazards in food safety. The dairy industry still faces important challenges, including strategies for the safety of artisanal raw milk cheeses and the development of pre- and post-processing contamination monitoring and control programs for pathogenic, spore forming, and spoilage bacteria.

CONCLUSION

Although, lactic acid bacteria predominated in all pools analyzed and were beneficial to the cheesemaking process,advances are necessary to reduce microbial hazards in food safety. Therefore, the dairy industry still faces important challenges, including strategies for the safety of artisanal raw milk cheeses and the development of pre- and post-processing contamination monitoring and control programs for pathogenic, spore forming, and spoilage bacteria.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analysis, or interpretation of the data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All the authors contributed equally to the manuscript.

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