Original Article

Microbial diversity in artisanal cheese produced and commercialized in Vale do Taquari in southern Brazil

Diversidade microbiana encontrada em queijos artesanais produzidos e comercializados no Vale do Taquari no sul do Brasil

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Abstract

Researchers have been utilizing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify bacteria and fungi directly from isolates obtained on culture plates; the resulting mass spectrum is then compared with spectra stored in the instrument software. Hence, a fast analytical response is obtained, and the more spectra are known to compare, the safer and more reliable the interpretation of the method will be. Thus, this study sought to identify the diversity of strains found in 10 samples of artisan cheese produced and commercialized in Vale do Taquari (Rio Grande do Sul State, Brazil) using MALDI-TOF MS. From the analyzed cheese, 22 strains were identified at the species level; one sample presented the pathogen *Staphylococcus aureus*, two showed the presence of lactic acid bacteria (*Lactococcus lactis*), and the vast majority (68.18%) of strains were composed of species of the *Enterobacteriaceae* family, with the prevalence of the genera *Escherichia*, *Enterobacter*, and *Klebsiella*. *Escherichia coli* was present in 50% of the samples analyzed. This demonstrates the need for greater control during all stages of artisanal cheese production and evaluation of the raw material, including safe practices during milking, so that the product meets the identity and quality parameters suitable for human consumption.

Keywords: cheese, identification, microbiota, mass spectrometry, lactic acid bacteria.

Resumo

MALDI-TOF MS vendo sendo utilizado em laboratórios para identificar bactérias e fungos diretamente de isolados obtidos em placas de cultura. O espectro de massa resultante é então comparado com espectros armazenados no software do equipamento. Assim, obtém-se uma resposta analítica rápida, sendo que, quanto mais espectros forem conhecidos para comparar, mais seguro e confiável será a interpretação do método. Desta maneira, o presente trabalho teve por objetivo, identificar por MALDI-TOF MS, a diversidade de cepas encontrados em 10 amostras de queijos artesanais produzidos e comercializados no Vale do Taquari, Rio Grande do Sul, Brasil. Dos queijos analisados, 22 cepas foram identificadas a nível de espécie, sendo uma (1) amostra apresentou o patógeno Staphylococcus aureus, duas a presença da bactéria ácido láctica (Lactococcus lactis) e a grande maioria (68,18%) das cepas era composta por espécies da família Enterobacteriaceae, com prevalência dos gêneros Escherichia, Enterobacter e Klebsiella. E. coli estava presente em 50% das amostras analisadas. Isso demonstra a necessidade de um maior controle durante todas as etapas de produção do queijo artesanal, bem como a avaliação da matéria-prima, incluindo práticas seguras durante a ordenha para que o produto atenda aos parâmetros de identidade e qualidade, sendo apto ao consumo humano.

Palavras-chave: queijo, identificação, microbiota, espectrometria de massas, bactérias ácido lácticas.

1. Introduction

The diversity of microorganisms and continuous discoveries in different areas of science have been the motivators for developing new methods for microbial identification (Nacef et al., 2017). In this context, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used to determine the genus, species, and even subspecies of bacterial isolates. In addition to being fast, this method requires less biological material and involves simpler preparation protocols than conventional molecular methods (Braga et al., 2018). In Brazil, few laboratories offer identification services using this technology. In addition to bacteria,

it also enables other microorganisms to be identified, including yeasts and molds isolated from various sources. Identification is performed by comparing the resulting mass spectra of the samples with a corresponding reference database (Quintela-Baluja et al., 2014; Rau et al., 2020). Researchers have been utilizing MALDI-TOF MS to identify species in milk (Barreiro et al., 2018; Nicolaou et al., 2012), dairy products (Rau et al., 2020; Kim et al., 2012; Lappa et al., 2021), among other food matrices.

The establishment of standards regulating the production and marketing of Brazilian artisanal cheese has stimulated small farmers regarding this activity (Camargo et al., 2021). For instance, in Rio Grande do Sul State in southern Brazil, Law no. 15615 regulates artisanal cheese produced with raw milk (Rio Grande do Sul, 2021). Artisanal cheese is defined as cheese made with raw milk from the farm itself using traditional methods, provided that it meets all relevant sanitary standards. Developing artisanal cheese from raw milk is restricted to cheese dairies located in rural establishments certified as free from tuberculosis and brucellosis; these facilities must implement good milking and manufacturing practices, control water potability, and guarantee care during commercialization, transportation, and registration of the product.

Siqueira et al. (2021) evaluated the hygiene conditions of equipment, workers' hands, and raw and pasteurized milk in goat dairies in the Cariri region (Paraíba State, northeastern Brazil). The researchers collected 32 samples from four dairies and analyzed mesophiles, total and thermotolerant coliforms, *Escherichia coli*, *Staphylococcus aureus*, *Samonella* spp., and *Listeria monocytogenes* in the receiving and pasteurization tanks, packaging machine, packaging, walls, workers' hand, and raw and pasteurized milk from each dairy. After isolation, 84 colonies were confirmed by MALDI-TOF MS, and the microorganisms of the family *Enterobacteriaceae* were the most frequent (77.38%), and within this family, *Escherichia coli*, *Klebsiella* spp., and *Enterobacter spp.* Were the most prevalent; Gram-positive bacteria accounted for 22.62% and consisted of *Bacillus* spp., *Staphylococcus* spp., *Enterococcus spp.*, and *Macrococcus caseolyticus*. The researchers did not detect *L. monocytogenes* and *Salmonella spp.* In the samples evaluated.

In another study, Souza et al. (2021) aimed to identify the bacteria and yeasts isolated from samples collected in the main stages of artisanal cheese production in southern Brazil using MALDI-TOF and DNA sequencing. In all, 58 bacterial strains were distributed in 10 species among the genera *Bacillus*, *Citrobacter*, *Klebsiella*, *Lactococcus*, *Paenibacillus*, *Staphylococcus*, and *Raoutella*. Three species were identified out of the 13 yeast strains analyzed: *Candida pararugosa*, *Meyerozima guilliermondii*, and *Rhodotorula mucilaginosa*. In three yeast isolates, the researchers only identified the genus *Candida* sp. And *Trichosporon* sp; the species *L. lactis* (48%) and *M. guilliermondii* (46%) were the predominant species of bacteria and yeasts isolated, respectively.

In Turkey, Kanak and Yilmaz (2018) evaluated 21 regional and traditionally made cheese and found 150 lactic acid bacteria (LAB) isolates from the cheese. The authors reported that 100 of them were identified at genus level as *Leunoconostoc* (10), *Lactobacillus* (46), and *Enterococcus* (44) by biochemical tests. A total of 71 strains were identified as *Enterococcus durans* (6), *E. faecalis* (18),

E. faecium (24), *E. italicus* (2), *L. short* (1), L. *paracasei* (2), *L. plantarum* (1), *Lactococcus lactis* (3), *Leuonostoc lactis* (1), Leu. Mesenteroides (11), and *Streptococcus parauberis* (2) at the species level using MALDI-TOF MS.

In another study, in order to evaluate the microbiological quality of traditional Slovak "Parenica" non-smoked and smoked cheese produced with cow milk, the microbiota was identified by MALDI-TOF MS (Kačániová et al., 2019). The researchers analyzed 100 cheese packages from four producers, including 50 smoked and 50 non-smoked cheese samples. The LAB were cultured on specific agars (Man Rogosa, All Purpose TWEEN®, and Mayeux, Sandine, and Elliker) at 30 °C, coliform bacteria on violet red bile lactose agar at 37 °C, total bacterial count on standard agar for counting at 30 °C, and enterococci on *Enterococcus* selective agar at 37 °C. Gram-positive and Gram-negative strains were identified by MALDI-TOF MS, and the identification revealed four genera belonging to LAB, including *Lactococcus*, *Lactobacillus*, *Enterococcus*, and *Leuconostoc*. *Lactobacillus* was the most represented genus with seven species: *Lactobacillus curvatus* (*L. curvatus*), L. *delbrueckii*, *L. fermentum*, *L. casei*, *L. paraplantarum*, *L. plantarum*, and *L. sakei*. *E. coli*, *Enterobacter asburiae*, *Klebsiella oxytoca*, and *K. pneumoniae* were the most identified bacterial species of the order *Enterobacteriales*.

Given the above, this study sought to identify the species found in 10 samples of artisanal cheeses produced and commercialized in Vale do Taquari (Rio Grande do Sul State, southern Brazil) using MALDI-TOF MS.

2. Materials and Methods

Ten artisanal cheese samples (~1 kg) were purchased from markets supplied by family agribusinesses in different municipalities of Vale do Taquari and packaged in an isothermal box with ice. The samples were aseptically portioned in laminar flow (QUIMIS, Aparelhos Científicos Ltda, Diadema, SP, Brazil) in the food analysis laboratory of the Universidade Estadual do Rio Grande do Sul (UERGS), Unidade Encantado (RS). Aliquots (200 g) were sent to a university laboratory to determine the total count of microorganisms and identify the isolates by MALDI-TOF MS.

2.1. Proteomics

Microbiological identification by MALDI-TOF-MS was performed by a university laboratory in São Paulo (Brazil) according to the method by Barcelos et al. (2019). For this, 10 colonies in each sample duplicates were randomly selected (20 per sample in total), being colonies present on the surface and inside the agar. The isolates were subjected to microbiological identification using the MBT Compass software (Brucker, v. 4.1.8). Identification scores ≥1.7 were considered reliable for genus-level identification and scores ≥2.0 were considered reliable for genus- and species-level identification.

3. Results and Discussion

The aerobic mesophilic population and different microorganisms identified by MALDI-TOF MS in each artisanal cheese sample are listed in Table 1.

Table 1. Diversity of microorganisms identified in the 10 samples of artisanal cheese.

Sample	Population (CFU/g)	Microorganism(s)
1	5.9×10^7	*Lactotoccus lactis*, *Glutamicibacter mysorens*, *Escherichia coli*
2	4.3×10^7	*Escherichia coli*
3	3.6×10^7	*Enterobacter bugandensis*, *Enterobacter kobei*
4	2.6×10^7	*Enterobacter cloacae*, *Enterobacter kobei*, *Klebsiella oxytoca*, *Klebsiella variicola*, *Enterococcus faecalis*
5	3.1×10^7	*Enterococcus faecalis*, *Klebsiella pneumoniae*
6	1.9×10^7	*Lactotoccus lactis*, *Escherichia coli*
7	2.2×10^6	*Klebsiella oxytoca*
8	1.8×10^7	*Enterobacter cloacae*, *Enterococcus faecium*, *Escherichia coli*
9	4.2×10^7	*Staphylococcus aureus*, *Klebsiella variicola*
10	4.5×10^7	*Escherichia coli*

CFU: colony forming unit.

The population found from 2.2×10^6 to 5.9×10^7 CFU/g (Table 1) does not represent the degree of contamination of the artisanal cheese per se, although it is related to the need to perform prior isolation to proceed with subsequent identification by MALDI-TOF MS. The bacterial isolate must be initially be adsorbed to some carrier material (matrix), followed by attaching the microorganism to the plate and irradiating with a UV laser so that the molecules (proteins/nucleic acids) of the bacteria are broken down into positively charged (ionized) fragments that are launched towards a detector. The time it takes for the fragment to reach the detector (time of flight) is measured. This time depends on the size and charge of the fragment, with large molecules generating numerous fragments, and a characteristic mass spectrum is used to proceed to microbial identification (Veterinary Bacteriology, 2022).

In total, 22 strains were identified from the 10 samples submitted for analysis. In only two samples (1 and 6), the LAB *Lactococcus lactis* was isolated. This bacterium was also isolated by Caro et al. (2020) in a type of artisanal cheese ('Oaxaca') produced in Mexico. When the authors phenotypically characterized the strains of the genus *Lactococcus*, they verified the acidifying activity and classified the species *L. lactis* subsp. *Lactis* as fast, medium, or slow acidifiers. Nine (45%) of the 20 strains isolated from this species were considered rapid acidifiers, reducing the pH of milk from 6.6 to 5.3 in less than 6 h at 30 °C. The researchers reported that one of the key issues in making artisanal 'Oaxaca' cheese is that milk acidification is achieved in a short time, highlighting that the isolated species is an important starter culture for industrial production. Despite LABs possessing desirable attributes for artisanal cheese, they can be contaminated by other microbial groups, including those that indicate hygienic failures during production, thus causing deterioration or even posing health risks to the consumer (Camargo et al., 2021).

The findings of Kačániová et al. (2019) and Siqueira et al. (2021) were quite similar to ours, as practically every sample presented some strain related to the *Enterobacteriaceae* family (genera *Escherichia*, *Enterobacter*, and *Klebsiella*), representing 15 (68.18%) of the total identified. *E. coli* was present in 50% of the samples analyzed and the most prevalent species (22.73%).

The saprophytic milk microbiota consists of Gram-negative bacteria that produce protease and lipase enzymes (Tronco, 1997). Wessels et al. (1989) investigated the proteolytic and lipolytic properties of *Enterobacteriaceae* isolated from milk and dairy products and reported that *K. oxytoca* strain has proteolytic activity at 25 °C and *K. pneumoniae*, *E. cloacae*, and *K. oxytoca* have lipolytic property. These strains were also isolated in this study, and microbial lipases can add to the natural milk lipase, which promotes the release of fatty acids and glycerol, consequently leading to a rancid smell and taste.

The pathogenic bacterium *S. Aureus* was only present in sample 9 (Table 1). Johler et al. (2018) characterized *S. aureus* isolated throughout the raw milk cheese production process in artisanal dairies in Italy. They collected 276 samples from different stages of production (raw milk, whey, curd, brine, drying benches, and cheese) in 36 artisanal dairies and found that 102 samples from 25 dairies tested positive for *S. aureus*, with 80% positive samples among the artisanal cheese tested. According to the authors, many strains were present in samples from various dairies in different regions, highlighting the pathogen's spread in small cheese production facilities. Moreover, the authors emphasized that their findings reinforce the need for effective measures to prevent staphylococcal food poisoning, limiting *S. aureus* and enterotoxin formation along the production chain and in the final product (Johler et al., 2018). In Brazil, the analysis of staphylococcal enterotoxin in cheese is already regulated in legislation through Normative Instruction No. 161 (Brasil, 2022).

Ansiliero et al. (2018) analyzed two samples of artisanal cheese collected in Santa Catarina State (southern Brazil) and, in one of them, detected the presence of *E. faecalis* and *E. coli*. In the current study, these pathogens that may be present in food due to lack of hygiene during production were also identified, since they are species commonly found in the intestinal flora of mammals. Margalho et al. (2020) investigated *Enterococcus* spp.

In Brazilian artisanal cheese and identified the presence of this genus as a natural component of the microbiota of Brazilian artisanal cheese, especially in 'Marajó,' 'Coalho,' and 'Manteiga' cheeses due to their resistance to heat and the curd cooking step applied during production in these regions. The authors also observed that some strains could inhibit *L. monocytogenes*, primarily isolates from 'Serrano,' 'Colonial,' and 'Minas' artisanal cheeses. In addition, they showed moderate to high proteolytic and lipolytic activity. The authors concluded that the antilisterial activity is of major interest, as it may contribute to reducing the risk of listeriosis due to the consumption of dairy products, while proteolysis and lipolysis are responsible for important characteristics for fermented foods, such as aroma, flavor, and texture.

4. Conclusion

The diversity found in the artisanal cheeses was basically composed of bacteria belonging to the *Enterobacteriaceae* family. In this sense, it is essential to elevate the importance of monitoring and control during artisanal cheese production by evaluating the raw materials, good milking and manufacturing practices, and respecting the maturation period in order for the product to be within the necessary identity and quality parameters, thereby guaranteeing safety for consumers. The isolates identified by MALDI-TOF MS were all at the species level, revealing that the method is quite discriminatory at this classification level. To date, this is one of the first studies to verify the microbial diversity in artisanal cheese produced and commercialized in the Vale do Taquari region by mass spectrometry.

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