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The main spoilage-related psychrotrophic bacteria included in the industrial slicing of mozzarella cheese under sanitation standard operating procedures

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Research Article

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Abstract

Sanitation Standard Operating Procedures (SSOP) are critical in key stages of food production and processing. After manufacturing, slicing process can serve as a point of contamination, potentially compromising the quality and shelf life of mozzarella. The objective of this study was to determine the effect of SSOP on the quantification and diversity of psychrotrophic bacteria with proteolytic and lipolytic potential in mozzarella before and after industrial slicing. Psychrotrophic bacteria were isolated, phenotypically assessed for spoilage potential under mesophilic and psychrotrophic conditions, analysed for diversity using dendrograms of genetic similarity and identified by partial sequencing of the 16S rRNA gene. The mean psychrotrophic counts were 3.77 (±0.83) log CFU/mL before slicing and 3.58 (±0.51) log CFU/mL in the sliced product, indicating a non-significant reduction ($p < 0.05$). Regarding spoilage potential, none of the 233 isolates evaluated exhibited proteolytic activity under psychrotrophic conditions. However, psychrotrophic lipolytic activity was predominant both before and after slicing. The species *Lactobacillus delbrueckii*, which is part of the saccharolytic inoculum used to reduce the pH of the curd during cheese production, was the main proteolytic bacteria under mesophilic conditions (35°C) in both before and after sliced samples. Although the bacterial counts indicated the full efficiency of the slicer's SSOP, the microbial diversity analysis revealed the inclusion of *Staphylococcus succinus, Staphylococcus hominis, Enterococcus faecalis* and *Klebsiella pneumoniae* during the slicing process, albeit at low levels. Therefore, relying solely on psychrotrophic quantification may not be sufficient to attest the efficiency of the slicer's SSOP. Even under controlled industrial conditions, spoilage bacteria from handling and environmental sources may be introduced into sliced mozzarella. Methods for improving the microbiological quality of the mozzarella pieces prior to slicing, as well as the intensification of sanitary procedures, must be reviewed and implemented to improve the shelf life and commercial potential of sliced mozzarella.

Introduction

Given its functional properties, mild flavour and excellent melting and slicing capabilities, mozzarella cheese is extensively utilized in various culinary applications (Ah and Tagalpallewar, 2017; Santos *et al.*, 2023). It stands as one of the most widely produced and consumed dairy products not only in Brazil but also in numerous other countries. In Brazil, several dairy enterprises specialize exclusively in the production of mozzarella cheese, making it their primary and often sole product offering.

The industrial production of cheese requires the implementation of self-control tools within manufacturing facilities to mitigate or eliminate health hazards to consumers (Owusu-Apenten and Vieira, 2022). For instance, the pasteurization of raw milk is identified as a biological critical control point for mozzarella production since no subsequent step in the process can adequately ensure the safety of the final product (Nunes *et al.*, 2024).

In these self-control programmes, Sanitation Standard Operating Procedures (SSOP) serve as essential documents that describe and systematize the procedures, inputs, frequency and personnel responsible for critical steps in the production process. These procedures are meticulously planned, executed, monitored and verified before, during and after the production process to control and prevent food contamination (Oliveira *et al.*, 2016; Sucipto *et al.*, 2020; Owusu-Apenten and Vieira, 2022; Santos *et al.*, 2024).

During the industrial slicing process of mozzarella cheese, failures in the SSOP for facilities and slicing equipment can introduce spoilage microorganisms that may compromise the quality and, therefore, the shelf life of sliced mozzarella cheese (Luiz *et al.*, 2022). The efficiency of SSOP execution may also reduce the need for additives to extend the cheese's shelf life (Jalilzadeh *et al.*, 2015).

Given that refrigeration is the primary method of storage and preservation for mozzarella cheese, psychrotrophic bacteria with proteolytic and/or lipolytic potential are especially important as these bacteria can impact cheese quality and shelf life (Luiz *et al.*, 2022; Tremonte *et al.*, 2024), and can compromise other characteristics such as shreddability (Rathod *et al.*, 2025).

Validating SSOPs through microbiological testing during their operation provides a technical foundation for their execution and can help improve an establishment's self-control plan (Awuchi, 2023). In this context, the objective of this study was to evaluate the impact of SSOP on the quantification and diversity of spoilage-related psychrotrophic bacteria in an industrial mozzarella cheese slicing process.

Material and methods

Sampling

Ten batches of mozzarella cheeses produced at a dairy plant in the State of Tocantins, Brazil, were randomly selected for this study. The dairy plant operates regularly by the State Inspection Regime (SIE) and complies with the Brazilian Inspection System for Products of Animal Origin (SISBI). Each batch comprised approximately 50 pieces, with each piece weighing 4 kg.

Between August and September 2023, one piece from each batch (one batch per collection day) was randomly selected for analysis before and after the slicing process. The slicing was performed using industrial automatic equipment (FTI-250; Equimatec Machinery Industry, Rio do Sul, SC, Brazil) at a rate of 250 slices/min with a plastic layer interleaved between the slices (interfoliation).

For each selected piece, an aliquot of approximately 250 g was aseptically collected from the beginning of the cheese piece immediately before it entered the slicer. Following the slicing and interfoliation process, another 200 g sample was collected from the same piece. All samples were kept refrigerated and transported to the Food Microbiology Laboratory at the Federal University of Northern Tocantins, in Araguaína, where they were analysed immediately upon arrival.

SSOP carried out

The slicing equipment was housed in a temperature-controlled room maintained at 16°C. It underwent preoperational (before starting work), operational (every 4 h of work), and post-operational (at the end of the work shift) SSOP. The specific room where the equipment was located was subjected only to pre- and post-operational SSOP.

For cleaning, the floor and walls were rinsed and scrubbed using a 3–5% neutral detergent. After 2 min, they were rinsed again and sprayed with 1% peracetic acid solution. The slicing equipment was subjected to the same sanitary procedure, which included thorough rinsing with pressurized industrial water, allowing the peracetic acid to act for 5 min. Additionally, preoperational cleaning of the slicing mat was performed with 70% ethanol. These procedures were approved by the Local Sanitary Inspection Service (SIE-TO).

Psychrotrophic counts

Aliquots of 25 g, representative of each sample, were aseptically processed and homogenized with 225 mL of buffered peptone water in a Stomacher for 180 s. This initial dilution (10^{-1}) was then sequentially diluted to 10^{-9} in sterile saline (0.89% NaCl) containing 0.01% peptone. From these dilutions, 0.1 mL was plated in duplicate onto the surface of Plate Count Agar (PCA) (Acumedia, CA, USA). The plates were incubated at 7°C (±1) for 10 days in a biological oxygen demand cabinet for psychrotrophic counts (Ribeiro Júnior *et al.*, 2019). The results of the counts were analysed using nonparametric statistical methods, specifically by χ^2 test in SAS v. 9.0 software (SAS Institute Inc., Cary, NC).

Spoilage potential

One of the duplicate plates used for bacterial counts was selected for the recovery of all psychrotrophic bacterial colonies. From each sample, approximately 25 isolates were recovered. Each of these isolates was purified by successive subcultures in PCA plates.

Each isolate was individually evaluated for proteolytic and lipolytic potential under mesophilic and psychrotrophic conditions, according to the protocol used by Ribeiro Júnior *et al.* (2018a). For proteolysis assessment, the isolates were subcultured on two milk agar plates (Acumedia) supplemented in a 1:9 ratio with a sterile solution of reconstituted skimmed milk powder at 10% (w/v). One plate was incubated at 35°C (±1) for 48 h and another at 7°C (±1) for 10 days to evaluate proteolysis under mesophilic and psychrotrophic conditions, respectively (Ribeiro Júnior *et al.*, 2018a). For the lipolytic potential, the same procedure was performed, replacing the culture medium with tributyrin agar (HiMedia, Mumbai, India) supplemented in a proportion of 1:99 with tributyrin (HiMedia).

Genetic diversity

All isolates showing spoilage potential were recovered in BHI broth (Acumedia) at 35°C for 48 h. Following incubation, the isolates were subjected to DNA extraction using the protocol described by Ribeiro Júnior *et al.* (2016), and the extracted DNA was quantified using a NanoDrop Lite Plus spectrophotometer (ThermoFisher, USA).

The diversity of psychrotrophs with spoilage potential was evaluated with two variables. First, the amplification profile of the internal transcribed spacer region was assessed according to the primers and conditions detailed by Ribeiro Júnior *et al.* (2019). Subsequently, the restriction profile was evaluated using

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Table 1. Identification of spoilage potential of psychrotrophic microbiota in mozzarella cheese before and after industrial slicing under SSOP conditions

	16S rRNA cluster identification	Isolates	Lipolytic		
		n	Proteolytic 35°C	7°C	35°C
Before slicing	*Lactobacillus delbrueckii*	36	28	9	–
	Bacillus spp.	16	1	15	4
	Staphylococcus spp.	8	4	5	1
	Staphylococcus epidermidis	1	–	1	–
	Staphylococcus equorum	1	–	1	–
	Moraxella osloensis	1	1	–	–
	Pantoea spp.	1	–	1	–
	Total	64	34	32	5
Sliced	*Lactobacillus delbrueckii*	35	19	13	9
	Bacillus spp.	13	3	10	4
	Staphylococcus spp.	8	1	4	4
	Staphylococcus equorum	4	–	4	–
	Staphylococcus succinus	3	–	3	–
	Staphylococcus hominis	1	1		–
	Staphylococcus epidermidis	1	–	1	–
	Pantoea spp.	2	–	2	1
	Enterococus faecalis	1	–	1	–
	Klebsiella pneumoniae	1	–	1	1
	Moraxella osloensis	1	1	–	–
	Total	70	25	39	19

the enzyme HhaI (Thermo Scientific, USA) according to the manufacturer's recommendations. (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012351_HhaI_10_UuL_2000U_UG.pdf).

These variables of each isolate were documented and analysed in the Bionumerics v. 1.50 software (Applied Mathematics, Kortrijk, Belgium) to construct genetic similarity dendrograms. The similarity matrix Dice coefficient (Dice, 1945) and the unweighted pair group mean algorithm (Sneath, 2005) were used. Clusters were determined based on a minimum of 70% phylogenetic similarity.

16S rRNA sequencing

One isolate from each cluster was randomly selected for identification by 16S rRNA gene sequencing. Primers 27f and 1492r were used for amplification (Osborne *et al.*, 2005) of an approximate 1,465-bp product, which was purified (PureLink™ Quick Gel Extraction and PCR Purification, Invitrogen) and quantified (NanoDrop) for partial sequencing of the 16S rRNA gene in both directions by the Sanger method (SeqStudio, Applied Biosystems). Sequence quality was analysed (http://lbi.cenargen.embrapa.br/phph/), and consensus sequences generated in Phred were analysed for similarity with GenBank using the Nucleotide Blast Tool of the National Center for Biotechnology Information.

Results and discussion

The mean psychrotrophic counts were 3.77 (±0.83) log CFU/mL for the whole mozzarella samples and 3.58 (±0.51) log CFU/mL for sliced samples. The results data did not follow a normal distribution. The median counts were 4.03 CFU/mL for whole mozzarella and 3.66 CFU/mL for sliced mozzarella, with no significant difference observed between the two ($p > 0.05$).

The absolute results in the psychrotrophic counts may have been influenced by the sampling locations on the 4 kg mozzarella bars. Samples from whole bars were taken from the surface, while sliced aliquots were collected from the interior. This difference in sampling points could explain the observed variation, as the surface is more prone to environmental contamination after the removal of the primary packaging. A similar effect of sampling location was reported in a recent study by Santos *et al.* (2024) on the industrial slicing of mozzarella and ham.

In total, 233 psychrotrophic isolates were recovered from all whole and sliced samples. Among these, 134 (57.5%) exhibited some spoilage activity under at least one condition evaluated, with 20 (8.58%) showing proteolysis and lipolysis or activity under both mesophilic and psychrotrophic conditions simultaneously. The remaining 114 (91.42%) presented only one type of spoilage activity under a single condition. The distribution of the spoilage potential of the isolates is presented in Table 1.

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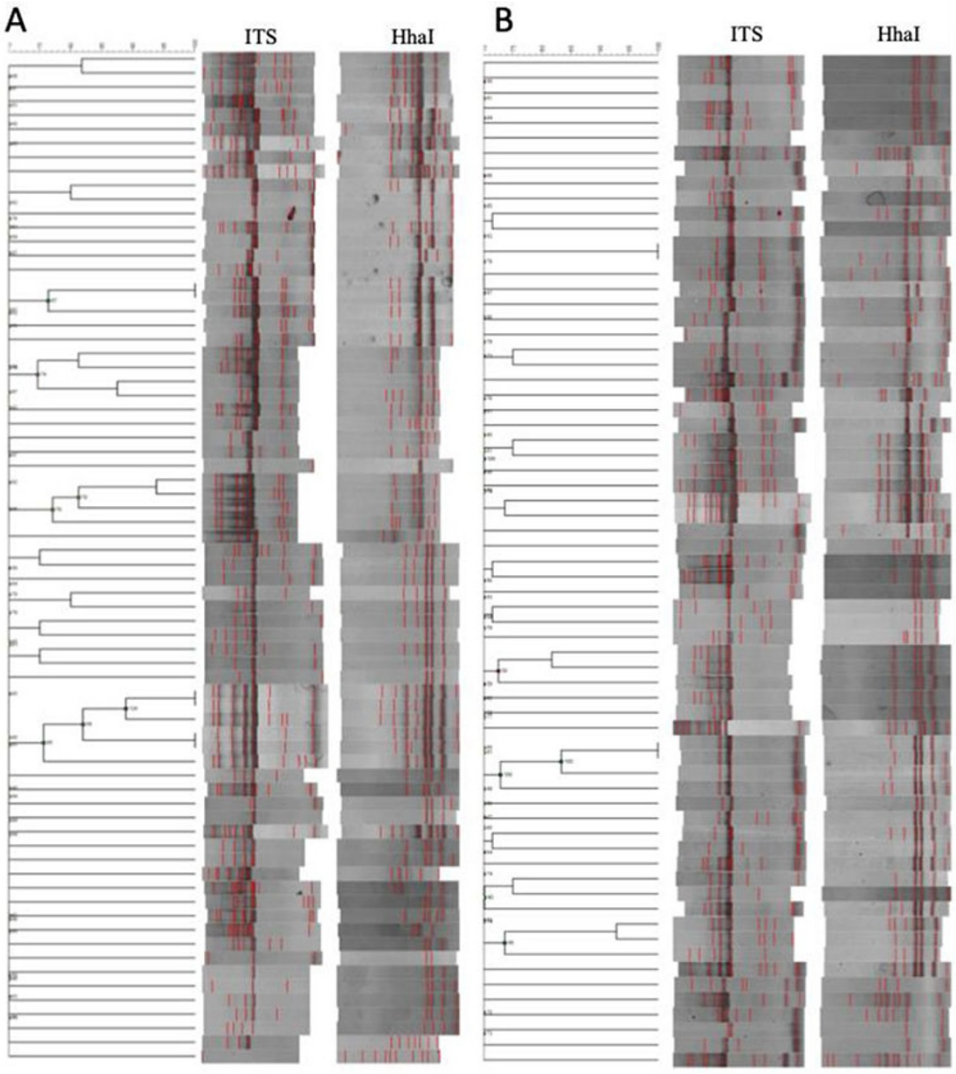

Figure 1. Genetic similarity dendrograms with the application variables of the internal transcription spacer (ITS) region and its restriction product with the enzyme Hhai, of 64 (A) and 70 (B) psychrotrophic spoilage isolates isolated in mozzarella cheese samples before and after slicing, respectively.

Satisfactorily, no isolate presented proteolytic activity at 7°C. Using only the criterion of psychrotrophic with proteolytic potential, the execution of SSOP can, therefore, be attributed to full efficiency in the control of technological problems/shelf life due to proteolysis, in association with refrigerated storage (Oštarić *et al.*, 2022).

The diversity of spoilage psychrotrophs before and after slicing is shown in Fig. 1A and B. Notably, the number of psychrotrophic clusters remained unchanged at 53 after slicing, despite an increase in the number of recovered isolates (from 64 before slicing to 70 after). This suggests that no distinct microbiota was introduced during the slicing process. However, sequencing of cluster representatives revealed a reduction in the number of clusters for *Lactobacillus delbrueckii* (three clusters) and *Bacillus* spp. (two clusters). Additionally, a single cluster of spoilage psychrotrophic microorganisms was introduced during slicing, even with the implementation of SSOPs. The identification of the isolates and their respective deteriorating potential is described in Table 1.

By analysing the counts independently, it was verified that automated slicing performed under SSOP conditions does not serve as a point of entry for psychrotrophic in the sliced mozzarella cheese. From this perspective, the SSOP of the slicer effectively minimized contamination of the equipment and the slicing process by bacteria that could otherwise compromise the quality, safety and shelf life of the mozzarella cheese. The psychrotrophs counts in the sliced product were more related to the amount present in the whole piece and its contamination during previous production and storage stages. A similar relationship was also observed by Santos *et al.* (2024) for the counts of other groups of quality indicators microorganisms, such as mesophilic aerobes and *Staphylococcus aureus*.

Regarding the diversity of spoilage psychrotrophic bacteria, it was observed that the species *Staphylococcus succinus, Staphylococcus hominis, Enterococcus faecalis* and *Klebsiella pneumoniae* were introduced during the slicing process, as these bacteria were not identified in the whole samples, despite the pieces before slicing had higher absolute bacterial counts. Each of these isolate species was identified in only one cluster, with a single isolate per species. Notably, these microorganisms are neither described as spoilage psychrotrophs in Brazilian milk

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(Ribeiro Júnior *et al*., 2018a) nor thermoduric (Ribeiro-Júnior *et al*., 2020). Therefore, it is suggested that the source of these bacteria may be linked to the environment, the production process or handling practices within the industry.

As the main result, despite the reduction in bacteria counts, microorganisms indicative of handling issues (*Staphylococcus* spp.) and environmental contamination (Gram-negative bacteria) were introduced during the slicing process. Although these microorganisms were recovered in low proportions, a significant compromise in the quality and shelf life of the sliced product is unlikely to be observed or expected. However, the isolation of this microbiota demonstrates that, even under industrial SSOP conditions of the slicer, it is essential to reinforce additional requirements within the self-control program. These include strict adherence to good food handling practices and rigorous pre- and post-operational hygiene procedures of the facilities.

Table 1 reveals that *L. delbrueckii* predominated in the microbiota of both whole (56.3%) and sliced (50%) samples. This species is part of the Starter Lactic Acid Bacteria (SLAB) culture used in the mozzarella manufacturing process, alongside with the species *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus helveticus* (Elian Comércio de Insumos, Vila Velha, ES, Brazil). The inclusion of these cultures after pasteurization is essential for the coagulation, fermentation and pH drop processes for the stretching process (Nunes *et al*., 2024).

Notably, no other SLAB isolates were recovered among the spoilage psychrotrophs. The predominance of *L. delbrueckii* among the psychrotrophs was somewhat unexpected, as these bacteria are typically characterized as mesophilic or thermophilic cultures (Coelho *et al*., 2022), consistent with the culture mixture used in the manufacture of the samples in the present study. Interestingly, *L. delbrueckii* has been reported to produce hydrogen peroxide, which exhibits antagonistic activity against pathogens and other bacteria when stored at 5°C (Villegas and Gilliland, 1998; Meloni *et al*., 2023).

Regardless of being proteolytic under mesophilic conditions, no isolate of *L. delbrueckii* was identified as proteolytic at 7°C, the storage temperature for mozzarella. However, they exhibited lipolytic potential at both 7°C and 35°C, indicating that they can remain active throughout the product's shelf life. Other SLAB species have also been identified within the autochthonous psychrotrophic microbiota with the potential to deteriorate Brazilian milk, such as *Lactococcus lactis* (Ribeiro Júnior *et al*., 2018a) as well as among the thermoduric spoilage bacteria such as *Streptococcus thermophilus* (Ribeiro Júnior *et al*., 2018b).

Proteolysis occurs during cheese production and ripening due to the hydrolysis of milk caseins. This process is initiated by the action of chymosin and other proteases naturally present in milk during the initial stage of cheese production (Nunes *et al*., 2024). These proteolytic enzymes produce large oligopeptides, which subsequently serve as substrate for proteinases and peptidases derived from SLAB (Bouroutzika *et al*., 2021; Oštarić *et al*., 2022).

Proportionally, *Bacillus* spp. was the second most frequently identified genus in the samples, both before and after slicing (Table 1). These microorganisms can also be considered environmental contaminants in cheese production facilities (Tirloni *et al*., 2022; Champidou *et al*., 2024). Additionally, they can persist and remain detectable from raw milk through to the final product, since they are thermoduric and, therefore, resistant to pasteurization and the stretching process in the mozzarella manufacturing (Montone *et al*., 2020; Ribeiro-Júnior *et al*., 2020; Qin *et al*., 2024).

All other spoilage psychrotrophic species identified in the whole samples were also detected after the slicing process. *Staphylococcus epidermidis, Staphylococcus equorum, Moraxella osloensis* and *Pantoea* spp. were also identified in the respective cheese samples after slicing. Since these microorganisms are recognized as contaminants in the production process (Woo *et al*., 2023; Nunes *et al*., 2024), it is essential to review and monitor the steps preceding mozzarella slicing to minimize the potential impact of these spoilage species on the quality and shelf life of the final product. Each step of the mozzarella production process individually influences its quality and microbiological safety (Nunes *et al*., 2024). Therefore validated SSOPs must be implemented to ensure the delivery of safe, high-quality products the consumers.

Conclusion

Industrial slicing under SSOP conditions did not significantly affect the quantification of psychrotrophic bacteria in mozzarella cheese. However, quantification alone was insufficient to attest the efficiency of the slicing SSOP, as the microbial diversity revealed the inclusion of environmental and handling-related bacteria with the potential to cause quality issues and reduce shelf life – mainly lipolytic bacteria under refrigerated storage conditions. Therefore, it is essential to review and increase the rigor of not only the slicing SSOP but also of all previous stages of mozzarella production and storage. Continuous training of handlers in good manufacturing and handling practices, along with intensified SSOP implementation in the industrial environment, is necessary to prevent the introduction of contaminants that could compromise the commercial potential of sliced mozzarella throughout the production process.

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Competing interests. 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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