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Staphylococcus aureus in artisanal Minas cheese produced in Serra da Canastra, Minas Gerais, Brazil

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Abstract

Artisanal cheeses bearing the ARTE seal are authorized for nationwide commercialization and are produced in accordance with quality standards established by specific regulations. In contrast, cheeses without this certification have their sale restricted to municipal or state-level commerce, subject to registration with official inspection services. This study evaluated the microbiological quality of Minas artisanal cheeses produced in the Serra da Canastra region, Minas Gerais, by comparing products from farms with and without the ARTE seal. A total of 69 samples were analyzed, including raw milk, pasteurized milk, curd, cheese, and swabs from cheese molds, collected from 16 farms, eight certified with the ARTE seal and eight uncertified. Microbiological quantification techniques, culture methods, and polymerase chain reaction assays were employed for pathogen identification, alongside phenotypic antimicrobial susceptibility testing and genetic similarity analysis of *Staphylococcus aureus* isolates via pulsed-field gel electrophoresis. Neither *Salmonella* spp. nor *Listeria monocytogenes* were detected. Elevated counts of Enterobacteriaceae and coagulase-positive staphylococci were more frequently observed in ARTE-certified farms; however, *Staphylococcus aureus* was isolated exclusively from uncertified properties. These findings suggest that the ARTE seal may contribute to more effective sanitary practices and enhanced microbiological safety of artisanal cheeses.

Keywords: sanitary certification; artisanal cheese; indicator microorganisms; bacterial resistance.

Practical Application: The ARTE certification seal strengthens good practices and quality monitoring in artisanal cheeses.

1 INTRODUCTION

Brazil produces over 36 types of artisanal cheese, classified according to the type of milk used (raw or pasteurized), manufacturing techniques, and region of origin (Chaves et al., 2021). Minas Gerais, the country's leading producer, is particularly known for Artisanal Minas Cheese (Queijo Minas Artesanal — QMA), with an annual output exceeding 78 thousand tons, primarily from small rural dairies (Instituto Brasileiro de Geografia e Estatística [IBGE], 2017). The sensory characteristics of this cheese are influenced by the local microbiota, leading to variations across different microregions (Monteiro & Matta, 2018).

Artisanal cheeses produced with raw milk are valued for their distinctive flavor, influenced by the presence of lactic acid bacteria (LAB) (Yoon et al., 2016). Although these bacteria can help inhibit pathogenic microorganisms, outbreaks of foodborne illnesses have been reported in association with the consumption of unpasteurized milk and cheese, involving pathogens such as *Staphylococcus aureus*, *Salmonella* spp., and *Listeria monocytogenes* (CDC, 2017; Jackson et al., 2018; Johler, Giannini, et al., 2015; Johler, Weder, et al., 2015; Robinson et al., 2020; Ung et al., 2019).

In addition to microbiological risks, animal-derived foods can serve as vectors for the dissemination of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARBs) throughout the food chain (European Food Safety Authority [EFSA], 2009; Titouche et al., 2022). Cases of methicillin-resistant *S. aureus* (MRSA) and extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae have been reported in the dairy production chain (Gelalcha & Dego, 2022; Silva et al., 2020), with resistance being promoted by improper antibiotic use and environmental contamination (Bava et al., 2024).

Given these challenges, regulations have been established to govern the production and sanitary quality of artisanal cheeses, such as the ARTE Seal (Law No. 13,680/2018), which, together with Law No. 13,860/2019 and Normative Instruction

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No. 161/2022, defines microbiological standards and control procedures for these products (Brazil, 2018, 2019, 2022). In Minas Gerais, complementary state legislation also regulates the production of raw milk cheeses (Law No. 23,157/2018 and Decree No. 48,024/2020) (Minas Gerais, 2018, 2020a).

In this context, the present study aimed to evaluate the microbiological quality and the occurrence of resistant bacteria in QMAs, comparing samples from farms with and without the ARTE Seal in the Serra da Canastra region, Minas Gerais, Brazil.

1.1 Relevance of the work

This study contributes to the understanding of the microbiological profile of Artisanal Minas Cheese produced in the Serra da Canastra region, highlighting the role of the ARTE seal in ensuring food safety.

2 MATERIALS AND METHODS

2.1 Sample collection

A total of 69 samples were collected between May 2022 and October 2024 from 16 dairies in the Serra da Canastra region, Minas Gerais, Brazil, producing QMA with an average maturation period of 14 days. Eight farms did not possess the ARTE Seal (P1–P8), while eight had the seal (P9–P16). On farms without the seal, five samples per site were collected: raw milk, pasteurized milk (except in P1, P7, and P8), curd, cheese, and mold swab, totaling 37 samples. On farms with the seal, four samples per site were collected: raw milk, curd, cheese, and mold swab, totaling 32 samples. Overall, 69 samples were obtained: 16 raw milk, five pasteurized milk, 16 curd, 16 cheese, and 16 mold swabs.

Sampling was conducted using sterilized materials, samples were stored in sealed tubes, and transported in insulated boxes to the Laboratório de Fisiologia de Microrganismos (LABFIS/UEMG, Passos-MG) for microbiological quantification. Complementary analyses were performed at the Laboratório de Biotecnologia e Resistência Bacteriana (BIOBAC/UNESP, FCAV, Jaboticabal-SP).

2.2 Microbiological quantification

Two groups of microorganisms were quantified using surface plating. For each sample, 25 g of solid food, 25 mL of liquid food, or 25 cm^2 of swab material were aseptically processed, diluted in peptone water (1:10), and subjected to serial decimal dilutions (Campbell & Campbell, 1986). From these dilutions, 0.1 mL was inoculated onto specific media: MacConkey Agar (MC — KASVI), used for the five pasteurized milk samples, for total Enterobacteriaceae quantification; and Mannitol Salt Agar (SM — KASVI), used for all 69 samples, for *Staphylococcus* spp. (Brazil, 1993). Inocula were spread using a Drigalski loop and incubated at 37 °C for 24 hours (Sanders, 2012), followed by colony-forming unit (CFU) counting.

Additionally, 0.1 mL of the 10^{-1} dilution from each sample was transferred into cryogenic tubes containing 0.5 mL of Brain Heart Infusion broth (BHI — Himedia, India) and incubated at 37 °C for 24 hours. After growth, 0.5 mL of 30% glycerol was added, and cultures were stored at –80 °C for future analyses.

2.3 Microbiological culturing for pathogen detection

The cultures stored at –80 °C were thawed and subjected to metabolic recovery in 5 mL of Brain Heart Infusion (BHI) broth, incubated at 37 °C for 24 hours. Subsequently, different procedures were applied to isolate *Staphylococcus aureus*, *Salmonella* spp., and *Listeria monocytogenes*, as described below.

For *S. aureus* isolation, 0.1 mL of the BHI broth was plated onto Mannitol Salt Agar (SM), spread using a Drigalski loop, and incubated at 37 °C for 24 hours. Typical colonies (identified by mannitol fermentation) were collected using a sterile swab, transferred to 5 mL of BHI broth, and incubated again at 37 °C for 24 hours. The resulting cultures were used for DNA extraction (Tallent et al., 2016).

Salmonella spp. isolation was performed by inoculating 0.1 mL of each sample into two tubes: one containing 10 mL of Selenite Cystine broth (KASVI) and the other containing 10 mL of Rappaport-Vassiliadis broth (KASVI), both supplemented with 0.1 mL of 4% novobiocin. The tubes were incubated at 37 °C and 42 °C, respectively, for 24 hours. After enrichment, a loopful from each broth was streaked onto MacConkey Agar (MC) and Xylose Lysine Deoxycholate Agar (XLD — KASVI), followed by incubation at 37 °C for 24 hours (Andrews et al., 2024). Typical colonies (colorless on MC and black-centered on XLD) were selected, transferred to 5 mL of BHI broth, and incubated at 37 °C for 24 hours for subsequent DNA extraction.

For *L. monocytogenes* detection, 0.1 mL of each sample was inoculated into Listeria Enrichment Broth (LEB — HIMEDIA) and incubated at 37 °C for 24 hours. Following this step, 0.1 mL of the broth was transferred to tubes containing 10 mL of Fraser broth (SIGMA-ALDRICH) and incubated at 35 °C for 24 hours (Hitchins et al., 2022). The cultures were then streaked onto PALCAM Listeria Agar (SIGMA-ALDRICH), supplemented with PALCAM Listeria Selective Supplement, and incubated at 37 °C for 48 hours (Van Netten et al., 1989). Typical colonies (small, black, with an esculin hydrolysis halo) were isolated, inoculated into BHI broth, and incubated at 37 °C for 24 hours prior to DNA extraction.

2.4 DNA extraction

DNA extraction from bacterial isolates followed the protocol described by Bag et al. (2016). For each presumptive isolate, 1.0 mL of BHI broth culture was centrifuged at 10,000 × g for 3 minutes, and the supernatant was discarded. The cell pellet was resuspended in 450 µL of extraction buffer (160 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0], 20 mM NaCl, 0.5% [w/v] SDS) and subjected to lysis in a dry bath (Thermomixer, Eppendorf, Germany) at 65 °C for 30 minutes.

Next, 180 µL of 5 M potassium acetate was added, and the solution was kept on ice for 15 minutes. DNA purification was performed using 400 µL of chloroform:isoamyl alcohol (24:1, v/v), followed by centrifugation at 12,000 × g for 10 minutes. The supernatant was transferred to new tubes, and 1,000 µL of cold absolute ethanol was added for DNA precipitation, followed by incubation at –20 °C for 12 hours. After centrifugation at 12,000 × g for 18 minutes, the DNA pellet was washed with 1,000 µL of 70% (v/v) ethanol, dried in an incubator, and resuspended in 30 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA quantification was performed using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

2.5 Genetic confirmation of pathogens

2.5.1 Screening polymerase chain reaction

The extracted DNA was used in a screening polymerase chain reaction (PCR) to detect *S. aureus*, *Salmonella* spp., and *L. monocytogenes*, through amplification of the *cydB*, *invA*, and *iap* genes, respectively (Almeida et al., 2018; Bubert et al., 1999; Fratamico & Strobaugh, 1998). Samples positive for at least one of these genes were selected for confirmatory PCR.

2.5.2 Confirmatory polymerase chain reaction and isolation

Samples testing positive in the screening PCR for S. aureus (*cydB*), *Salmonella* spp. (*invA*), and *L. monocytogenes* (*iap*) were streaked for isolation onto Mannitol Salt Agar, MacConkey Agar, and PALCAM Listeria Agar, respectively, and incubated at 37 °C for 24 to 48 hours.

From each selective medium, ten colonies with characteristic morphology were selected and individually subcultured on BHI agar. After incubation at 37 °C for 24 hours, a loopful of each culture was transferred into tubes containing 1 mL of BHI broth (ten tubes per positive sample). The cultures were then subjected to DNA extraction and PCR to confirm the presence of the target genes. Colonies confirmed by PCR were considered as isolates.

Confirmed isolates were transferred to cryogenic tubes containing 0.5 mL of BHI broth, incubated at 37 °C for 24 to 48 hours, and stored in 30% glycerol at –80 °C for further analysis. The workflow for detection and isolation of *S. aureus*, *Salmonella* spp., and *L. monocytogenes* is illustrated in Figure 1.

2.6 Phenotypic antimicrobial susceptibility testing

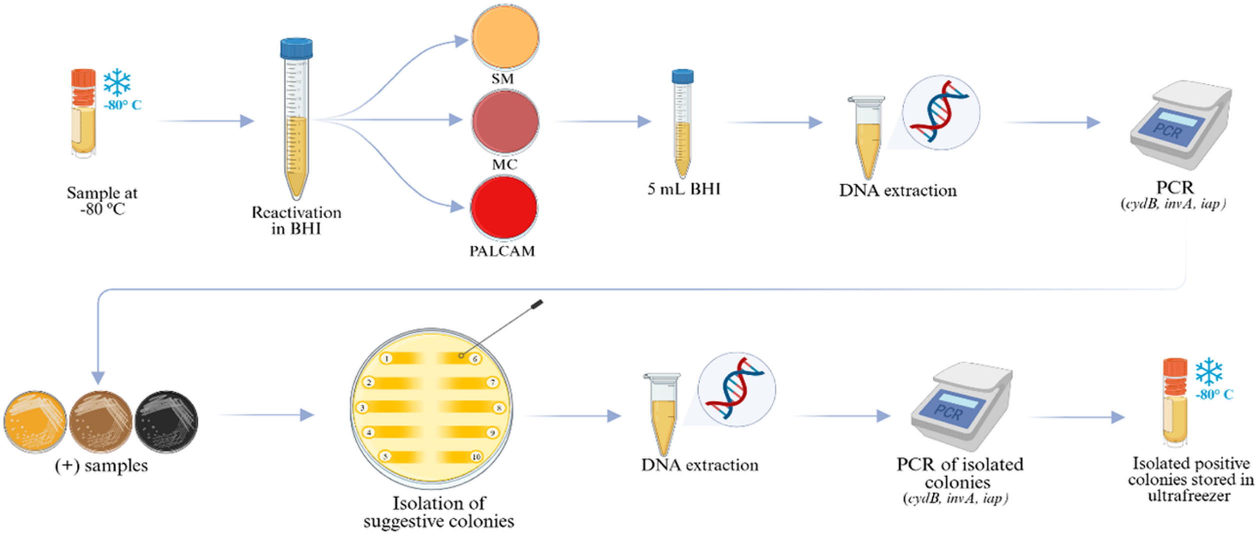
2.6.1 Selective media screening with antibiotics

For *S. aureus*, positive colonies were resuspended in BHI broth (0.5 McFarland standard) and plated onto Mannitol Salt Agar supplemented with oxacillin (6 µg/mL), following Clinical and Laboratory Standards Institute (CLSI, 2024) guidelines, to assess growth at 35 °C. For Enterobacteriaceae, all 69 samples were plated on MacConkey Agar supplemented with ceftazidime (16 µg/mL) and incubated at 37 °C for 24 hours. Colonies exhibiting growth were stored and sent to the Instituto Biológico for identification via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

2.6.2 Disk diffusion test

Antimicrobial susceptibility was assessed using the Kirby-Bauer disk diffusion method (Bauer et al., 1966). PCR-confirmed isolates were subcultured on BHI agar and incubated at 37 °C. Isolated colonies were suspended in 0.9% saline solution, adjusted to the 0.5 McFarland standard, and inoculated onto Mueller-Hinton Agar using a sterile swab. After 3 minutes, antibiotic disks recommended by CLSI were applied, and inhibition zone diameters were compared to interpretive standards from CLSI (2024, 2025), and Brazilian Commitee on Antimicrobial Susceptibility Testing (BrCAST, 2025).

The antimicrobials tested for Enterobacteriaceae were: amoxicillin + clavulanate (30 µg), cefoxitin (30 µg), cefepime (30 µg), ceftazidime (10 µg), imipenem (10 µg), ampicillin



PCR: polymerase chain reaction.

Figure 1. Workflow for the isolation and identification of *Staphylococcus aureus*, *Salmonella* spp., and *Listeria monocytogenes*.

(10 µg), cephalothin (30 µg), cefazolin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), and cephalexin (30 µg).

For *S. aureus*, the antimicrobials tested were: erythromycin (15 µg), doxycycline (30 µg), norfloxacin (10 µg), penicillin (10 µg), sulfamethoxazole + trimethoprim (25 µg), tetracycline (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), rifampicin (5 µg), chloramphenicol (30 µg), cefoxitin (30 µg), and azithromycin (15 µg).

2.7 Genetic detection of resistance and virulence in Staphylococcus aureus

PCRs were performed on *S. aureus* isolates to detect the *mecA* gene (associated with methicillin resistance — MRSA) and the *seA* gene (associated with the production of staphylococcal enterotoxin A), following the protocols described by Kim et al. (2011) and Mehrotra et al. (2000). For the remaining target genes used for species identification, PCR assays were performed according to the protocols described in Table 1.

Genetic similarity

The genetic similarity among *S. aureus* isolates was assessed by pulsed-field gel electrophoresis (PFGE) using 1% Pulsifield Certified agarose gel (Bio-Rad, USA) and the CHEF DR-III system (Bio-Rad, USA). Chromosomal DNA was digested with the enzyme *XbaI* (Invitrogen, USA) for Enterobacteriaceae and *SmaI* for *Staphylococcus*, according to the Centers for Disease Control and Prevention (CDC) protocol (Ribot et al., 2006). Electrophoresis was conducted at 6 V/cm with a 120° angle, an initial switch time of 2.2 s, and a final switch time of 54.2 s, over 18 h at 14 °C. The *Salmonella* Braenderup strain (H9812) from the Adolfo Lutz Institute was used as a molecular size standard and normalization control. Band pattern similarity was calculated using the Dice coefficient with a 1% position tolerance and optimization. A dendrogram was generated using the UPGMA method in BioNumerics software version 7.5 (Applied Maths). Isolates displaying ≥ 90% similarity were considered genetically related. The diversity ratio (DR) was calculated using Equation 1 in WPS Office spreadsheet software.

$$DR\ (\%) = \left(\frac{number\ of\ PFGE\ banding\ patterns}{total\ number\ of\ isolates}\right) x\ 100 \qquad (1)$$

3 RESULTS

3.1 Microbiological quantification

Colony-forming unit (CFU) counts were evaluated according to the criteria established by Instruction Norm No. 161 (Brazil, 2022). Figure 2 presents the total number of samples from each farm that exceeded the legal limits for coagulase-positive staphylococci counts.

For Enterobacteriaceae, among the five non-certified (non-ARTE) farms that performed milk pasteurization, three (P2, P4, and P6) showed bacterial counts above the regulatory threshold of 1.0×10^1 CFU/mL for pasteurized milk, with values of 1.0×10^2 CFU/mL (P2 and P4) and 2.8×10^3 CFU/mL (P6). In ARTE-certified farms, pasteurized milk was not analyzed, as these producers used raw milk in their production processes.

Regarding coagulase-positive staphylococci, among the non-certified farms, P1 exhibited elevated counts in curd (> 1,000 CFU/g), cheese (> 1,000 CFU/g), and surface swabs from cheese molds (> 1,000 CFU/cm²). Farms P4 and P7 showed excess levels in both curd and cheese, while P8 exceeded the limits in cheese only. Among the certified farms, P9 and P11 exceeded the limits in both curd and cheese; P10, P12, P13, and P16 showed high counts in cheese only; and P14 and P15 exceeded limits in curd, cheese, and mold surface swabs.

Among ARTE-certified samples, 43.8% (14/32) exceeded the legal limits for coagulase-positive staphylococci. In contrast, among the non-certified samples, the percentage was 21.6% (8/37).

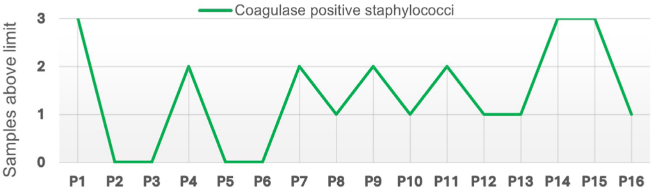


Figure 2. Farms and the total number of samples exceeding the legal limits for coagulase-positive staphylococci counts, according to Instruction Norm No. 161 (Brazil, 2022).

Table 1. Primers used in the polymerase chain reaction assays.

Gene	Nucleotide sequence (5' – 3')	Size (pb)	Reference
cydB	CCCATTTGCTTGGTCTGTAGTA GTCCAGCCCATTTCTGGATTA	432	Almeida et al. (2018)
invA	CGGTGGTTTTAAGCGTACTATT CGAATATGCTCCACAAGGTTA	796	Fratamico and Strobaugh (1998)
iap	CAAACTGCT TTATACGCG	660	Bubert et al. (1999)
mecA	ACTGCTATCCACCCTCAAAC CTGGTGAAGTTGTAATCTGG	163	Mehrotra et al. (2000)
sea	CCTTTGGAAACGGTTAAAACG TCTGAACCTTCCCATCAAAAAC	127	Kim et al. (2011)

3.2 Pathogen detection

Of the 69 samples analyzed, four (5.8%) tested positive for *Staphylococcus aureus*, confirmed by the presence of the *cydB* gene. All positive samples originated from non-ARTE-certified farms. Samples 11 (P3), 16 (P4), and 50 (P7) were obtained from raw milk, while sample 55 (P8) was from cheese curd. A total of 30 *S. aureus* isolates were obtained following isolation and PCR: eight isolates from sample 11, seven from sample 16, eight from sample 55, and seven from sample 50. The *invA* (for *Salmonella* spp.) and *iap* (for *Listeria monocytogenes*) genes were not detected in any sample.

3.3 Phenotypic antimicrobial susceptibility testing

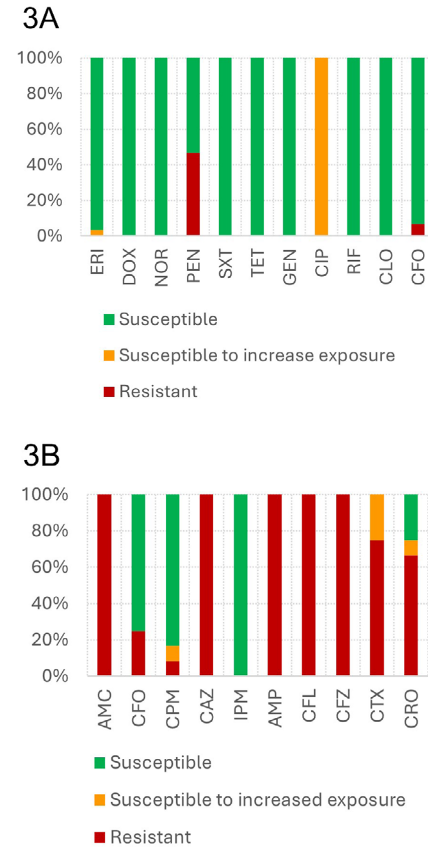
3.3.1 Screening on selective media containing antibiotics

Samples 11, 16, and 50, which were positive for *S. aureus*, exhibited phenotypic resistance, demonstrated by growth on Mannitol Salt Agar containing 6 mg/mL of oxacillin (4.35%). Regarding Enterobacteriaceae, nine samples (13.04%) exhibited phenotypic resistance to ceftazidime: samples 21, 25, 26, 27, 31, 35, 38, 50, and 55. These yielded a total of 11 isolates, which were identified by MALDI-TOF as the species described in Table 2.

3.3.2 Disk diffusion test

Among the *Staphylococcus aureus* isolates, resistance was observed to penicillin (46.67% [14/30]) and cefoxitin (6.67% [2/30]). However, the overall susceptibility profile was broad, with all isolates showing sensitivity to norfloxacin, doxycycline, sulfamethoxazole + trimethoprim, tetracycline, gentamicin, rifampin, chloramphenicol, and azithromycin (Figure 3A).

For Enterobacteriaceae isolates, resistance patterns were species-intrinsic, with 100% resistance observed for amoxicillin + clavulanate, ceftazidime, ampicillin, cephalothin, cefazolin, cephalexin, and cefotaxime (80% [12/15]), as well as ceftriaxone (73.33% [11/15]). In contrast, high susceptibility was noted for



AMC: Amoxicillin + Clavulanic Acid; AMP: Ampicillin; AZM: Azithromycin; CAZ: Ceftazidime; CFL: Cefalotin; CFE: Cephalexin; CFZ: Cefazolin; CIP: Ciprofloxacin; CFO: Cefoxitin; CLO: Chloramphenicol; CPM: Cefepime; CRO: Ceftriaxone; CTX: Cefotaxime; DOX: Doxycycline; ERI: Erythromycin; FOX: Cefoxitin; GEN: Gentamicin; IPM: Imipenem; NOR: Norfloxacin; PEN: Penicillin; RIF: Rifampicin; SXT: Sulfamethoxazole + Trimethoprim.; TET: Tetracycline.

Figure 3. Percentage distribution of antimicrobial susceptibility determined by the disk diffusion method in *Staphylococcus aureus* **(A)** and Enterobacteriaceae **(B)** isolates obtained from raw milk, pasteurized milk, curd, and cheese samples during the production of *Queijo Minas Artesanal* in the Serra da Canastra region, Minas Gerais, Brazil.

Table 2. Ceftazidime-resistant Enterobacteriaceae isolates identified by MALDI-TOF, and oxacillin-resistant *S. aureus* isolates, obtained from different farms and sample types.

Microorganism	Isolated	Property	Selo ARTE	Origin
Enterobacteriaceae				
Hafnia alvei	21.1	P5	No	Pasteurized milk
Hafnia alvei	21.2	P5	No	Pasteurized milk
Hafnia alvei	25	P9	Yes	Raw milk
Hafnia alvei	26.1	P9	Yes	Cheese curd
Hafnia alvei	26.2	P9	Yes	Cheese curd
Hafnia alvei	27	P9	Yes	Cheese
Hafnia alvei	31	P10	Yes	Cheese
Hafnia alvei	35	P11	Yes	Cheese
Hafnia alvei	38	P12	Yes	Cheese curd
Enterobacter asburiae	50	P7	No	Raw milk
Citrobacter freundii	55	P8	No	Cheese curd
***Staphylococcus* sp.**				
Staphylococcus aureus	11	P3	No	Raw milk
Staphylococcus aureus	16	P4	No	Raw milk
Staphylococcus aureus	50	P7	No	Raw milk

cefepime (80% [12/15]) and cefoxitin (60% [9/15]), with all isolates being fully susceptible to imipenem (Figure 3B).

3.4 Detection of resistance and virulence genes

The *seA* and *mecA* genes were not detected in any of the *S. aureus* isolates

3.5 Genetic similarity

PFGE analysis revealed six distinct genetic profiles, using a 90% similarity cutoff in the dendrogram (Figure 4). The DR was 20% (6/30). Genetic similarity among isolates ranged from 55 to 95%, with well-defined clustering patterns.

4 DISCUSSION

Normative Instruction No. 161 (Brazil, 2022) establishes microbiological standards for food products. In cheese, it prohibits the presence of *Salmonella* spp. and staphylococcal enterotoxins; it allows up to 10^3 CFU/g of coagulase-positive staphylococci in a maximum of two samples when moisture content is below 46%. For pasteurized milk, no sample may exceed 10^1 CFU/mL of Enterobacteriaceae. Artisanal cheeses must comply with these limits or follow specific regulations.

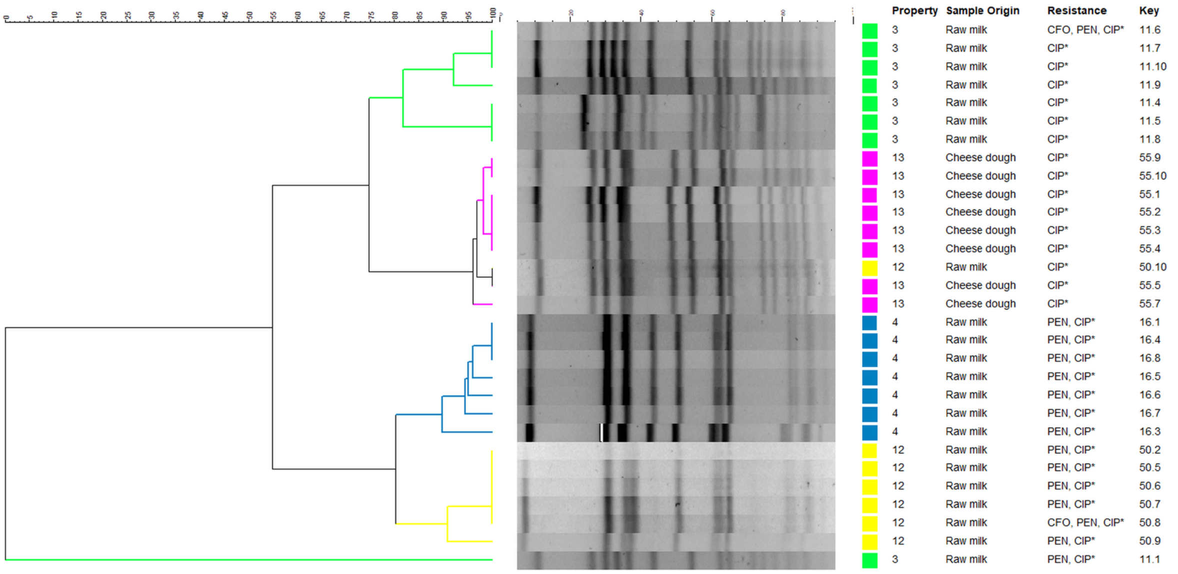
In Brazil, RDC No. 216/2004 requires environmental monitoring but does not specify quantitative limits for utensils (Brazil, 2004). In contrast, the European Union adopts a limit of ≤ 10 CFU/cm^2 (European Commission, 2001, 2004), and the United States Pharmacopeia (USP) sets a limit of ≤ 5 CFU/25 cm^2 in ISO 7 classified environments (USP, 2021). Although there is no specific standard for swab sampling of utensils used in artisanal cheese production, a limit of $\leq 1{,}000$ CFU/cm^2 for total staphylococci was adopted as a reference, considering the high degree of manual handling and low automation of the process.

High counts of Enterobacteriaceae in pasteurized milk from farms P2, P4, and P6 may be attributed either to thermal processing failures or recontamination via biofilms in pasteurization systems with CIP (clean-in-place) cleaning, where inadequate sanitation in closed circuits favors bacterial adhesion and compromises milk quality (Maifreni et al., 2015; Nasiri & Hanifian, 2022).

Artisanal cheese from the Serra da Canastra microregion undergoes a minimum maturation period of 22 days on ventilated shelves with insect protection, remaining exposed to the environment for an extended duration (Minas Gerais, 2020b). This exposure facilitates microbial colonization despite all adopted precautions.

Intensive management and the use of raw milk from animals with subclinical mastitis are critical factors contributing to contamination by coagulase-positive staphylococci, reflecting shortcomings in hygienic practices (Andretta et al., 2019). *S. aureus*, the most common species in this group, is frequently detected in high counts due to its association with mastitis and improper milk handling (Ribeiro Júnior et al., 2024). In this study, 73.33% (22/30) of *S. aureus* isolates were obtained from raw milk, and 26.67% (8/30) from cheese curd, all originating from farms without the ARTE seal.

The detection of *S. aureus* in three samples (11, 16, and 50), exhibiting resistance to oxacillin—including two isolates (11.3 and 50.8) resistant to cefoxitin—was not accompanied by the presence of the *mecA* gene or enterotoxin-encoding genes. Similar findings were reported by Aragão et al. (2019), who observed 7.4% methicillin resistance in *S. aureus* by phenotypic testing without detection of *mecA* or enterotoxin genes, and by



	Property	Sample Origin	Resistance	Key
🟩	3	Raw milk	CFO, PEN, CIP*	11.6
🟩	3	Raw milk	CIP*	11.7
🟩	3	Raw milk	CIP*	11.10
🟩	3	Raw milk	CIP*	11.9
🟩	3	Raw milk	CIP*	11.4
🟩	3	Raw milk	CIP*	11.5
🟩	3	Raw milk	CIP*	11.8
🟪	13	Cheese dough	CIP*	55.9
🟪	13	Cheese dough	CIP*	55.10
🟪	13	Cheese dough	CIP*	55.1
🟪	13	Cheese dough	CIP*	55.2
🟪	13	Cheese dough	CIP*	55.3
🟪	13	Cheese dough	CIP*	55.4
🟨	12	Raw milk	CIP*	50.10
🟪	13	Cheese dough	CIP*	55.5
🟪	13	Cheese dough	CIP*	55.7
🟦	4	Raw milk	PEN, CIP*	16.1
🟦	4	Raw milk	PEN, CIP*	16.4
🟦	4	Raw milk	PEN, CIP*	16.8
🟦	4	Raw milk	PEN, CIP*	16.5
🟦	4	Raw milk	PEN, CIP*	16.6
🟦	4	Raw milk	PEN, CIP*	16.7
🟦	4	Raw milk	PEN, CIP*	16.3
🟨	12	Raw milk	PEN, CIP*	50.2
🟨	12	Raw milk	PEN, CIP*	50.5
🟨	12	Raw milk	PEN, CIP*	50.6
🟨	12	Raw milk	PEN, CIP*	50.7
🟨	12	Raw milk	CFO, PEN, CIP*	50.8
🟨	12	Raw milk	PEN, CIP*	50.9
🟩	3	Raw milk	PEN, CIP*	11.1

CFO: Cefoxitin; CIP: Ciprofloxacin; PEN: Penicillin.

Clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method in BioNumerics software version 7.5, with the Dice coefficient and optimization set at 1%. A PFGE similarity cutoff of 90% was applied (indicated by the red dashed line).

Figure 4. Dendrogram generated from the comparison of pulsed-field gel electrophoresis banding patterns of *Staphylococcus aureus* isolates from raw milk and cheese curd samples.

Silva et al. (2021), who identified 10 *S. aureus* strains in Minas Frescal cheese, also lacking the *SEA*, *SEB*, *SEC*, *SED*, and *SEE* genes. Other studies have reported cefoxitin-resistant isolates negative for *mecA*, suggesting the possible presence of the *mecC* gene (Badawy et al., 2022; García-Álvarez et al., 2011) or variants with low similarity to classical *mecA*, undetectable by conventional molecular methods, highlighting limitations in genotypic detection of methicillin resistance (Paterson et al., 2014; Stegger et al., 2012).

The absence of *Salmonella* spp. may be explained by the inhibitory action of lactic acid bacteria (LAB) and coliforms, particularly in acidic environments with low pH (< 4.7) after maturation or when initial contamination occurs at low concentrations (Donegá et al., 2023; Martins et al., 2015). In another study, LAB also demonstrated anti-listerial activity in milk contaminated with *L. monocytogenes*, reducing the infection risk by approximately 1.5-fold in the general population (Campagnollo et al., 2018). These bacteria produce bacteriocins—natural compounds stable at high temperatures, resistant to pH variations, colorless and odorless—, which are effective against pathogens (Daba & Elkhateeb, 2020; Saragoça et al., 2024).

The exclusive occurrence of *S. aureus* in uncertified farms suggests that the ARTE Seal may be associated with improved hygienic-sanitary practices. However, continuous training and inspection measures remain necessary to ensure microbiological safety.

The high prevalence of *Hafnia alvei* in the samples, coupled with resistance to β-lactam antibiotics, is consistent with the findings of Merchán et al. (2022), who also identified this species as one of the most predominant in cheeses from various origins. Nevertheless, *H. alvei* is known to exhibit intrinsic resistance to these antimicrobials. *H. alvei* and *Citrobacter freundii* are common inhabitants of the gastrointestinal tract but rarely cause human infections (Pasquali et al., 2022). This study corroborates previous findings that detected *Enterobacter* spp. and *Citrobacter* spp. in cheeses, including strains producing ESBL and AmpC enzymes, suggesting their role as reservoirs of antimicrobial resistance (Ritschard et al., 2022). Pasquali et al. (2022) also reported the predominance of *E. cloacae*, *H. alvei*, and *C. freundii* in Italian artisanal cheeses, underscoring the importance of milk microbiological quality and hygiene during production stages, especially maturation.

In the genetic diversity analysis, a notable finding was the clustering of the *S. aureus* isolate from raw milk at farm P7 (key 50.10) with isolates from cheese curd at farm P8. The genetic proximity of these strains indicates possible transmission between farms, potentially related to geographic proximity and shared management practices, including herd movement, common equipment use, and dissemination of antimicrobial-resistant strains (Dendani Chadi & Arcangioli, 2023).

5 CONCLUSIONS

Although more samples from ARTE-certified farms exhibited counts outside microbiological standards, *S. aureus* isolates were detected exclusively in products from uncertified farms. These findings indicate that the ARTE seal may be associated with more effective sanitary practices, reinforcing its importance not only as a commercial differentiator but also as a tool for microbiological quality monitoring and the promotion of good practices in artisanal cheese production.

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