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Microbiological Safety of Raw Sheep's Milk and Cheese Produced in Southern Brazil: An Evaluation of Enterotoxins and Antimicrobial Resistance in Isolated Staphylococcus Species

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Abstract: This work highlights that monitoring the microbiological quality of animal products, such as raw sheep's milk and cheese, is necessary for food safety. Sheep's milk and its derivatives are not legislated in Brazil. Thus, present study aimed to evaluate: (i) the hygienic-sanitary quality of raw sheep's milk and cheese produced in southern Brazil; (ii) the presence of enterotoxins and *Staphylococcus* spp. in these products; and (iii) the antimicrobial susceptibility profile and resistance genes of the isolated *Staphylococcus* spp. Thirty-five raw sheep's milk and cheese samples were evaluated. Microbiological quality and the presence of enterotoxins were determined by Petrifilm and VIDAS SET2 methods, respectively. Antimicrobial susceptibility tests were conducted using VITEK 2 equipment and the disc diffusion method. The presence of resistance genes *tet*(L), *sul*1, *sul*2, *erm*B, *tet*M, AAC(6)', *tet*W, and *str*A were evaluated by PCR. In total, 39 *Staphylococcus* spp. were isolated. The *tet*M, *erm*B, *str*A, *tet*L, *sul*1, AAC(6)', and *sul*2 resistance genes were detected in 82%, 59%, 36%, 28%, 23%, 3%, and 3% of isolates, respectively. The results showed that both products contained *Staphylococcus* spp. and these strains were resistant to antimicrobials as well carriers of resistance genes. These results highlight Brazil's need for specific legislation regarding the production and marketing of these products.

Keywords: raw sheep's milk; Cheese; Staphylococcal enterotoxin. microbiological safety; antimicrobial resistance

1. Introduction

Sheep's milk and cheese production is a recent activity in Brazil in comparison with European countries. Brazilian production of sheep's milk reached 1.72 million liters in 2017 [1], and most of this milk was used for cheese-making [2]. The microbiological quality of milk is related to its natural microbiota and contamination, usually by viruses, bacteria, and fungi [3]. Thus, evaluating microorganisms that indicate the hygienic-sanitary quality of milk can prevent foodborne illness outbreaks. Foodborne illnesses are a major public health problem arising from consuming contaminated food, affecting ~600 million people annually worldwide [4]. Such illnesses are caused by pathogenic microorganisms such as *Staphylococcus aureus* (*S. aureus*), which stands out due to

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enterotoxin production, since these toxins have been implicated in foodborne illness outbreaks resulting from consuming cheese [5].

Cheese produced from sheep's milk is rich in proteins, fats, and carbohydrates, which often favors toxin production by *S. aureus* [6]. Many *S. aureus* contamination sources are associated with human management, water, milking equipment, and the environment [7]. *S. aureus* causes mastitis in animals and is thus a common contaminant of raw milk [8]. Therefore, one of the main challenges in the dairy industry is producing milk and derivatives with the lowest contamination level possible to guarantee product conservation and consumer safety [9]. Few studies have been conducted on the quality of raw sheep's milk and the cheese produced from it in Brazil, where there is no specific legislation regarding these products.

Another relevant issue in food safety is antimicrobial resistance (AMR). The use of antimicrobial agents in humans and animals has led to the selection of antimicrobial-resistant microorganisms [10]. According to [11]), in 2019, antibiotic-resistant bacterial infections were responsible over 1.2 million deaths, 100,000 of which occurred due to methicillin-resistant *S. aureus* (*MRSA*), a major agents causing serious foodborne outbreaks [4]. Thus, the objectives of the present study were to evaluate: (i) the microbiological quality of raw sheep's milk and cheese produced on farms in southern Brazil; (ii) the presence of *Staphylococcus* spp. and their enterotoxins in these samples; and (iii) the antimicrobial susceptibility profiles of *Staphylococcus* spp. and the resistance genes of these isolates.

2. Materials and Methods

2.1. Sampling and processing

Fifteen raw sheep's milk samples were collected in three producing farms (F1, F2 and F3, n = 5 each), and 20 cheese samples from different farms and cheese types (colonial, fresh, feta-type and pecorino-type, n = 5 each) were purchased from the local commerce (Figure 1). All samples were within the expiration date established by their manufacturers. After collection, the samples were transported to the laboratory in Styrofoam boxes with ice and submitted to microbiological analysis under aseptic conditions within 24 hours.

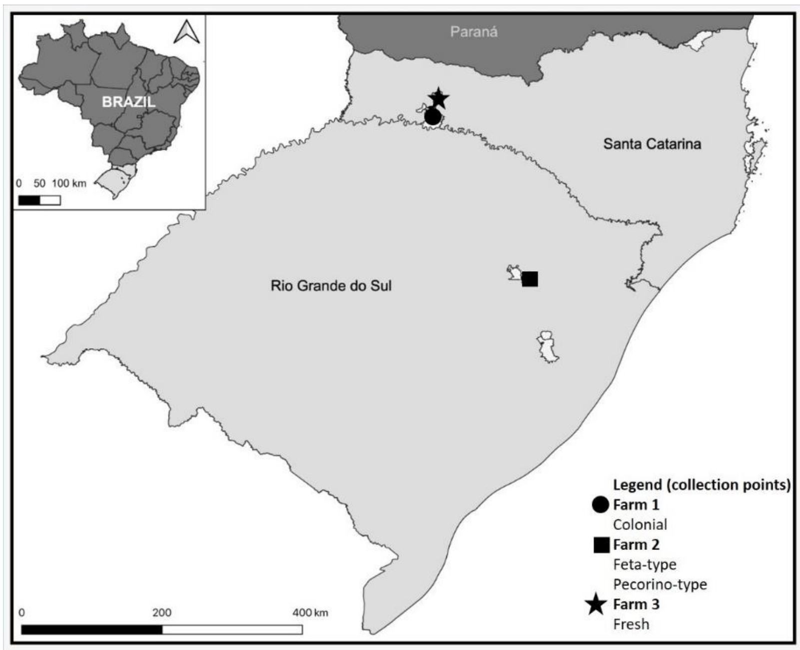

Figure 1. Sampling sites of sheep's raw milk and cheese.

For enterotoxins' analysis, *Staphylococcus* spp. isolation, and antimicrobial susceptibility and resistance genes of the isolates, only 15 milk and 15 cheeses samples were evaluated, and the fresh cheese did not undergo these analyses.

2.2. Sampling and processing

Microbiological analysis of aerobic mesophilic microorganisms (AM), total coliforms (TC), *Escherichia coli* and *S. aureus* were performed by the petrifilmTM system (3M Company, St, Paul, MN, USA). An aliquot (10 g) of each cheese sample was transferred to a sterile bag, in which 90 mL of buffered peptone saline (Scharlau) were added and homogenized in a stomacher for 5 min. Then, serial decimal dilutions were carried out in 0.85% saline solution (up to 10-4), for the enumeration of AM, TC, *E. coli* and *S. aureus*. For milk, 1 mL was inoculated into 1 tube containing 9 mL of 0.85% saline solution and followed with the serial dilutions (up to 10-4).

Petrifilm™ plates were used to count AM, TC, *E. coli*, and *S. aureus*. A 1.0 mL volume of each dilution was inoculated in the center of the lower film, after which the upper film was carefully positioned, avoiding the formation of air bubbles. Diffusers indicated for each type of plate were used to distribute the inoculum. The plates were incubated for 24 – 48 h at 35 ± 1 °C and the results were expressed in CFU/mL [12].

2.3. Analysis of staphylococcal enterotoxins

The VIDAS SET enzyme-linked fluorescent immunoassay (ELFA) assay (bioMérieux, RCS Lyon, France) was used to detect enterotoxin production from SEA to SEE without distinguishing the individual toxins. The analysis was performed according to the manufacturer's guidelines.

2.4. Staphylococcus spp. Isolation

For each cheese sample, 25 g were placed in a sterile plastic bag with 225 mL of buffered peptone water (Scharlau, Spain). After homogenization in a mixer (Smasher™ AES Blue line) for 3 min, 1 ml of the initial 10-1 suspension divided into 0.3, 0.3 and 0.4 mL was seeded on 3 Baird-Parker agar plates (BP) (Neogen), supplemented with egg yolk tellurite emulsion (Neogen, United States).

Milk samples were inoculated directly into BP plates (0.3, 0.3 and 0.4 mL). Dilutions of up to 10-3 were performed for each sample. It was then incubated at 37°C for 48 hours. After 48 h, 5 typical colonies that appeared black, shiny, and convex and surrounded by zones of 2 to 5 mm and 5 atypical colonies were selected.

The isolates obtained were confirmed using Gram color to observe the morphology of the colonies. Subsequently, tests of catalase, coagulase and fermentation in mannitol salt were carried out. *Staphylococcus aureus ATCC* 25923 was used as a positive control in each of the biochemical test protocols.

The isolates were then seeded on TSA agar plates (Kasvi) and incubated at 37 °C for 24 hours. After this time, the colonies were suspended in a solution of 3 mL of 0.45% saline solution. A turbidity of 0.5 – 0.63 standard McFarland was established using VITEK DensiCHEK Plus (bioMérieux, Nürtingen, Germany). Isolates were identified to the species level using the VITEK 2 system (bioMérieux, Nürtingen, Germany) using GP cards (for analysis of gram-positive bacteria). The isolates identified as *Staphylococcus* spp. they were removed with a sterile loop from the TSA plate and placed in Eppendorf containing 1mL of TSB broth (Oxoid) with 10% glycerol and despaired at -20 ºC.

2.5. Antimicrobial susceptibility analysis

The *Staphylococcus* spp. isolates were tested for antibiotic resistance by the VITEK 2 method and the disk diffusion test using Mueller-Hinton agar (KASVI). A panel of 23 antimicrobial agents were evaluated. The antibiotics evaluated the VITEK 2 method as follows: benzylpenicillin (BENPEN) oxacillin (OXA), cephalothin (CFL), cefovecin (CVN), ceftiofur (CEF), enrofloxacin (ENR), marbofloxacin, pradofloxacin (PRA), amoxicillin/clavulanic acid (AUG), kanamycin (K), gentamicin (GEN), neomycin (N), erythromycin (ERI), clidamycin (CLI), tetracycline (TET), doxycycline (DXT), chloramphenicol (CLO), nitrofurantoin (F) and Trimethoprim/Sulfamethoxazole (SXT), according to the concentration established in the analysis cards. For this, 280 μL were taken from the McFarland standardized solution and added to 3 mL of 0.45% saline solution. The antibiogram of the isolates

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was performed with a VITEK 2 system (bioMérieux, Nürtingen, Germany) using AST – GP80 cards. For antibiotics that were not included in the AST-GP80 card, the agar disk-diffusion method was used, as recommended by the Clinical and Laboratory Standards Institute [13].

The antimicrobial agents tested by disc-diffusion method were based on *Staphylococcus* infection treatment. The antimicrobials tested were ampicillin (AMP) (10 µg), linezolid (LNZ) (30 µg), rifampicin (RIF) (5 µg), sulfazotrim (SUT) (25 µg). *Staphylococcus aureus* strain *ATCC*25923 was used as control. The isolates were categorized as susceptible (S), intermediate (I), or resistant (R), according to Clinical and Laboratory Standards documents [13]. Multidrug resistance was considered if the strain was resistant to 3 or more antimicrobial classes.

2.6. Detection of resistance genes

Staphylococcus spp. DNA was extracted using PureLink Genomic DNA Mini Kit, according to the manufacturer's instructions and described by Endres et al. 2021.   The extracted DNA was stored at -20 °C until analysis.

Polymerase Chain Reaction (PCR) was performed to detect the genes *erm*B, AAC(6)', *tet*L, *tet*M, *tet*W, sul1, *sul*2, *str*A (Table 1). PCR for *16S rRNA* gene was used as internal control. Amplicons were submitted to electrophoresis using 1% agarose gels stained with ethidium bromide. Strains available in laboratory harboring the mentioned resistance genes were used as a positive control.

Table 1. Nucleotide sequences used as primers in PCR for identification of resistance genes.

Target	Annealing temperature (°C)	Amplicon size (bp)	Sequence (5′ to 3′)	Reference
16S rRNA	55	375	F CACGGTCGKCGGCGCCATT R GGACTACHVGGGTWTCTAAT	[14]
*erm*B	52	639	F GAAAAGGTACTCAACCAAATA R AGTAACGGTACTTAAATTGTTTAC	[15]
AAC(6)'	60	219	F CCAAGAGCAATAAGGGCATA R CACTATCATAACCACTACCG	[16]
*tet*L	58	628	F ACTCGTAATGGTTGTAGTTGC R TGTAACTCCGATGTTTAACACG	[17]

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*tet*M	52	657	F GTTAAATAGTGTTCTTGGAG R CTAAGATATGGCTCTAACAA	[18]
*tet*W	60	168	F GAGAGCCTGCTATATGCCAGC R GGGCGTATCCACAATGTTAAC	[19]
*Sul*1	60	99	F GGATCAGACGTCGTGGATGT R GTCTAAGAGCGGCGCAATAC	[20]
*Sul*2	57	99	F CGCAATGTGATCCATGATGT R GCGAAATCATCTGCCAAACT	[20]
*str*A	59	99	F CCAGTTCTCTTCGGCGTTAG R ACTCTTCAATGCACGGGTCT	[20]

2.7. Statistical analysis

The comparison of microorganisms' counts was performed using the Kruskal-Wallis test. The different types of cheese were compared, and the raw sheep's milk samples were compared between the different farms. The level of significance was set as $p<0.05$. Afterwards, the Dunn test for multiple comparisons was performed. The enterotoxin detection, *Staphylococcus* spp. isolation, and resistance studies were shown as descriptive statistics.

3. Results and discussion

3.1. Microbiological quality of milk samples

No difference in total mesophilic aerobic microorganism and S. aureus counts were observed across the milk samples from the evaluated farms (Supplementary Table S1 and Figure 2 a and d). Regarding total coliforms, Farm 1 had lower counts than Farms 2 and 3 ($p = 0.022$, and 0.027 respectively; Figure 2b). For E. coli, Farm 3 had higher counts than Farms 1 and 2 ($p = 0.013$, and 0.031 respectively; Figure 2c). As Farms 1 and 3 are in Santa Catarina state and Farm 2 in Rio Grande do Sul state, the difference in microorganism counts do not appear associated with geographic location. Instead, the factors involve likely include hygiene during the milking process and milk storage on each farm. However, no follow-up was conducted to assess the hygiene conditions at the farms.

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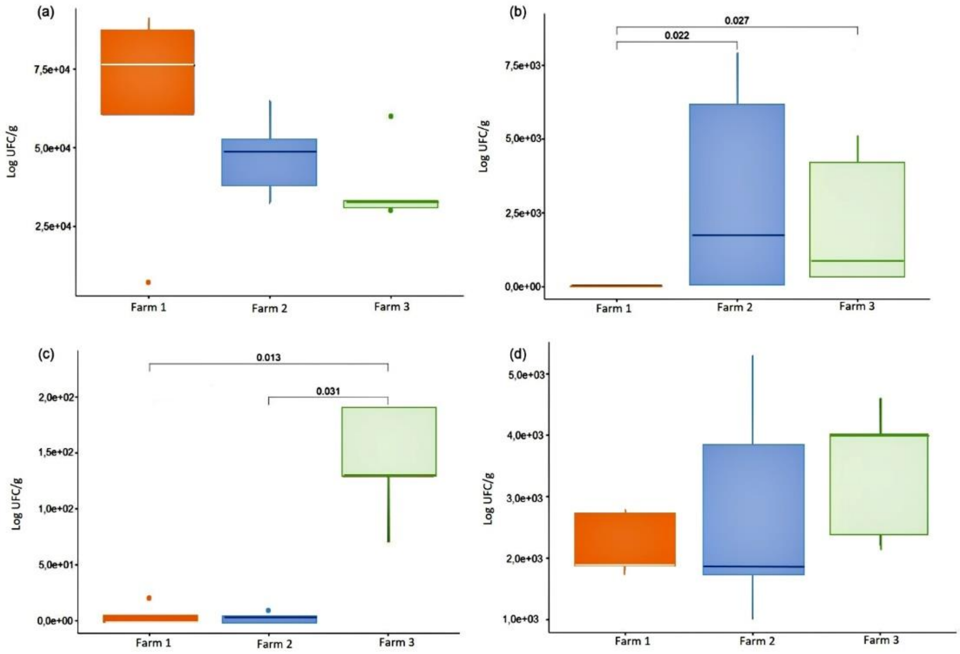

Figure 2. Microbiological quality of raw sheep's milk. (a) Aerobic mesophilic microorganism counts (AM); (b) total coliforms (TC); (c) *Escherichia coli*; and (d) *Staphylococcus aureus*.

All samples of raw sheep's milk showed high aerobic mesophilic bacteria and *S. aureus* counts, regardless of the farm. *E. coli* was absent from 5 samples (three from Farm 1 and two from Farm 2). Other studies have investigated *E. coli*, total coliform, and mesophilic bacterial contamination in milk used to make cheese [21] [22]. Contamination by these microorganisms impacts the milk's shelf life, quality, and ability to transmit diseases, especially when consumed raw or used to produce derivatives without pasteurization or minimum maturation required by legislation [23].

S. aureus was observed in all raw sheep's milk analyzed by Petrifilm plates. The presence of *S. aureus* in raw milk is often associated with subclinical mastitis and poor hygiene practices. *S. aureus* may be an opportunistic pathogen in humans [21] [22] and was found in raw sheep's milk in previous studies [23] [24], indicating the need for microbial control and handler training. *S. aureus* may be responsible for producing thermostable enterotoxins that remain in the product after pasteurization, causing problems for consumers [21] and shortening the product shelf life [25].

3.2. Microbiological quality of cheese samples

The multiple comparison analysis performed using Dunn's test showed statistical differences in the microorganism counts of some types of cheese (Figure 3). These comparisons must be carefully evaluated, considering the manufacturing technology, milk composition, and maturation time used for each type of cheese. The evaluated cheese types undergo different maturation processes. In addition, these cheeses are produced from pasteurized milk with starter cultures added, shaping the microbiota of the final product [22]. Most of the evaluated cheeses had low total coliform, *E. coli*, and *S. aureus* counts (Supplementary Table S2). Colonial cheeses had lower total coliform counts than those of fresh cheeses ($p<0.05$; Figure 3b); this finding is related to the maturation process of colonial cheeses, which inhibits microorganism growth. No significant difference in *E. coli* counts was observed between the cheese types (Figure 3c). The low coliform count indicates adequate milk pasteurization and manufacturing practices.

All cheese samples showed significant mesophilic bacteria counts, and feta-type cheeses had higher counts than colonial and fresh cheeses (Figure 3a). *S. aureus* was found in fresh cheese but not in colonial and feta-type cheeses (Figure 3d). This finding may be associated with the pH reduction process, the microbiological load of the raw material, and good manufacturing practices at the

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production sites. Moreover, the use of starter cultures and their metabolites are the main contributors to controlling pathogens during the maturation process [26].

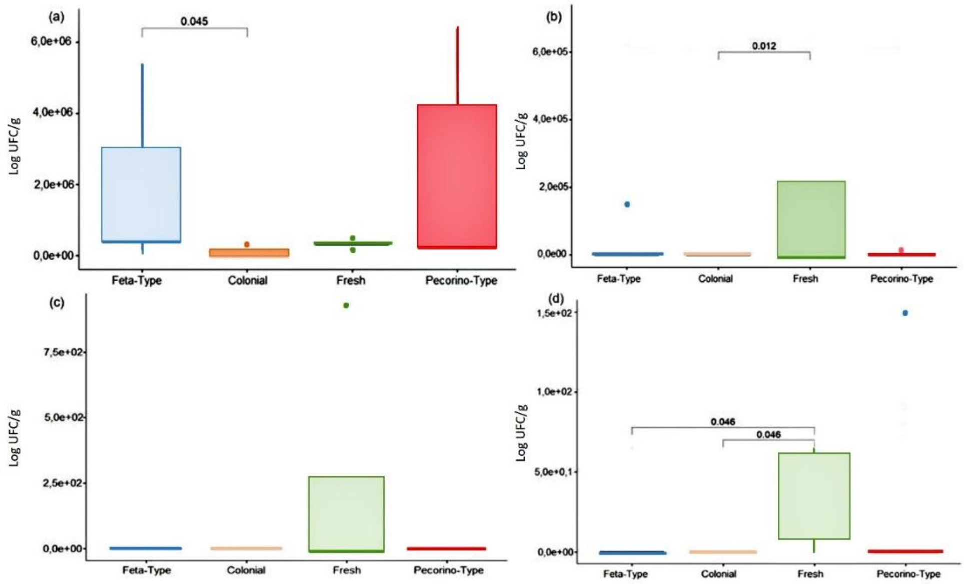

Figure 3. Microbiological quality of sheep's cheese. (a) Aerobic mesophilic microorganism counts; (b) total coliforms; (c) *Escherichia coli*; and (d) *Staphylococcus aureus*.

S. aureus and *E. coli* are the most important zoonotic pathogens causing bacterial death and foodborne illnesses worldwide [27]. These microorganisms have been described in chicken meat and offal, eggs, beef, sheep and goat meat, buffalo meat, raw bovine milk, raw sheep's and goat's milk, raw buffalo milk, cheese, and fish [28]–[35]. The Health Surveillance Secretariat in Brazil recorded 2,504 outbreaks of Foodborne Diseases between 2016 and 2019, affecting 37,247 patients and causing 38 deaths. However, this number may be lower than the actual number of affected individuals because reporting foodborne outbreaks is not mandated in Brazil [34]. In 11.5% of these outbreaks, the etiological agent was identified as *S. aureus*, and 9.06% were caused by consuming milk and its derivatives [36]. Nonetheless, no specific information is available on the frequency at which cheeses are related to staphylococcal food poisoning.

According to the Brazilian legislation for raw milk and dairy products (Ordinance 146 of March 7, 1996), microbiological standards are established according to the cheese's moisture content. However, this legislation is not specific to sheep's milk and its dairy products, for which there is a lack of regulation. The cheeses assessed in this study have a moisture content between 35.9% and 45.9%, characterizing them as cheeses with medium moisture. The counts were within the established legislation for microorganisms, such as the total coliform and *S. aureus* count. Only one fresh cheese sample had thermotolerant coliform content above the acceptable level (5.00×10^2 CFU/mL).

In general, higher microorganism counts were observed in raw sheep's milk than in cheeses, possibly because of the use of pasteurized milk for cheese fabrication and the cheese maturation process. The microbiological quality of cheeses does not necessarily depend on milk pasteurization since some microorganisms can produce thermoresistant toxins [37]. However, pasteurization can eliminate potential pathogenic and spoilage bacteria that may be present in raw milk. In addition, the sanitary control of the herd, standard manufacturing process, pH reduction, removal of water, and addition of salt in cheese production favors microbiological safety [38].

3.3. Enterotoxin investigation

Although milk pasteurization, fermentation, and cheese maturation delay the growth of S. aureus in cheese, it is important to investigate the presence of enterotoxins [21]. Food poisoning by S. aureus is caused by ingesting staphylococcal enterotoxin (SE) produced during microorganism growth in contaminated food. In the present study, staphylococcal enterotoxin was found in one sample of raw sheep's milk; this sample had the highest S. aureus count (5.30 × 10^3 CFU/mL) by the traditional culture method. Several studies have revealed enterotoxins in milk samples [39], [40]. It is important to point out that although the present work only characterizes S. aureus, coagulase-positive (CoPS) and coagulase-negative (CoNS) Staphylococcus spp. possess enterotoxin-producing genes similar to that of S. aureus [41]–[43].

3.4. Staphylococcus spp. isolation

Four hundred sixty-one typical and atypical colonies were selected from 15 samples of raw sheep's milk and 15 samples of cheese and analyzed using the catalase test. The positive catalase strains (40%; n = 186) were subjected to the mannitol salt fermentation test and Gram staining. A total of 39 isolates of Staphylococcus spp. were then selected (24 from sheep's milk and 15 from cheese). Of the 15 cheese strains, 8 (53%) were isolated from feta-type cheese, 4 (27%) from pecorino-type cheese, and 3 (20%) from colonial cheese. Of the 24 raw sheep's milk strains, 13 (54%) were isolated from Farm 1, 1 (4%) from Farm 2 and 10 (42%) from Farm 3. All the isolates were identified (Table 2), and the main Staphylococcus species isolated in raw sheep's milk was S. sciuri (66.7%), while in cheese, the predominant species was S. lentus (60%). Most isolated strains were characterized as CoNS species (Table 2).

Table 2. *Staphylococcus* species isolated in the present study.

Sample	Species	Frequency (%)	Coagulase test	AMR frequency (%)*
Milk	*S. sciuri*	16 (67)	CoNS	15 (94)
	S. Simulans	4 (17)	CoNS	3 (75)
	S. aureus	3 (13)	CoPS	3 (100)
	S. lentus	1 (4)	CoNS	1 (100)
	Total	**24 (100)**		22 (92)
Cheese	*S. lentus*	9 (60)	CoNS	8 (89)
	S. warneri	2 (13)	CoNS	2 (100)
	S. pseudintermedius	2 (13)	CoPS	2 (100)
	S. chromogenes	1 (7)	CoNS	1 (100)
	S. sciuri	1 (7)	CoNS	1 (100)
	Total	**15 (100)**		14 (93)

* Resistant to at least one antimicrobial.

Staphylococcus aureus was detected only in raw sheep's milk samples. As commented above, cheese preparation involves processes that can reduce the microbial count. The organic acids (lactic, acetic, propionic, and butyric acids) produced by *Lactobacillus* spp. in the cheese ripening process are responsible for the lower pH [44], inhibiting bacterial growth, although some pathogens can survive in the acidic conditions. However, the possibility of contamination during forming and packaging remains.

A few researchers have assessed *Staphylococcus* spp. in sheep's milk, isolating *S. chromogenes, S. epidermidis, S. haemolyticus, S. pseudintermedius, S. aureus*, and *S. agnetis* [24], [45], [46]. This difference in species profile may be associated with the animals' microbiota, management techniques, and other factors not assessed in this study.

Staphylococcus pseudintermedius is a common coagulase-positive *Staphylococcus* and was detected in the cheese samples. *S. pseudintermedius* is mainly associated with infection in dogs, cats, and humans [47]. Regarding coagulase-negative isolates, *Staphylococcus chromogenes* has been identified in bovine, sheep's, and goat's milk and is responsible for intramammary infections characterized as mastitis [48]–[50]. *S. sciuri* is also associated with mastitis and is often described as methicillin-resistant [51], [52]. *S. warneri* is common in humans and animals and causes meningitis, endocarditis,

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and septic arthritis in humans [53]. *S. warneri* has been isolated from fish, and antimicrobial resistance is a concern [54].

A previous study in northern Italy also detected CoNS (*S. equorum, S. lentus, S. simulans, S. sciuri*, and *S. xylosus*) in raw milk and cheese [55]. CoNS are often present in fermented foods as part of the normal microbiota and can positively contribute to the development of flavor and aroma due to the production of proteolytic and lipolytic enzymes. CoNS are also salt and acid tolerant, often recovered from sheep's milk-derived cheeses.

3.5. Antimicrobial susceptibility tests and the detection of resistance genes

The antimicrobial susceptibility of the 39 Staphylococcus isolates was evaluated using 23 antibiotics, and resistance was detected in 87% of the evaluated Staphylococcus strains, with 46% being multidrug-resistant. All isolates from raw sheep's milk samples were susceptible to linezolid, sulfazotrim, gentamicin, and nitrofurantoin, and a high frequency of AMR to oxacillin was observed (Figure 4). The isolates obtained from cheese samples showed a lower AMR frequency than those obtained from milk. The cheese isolates were susceptible to ampicillin, linezolid, sulfazotrim, amoxicillin/clavulanic acid, gentamicin, kanamycin, neomycin, doxycycline, chloramphenicol, and trimethoprim/sulfamethoxazole; the greater resistance frequency was to rifampicin (Figure 4).

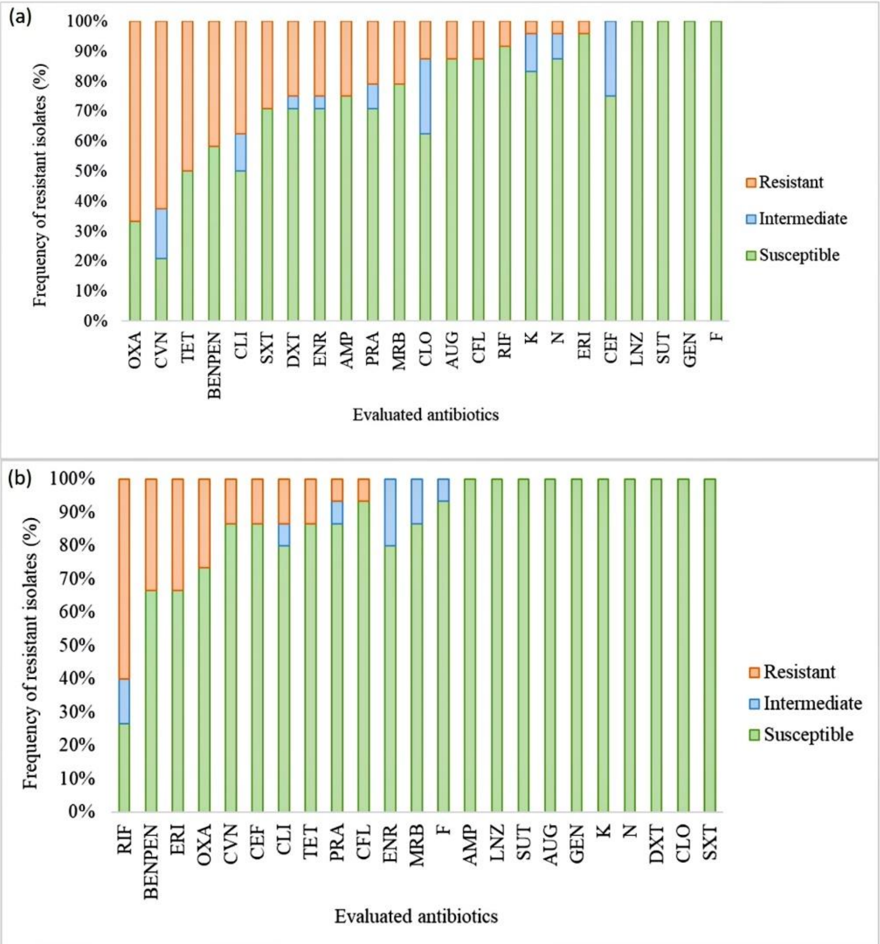

Figure 4. Antimicrobial susceptibility profile of *Staphylococcus* spp. isolates to different antimicrobials. Raw sheep milk isolates are shown in (a), and sheep cheese isolates in (b). Susceptible isolates did not show resistance to any class of antimicrobials; resistant isolates showed resistance to at least one class, and multi-site isolates showed resistance to 3 or more classes of antimicrobials.

Regarding the antimicrobial classes, all strains were susceptible to oxazolidinones. At least one *Staphylococcus* isolate was resistant to rifamycins, sulfonamides, β-lactams, macrolides, fluoroquinolones, lincosamides, and tetracyclines (Figure 5).

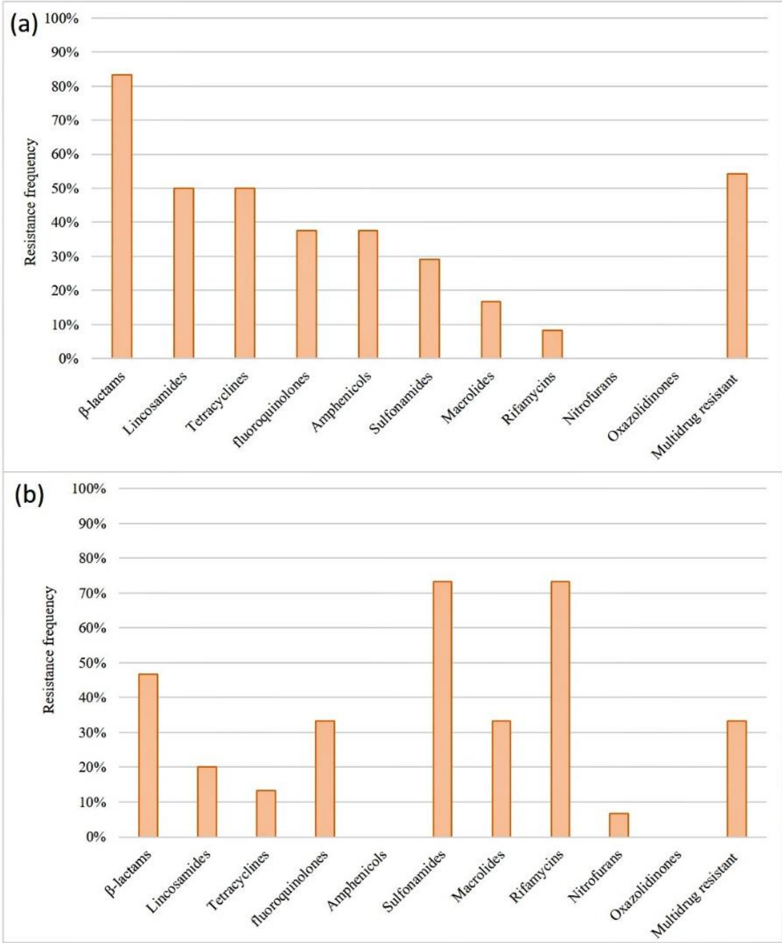

Figure 5. Frequency of *Staphylococcus* spp. resistance according to antimicrobial class. (a) Strains isolated from raw sheep's milk. (b) Strains isolated from cheeses.

All *S. aureus* isolates were multidrug-resistant; these isolates were from the same farm (Farm 3). This result is worrisome since multidrug-resistant bacteria represent a serious public health problem worldwide, causing ~700,000 deaths per year [56]. The resistance mechanisms are diverse and depend on resistance genes, which are of particular concern when located in mobile genetic elements that can be transmitted via horizontal gene transfer [57]. Thus, regardless of their pathogenicity, resistant microorganisms may impact public health, leading to increased hospitalization periods and complicating treatment [58]. Resistance to up to 15 antimicrobials was detected in one of the isolates identified as *S. aureus* in the present study (Figure 6).

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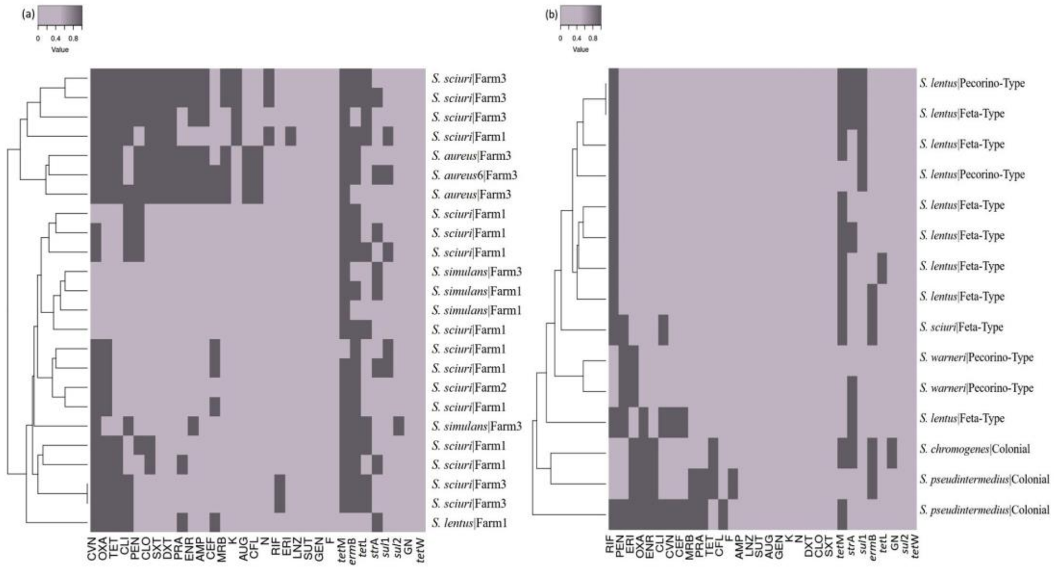

Figure 6. Presence/absence matrix and dendrogram cluster showing the antimicrobial susceptibility profile of each *Staphylococcus* spp. isolate and its resistance genes. Hierarchical groupings are presented for milk (a) and cheese (b) samples. The light gray represents antimicrobial susceptibility and the absence of investigated genes, while dark gray shows the presence of antimicrobial resistance and resistance genes.

Antimicrobial resistance can interfere with clinical therapies and even cause them to fail. The concern related to food consumption relies on the possibility of acquiring resistance genes and microorganisms present in food due to environmental contamination such as contaminated water, air, soil or manure. Moreover, the use of antibiotics in animal production is also a pressure for resistant microorganism selection. The three farms enrolled in this study were asked about the use of antibiotics in the animals and about the presence of other animals in the production environment. Farm 1 replied that it does not use antibiotics and has Maremano shepherd dogs on the property. Farm 2 indicated the use of gentamicin and does not raise other types of animals on the property. Farm 3 reported the use of sulfonamides and terramycins and has bovine milk production on the property. This assessment corroborates that antibiotic use may favor resistance [59], along with the presence of other breeding animals from which a transfer of microorganisms and resistance genes may occur [60]. However, other non-measured factors may be related to AMR, especially in Farm 1, which claims no antibiotic use. Notably, cross-resistance can occur, defined as resistance to antimicrobial agents that act through a common pathway or on the same targets [61].

Importantly, three isolates were identified as MRSA, recognized by the World Health Organization as one of the highest-priority antibiotic-resistant pathogens, causing over 100,000 deaths worldwide in 2019 [4]. MRSA has been isolated from foods in several countries, including Brazil, and is also associated with foodborne disease occurrence [28], [62], [63]. MRSA has been detected in milk and dairy products from different species and locations [64]–[69]. Moreover, MRSA is a prominent cause of nosocomial infection [70] and has been identified in food handlers in public hospitals, emphasizing the need for better food-handling practices to prevent these strains from being transmitted to the community.

This study also investigated some resistance genes for which the protocols and primers were available at the laboratory. The isolated *Staphylococcus* spp. were found to harbor various resistance genes, regardless of their pathogenicity (Figure 6). A total of 82% of isolates had the *Tet*M gene, 59% had *erm*B, 36% had *str*A, 28% had *tet*L, 23% had *sul*1, and 3% had *sul*2 and AAC(6)′. The *tet*W gene was not identified in any strain. The *tet*M gene (related to tetracycline resistance) is commonly located on the Tn916 transposon, which can be transferred naturally between various Gram-positive and Gram-negative bacteria. The Tn916 transposon has been identified in coliform strains isolated from raw cow's milk [71]. The *sul*1, *erm*B, *str*A, and *sul*2 genes have been described in *Salmonella* spp. isolated from leafy vegetables, chicken carcasses, and raw milk processing environments [72], [73]. Studies have shown that the horizontal transfer of these genes between *Staphylococcus* strains is a potential risk for expansion and distribution of multidrug-resistant strains [74].

4. Conclusions

High total coliform, *S. aureus* and *E. coli* numbers were detected in raw milk in this study. However, most of the cheese samples showed low counts. The presence of enterotoxin was verified in 1 sample of raw sheep's milk. This finding is concerning since enterotoxins are heat-resistant and can remain after pasteurization, damaging consumer health. Furthermore, this study revealed different *Staphylococcus* spp. in raw sheep's milk and cheese. These isolates were resistant to several antimicrobial agents, with 46% being multidrug-resistant. Most had at least one resistance gene, a worrying result since resistance is considered a public health problem. These results suggest that sanitary control and the rational use of antimicrobials should be the subject of regulations and monitoring by regulatory agencies. In addition, it is necessary to present guidelines for producing sheep's milk derivatives to establish quality measures for the food produced.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Quartiles and sample counts for the milk collection farms; Table S2: Quartiles and counts of the different cheese types.

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