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Staphylococcus aureus from Minas Artisanal Cheeses: Biocide Tolerance, Antibiotic Resistance and Enterotoxin Genes

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Abstract: *Staphylococcus aureus* is a common contaminant in artisanal raw-milk cheeses. Tolerance of *S. aureus* to biocides is a threat to disinfection in the cheese production environment, while antibiotic resistance and enterotoxin production are additional health concerns. This study aimed to evaluate the tolerance of *S. aureus* isolated from Minas artisanal cheeses to the biocides benzalkonium chloride, hexadecylpyridinium chloride, cetrimide, triclosan, hexachlorophene, and chlorhexidine, and the simultaneous occurrence of genes coding for antibiotic resistance (*mecA*, *aacA-aphD*, and *tetK*), efflux pumps [*qacA/B* and *smr* (*qacC/D*)], and enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*). Among the tested isolates, 38.2% were resistant to at least one biocide, and 73.1% were positive for one or more antibiotic resistance gene. Most of the biocide-tolerant and antibiotic-resistant isolates harbored efflux pump genes, and were positive for at least one staphylococcal enterotoxin gene. The study highlights the need for correct hygiene monitoring programs to ensure the safety of these products.

Keywords: artisanal cheese; *Staphylococcus aureus*; biocide; antibiotic; efflux pump; enterotoxins



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1. Introduction

Coagulase-positive staphylococci are commensal colonizers of the skin and mucous membranes of humans and other warm-blooded animals. They are potentially pathogenic, especially *Staphylococcus aureus*, which can cause a variety of infections, such as superficial infections of the skin and soft tissues, osteomyelitis, and septicemia [1]. They also cause staphylococcal food poisoning (SFP), one of the most frequent foodborne illnesses in the world [2]. SFP is a non-contagious acute gastrointestinal disease that results from the ingestion of foods containing preformed staphylococcal enterotoxins (SE), secreted mainly by coagulase-positive staphylococci, especially *S. aureus*. Several SE have been described, including the classical SEA and SED, responsible for the majority of SFP cases, in addition to the less common SEB, SEC, SEE, SEG, SEH, and SEI [3,4]. The incidence of SFP cases caused by methicillin-resistant *S. aureus* (MRSA) has increased in recent years, raising significant public health concerns [1].

Research and discovery of new antimicrobial compounds for use as stand-alone or combination therapy options must consider preventive strategies and measures to reduce the occurrence of resistant microorganisms. In this sense, effective disinfection and sanitation are critical in preventing the transmission of communicable diseases in human and animal environments [5].

Biocides have been used for centuries for many different applications, including, after the discovery of the microbe, as disinfectants, sanitizers, antiseptics, and preservatives [6]. They include alcohols, acids and alkalis, aldehydes, anilids and biguanides, diamides, halogen releasing compounds, oxidizing agents, organic acids, peroxygens, phenolics

(phenols, bisphenols, and halophenols), quaternary ammonium compounds, and vapor phase sterilants [7].

The main biocides are quaternary ammonium compounds (QACs), such as benzalkonium chloride, cetrимide, and hexadecyl pyridinium chloride, as well as biguanides, such as chlorhexidine, and bisphenols, such as triclosan and hexachlorophene [6]. These antimicrobial compounds are used as active ingredients in specific formulations for the control of pathogenic organisms in livestock, food and beverage production, the pharmaceutical and textiles industries, consumer products, and health environments [8,9].

The incorrect use of biocides in the food production chain for the reduction of microorganisms of public health importance to safe levels may result in an increase in prevalence and spread of antimicrobial resistance [6]. When cleaning before disinfection is insufficient, and/or rinsing after disinfection is inadequate, bacteria may be exposed to sublethal concentrations of biocides, generating selective pressure for adaptation or the acquisition of tolerance genes, hampering the eradication of these bacteria from food processing environments [10–12].

Microbial tolerance to biocides is a complex network that includes phenotypic alterations, such as viable but not culturable state (VBNC) and cell surface modifications, as well as genetic factors, such as the activation of efflux pump systems and/or the acquisition of mobile genetic elements, such as plasmids and transposons carrying resistant genes [12]. The selection of biocide-tolerant microorganisms increases their ability to form biofilms, requiring treatments with increasingly higher concentrations of biocides for their elimination from the environment [13,14].

Thus, microbial tolerance to biocides compromises food safety and threatens public health, mainly because the mechanisms of transmission of this tolerance are similar to those observed for clinically important antimicrobials [12]. Biocides also activate efflux pump systems, and there is concern that this activation could lead to antibiotic resistance [6,15]. The intensive use of QACs in food processing environments can provide a natural selection of more resistant strains with enhanced multi-drug resistance efflux pump activity [16].

In food production systems, microorganisms from the environment and handlers may contaminate final products, endangering their safety and compromising their quality [17]. *S. aureus* is the most common causative agent of mastitis in dairy cows, and a frequent contaminant in raw milk [18]. In this sense, coagulase-positive staphylococci in the artisanal cheese production system deserve special attention, as these microorganisms are frequent contaminants in cheeses manufactured with raw milk, such as the Brazilian Minas artisanal cheese, one of the best known and most-consumed cheeses in the country [19–21].

As occurrence of *S. aureus* in Minas artisanal cheeses is frequently reported [19–21], this study aimed to investigate if *S. aureus* isolates, obtained from samples collected from retail establishments in the city of São Paulo, Brazil, present tolerance to biocides and/or harbor antibiotic resistance and staphylococcal enterotoxin genes. The final objective was to contribute to a better understanding of the role of good hygiene and manufacturing practices in safeguarding consumer health, not only at cheese production level, but also in retail environments.

2. Materials and Methods

2.1. Bacterial Strains

One hundred samples of Minas artisanal cheeses, collected from randomly selected markets in the city of São Paulo, Brazil, were submitted to testing for coagulase-positive staphylococci using Petrifilm™ STX plates (3M, Sumaré, Brazil). As Minas artisanal cheeses are usually sold in pieces, as parts of the same cheese, the cheese samples selected for the study represented different lot numbers and manufacture/expiration dates, ensuring that they corresponded to different cheeses, and not pieces of the same cheese. For plating, cheese samples were homogenized with 0.1% peptone water (PW, Oxoid, Basingstoke, Hampshire, UK) and submitted to decimal dilutions in 0.1% PW. One to five typical colonies per cheese sample, obtained before and after the insertion of the revelation Petrifilm™ Staph

Express Disk (3M, Sumaré, Brazil), were submitted to the coagulase test [22]. Prior to the coagulase test, colonies were transferred to tubes containing Brain Heart Infusion (BHI, Becton-Dickinson, Franklin Lakes, NJ, USA), incubated overnight at 35 °C, plated on Baird-Parker agar (Oxoid, Basingstoke, Hampshire, UK) and incubated overnight at 35 °C. Only typical colonies (shiny black, with and without halos) were submitted to the coagulase test.

For identification of coagulase positive colonies to species level, total DNA of five isolates per positive cheese sample was extracted using the GenElute™ kit (Sigma-Aldrich, Burlington, MA, USA) and submitted to 16S ribosomal DNA sequencing at the Center for Human Genome and Stem Cell Studies in the University of Sao Paulo, SP, Brazil. The obtained genomic sequences were analyzed with the BLAST algorithm, available at the National Centre for Biotechnology Information (NCBI, Bethesda, MA, USA).

2.2. Determination of Biocide Tolerance

All *S. aureus* isolates were tested for susceptibility to benzalkonium chloride (BC), hexadecylpyridinium chloride (HDP), cetrimide (CT), triclosan (TC), hexachlorophene (HC), and chlorhexidine (CD) (all from Sigma Aldrich, Spain) using the broth microdilution method and 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ, USA) [23]. Biocides BC, HDP, CT, and CD were dissolved in Tryptic Soy Broth (TSB, Oxoid, Basingstoke, Hampshire, UK), while TC and HC were dissolved in 96% ethanol. These solutions were diluted to achieve concentrations of 256 mg/L, 128 mg/L, 64 mg/L, 32 mg/L, 16 mg/L, 8 mg/L, 4 mg/L, and 2 mg/L, respectively. For the tests, the isolates were activated in TSB at 37 °C for 24 h, and the cultures were diluted 1:10 in TSB. The biocide solutions, at the tested concentrations, were added to the wells of the microplates (180 µL per well), and 20 µL of each 1:10 diluted culture was added to the wells. The optical density (OD) at 620 nm was measured in a Sunrise microplate reader (Tecan, Grödig, Austria), at time zero and after 24 h at 30 °C. An isolate was classified as tolerant to a biocide when the difference between the ODs measured at time zero and after 24 h was ≥ 0.05 [17]. The MIC corresponded to the minimum concentration required to maintain the difference between the ODs measured at time zero and after 24 h below 0.05 [17]. Three controls were included in the tests: positive controls (pure cultures), negative controls (biocides at the tested concentrations, without added cultures), and sterility of culture medium (TSB). All tests were performed in triplicate.

2.3. Screening for Antibiotic Resistance, Efflux Pumps and Enterotoxin Genes by PCR

2.3.1. Extraction of DNA

The DNA of the isolates was extracted using Quick-DNA™ Fungal/Bacterial Miniprep (Zymo Research, Irvine, CA, USA), following the recommendations of the manufacturer. The quality of the extracted DNA was considered satisfactory when the $OD_{260\text{ nm}}/OD_{260\text{ nm}}$ ratio, measured with a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA), was in the range of 1.7 to 2.0. PCR was performed in a Mastercycler® nexus thermocycler (Eppendorf, Hamburg, Germany).

2.3.2. Antibiotic Resistance Genes

Potential resistance of the *S. aureus* isolates to beta-lactams, aminoglycosides, and tetracycline was determined by investigation of genes *mecA*, *aacA-aphD*, and *tetK*, respectively [24]. PCR was performed by mixing 1 µL (20–100 ng) of extracted DNA, 1 µL (400 nM) of each primer listed in Table 1, 10 µL of master mix (Promega, São Paulo, Brazil), and ultrapure water (Promega, São Paulo, Brazil) to complete 20 µL. Conditions of the PCR tests were 5 min at 95 °C, 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 5 min at 72 °C. Amplicons and a 100 bp marker (Promega, São Paulo, Brazil) were submitted to 2% agarose gel electrophoresis for 30 min/100 V, stained with SYBR Safe (Invitrogen, Waltham, MA, USA), and visualized using the Gel Doc XR+ system (BioRad, Hercules, CA, USA). *S. aureus* ATCC700699, resistant to methicillin and positive for genes *mecA* and *aacA-aphD*, and *S. aureus* ATCC 25923, positive for gene *tetK*, were used as positive controls.

Table 1. Primers used in the investigation of antibiotic resistance and efflux pump genes.

Gene	Primers	Sequences	Amplicon (bp)	Reference
<i>mecA</i>	MEC-1	AAAATCGATGGTAAAGGTTGGC	532	[24]
	MEC-2	AGTTCTGCAGTACCGGATTTGC		
<i>aacA-aphD</i>	<i>aacA-aphD</i> -1	TAATCCAAGAGCAATAAGGGC	227	[24]
	<i>aacA-aphD</i> -2	GCCACACTATCATAACCACTA		
<i>tetK</i>	TETK-1	GTAGCGACAATAGGTAATAGT	360	[24]
	TETK-2	GTAGTGACAATAAACCTCCTA		
<i>qacA/B</i>	SEA-1	GCAGAAAGTGCAGAGTTTCG	361	[25]
	SEA-2	CCAGTCCAATCATGCCTG		
<i>smr (qacC/D)</i>	SEB-1	GCCATAAGTACTGAAGTTATTGGA	195	[25]
	SEB-2	GACTACGGTTGTAAAGACTAAACCT		

mecA = gene encoding resistance to beta-lactams; *aacA-aphD* = gene encoding resistance to aminoglycosides; *tetK* = gene encoding resistance to tetracycline; *qacA/B* and *smr (qacC/D)* = efflux pump genes encoding resistance to quaternary ammonium biocides.

2.3.3. Efflux Pumps Genes

All isolates were screened for efflux pumps genes *qacA/B* and *smr (qacC/D)*, responsible for conferring resistance to cationic antiseptic agents in *S. aureus* [25]. PCR was performed by mixing 2 µL (40–100 ng) of extracted DNA, 2.5 µL (400 nM) of each primer (Exxtend, Paulinia, Spain) listed in Table 1, 6 µL of dNTP's (200 pM), 0.75 µL (100 nM) of MgCl₂, 5 µL of *Taq* buffer, 0.2 µL of *Taq* polymerase, and ultrapure water (MilliQ, Kenilworth, NJ, USA) to complete 50 µL. Amplifications were comprised of initial denaturation at 94 °C for 2 min, secondary denaturation at 94 °C for 2 min, annealing at 55 °C for 1 min, 72 °C for 1 min, and extension at 72 °C for 5 min. The amplification products, and a 100 bp marker (Promega, Madrid Spain), were submitted to 2% agarose gel electrophoresis for 30 min/100 V, stained with SYBR Safe (Invitrogen, Waltham, MA, USA), and visualized using the Gel Doc XR⁺ system (BioRad, Hercules, CA, USA). The positive controls were *Enterococcus faecalis* ATCC 29212, for the *qacA/B* gene, and *S. aureus* CECT4465, for the *smr* gene.

2.3.4. Staphylococcal Enterotoxin Genes

All *S. aureus* isolates were tested for the enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *she*, *sei*, and *sej* by mixing 1 µL (20–100 ng) of extracted DNA, 1 µL (400 nM) of each primer (Table 2), 10 µL of master mix (Promega, São Paulo, Brazil), and ultrapure water (Promega, São Paulo, Brazil) to complete 20 µL. Conditions of the PCR tests were 5 min at 95 °C, 1 min at 95 °C, 1 min at 53 °C to 63 °C, depending on the toxin, 45 s at 72 °C, and 5 min at 72 °C. Amplicons and a 100 bp marker (Promega, São Paulo, Brazil) were submitted to 2% agarose gel electrophoresis for 30 min/100 V, stained with SYBR Safe (Invitrogen, Waltham, MA, USA), and visualized using the Gel Doc XR⁺ system (BioRad, Hercules, CA, USA). The positive controls were *S. aureus* ATCC 13565, for gene *sea*, *S. aureus* ATCC 19095, for genes *seb* and *sec*, *S. aureus* ATCC 23235, for genes *sed*, *seg*, *sei*, and *sej*, *S. aureus* ATCC 23235, for gene *seh*, and *S. aureus* ATCC 27664 for gene *see*.

Table 2. Primers used in the investigation of staphylococcal enterotoxin genes.

Gene	Primers	Sequences	Amplicon (bp)	Reference
<i>sea</i>	SEA-1	GAAAAAAGTCTGAATTGCAGGGAACA	560	[26]
	SEA-2	CAAATAAATCGTAATTAACCGAAGGTTC		
<i>seb</i>	SEB-1	ACACCCAACGTTTTAGCAGAGAGTCA	633	[27]
	SEB-2	TCCTGTGCAGGCATCATGTCA		
<i>sec</i>	SEC-1	GTAAAGTTACAGGTGGCAAAACTTG	297	[26]
	SEC-2	CATATCATACCAAAAAGTATTGCCGT		
<i>sed</i>	SED-1	GTGGTGAAATAGATAGGACTGC	384	[28]
	SED-2	ATATGAAGGTGCTCTGTGG		
<i>see</i>	SEE-1	CAAAGAAATGCTTTAAGCAATCTTAGGC	482	[26]
	SEE-2	CACCTTACCGCCAAAGCTG		
<i>seg</i>	SEG-1	AATTATGTGAATGCTCAACCCGATC	642	[29]
	SEG-2	AAACTTATATGGAACAAAAGGTACTAGTTC		
<i>seh</i>	SEH-1	CAATCACATCATATGCGAAAGCAG	376	[29]
	SEH-2	CATCTACCCAAACATTAGCACC		
<i>sei</i>	SEI-1	CTCAAGGTGATATTGGTGTAGG	576	[26]
	SEI-2	AAAAAACTTACAGGGAGTCCATCTC		
<i>sej</i>	SEJ-1	TAACCTCAGACATATATACTTCTTAACG	300	[26]
	SEJ-2	AGTATCATAAAGTTGATTGTTTTCATGCAG		

3. Results

Based on the combinations of results of the tests for biocide tolerance, antibiotic resistance, efflux pump genes, and enterotoxin genes, 136 distinct *S. aureus* isolates were obtained. Isolates from the same cheese sample presenting identical results were considered as one isolate.

3.1. Tolerance to Biocides

Tolerance to at least one of the tested biocides was observed in 52 (38.2%) *S. aureus* isolates. The most tolerated biocides were those belonging to the QAC group: 39 (28.6%) were resistant to BC, 11 (8.0%) to HDP, and 3 (2%) to both. Among the isolates tolerant to BC ($n = 39$), the MIC was 32 mg/L for 35 isolates and 16 mg/L for four isolates. Five isolates were not inhibited by HC, four of which were also not inhibited by BC. The MIC for HDP and HC was 16 mg/L for both biocides. The other four isolates were tolerant to CD (MIC = 4 mg/mL). None of the isolates presented tolerance to TC or CT in the tested concentrations.

3.2. Antibiotic Resistance

At least one antibiotic resistance gene was detected in 113 (83.0%) *S. aureus* isolates, and almost half ($n = 64$, 47.1%) presented more than one. The most detected resistance genes were *tetK* ($n = 74$, 54.4%) and *mecA* ($n = 71$, 52.2%), followed by *aacA-aphD*, detected in 41 (30.0%) isolates. The occurrence of antibiotic resistance genes in the biocide-tolerant *S. aureus* isolates was high: 38 (73.1%) were positive for at least one gene, 23 (44.2%) were positive for two genes, and four (7.7%) harbored all three investigated genes (two isolates were tolerant to HDP, one isolate was tolerant to BC only, and one isolate was tolerant to BC and HDP, simultaneously).

3.3. Screening for Efflux Pump Genes

The great majority of biocide-tolerant isolates ($n = 48$, 92.3%) presented at least one of the two QAC efflux genes (*smr-qacC/D* and *qacA/B*), with greater prevalence of *smr-qacC/D*, detected in 43 (89.6%) of these isolates. The *qacA/B* gene was observed in 14 (29.2%) of the isolates harboring efflux pump genes. Ten isolates (20.1%) presenting an efflux pump gene were simultaneously positive for the two tested genes.

Thirty eight out of 39 BC-tolerant isolates were positive for the *smr-qacC/D* gene, and 10 (25.6%) of them were also positive for the *qacA/B* gene. Both genes were detected in nine (23.1%) BC-tolerant isolates.

Eight HDP-tolerant isolates ($n = 11$) were positive for at least one of the two QAC efflux genes (72.8%). Among these isolates, *smr-qacC/D* was detected in four, *qacA/B* in five, and one isolate was positive for both genes.

All HC-tolerant isolates ($n = 5$) were positive for the *qacA/B* or *smr* genes. Three (60%) isolates contained only *smr*, and two (40%) were positive for both markers.

Among the isolates presenting at least one antibiotic resistance gene ($n = 112$), efflux pump genes were detected in 99 (88.4%), mainly *smr*, present in 92.9% of them. The *qacA/B* was much less frequent, detected in 26 of these isolates (26.3%). Nineteen isolates (19.1%) were positive for both genes simultaneously.

The majority of *S. aureus* presenting tolerance to biocides and antibiotic resistance genes, simultaneously ($n = 40$), were positive for the *smr-qacC/D* or *qacA/B* genes ($n = 37$, 92.5%), with greater prevalence of *smr-qacC/D*, detected in 33 (82.5%). The *qacA/B* gene was observed in 13 (32.5%) of these isolates. Nine isolates (22.5%) were positive for both genes.

3.4. Staphylococcal Enterotoxin Genes

A high prevalence of enterotoxin genes was detected in the 136 *S. aureus* isolates (Table 3): 129 (94.9%) were positive for at least one enterotoxin gene, and 104 (80.7%) presented more than one gene. The *she* gene was the most prevalent, found in 119 (92.2%) of the isolates, followed by *seg*, found in 97 (75.2%), and *sei*, detected in 35 (27.1%). The classical enterotoxin genes *sea*, *seb*, *sec*, and *see* were much less frequent, observed in 15 (11.6%), 4 (3.1%), 5 (3.8%), and 7 (5.4%) isolates, respectively. None of the *S. aureus* isolates were positive for genes *sed* or *sej*.

Table 3. Distribution of enterotoxin genes among 129 *Staphylococcus aureus* isolates positive for at least one gene.

Enterotoxin Gene	Prevalence %
<i>sea</i>	15 (11.6%)
<i>seb</i>	4 (3.1%)
<i>sec</i>	5 (3.9%)
<i>sed</i>	0
<i>see</i>	7 (5.4%)
<i>seg</i>	97 (75.2%)
<i>seh</i>	119 (92.2%)
<i>sei</i>	35 (27.1%)
<i>sej</i>	0

3.5. Tolerance to Biocides and Presence of Antibiotic Resistance and SE Genes

The simultaneous tolerance to at least one biocide and presence of at least one antibiotic resistance and one SE enterotoxin gene was observed in 38 (27.9%) isolates. The most frequent combination was tolerance to BC and positivity for *tetK* and *seg* and/or *seh* genes, observed in 22 isolates, corresponding to 57.9% of the isolates belonging to this group and 16.2% of the total number of isolates included in the study.

4. Discussion

In addition to the use of raw milk for production, artisanal cheeses undergo several processing steps where contact with handlers is unavoidable, and so, contamination with *S. aureus* can be expected [20,23]. Cross-contamination at retail locations can also be a source of *S. aureus*, as whole cheeses are cut into smaller pieces for sale, and cutting boards and utensils can transfer microorganisms from one cheese to another. Handlers with poor personal hygiene practices, in retail environments, can be additional sources of contamination [30].

A worrisome percentage (38.2%) of the *S. aureus* isolates obtained from Minas artisanal cheese samples collected from retail establishments in Sao Paulo was tolerant to at least one biocide, mainly to quaternary ammonium compounds (QACs), benzalkonium chloride (BC), and hexadecylpyridinium chloride (HDP). The wide use of BC in the formulations of products destined for cleaning utensils and other surfaces in the food industry may explain these results [31]. BC is also used as a component in pesticides, disinfectants, and detergents in animal husbandry, mainly dairy cattle [32]. HDP is used in personal hygiene products, and is also approved for decontaminating raw poultry, so its use in food processing environments is possible [33].

The frequent use and overuse of biocides in the food processing system can create selective pressure, resulting in the proliferation and abundance of disinfectant-tolerant strains [9], besides the appearance of strains tolerant to multiple biocides, as evidenced in this study and reported by other authors [34,35]. The reported tolerance to biocides suggests that any use that may provide a sublethal exposure represents a risk of developing adaptation to these compounds.

A high prevalence of the QAC efflux pump *smr-qacC/D* gene among the *S. aureus* isolates tolerant to biocides (89.6%) was detected, confirming previously published results [36,37]. Some studies have shown that efflux pumps decreased the effectiveness of QAC biocides, phenolic parabens, and intercalating agents, most notably in *S. aureus* expressing pumps such as QacA-D, QacG, and QacH [38]. In this study, none of the CD-tolerant isolates were positive for the *qacA/B* and *smr* genes, suggesting that these genes do not play an important role in tolerance to this biocide, as also reported in other studies [35].

Most biocide-tolerant *S. aureus* isolates harbored at least one antibiotic resistance gene (92.3%). This phenomenon has been observed for *Salmonella* from chicken eggshells: isolates tolerant to benzalkonium chloride, cetrimide, hexadecylpyridinium chloride, triclosan, hexachlorophene, and P3-oxonia were resistant to ampicillin (90.5%), chloramphenicol (61.9%), tetracycline (47.6%), and trimethoprim-sulfamethoxazole (38.1%) [17]. *Salmonella* sp. (*S. derby*, *S. typhimurium* and *S. infantis*) isolates from a pig slaughterhouse, presenting multidrug resistance (amoxicillin, chloramphenicol, florfenicol, doxycycline, cefaclor, azithromycin, and enrofloxacin), were also tolerant to 0.5% benzalkonium chloride [39].

The co-existence of tolerance to biocides and potential resistance to antibiotics found in the *S. aureus* isolates obtained from artisanal cheeses can be explained by the positivity for *smr* and *qacA/B* genes encoding QAC efflux pumps. Despite having different mechanisms of action, studies indicate that the genetic systems for antibiotic and biocide resistance are similar [40]. Since similar genetic mechanisms are at play in antibiotic resistance, selection pressure may eventually contribute to the emergence of multi-resistant bacteria with the potential to impact public health [41], as evidenced in this study.

Previous studies have shown that decreased susceptibility to chlorhexidine in methicillin-resistant *S. aureus* (MRSA) was associated with the *qac A*, *qac B*, and *smr* genes [40]. The mechanisms underlying tolerance/resistance indicate that biocide tolerance genes and antibiotic resistance genes are located on the same mobile genetic elements [42]. Mutations in *pmrB* and the upregulation of efflux pump genes contributed to antibiotic resistance in *Pseudomonas aeruginosa* after exposure to benzalkonium chloride [42].

The most recent studies suggest that biocide-induced antibiotic resistance is more likely to become a contributing factor when bacteria are repeatedly exposed to sub-inhibitory concentrations of biocidal agents in disinfectants, increasing the chances of the co-selection of antibiotic-resistant bacteria [41,43]. Furthermore, these resistant bacteria were shown to be able to form strong biofilms on surfaces in the cheese production environment, with consequent hazards for public health and for the quality of the final products [37,44]. However, biocide-induced antibiotic resistance seems to be bacterial species- or strain-dependent: *Listeria monocytogenes* isolates obtained from food production plants and tolerant to BC did not present cross-resistance to clinically relevant antibiotics [45].

Most of the *S. aureus* strains isolated from the cheeses presented enterotoxin genes, predominantly for non-classical enterotoxins such as SEH and SEG. Despite the knowledge

that the presence of these genes does not mean that the corresponding enterotoxins are produced, as genes require complex machinery for expression, these results are worrisome, and confer additional preoccupation to the current knowledge that the occurrence of enterotoxigenic *S. aureus* in Minas artisanal cheeses is common [26,46–48]. The prevalence of non-classical enterotoxin *seh* gene in *S. aureus* isolated from Brazilian artisanal raw-milk cheeses, reported by previous studies, corroborates our findings [26,49]. The increasing predominance of non-classical staphylococcal enterotoxin genes demonstrates the importance of investigating these new enterotoxins in dairy foods when testing their safety.

Also worrisome is that nearly one third of the *S. aureus* isolates harboring enterotoxin genes were resistant to at least one biocide, and harbored genes of resistance to the main antibiotics used in clinical medicine. Tetracycline and methicillin are used in intensive livestock production, which may explain the resistance observed in bacteria obtained from animal products, such as cow's milk [26]. As pointed out for other types of cheese, *S. aureus* in these products deserves attention due to the potential risk to public health, especially when it accumulates resistance to antimicrobials, tolerance to biocides and production of enterotoxins that cause serious food poisoning [23]. The occurrence of *S. aureus* is mainly attributed to the inappropriate use of antimicrobial drugs and biocides in human and veterinary medicine, such as unnecessary use, incorrect choice, wrong dosage, short contact time, or irregular application [38]. Within the One Health perspective, it is necessary to address the possible transfer of microbial resistance genes between humans, animals, wildlife, plants, and the environment [50].

5. Conclusions

This study evidenced that tolerance to biocides in *S. aureus* in Minas artisanal cheese from retail establishments is quite common, and that these isolates may also be resistant to antibiotics and harbor efflux pump genes that favor tolerance to biocides. Furthermore, the majority of the tested isolates were potentially enterotoxigenic. Improper use of biocides in artisanal cheese processing may result in the selection of enterotoxigenic *S. aureus* that are resistant to antibiotics. Biocide-tolerant strains may form biofilms on surfaces, constituting a threat to disinfection. The presence of antibiotic resistance and enterotoxin genes may be an additional health concern for consumers of artisanal cheeses. The implementation of correct monitoring programs is crucial to safeguard the safety of these products, not only at production level, but also in retail environments.

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