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Microbial safety status of Serro artisanal cheese produced in Brazil

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ABSTRACT

Considering the growing consumption of artisanal foods worldwide, we aimed to evaluate the microbial safety of Serro artisanal cheese (SAC), produced in Minas Gerais State, Brazil. This cheese is produced with raw milk using 1 of 2 natural starter cultures: "pingo" and "rala." A total of 53 SAC samples (pingo = 8; rala = 45) were obtained from different farmers and subjected to conventional and molecular assays to detect and enumerate *Listeria monocytogenes*, *Salmonella* spp., coagulase-positive staphylococci (CPS), diarrheagenic *Escherichia coli*, *Mycobacterium tuberculosis*, and *Brucella abortus*. The SAC samples were also subjected to an ELISA to detect classical staphylococcal enterotoxins (CSE: SEA, SEB, SEC, SED, SEE) and to PCR assays to detect staphylococcal enterotoxin-related genes (*sea*, *seb*, *sec*, *sed*, *see*). Coagulase-positive staphylococci isolates were obtained and tested by the same assays to detect their potential in CSE production and presence of CSE-related genes. None of the SAC samples showed any of the screened food-borne pathogens and zoonotic agents, and none showed the presence of CSE by phenotypic and genotypic approaches. Despite the absence of microbial hazards, mean counts of CPS in SAC samples were 5.2 log cfu/g (pingo starter) and 4.6 log cfu/g (rala starter), indicating poor hygiene practices during production. None of the tested CPS isolates (n = 116) produced CSE or presented CSE-related genes. Despite the relative microbial safety, hygienic conditions during SAC production must be improved to meet official guidelines established in Brazil.

Key words: artisanal cheese, Serro, hazard, good manufacturing practices, *Staphylococcus*

INTRODUCTION

The consumption of artisanal cheeses is increasing worldwide because of consumer demand for natural foods without chemical additives, and because of valorization of cultural and historical aspects of different countries (Claeys et al., 2013). The cheeses produced in Minas Gerais state represent one of the oldest and most traditional artisanal products made in Brazil, and they are named according to their region of origin; for example, Serro, Salitre, Campos das Vertentes, Canastra, Araxá, and Triângulo (EMATER, 2019). The production of these artisanal cheeses has socioeconomic relevance for the dairy farmers, leading to official support from the Minas Gerais state government that established specific guidelines for production in 2002, including the health of the producing animals, hygiene during production, and microbiological parameters for quality and safety of end products (Minas Gerais, 2002a,b). Minas artisanal cheeses have been recognized as a Brazilian Historical Patrimony (IPHAN, 2006), and artisanal cheeses produced in the Serro region (Figure 1) received an indication of Geographic Origin Denomination (INPI, 2011), improving their value in retail sales.

Serro artisanal cheese (**SAC**) is traditionally produced with raw milk and a natural starter culture called "pingo," which is obtained from SAC whey produced after molding. Then, SAC is subjected to ripening for 17 to 22 d, resulting in a semi-hard cheese with a compact texture, white to yellowish, salty, and characterized by floral notes and a spicy flavor (INPI, 2011). Since the 1990s, the pingo starter has been replaced by an alternative starter culture, named "rala," which is the grated cheese resulting from the SAC cleaning, after ripening; this procedure is supposed to ensure a low microbial contamination in SAC (personal communication, Cooperativa dos Produtores Rurais do Serro - CooperSerro, Serro, MG, Brazil). Although SAC is well accepted by consumers, few scientific studies have assessed its quality and safety or the differences between "pingo" and "rala" starters. It is generally accepted that SAC production lacks standardization, resulting in poor hygienic quality of the end products (Brant et al., 2007; Vale et al., 2018).

Thus, proper scientific studies are needed to assess the microbiological characteristics of SAC in order to generate reliable data that can support enhanced

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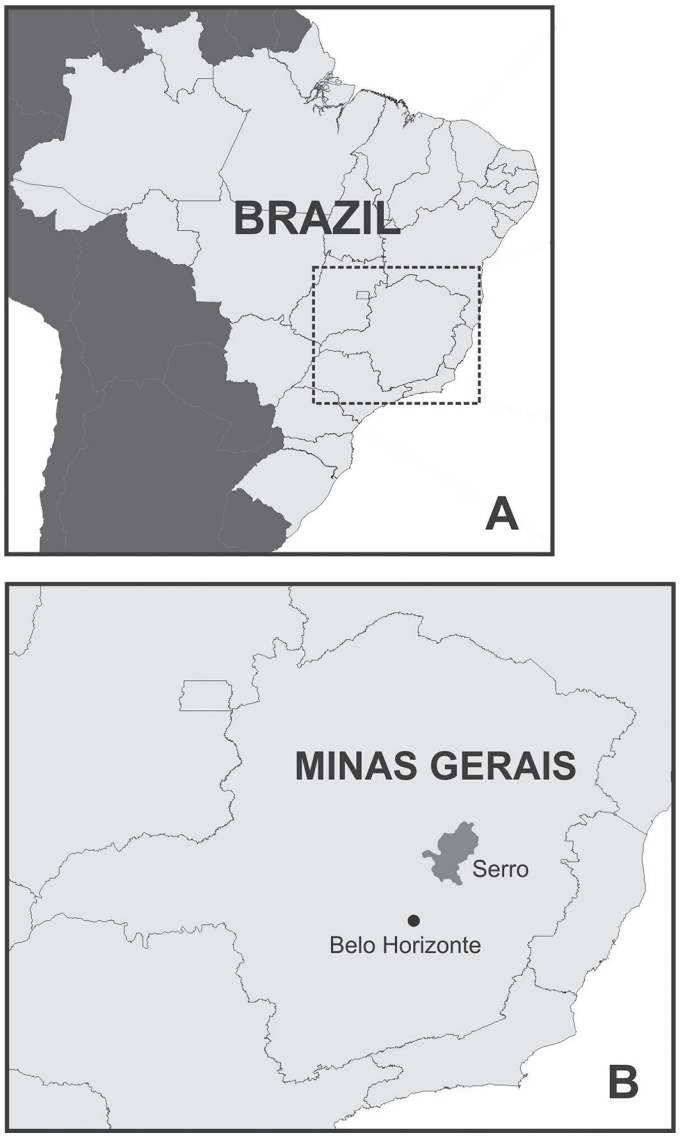

Figure 1. Localization of Minas Gerais state in Brazil (A) and the Serro region in Minas Gerais (capital: Belo Horizonte) (B; dotted area in A).

SAC quality and safety. As SAC is produced with raw milk, it is susceptible to contamination by pathogenic bacteria that pose hazards and risks to human health (Gould et al., 2014). A variety of food-borne pathogens associated with artisanal foods present hazards, such as *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., pathogenic *Escherichia coli*, enterotoxins produced by *Staphylococcus* spp., and microorganisms with zoonotic potential, such as *Brucella abortus* and *Mycobacterium* spp. All of these are commonly associated with consumption of raw milk cheeses (Claeys et al., 2013). For these reasons, in the present study, we aimed to assess the microbial safety of SAC.

MATERIALS AND METHODS

Samples and Dilutions

Serro artisanal cheese samples (n = 53) from different farmers were obtained from the distribution center of the SAC Producers Association (CooperSerro, Belo Horizonte, MG, Brazil) and kept under refrigeration pending microbiological analysis. Portions of 25 g of each sample were aseptically obtained, transferred to sterile bags with 225 mL of citrate solution (0.85%, wt/vol), and diluted 10-fold in NaCl 0.85% (wt/vol). The total DNA from SAC samples was obtained by using the PureLink Microbiome DNA Purification Kit (Invitrogen, ThermoFisher Scientific, Waltham, MA) and subjected to PCR. The SAC samples were categorized based on their production procedures, depending on whether the pingo (n = 8) or rala (n = 45) starter was used.

Listeria monocytogenes

Listeria monocytogenes was assayed based on Wehr and Frank (2004). Portions of 25 g of each sample were transferred to sterile bags, with 225 mL of *Listeria* Enrichment Broth (Oxoid Ltd., Basingstoke, UK) and incubated at 30°C for 48 h. Then, the obtained cultures were streaked onto plates containing Palcam and Oxford agars (Oxoid Ltd.) and incubated at 35°C for 48 h. Colonies that presented typical *Listeria* morphology (small, black, and surrounded by black halos) were purified on trypticase soy agar (Oxoid), subjected to DNA extraction (Wizard Genomic DNA Purification kit, Promega Corp., Madison, WI) and PCR assays targeting *prs* for *Listeria* spp., and *hly* for *L. monocytogenes* (Hudson et al., 2001; Doumith et al., 2004). The total DNA obtained from SAC samples was subjected to the same PCR assay targeting *hly* for detection of *L. monocytogenes* (Hudson et al., 2001). Primers, product sizes, PCR conditions, and positive controls are detailed in Table 1. Based on the obtained results, the presence or absence of *L. monocytogenes* was expressed per 25 g of SAC.

Salmonella spp.

The presence of *Salmonella* spp. was assessed based on the protocol described in ISO 6579 (ISO, 2017). Portions of 25 g of each SAC samples were transferred to sterile bags with 225 mL of buffered peptone water (Oxoid Ltd.) and incubated at 35°C for 18 h. Then, aliquots of the obtained cultures were transferred to Rappaport-Vassiliadis (Oxoid Ltd.) and Muller Kauffmann tetrathionate (Oxoid Ltd.) broth, followed by

Table 1. Oligonucleotides and conditions of PCR reactions conducted to identify and characterize microbial hazards (food-borne pathogens, zoonotic agents, and classical staphylococcal enterotoxins) in Serro artisanal cheeses (SAC) and bacterial isolates obtained from SAC

			PCR[1]					
Hazard	Target	Oligonucleotide (F, forward; R, reverse)	Denaturation	Annealing	Extension	Product (bp)	Positive control[2]	Reference
Listeria spp.	*prs*	F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG	95°C, 1 min	53°C, 1 min	72°C, 1 min 15 s	370	*L. monocytogenes* ATCC 49494	Doumith et al. (2004)
Listeria monocytogenes	*hly*	F: GCCTGCAAGTCCTAAGACGCCAATC R: CTTGCAACTGCTCTTTAGTAACAGC	95°C, 40 s	62°C, 1 min	72°C, 1 min	706	*L. monocytogenes* ATCC 49494	Hudson et al. (2001)
Salmonella spp.	*invA*	F: TTGTTACGGCTATTTTGACCA R: CTGACTGCTACCTTGCTGATG	95°C, 20 s	42°C, 20 s	72°C, 30 s	521	*Salmonella* Enteritidis ATCC 13076	Swamy et al. (1996)
Coagulase-positive staphylococci	*nuc*	F: TGCTATGATTGTGGTAGCCATC R: TCTCTAGCAAGTCCCTTTTCCA	95°C, 30 s	55°C, 30 s	72°C, 30 s	420	*Staphylococcus aureus* ATCC 8095	Baron et al. (2004)
	rep-PCR[3]	TCGCTCAAAACAACGACACC	95°C, 1 min	54°C, 1 min	72°C, 2 min	—	—	Del Vecchio et al. (1995)
	sea	F: ACGATCAATTTTTACAGC R: TGCATGTTTTCAGAGTTAATC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	544	*Staph. aureus* ATCC 8095	Rosec and Gigaud (2002)
	seb	F: GAATGATATTAATTCGCATC R: TCTTTGTCGTAAGATAAACTTC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	416	*Staph. aureus* ATCC 12588	Rosec and Gigaud (2002)
	sec	F: GACATAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCCA	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	257	*Staph. aureus* ATCC 14458	Rosec and Gigaud (2002)
	sed	F: TTACTAGTTTGGTAATATCTCCTT R: CCACCATAACAATTAATGC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	334	*Staph. aureus* FRI 326	Rosec and Gigaud (2002)
	see	F: ATAGATAAAGTTAAAACAAGCAA R: TAACTTACCGTGGACCC	95°C, 45 s	46.2°C, 45 s	72°C, 30 s	702	*Staph. aureus* FRI 196	Rosec and Gigaud (2002)
Diarrheagenic *Escherichia coli*	*eae*	F: CCCGAATTCGGCACAAGCATAAGC R: CCCGGATCCGTCTCGCCAGTATTCG	95°C, 30 s	52°C, 30 s	72°C, 30 s	881	*E. coli* wild strain	Toma et al. (2003)
	stx	F: TGATGATGGCAATTCAGTAT R: GAGCGAAATAATTTATATGTG	95°C, 30 s	52°C, 30 s	72°C, 30 s	518	*E. coli* ATCC 43895	Toma et al. (2003)
	agg	F: GTATACACAAAAGAAGGAAGC R: ACAGAATCGTCAGCATCAGC	95°C, 30 s	52°C, 30 s	72°C, 30 s	253	*E. coli* wild strain	Aranda et al. (2007)
	ipaH	F: GTTCCTTGACCGCCTTTCCGATACCGTC R: GCCGGTCAGCCACCCTCTGAGAGTAC	95°C, 30 s	52°C, 30 s	72°C, 30 s	600	*E. coli* ATCC 43893	Aranda et al. (2007)
Brucella abortus	IS711	F: GAGAATAAAGCCAACACCCG R: GATGGACGAAACCCACGAAT	95°C, 30 s	58°C, 30 s	72°C, 30 s	317	DNA from *B. abortus* DDB/2018/1306T1, wild strain[4]	Ning et al. (2012)
Mycobacterium tuberculosis	IS6110	F: CGTGAGGGCATCGAGGTGGC R: GCGTAGGCGTCGGTGACAAA	94°C, 1 min	68°C, 1 min	72°C, 1 min	500	DNA from *M. tuberculosis* H37RO.CRNC25, wild strain[4]	de Souza Figueiredo et al. (2009)

[1]All reactions were conducted with initial denaturation at 95°C for 5 min; amplification was conducted with 30 (*nuc*, IS711, IS6110, *invA*, *eae, stx, agg, ipaH*), 35 (*sea*, *seb*, *sec*, *sed*, *see*, *prs*) and 40 (*hly*, rep-PCR) cycles, and final extension at 72°C for 5 to 10 min.

[2]ATCC = American Type Culture Collection (Manassas, VA); FRI = Food Research Institute (Madison, WI).

[3]Repetitive element PCR.

[4]DNA provided by LANAGRO MG (Laboratório Federal de Defesa Agropecuária - LFDA MG), Pedro Leopoldo, MG, Brazil.

incubation at 41°C and 35°C, respectively, for 24 h. The obtained cultures were then streaked onto plates containing mannitol lysine crystal violet brilliant green agar (MLCB; Oxoid Ltd.) and xylose lysine deoxycholate agar (XLD; Oxoid Ltd.) and incubated at 35°C for 24 h. Colonies that presented typical *Salmonella* morphologies (MLCB: dark gray, rounded; XLD: black centered, rounded) were purified on TSA (Oxoid Ltd.), subjected to DNA extraction (Wizard Genomic DNA Purification kit, Promega), and then to a PCR assay targeting *invA* (Swamy et al., 1996). Total DNA from SAC samples was subjected to the same PCR protocol targeting *invA* for *Salmonella* spp. detection (Swamy et al., 1996). Primers, product sizes, PCR conditions, and positive controls are detailed in Table 1. Based on the obtained results, the presence or absence of *Salmonella* was expressed per 25 g of SAC.

Coagulase-Positive Staphylococci and Classical Staphylococcal Enterotoxins

Selected dilutions of SAC samples were surface plated onto Baird Parker agar plates (Oxoid Ltd.), according to ISO 6888-1 (ISO, 1999). Plates were incubated at 35°C for 48 h, when colonies were analyzed by their morphology (typical: circular, smooth, convex, gray to jet-black, with a light-colored margin surrounded by 2 halos, and atypical), enumerated, and selected for additional characterization. The selected colonies were subjected to Gram staining and tested for production of catalase, coagulase, and DNase (ISO, 1999). Isolates with biochemical characteristics consistent with coagulase-positive staphylococci (**CPS**) were subjected to DNA extraction (as described above) and a PCR assay targeting *nuc*, according Baron et al. (2004), for *Staph. aureus* identification. Primers, product sizes, PCR conditions, and positive controls are detailed in Table 1. Based on the obtained results, counts of CPS were obtained and expressed as colony-forming units per g (cfu/g) of SAC.

Aliquots of SAC samples (10 g) were subjected to an ELISA to detect the presence of classical staphylococcal enterotoxins (**CSE**; SEA, SEB, SEC, SED, and SEE), by using the Ridascreen Set Kit SEA-SEE (R-Biopharm, Darmstadt, Germany). Based on the obtained results, the presence or absence of CSA was expressed per 10 g of SAC.

The DNA from isolates carrying the *nuc* gene was subjected to a repetitive element (**rep**)-PCR protocol using the single primer RW3A (Del Vecchio et al., 1995). Primers and PCR conditions are detailed in Table 1. The obtained genetic profiles were analyzed by using the Bionumerics 6.6 software (Applied Maths, Gand, Belgium), considering Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) method (optimization/tolerance of 5% and similarity index of 100%). Based on their genetic profiles and origin (SAC sample and starter culture), isolates were selected, transferred to brain heart infusion agar (Oxoid), incubated at 35°C for 24 h, and centrifuged at 3,500 × *g* at 10°C. Then, the supernatant was subjected to the same ELISA protocol (Ridascreen Set Kit SEA-SEE, R-Biopharm) to detect production of CSE. Based on the obtained results, *Staph. aureus* isolates were categorized as enterotoxin producers or not.

The DNA from the selected *Staph. aureus* isolates and from the SAC samples was subjected to PCR assays targeting genes related to production of SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), and SEE (*see*), as described by Rosec and Gigaud (2002). DNA from SAC samples was additionally subjected to the PCR assay described above for *nuc* (Baron et al., 2004), as a control for the presence of *Staph. aureus*. Based on the obtained results, *Staph. aureus* isolates and SAC were categorized as having, or not, the potential to produce CSE.

Molecular Detection of Diarrheagenic E. coli, B. abortus, and M. tuberculosis

The total DNA from SAC samples was subjected to a multiplex PCR assay to identify the potential presence of the 4 groups of diarrheagenic *E. coli*: (1) enteropathogenic *E. coli* (EPEC), targeting the gene *eae*; (2) Shiga-toxin producing *E. coli* (STEC), targeting *stx*; (3) enteroaggregative *E. coli* (EAEC), targeting *agg*; and (4) enteroinvasive *E. coli* (EIEC), targeting *ipaH* (Toma et al., 2003; Aranda et al., 2007). Also, total DNA from SAC was subjected to PCR assays to identify the potential presence of (1) *B. abortus* targeting the IS711 genome region, as described by Ning et al. (2012), and (2) *M. tuberculosis*, targeting the IS6110 genomic region, as described by de Souza Figueiredo et al. (2009). Primers, product sizes, PCR conditions, and positive controls are detailed in Table 1. Based on the obtained results, the potential presence or absence of the target pathogens was expressed per SAC.

Data Analysis

The SAC samples were categorized based on the presence or absence of the target pathogens, as described above. Counts of CPS were converted to $\log_{10}$ and compared considering SAC production (pingo and rala) by ANOVA ($P < 0.05$); SAC with CPS counts <100 cfu/g were not considered in this analysis. Also, SAC samples were categorized based on their CPS counts and recorded frequencies compared by χ^2 ($P < 0.05$) considering

Table 2. Frequencies of Serro artisanal cheese (SAC) samples produced with "pingo" and "rala" starters with different contamination levels by coagulase-positive staphylococci (CPS)

		SAC		
Category[1]	Count (cfu/g)	Pingo	Rala	*P*-value[2]
Contamination level	<100	3	9	0.576
	100 to 1,000	0	1	1.000
	1,000 to 10,000	0	6	0.239
	10,000 to 100,000	2	17	0.749
	>100,000	3	12	0.850
Minas Gerais (2002a)	>1,000	5	35	0.664
European Union (2005)	>100,000	3	12	0.850

[1]Contamination levels of CPS in all SAC samples, and considering upper limits of CPS in raw milk cheeses established in Minas Gerais (2002a) and European Union (2005).

[2]*P*-values > 0.05 indicate absence of significant differences by χ^2 test.

SAC production (pingo and rala) and reference values for raw milk cheeses in Minas Gerais State (upper limit = 1,000 cfu/g; Minas Gerais, 2002a) and the European Union (upper limit = 100,000 cfu/g; European Union, 2005). Statistical analysis was conducted by using the software XLStat 19.01 (AddinSoft, New York, NY).

RESULTS

The results for the pathogenic bacteria assessed by conventional and molecular methods in each SAC sample are presented in Supplemental Table S1 (https://doi.org/10.3168/jds.2019-16967). Considering the results obtained by conventional methods, none of the SAC samples showed *L. monocytogenes* or *Salmonella* spp.; *Listeria* spp. presumptive colonies were obtained from just one SAC sample, and they were positive only for *prs* (negative for *hly*), allowing their identification only to the genus level. In agreement, none of the SAC samples were positive for *L. monocytogenes* or *Salmonella* spp. by PCR. In addition, no SAC sample had PCR amplification products for diarrheagenic *E. coli*, *B. abortus*, or *M. tuberculosis*.

The mean CPS count in pingo SAC was 5.2 log cfu/g (SE: 0.3) and in rala SAC was 4.6 cfu/g (SE: 0.1); these counts did not differ ($P = 0.139$). The frequencies of SAC samples with different levels of contamination by CPS are presented in Table 2. Based on these results, SAC samples showed high levels of contamination by CPS, but no statistical difference was detected among the SAC produced with pingo and rala (see mean counts, Table 2). Based on the reference values established in Minas Gerais for CPS counts in artisanal cheeses (Minas Gerais, 2002a), 40 (75.5%) SAC samples would not be considered suitable for retail sale because of counts $>10^3$ cfu/g; of these, 5 (62.5%) were produced using pingo and 35 (77.8%) using rala ($P = 0.664$, Table 2). Similarly, based on the European Union reference value of 10^5 cfu/g of CPS in raw milk cheeses (European Union, 2005), 15 (28.3%) SAC samples would not be adequate for human consumption, 3 (37.5%) pingo SAC and 12 (26.7%) rala SAC ($P = 0.850$). In addition, the same 15 (28.3%) SAC samples would be subjected to further analysis for staphylococcal enterotoxins once they presented CPS counts $>10^5$ cfu/g (European Union, 2005).

Despite the high counts of CPS observed here, none of the SAC samples were positive for CSE assayed by conventional (ELISA) or molecular (PCR) methods. A total of 866 colonies were obtained from Baird-Parker plates, 155 being typical and 711 atypical. Of this total, 350 isolates were characterized as CPS and 427 as DNase-positive. A total of 316 of the CPS isolates were positive for *nuc*, being identified as *Staph. aureus*. Based on their genetic profiles obtained by rep-PCR and SAC origin, 116 isolates were selected for CSE assays: none showed positive results by ELISA or PCR. Supplemental Figure S1 (https://doi.org/10.3168/jds.2019-16967) shows the genetic profiles of the 116 isolates selected, based on rep-PCR.

DISCUSSION

The presence of food-borne pathogens and zoonotic agents in artisanal food produced with raw milk is a source of concern to producers, consumers, and official inspection services. Considering the widespread acceptance of artisanal food products by consumers, assessment of their safety is mandatory, through proper inspection of producer farms and establishment of adequate guidelines for production, thus assuring the safety to consumers. Contamination by pathogenic bacteria can occur at different stages of artisanal cheese production, starting with the raw milk obtained from potentially infected animals and from the milking and producing environment (Melo et al., 2015). *Listeria monocytogenes* and *Salmonella* spp. are food-borne pathogens usually associated with raw milk and dairy

products, and they are commonly screened in dairy products as safety indicators (Torres-Vitela et al., 2012; Almeida et al., 2013). None of the SAC samples included in this study were positive for these pathogens, by conventional or molecular methods, as also observed in previous studies with raw milk cheeses (Esho et al., 2013; Amorim et al., 2014; Martins et al., 2015; Dias et al., 2016; Soares et al., 2018).

Escherichia coli is an important indicator to assess risk and hygiene failures in the cheese production system, because it is present in the gastrointestinal tract of humans and healthy animals and consequently can be present in the milk production environment (Cardoso and Marin, 2017). Some strains can be pathogenic and cause foodborne illness in humans and animals. Among these, the 6 most important categories of pathogenic *E. coli* vary in infective and toxin-producing mechanisms: STEC, EPEC, enterotoxigenic *E. coli*, EAEC, EIEC, and diffusely adherent *E. coli* (Farrokh et al., 2013). In this study, none of the SAC samples were positive for any category of pathogenic *E. coli*. In contrast, some studies have found diarrheagenic *E. coli* in samples of artisanal cheeses (Paneto et al., 2007; Marozzi et al., 2016; de Campos et al., 2018; Parussolo et al., 2019), but all have made an association between the microbiological culture and the use of the PCR technique, suggesting that the food matrix may interfere in the direct detection of these pathogenic strains.

Considering the concern surrounding *M. tuberculosis* and *B. abortus* as zoonotic agents, as direct transmission from animals to humans may occur, the ingestion of dairy products contaminated with these bacteria is considered the greatest risk to human health: the World Organization for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO) consider brucellosis to be the world's most neglected zoonosis (Dadar et al., 2019). Because of this, the Brazilian Ministry of Agriculture started a national program in 2001 to control and eradicate animal brucellosis and tuberculosis, focusing mainly on cattle (dairy and beef); as a consequence, all current Brazilian legislation related to dairy production, including raw milk and artisanal cheeses, is included in the program, ensuring the proper control of these diseases in producing animals (Minas Gerais, 2002b; Brasil, 2017). As expected, no SAC samples were positive for *B. abortus* or *M. tuberculosis* by PCR. Few studies have been conducted on the presence of these zoonotic agents based on PCR assays in artisanal cheeses, but they have been found in this matrix using highly sensitive molecular assays, such as quantitative PCR and nested PCR (Ongör et al., 2006; Cezar et al., 2016; Silva et al., 2016, 2018).

The presence of *Staphylococcus* in SAC samples was expected, because isolates of this genus are usually found on the skin and mucous membranes of animals and humans as well as the surfaces of dairy equipment and the milking environment (Basanisi et al., 2016). Cheese production, in particular, is characterized by direct manipulation, increasing the potential of transferring of *Staphylococcus* spp. from utensils, equipment, and employees to the end products (Johnson, 2017). Because of these potential contamination routes, *Staphylococcus* spp. are usually considered good indicators of proper handling and good manufacturing practices during food processing (Novakovic and Grujic, 2017). The intrinsic conditions of the cheese, such as pH, water activity, and NaCl concentrations, can favor the growth of *Staphylococcus* spp. (Viana et al., 2009; Borelli et al., 2011). Although controversial, the enterotoxigenic potential of *Staphylococcus* has always been associated with its pathogenic potential, which is usually characterized by its ability to produce the enzyme coagulase (Rodríguez et al., 2016). Therefore, official guidelines in Brazil and Europe consider counts of CPS as a reference for safety (Minas Gerais, 2002a; European Union, 2005). Thus, enumeration and characterization of *Staphylococcus* spp. is an important step to evaluate potential factors that may pose a risk to consumers and a reliable tool to support quality control programs, such as hazard analysis and critical control points (HACCP) and good manufacturing practices (GMP; Ding et al., 2016).

The high counts of CPS observed in the present study indicate hygienic failures in SAC production, in agreement with similar studies with artisanal cheeses (Brant et al., 2007; Dores et al., 2013; Amorim et al., 2014; Dias et al., 2016; Vinha et al., 2018). Information on the microbiological influence of the type of starter culture (pingo or rala) used for the production of SAC is scarce; in the current study, pingo SAC had higher CPS counts than rala SAC, albeit not significantly different. In addition, no significant differences were observed when contamination levels by CPS were considered according to SAC starter culture ($P > 0.05$, Table 2). These results indicate poor hygienic conditions during SAC production, independently of the starter culture used. To date, no study has addressed the influence of starter cultures (pingo or rala) on the microbiological characteristics of SAC.

The production of staphylococcal enterotoxins is one of the main virulence mechanisms of *Staph. aureus*, and 23 different enterotoxins have been identified so far; however, 95% of confirmed outbreaks are due to the classical enterotoxins (SEA, SEB, SEC, SED and SEE; Rahimi and Alian, 2013). Based on European legislation, only artisanal cheese batches that have samples

with CPS counts $>10^5$ cfu/g must be assayed for the presence of CSE (European Union, 2005), as observed in some studies (Pelisser et al., 2009; Ciupescu et al., 2018). In Brazil, no current legislation addresses the evaluation of CSE in artisanal cheeses (Brasil, 2001; Minas Gerais, 2002a). Even though the SAC samples had relatively high counts of CPS, none were positive for the presence of the CSE assayed by ELISA, indicating that the SAC samples did not pose a direct risk to consumers related to CSE (Johler et al., 2015; Rola et al., 2016).

Despite the absence of CSE in SAC samples, it is necessary to study *Staph. aureus* with enterotoxigenic potential, because the genes encoding different enterotoxins are transported and disseminated by different genetic elements and can be expressed according to the intrinsic and extrinsic conditions of the food, such as temperature, pH, water activity, and salt concentration (Denayer et al., 2017). In this study, none of the SAC samples showed amplification by PCR of *sea*, *seb*, *sec*, *sed*, or *see*, as also observed previously in similar studies (Martins et al., 2014; Johler et al., 2015). In addition, none of the *Staph. aureus* isolated in this study were positive for the screened CSE genes or showed CSE production by ELISA. Safety related to CSE in *Staphylococcus* stains must be assessed by phenotypic and genotypic approaches to detect potential CSE producers that express this characteristic under specific conditions and might transfer these genetic elements to non-CSE-producing strains. The identification of *Staphylococcus* strains from artisanal cheeses that present CSE-related genes despite not being CSE producers is not uncommon (Carfora et al., 2015; Grispoldi et al., 2019). However, the CSE results of *Staph. aureus* isolates obtained in the present study might indicate a particular profile in the regional staphylococcal microbiota, leading to further studies to investigate their genetic profiles and other CSE-related genes and production.

CONCLUSIONS

The absence of *Staphylococcus* with potential to produce CSE and the absence of pathogens such as *Salmonella*, *L. monocytogenes*, diarrheagenic *E. coli*, *M. tuberculosis*, and *B. abortus*, indicate that SAC is relatively safe, not posing a risk for transferring foodborne pathogens and zoonotic agents to consumers. However, the high CPS counts indicated poor hygiene conditions during processing of SAC with different natural starter cultures ("pingo" and "rala"), demanding proper control of processing and establishment of quality and production parameters, in order to standardize production and ensure a high-quality end product.

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