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PROFILE OF THE ANTIMICROBIAL RESISTANCE OF *STAPHYLOCOCCUS* SPP. FROM GOAT MILK AND ARTISANAL DAIRY PRODUCTS MADE ON FAMILY FARMS OF RIO DE JANEIRO, BRAZIL

Perfil da resistência antimicrobiana de *Staphylococcus* spp. provenientes de amostras de leite de cabra e derivados lácteos artesanais produzidos em fazendas familiares do Rio de Janeiro, Brasil

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ABSTRACT

The aim of this study was to investigate the species of *Staphylococcus*, and their profile, of refrigerated raw goat milk and goat dairy product samples as a way of evaluating the sanitary and pathogenic profile of these bacterial species that act as markers. A total of 25 samples were evaluated, including six of refrigerated raw goat milk, 16 of artisanal goat cheese with or without spices, and three of goat milk caramel, from a family farm located in the state of Rio de Janeiro, Brazil. Microbiological analyzes included enumeration and biochemical characterization of *Staphylococcus* spp.; disk diffusion test and amplification of *mec*A gen to scan antimicrobial susceptibility of *S. aureus* isolates. Only six of the 25 samples were positive for *Staphylococcus aureus*, being five of goat milk and one of goat artisanal cheese. Coagulase negative *Staphylococcus* prevalent species were *S. epidermidis*, *S simulans*, *S. xylosus*, and *S. caprae.* Five *Staphylococcus aureus* strains presented a multidrug resistance profile, and one isolate of raw milk sample was *mec*A gen positive.

Keywords: *Staphylococcus*, goat milk; artisanal dairy products.

RESUMO

O objetivo do presente estudo foi investigar as espécies de *Staphylococcus*, e

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seu perfil, em amostras de leite de cabra cru refrigerado e produtos lácteos de cabra, como forma de avaliar o perfil higienicossanitário e patogênico a partir desta espécie bacteriana que atuam como marcadoras. Foram avaliadas 25 amostras, incluindo seis de leite de cabra cru refrigerado, 19 de queijo de cabra artesanal, com ou sem especiarias, e três de doce de leite de cabra, produzidos em fazendas de produção familiar, localizadas no estado do Rio de Janeiro, Brasil. As análises microbiológicas incluíram enumeração e caracterização bioquímica de *Staphylococcus* spp., teste de difusão em disco e amplificação do gene *mec*A, pela técnica de PCR, para verificar o perfil de susceptibilidade antimicrobiana em isolados de *S. aureus*. Apenas seis das 25 amostras foram positivas para *Staphylococcus aureus*, sendo cinco de leite de cabra cru e uma de queijo de cabra artesanal. As espécies prevalentes de *Staphylococcus* coagulase negativa foram: *S. epidermidis, S. simulans, S. xylosus* e *S. caprae.* Cinco cepas de *Staphylococcus aureus* apresentaram perfil de multirresistência, e apenas uma amostra de leite cru foi detectado o gene *mec*A.

Palavras-chave: *Staphylococcus*, leite de cabra; laticínios artesanais, produção familiar.

INTRODUCTION

In Brazil, dairy goat farming has become a viable alternative for Brazilian agribusiness, mainly for small and medium producers. Dairy goat has been present in Brazil since the days of the Portuguese colonizers when the consumption of milk was more for subsistence than for trade. Recently, goat milk production came to be seen as an income source for family farming through the intervention of government programs, considering the low cost of production, small areas for production, and the growing search for healthier and more sophisticated foods (OLIVEIRA *et al*., 2017; SILVA; FAVARIN, 2019). Goat milk and its derivatives, such as yogurts, ice cream, and cheeses began to be appreciated not only for gastronomic quality but also for its nutritional quality. Goat milk has become an alternative to bovine milk for those allergic consumers, due to the lower levels of α-s1 casein, but also for the elderly and children due to its better digestibility (VERRUCK *et al*., 2019).

Even though dairy goats in Brazil have increased production and representativeness in the market, family producers still present a great demand in terms of adopting standards food safety protocols in the entire production chain. The lack of infrastructure, tools, and hygienic-sanitary practices end up not allowing these producers to meet the requirements of the current legislation, which includes the mandatory physical-chemical and microbiological analyzes to certify food safety.

According to the World Health Organization (WHO, 2020), raw milk can be responsible for the transmission of all over sixteen bacterial and seven viral diseases, including tuberculosis, brucellosis, and gastroenteritis. Thus, goat milk constitutes excellent culture media for the development of undesirable, spoiling and pathogenic microorganisms, when there is no quality control during the obtaining and handling of raw material, nor the adoption of good hygienic manufacturing practices. Dairy goat farming became a viable activity for small and medium family producers, but besides the increased representativeness in the Brazilian market, family producers still present great demand in terms of adopting standard and food safety protocols in the entire production chain.

Dairy product consumers are increasingly demanding the quality of foodstuffs. This generated profound changes in the milk quality criteria that were reproduced in sanitary legislation (BRASIL, 2001; BRASIL, 2011; BRASIL, 2018; BRASIL, 2019). Changes in quality parameters legislation included: the somatic cell count, the total bacterial count and the mandatory cooling system for raw milk on the farm (BRASIL, 2018), limits of 100 UFC/ml positive-coagulase *Staphylococcus,* counts and absence of Staphylococci Enterotoxin (BRASIL, 2019). Such parameters reflect the health of the herd and mammary gland, the general conditions of animal management, and good practices for obtaining and processing raw milk (PONCE CEBALLO; HERNÁNDEZ, 2001).

In addition, *Staphylococcus* spp. comprises a variety of Gram-positive cocci, which are the etiological agents of severe animal diseases, such as suppurative disease, mastitis, arthritis, and urinary tract infections, because of different virulence factors, such as the production of extracellular toxins and enzymes. Most staphylococci species can present genes that own resistance to β-lactams, aminoglycosides, and macrolides antimicrobials, such as the worldwide spread *mec*A gene expressed by *Staphylococcus aureus* Methicillin Resistant strains (MRSA) (COELHO *et al*., 2007). Staphylococci group also compounds the normal cutaneous and mucosal microbiota of animals and humans which may facilitate zoonotic transmission in cases of low immunity when they can act as opportunistic pathogens. Emergence and dissemination of pathogenic bacteria is an important problem in human and veterinary medicine, especially amongst the staphylococci group, and represents a worldwide challenge because several infection strains have acquired resistance toward most available antibiotics and therapeutic options have been reduced over time.

Investigations focused on detecting potential pathogenic bacteria, such as *Staphylococcus* spp. are of great importance to accessing microbiological profile information of goat milk and artisanal dairy derivates produced by family farms as their importance to Brazil economic and social scenario. The present work aimed to analyze *Staphylococcus* species in 25 samples of raw goat milk and dairy goat products as indicator bacteria to access hygienic-sanitary and pathogenic profile and antimicrobial resistance profiles.

MATERIAL AND METHODS

In this study, a total of 25 samples were evaluated, including six monthly samples of refrigerated raw goat milk from family farms located in cities of the state of Rio de Janeiro, Brazil), and 16 samples of artisanal goat cheese (ricotta, Boursin and Chancliche type), with and without spices, and three samples of goat milk caramel, during the period from August/2018 to October/2019. A volume of 500 mL of milk samples was collected in sterile bottles and obtained directly from the expansion tanks and dairy derivatives were obtained in their commercial packaging. The samples were transferred to the Food Microbiology Laboratory of UFRJ, Campus Macaé, in an insulated ice box with a minimum delay to be immediately submitted to enumeration and biochemical characterization protocols. Conventional method protocols were established initially by serial decimal dilutions in 1% peptone solution (m/v) (Difco, Detroit, MI). After, 0.1 mL of every selected dilution was inoculated on Agar Baird-Parker (BP) by spread plate technique and subsequently incubated at 36±1°C from 30 to 48 hours. Typical (Opaque ring shiny black, surrounded by a clear transparent halo highlighted over the opacity from the environment) and atypical

colonies growth on BP were enumerated (ISO 6888-1: 2003).

All typical and atypical *Staphylococcus* isolates were enriched in Muller Hinton Broth and then streaked onto Brain and Heart Infusion agar and Mannitol Salt agar and sequentially tested by coagulase production on rabbit plasma. According to morphologic and Gram-staining characteristics, *Staphylococcus* species were distinguished by phenotypic tests, including catalase and coagulase production, detection of hemolysis in Blood Agar, bacitracin, polymyxin B and novobiocin susceptibility, acetoin production, ureases, nitrate reduction, ornithine decarboxylation and mannitol, maltose, fructose, xylose, arabinose, raffinose, and trehalose fermentation (KONEMAN e*t al*., 2008).

Antimicrobial susceptibility tests were executed at National Reference Laboratory of Enterobacteria Infections (Oswaldo Cruz Institute – FIOCRUZ, Rio de Janeiro, Brazil). *Staphylococcus aureus* isolates were submitted to disk diffusion assays performed by standard CLSI methodology using Muller-Hinton plates. The density of inoculum was adjusted to 0.5 MacFarland scale by Vitekturbidimetri (BioMérieux®). After overnight incubation on 35 oC, zone diameters were read, and interpretation criteria were established according to CLSI (2019). Antimicrobial drugs (OXOID®) representative of each class was used, including β-lactamics (ampicillin, oxacillin, cefoxitin), macrolides (erythromycin), lincosamides (clindamycin), chloramphenicol, quinolone (ciprofloxacin), tetracycline, aminoglycosides (gentamicin) and glycopeptides (vancomycin). *Staphylococcus aureus* ATCC29213 and Escherichia coli ATCC25922 were used as quality controls.

β-lactamic resistance due to PBP2a expression regulated by the mecA gene was detected by PCR amplification using primers and amplification protocol as previously described (MATIAS *et al*., 2018). Briefly, DNA was extracted from overnight cultures in 10 mL of Brain Heart Infusion Broth. Bacterial cells were collected by centrifugation for 30s at 14.000 rpm, washed in 1mL of TE buffer (10 mM Tris HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl), and recentrifuged. The pellet was resuspended in 400 µL of TE buffer including 5µL of lysostaphin (stock concentration 1mg/mL; Sigma-Aldrich®) and incubated for 30 min at 37 ºC. Lysis was completed with 10 min of water incubation at 100 ºC. Simplex PCR was executed to genotypically identify *Staphylococcus aureus* using rDNA 23S primer (5'-ACG GAG TTA CAA AGG ACG AC-3' and 5'- AGC TCA GCC TTA ACG AGT AC-3') and amplification conditions according to STRAUB *et al*. (1999). Oxacillin resistance due to PBP2a expression regulated by the mecA gene was detected by the construction of the primer: 5'-AAAATCGATGGTAAAGGTGGC-3' and 5'-AGTTCTGCAGTACCGGATTTGC-3'as described by ROSATO *et al*. (2003). Amplification products were analyzed by electrophoresis through a 0.8-1.5% agarose followed by impregnation with ethidium bromide solution (0.5 mg/mL) and visualized on an UV Transilluminator ImageQuant®.

RESULTS AND DISCUSSION

Detection of Coagulase-positive *Staphylococcus* (CPS) was observed in four raw goat milk and one Boursin Cheese with tomato samples (Table 1). The verified enumeration values, in general, were under 6.0×10^2 CFU/mL. All other goat cheese samples had CPS enumeration under 1×10^2 CFU/g, which was by RDC Nº 12, 2001 (BRASIL, 2001) which establishes a maximum limit of 1×10^3 CFU/g. No growth of *Staphylococcus* spp. was observed in goat milk caramel samples.

Present results of raw refrigerated

Table 1 – *Staphylococcus* spp. profile: food samples, origin location, species, counts, and *mec*A gene PCR detection

Samples/Type of food	*Staphylococcus* spp.	Counts of *Staphylococcus* (CFU/g,mL)	Presence of *mec*A gene
Refrigerated raw goat milk (city 1)	*S. aureus*	$6,0\times10^2$	Negative
Refrigerated raw goat milk (city 1)	*S. aureus, S. caprae*	$4,5\times10^1$	Negative
Refrigerated raw goat milk (city 2)	*S. simulans*	$<1\times10^1$	Negative
Refrigerated raw goat milk (city 2)	*S. aureus, S. epidermidis*	$1,6\times10^2$	Negative
Refrigerated raw goat milk (city 3)	*S. aureus, S. xylosus*	$3,7\times10^2$	Positive
Refrigerated raw goat milk (city 3)	*S. aureus*	$2,8\times10^2$	Negative
Ricota Cheese	-	NG	-
Chancliche Cheese with oregano	*S. chromogenes*	$< 1\times10^1$	Negative
Chancliche Cheese with oregano	-	NG	-
Chancliche Cheese with papikra	CNS	$< 1\times10^1$	Negative
Chancliche Cheese with papikra	CNS	$< 1\times10^1$	Negative
Chancliche Cheese with zattar	CNS	$< 1\times10^1$	Negative
Chancliche Cheese	*S. caprae*	$< 1\times10^1$	Negative
Boursin Cheese with basil	CNS	$< 1\times10^1$	Negative
Boursin Cheese with basil	*S. simulans*	$< 1\times10^1$	Negative
*Bours*in Cheese with tomato	*S. aureus, S. xylosus*	1×10^2	Negative
Boursin Cheese with tomato	CNS	$< 1\times10^1$	
Boursin Cheese with garlic and fine herbs	-	NG	-
Boursin Cheese with garlic and fine herbs	CNS	$< 1\times10^1$	Negative
Boursin Cheese with fine herbs	*S. epidermidis*	$< 1x10^1$	Negative
Boursin Cheese	CNS	$< 1\times10^1$	Negative
Boursin Cheese	CNS	$< 1\times10^1$	Negative
Goat milk caramel	-	NG	-
Goat milk caramel	-	NG	-
Goat milk caramel	-	NG	-

CNS: Coagulase-negative *Staphylococcus* non typeable by phenotypic tests; NG: No growth of *Staphylococcus* spp.; $< 1x10^1$: Positive *Staphylococcus* growth, but with count under 25 UFC/mL; Limit count of positive-coagulase *Satphylococcus* is 100 UFC/ml according to Brazilian law (BRASIL, 2019).

goat milk CPS enumeration were similar to Gottardi *et al*. (2008) study, in which observed 1.0×10^3 CFU/mL and Picoli *et al*. (2006) also obtained a count of 8.47×10^3 CFU/mL. Souza *et al*. (2011) studied 20 samples of spice Coalho goat cheese type and detected a score of 8.0×10^5 CFU/g of SCP enumeration, and Celia *et al*. (2016) that related scores varying between 2×10^2 to $4{,}2\times10^3$ CFU/g. *Staphylococcus* spp. contamination of raw goat milk is related to its origin from animals with mastitis, as well as contamination during processing, mainly by handlers, since human and animal skin and mucosa represent their main reservoirs.

In the case of artisanal goat cheeses, staphylococci can be introduced into the food by the act of the handler touching the mouth or nose, precarious or insufficient hand washing, and through staphylococcal skin lesions of the employee who works directly with food (PEREIRA *et al*., 2019). Favorable intrinsic and extrinsic cheese conditions can induce staphylococci enterotoxins production which is responsible for food poisoning (MACHADO *et al*., 2018). The artisanal cheeses present evaluated are heat treated to slow pasteurization (60°C to at least 30 minutes) which may partly be related to the significant reductions in *Staphylococcus* spp. counts observed between samples of raw milk and artisanal cheese. Spices, seasonings, and condiments may also represent significant contamination sources of CNS since they are not under strict quality control in artisanal cheese production.

All samples of goat milk caramel did not present growth of typical staphylococci colonies, due to heat treatment and sugar addiction of artisanal processes which the candy went through. This result is in accordance to a previous study described by Agibert (2013) who affirmed that the candy process was efficient to eliminate microorganisms even when goat milk origin was in bad hygienic conditions.

A total of 50 isolates that represented both typical and atypical colonies were tested for coagulase and the presence of the enzyme was confirmed in 16 (32%, 16/50) isolates, and those were subsequently biochemical characterized by *Staphylococcus aureus* (Table 1). All over 34 coagulase-negative

Table 2 – *Staphylococcus aureus* antimicrobial resistance profile

N*	AMP	OX	FOX	CTX	MEM	DA	ERI	CHL	CIP	TCY	GEN	VAN
1	R	S	S	R	S	S	S	S	S	S	S	S
1	R	R	I	R	R	R	S	I	S	S	S	S
1	I	S	S	R	S	I	S	S	S	R	S	S
2	S	S	S	S	S	S	S	S	S	S	R	S
1**	R	R	S	R	S	R	S	S	S	S	R	S
10	S	S	S	S	S	S	S	S	S	S	S	S

* Number of *S. aureus* isolates; ***S. aureus mec*A positive; AMP: ampicillin; OX: oxacillin; FOX: cefoxitin; CTX: cefotaxime; MEM: meropenem; DA: clindamycin; ERI: erythromycin; CHL: chloramphenicol; CIP: ciprofloxacin; TCY: tetracycline; GEN: gentamicin; VAN: vancomycin; S: susceptible; R: resistant; I: intermediary.

staphylococci isolates were biochemically characterized and the prevalent species were: *S. epidermidis, S simulans*, *S. xylosus, S. caprae,* and *S. chromogenes*. Results corroborate the relevance of CNS in food chain production due to their recognized genetic ability to produce enterotoxins (ONNI *et al*., 2012). However, for normative purposes, *S. aureus* is considered the gold standard specie for enterotoxin potential risk in dairy products; Because of that, antimicrobial susceptibility of 16 strains of *S. aureus* was evaluated, and 37,5% (6/16) isolates were resistant for at least one antimicrobial, in the other hand, 62,5% (10/16) were sensible to all antimicrobials tested. No resistance to erythromycin, vancomycin, and ciprofloxacin was detected. PCR assays detected only one *mec*A positive isolate represented by *S. aureus*, which also presented a multidrug resistance profile (Table 2).

CONCLUSION

The results obtained in our study established that *Staphylococcus* hygienic sanitary pattern of goat milk and dairy products are extremely important due to the potential risk of contamination. Multidrug resistant *Staphylococcus aureus* strain detected in raw goat milk and prevalence of coagulase-negative *Staphylococcus* species detected in artisanal goat cheese should not be ignored due to its genetic virulence and antimicrobial resistance spread potential.

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