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Identification of *Staphylococcus* spp. isolated during the ripening process of a traditional Minas cheese

[*Identificação de* Staphylococcus *spp. isolados durante o processo de maturação de um queijo-de-minas tradicional*]

B.M. *Borelli*[1], I.C.A. *Lacerda*[1], L.R. *Brandão*[1], C.R. *Vianna*[1], M.C. *Ferreira*[1], F.C.O. *Gomes*[2], L.S. *Carmo*[1], L.G.D. *Heneine*[3], C.A. *Rosa*[1]*

[1]Departamento de Microbiologia - ICB-UFMG
Caixa Postal 486
31270-901 Belo Horizonte, MG
[2]Departamento de Química - Centro Federal de Educação Tecnológica de Minas Gerais - CEFET-MG, Belo Horizonte, MG
[3]Laboratório de Imunologia - Fundação Ezequiel Dias - FUNED - Belo Horizonte, MG

ABSTRACT

The population dynamics of *Staphylococcus* spp. was studied during the ripening of Canastra Minas cheese at three farms located in the State of Minas Gerais, Brazil. The presence of coagulase (*coa*), thermonuclease *(nuc*), and enterotoxin (*sea*, *seb*, *sec*, and *sed*) genes was investigated in *Staphylococcus* strains isolated during the 60-day cheese-ripening period. The presence of the staphylococcal enterotoxins A, C, and D was also investigated in the cheese samples. Cheese samples that were matured for 0, 7, 15, 30, and 45 days presented staphylococci counts from 10^3 to 10^8cfu/g. All isolates considered coagulase-positive by physiological tests had the *coa* gene. However, no association was observed between the results obtained with biochemical tests and those obtained by PCR using gene-specific primers for coagulase-negative strains. Coagulase and thermonuclease genes occurred simultaneously in 41.3% of *Staphylococcus* spp. tested. None of the investigated *Staphylococcus* strains expressed enterotoxins SEA, SEB, SEC, and SED. Enterotoxins A, C, and D were not detected in any of the cheese samples.

Keywords: Canastra Minas cheese, *Staphylococcus*, enterotoxin

RESUMO

Estudou-se a dinâmica das populações de Staphylococcus *spp. durante a maturação do queijo Canastra, em três fazendas localizadas no estado de Minas Gerais. A presença dos genes que codificam para a produção das enzimas coagulase* (coa), *termonuclease* (nuc) *e produção de enterotoxinas* (sea, seb, sec *e* sed), *em linhagens de Staphylococcus isoladas durante os 60 dias de maturação do queijo foi analisada. Também foi investigada a presença de enterotoxina estafilocócica A, C e D nas amostras de queijo. As amostras de queijo com 0, 7, 15, 30 e 45 dias de maturação apresentaram contagens de* Staphylococcus *spp. entre* 10^3 *e* 10^8*ufc / g. Todos os isolados coagulase positivo nos testes fisiológicos apresentaram o gene* coa. *Não foi observada associação entre os resultados obtidos com os testes bioquímicos e aqueles obtidos com a PCR usando iniciadores gene-específicos para linhagens coagulase negativa. Os genes da coagulase e termonuclease ocorreram simultaneamente em 41,3% dos* Staphylococcus *spp. testados. Nenhum dos isolados de* Staphylococcus *apresentou os genes que codificam para a produção das enterotoxinas SEA, SEB, SEC ou SED. As enterotoxinas A, C ou D não foram detectadas em nenhuma das amostras de queijo analisadas.*

Palavras-chave: queijo Minas Canastra, Staphylococcus, *enterotoxinas*

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Autor para correspondência (*corresponding author*)
E-mail: carlrosa@icb.ufmg.br

INTRODUCTION

The traditional Minas cheese is one of the most important varieties of cheese produced in Brazil. Canastra cheese is the original name of a variety of Minas cheese produced from raw cow milk in Serra da Canastra, a region located in the southeast of Minas Gerais State (Borelli et al., 2006a). It is a semi-hard cheese that is made at the farm house level using traditional procedures for the last 200 years. The region produces about 375.5 tons of cheese per month. The milk is coagulated by employing natural whey cultures as starters (which contain indigenous lactic acid bacteria) and commercial rennet, and ripening occurs via the natural microbiota present in the milk and in the environment (Borelli et al., 2006a). After being manufactured, Canastra cheese presents a pale cream color that becomes yellow during the ripening process.

Cheeses made with unpasteurized milk following traditional procedures may possess a diverse and rich microbiota (Lima et al., 2009). Raw cow milk can also be a source of microbial pathogens, such as *Salmonella* spp., *Escherichia coli, Listeria monocytogenes,* and *Staphylococcus aureus*. The use of raw milk is an important characteristic of the production of traditional Minas cheeses. The Brazilian legislation prohibits the commercialization of Canastra Minas cheese from raw milk except for cheeses that have a maturation period longer than 60 days (Brasil, 1996). Although the use of raw milk in the production of cheese with a long ripening period does not create a risk of pathogenic contamination by itself, it is not recommended because the Brazilian climate and the conditions involved in cheese production are extremely favorable for contamination by and development of microorganisms (Paciulli, 1996).

Outbreaks of food poisoning caused by *Staphylococcus* toxin were previously associated with the consumption of traditional cheeses in Brazil (Carmo et al., 2002), resulting from the ingestion of low levels of staphylococcal enterotoxins. Some strains of *Staphylococcus* can cause food poisoning by producing enterotoxins (SE) when growing in foods, and these toxins have been classified into different serological types (Monday and Bohach, 1999; Chiang et al., 2008). There are 18 serologically distinct staphylococcal enterotoxins (SE) and enterotoxin-like (SEl) toxins of *S. aureus* known so far. These enterotoxins are designated in alphabetical order as A–U, excluding F, S, and T. SEA is the most important enterotoxin in staphylococcal poisoning outbreaks (>75% of outbreaks), followed by SED, SEC, and SEB (Vernozy-Rozand et al., 1998). The aim of this work was to study the dynamics of populations of *Staphylococcus* spp. during the ripening process in Canastra cheese. The presence of the genes encoding coagulase (*coa*), thermonuclease *(nuc*), and enterotoxins (*sea*, *seb*, *sec*, and *sed*) in *Staphylococcus* spp. strains isolated during the 60-day period of Canastra Minas cheese ripening was also analyzed. The cheese samples were also investigated for the presence of the staphylococcal enterotoxins A, C, and D.

MATERIAL AND METHODS

Cheese samples were obtained from three different farms located in the city of São Roque de Minas, State of Minas Gerais, Brazil, in August, September, and October. One sample from the same cheese production lot was collected from each farm. Cheese samples were aseptically collected after salting on days 0, 7, 15, 30, 45, and 60 of the ripening period, cooled down and transported to the laboratory. Microbiological analyses were performed within a 24h period.

To isolate *Staphylococcus* spp., 25g portions of cheese samples were homogenized with 225mL of 0.1% buffered peptone water in a Stomacher 400 Lab Blender (London, UK) for 1 minute. The homogenate was serially diluted and plated onto Baird-Parker agar (Biobrás, Brazil) supplemented with egg yolk solution (12.5mL egg yolk in 25mL 0.85% saline solution) and 1% potassium tellurite. After incubating at 37ºC for 48h, typical (jet black to dark grey with smooth, convex, entire margins presenting an opaque zone, clear halo beyond the opaque zone) and atypical (jet black to dark grey with an entire margin and without a halo) *Staphylococcus* colonies from the appropriate dilutions were counted. Ten colonies from each sample (five typical and five atypical) were selected, purified, and transferred to individual tubes with nutrient agar (stock culture). The purified *Staphylococcus* colonies were tentatively identified by Gram staining, catalase activity, heat stable nuclease (TNase), coagulase test, anaerobic fermentation

of glucose and mannitol, and hemolysis on sheep blood agar (Downes and Ito, 2001). *Staphylococcus aureus* identification was confirmed by PCR using specific primers for the coagulase and thermonuclease genes (Table 1) and using gene-specific primers as described by Martineau et al. (1998).

Table 1. Gene-specific primers used for identification of *Staphylococcus aureus* and primers for detection of the genes for staphylococcal enteroxins (*sea*, *seb*, *sec*, and *sed)*, coagulase (*coa*), and thermonuclease (*nuc*) in *Staphylococcus* spp. strains isolated during the Canastra cheese ripening

Gene	Oligonucleotide sequence (5'-3')	Tm (^oC)[a]	Size of PCR product (bp)	Reference
Gene-specific primers for *S. aureus*	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA	58	102	Martineau et al. (1998)
Sea	GCAGGGAACAGCTTTAGGC GTTCTGTAGAAGTATGAAACACG	57	520	Monday and Bohach (1999)
Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	57	164	Mehrotra et al. (2000)
Sec	CTTGTATGTATGGAGGAATAACAA TGCAGGCATCATATCATACCA	60	283	Monday and Bohach (1999)
Sed	GTGGTGAAATAGATAGGACTGC ATATGAAGGTGCTCTGTGG	60	384	Monday and Bohach (1999)
Coa	ACCACAAGGTACTGAATCAACG TGCTTTCGATTGTTCGATGC	60	820-1000	Aarestrup et al. (1995)
Nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	58	270	Brakstad et al. (1992)

a: primer annealing temperature

Staphylococcal isolates with different physiological and biochemical profiles were selected from each sample and subjected to molecular characterization. DNA was extracted from isolates using the method described by Tannock et al. (1999). DNA aliquots were individually tested by PCR with each gene-specific primer pair (Table 1). The following *S. aureus* enterotoxigenic reference strains were used as standards: *S. aureus* FRI 722, FRI S6, FRI 361, and FRI 1151 (obtained from the Food Research Institute, USA), which represent strains producing enterotoxins SEA, SEB, SEC, and SED, respectively. The strain *S. aureus* FRI 361 was also used as an internal positive control in PCR to identify the presence of coagulase (*coa*) and thermonuclease (*nuc*) genes. *Staphylococcus epidermidis* ATCC 12228, a strain that does not produce enterotoxin, was used as a negative control. Total DNA (50-500ng) was added to the PCR mix (25µL), which contained 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mM of each deoxynucleoside triphosphate, 0.5mM of each primer, and 2.5U of recombinant Taq DNA polymerase (Invitrogen, Brazil). The PCR conditions were as follows: an initial denaturation at 92°C for 2 min was followed by 30 cycles of amplification [denaturation at 92°C for 1 min; an annealing temperature specific to each primer pair (Table 1), and an extension at 72°C for 1min for the *coa* gene and 30s for the other genes], followed by a final extension at 72°C for 10min. The amplified DNA products were separated by agarose gel electrophoresis, stained with ethidium bromide, visualized under UV-light, and photographed.

The qualitative detection of staphylococcal enterotoxins SEA, SEC, and SED in cheese samples was performed using an enzyme-linked immunosorbent assay (ELISA) following the procedures described by Campos (2004).

RESULTS AND DISCUSSION

Table 2 shows the results of the identification and enumeration of *Staphylococcus* spp. isolated from samples of cheese during the ripening. The cheeses that were matured for 60 days had lower counts of *Staphylococcus* spp. Cheese samples with 0 ("frescal" cheese), 7, 15, 30, and 45 days of maturation presented counts of *Staphylococcus* spp. from $<10^3$ to 10^8cfu/g.

Table 2. Microbial counts (cfu g^{-1}) of *Staphylococcus aureus* and *Staphylococcus* spp. obtained from samples collected during the ripening process in three Minas cheese-producing farms in the region of Serra da Canastra, State of Minas Gerais, Brazil

	Days into the ripening process					
Staphylococcus species	Fresh cheese	7 days	15 days	30 days	45 days	60 days
Farm A						
S. aureus	$5.2x10^5$	$1.6x10^5$	$4.6x10^7$	$<10^3$	$<10^3$	$<10^3$
Staphylococcus spp. *coa*$^{+}$ [a]	$5.0x10^3$	$<10^3$	$5.0x10^3$	$<10^3$	$<10^3$	$<10^3$
Staphylococcus spp. *coa*$^{-}$ [b]	$<10^3$	$2.1 x 10^8$	$1.6x10^5$	$6.9 x10^5$	$1.2x10^4$	$1.8 x10^3$
Farm B						
S. aureus	$4.5x10^4$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$
Staphylococcus spp. *coa*$^{+}$	$4.4x10^5$	$4.4x10^6$	$4.2x10^6$	$2.0x10^5$	$<10^3$	$<10^3$
Staphylococcus spp. *coa*$^{-}$	$<10^3$	$4.5x10^6$	$1.1x10^7$	$1.2x10^6$	$2.0x10^4$	$9.5x10^2$
Farm C						
S. aureus	$2.9x10^5$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	ND[c]
Staphylococcus spp. *coa*$^{+}$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$	ND
Staphylococcus spp. *coa*$^{-}$	$2.0x10^6$	$6.1x10^7$	$4.8x10^7$	$6.8x10^6$	$4.8x10^5$	ND

[a] *Staphylococcus* spp. *coa*$^{+}$: Non-*aureus Staphylococcus* spp. strains that were positive for coagulase gene amplification.
[b] *Staphylococcus* spp. *coa*$^{-}$: *Staphylococcus* spp. strains that were negative for coagulase gene amplification.
[c] ND: not determined

S. aureus contamination over the limit of 10^3cfu/g established by the Brazilian legislation was observed in the cheese samples from all three farms at day zero of ripening (Table 2). The cheese sample from farm A at 7 and 15 days of maturation had *S. aureus* counts above 10^5cfu/g. The occurrence of *S. aureus* populations with counts above 10^5cfu/g in foods indicates the potential presence of a sufficient amount of enterotoxin to cause food poisoning (Carmo and Bergdoll, 1990). Non-*aureus Staphylococcus* coagulase-positive strains were observed in farm A, with 0 and 15 days of ripening showing low counts. In farm B, counts above 10^5cfu/g of these bacteria were found at 0, 7, 15, and 30 days of ripening. Enumeration of coagulase-positive *Staphylococcus* strains is recommended by the Brazilian legislation in order to determine the hygienic and sanitary conditions of foods, including cheeses. Coagulase-negative *Staphylococcus* strains were found in all farms, and were found at high counts in farms A and B after seven days of the ripening process (Table 2). These microorganisms were isolated with more frequency than *S. aureus* in all farms. Lima et al. (2008) detected the presence of coagulase-negative *Staphylococcus* in all Minas cheese samples from Serra do Salitre, region of Minas Gerais state. This contamination may have originated from the hands of the workers or from contaminated utensils that may have come in contact with the cheese (Borelli et al., 2006b). The isolation of coagulase-positive and -negative *Staphylococcus* from the hands of the workers has frequently been reported (Bergdoll, 1989; Carmo et al., 2004). Staphylococci can contaminate cheeses through excessive handling, storage conditions, and milk collection, and the pH, water activity, and salt concentrations found in cheeses during their production are favorable for the growth of these microorganisms (Paciulli, 1996; Viana et al., 2009).

A total of 95 *Staphylococcus* spp. isolates (24 coagulase-positive and 71 coagulase-negative) were subjected to molecular analyses. All isolates considered to be coagulase-positive by the physiological tests had the *coa* gene. For coagulase-negative strains, there was no observed association between the results obtained by the physiological tests and those obtained by PCR using gene-specific primers. Thirty-five coagulase-negative strains had the coagulase gene (data not shown). These results show that the *coa* gene may be present but remain unexpressed by some staphylococci strains during the physiological tests (Veras et al., 2008). Coagulase and thermonuclease genes were found together in 41.3% of the *Staphylococcus* spp. tested; therefore, some

coagulase-positive isolates did not harbor the *nuc* gene. Some studies found a positive correlation between the presence of thermonuclease and coagulase in *S. aureus* strains (Brakstad et al., 1992).

The *coa* PCR product size varied among the strains from 850bp to about 1,000bp (Fig. 1). The polymorphisms of the *coa* gene were also observed in the PCR product of DNA amplified from *Staphylococcus* strain UFMG-QJ6. This strain has multiple copies of *coa* as shown by the different fragment sizes (Fig. 1, lane 6). Scherrer et al. (2004) have also shown the polymorphisms of the *coa* gene among 293 coagulase-positive *S. aureus* isolates investigated from samples of refrigerated raw milk, from which only 285 were *coa*-positive, and five fragment sizes were observed. The genetic polymorphisms of different amplicons can be due to small 81-bp tandem repeated sequences, and the presence of multiple *coa* amplification products could be explained by multiple-allelic polymorphisms and mutations within the strain (Goh et al., 1992).

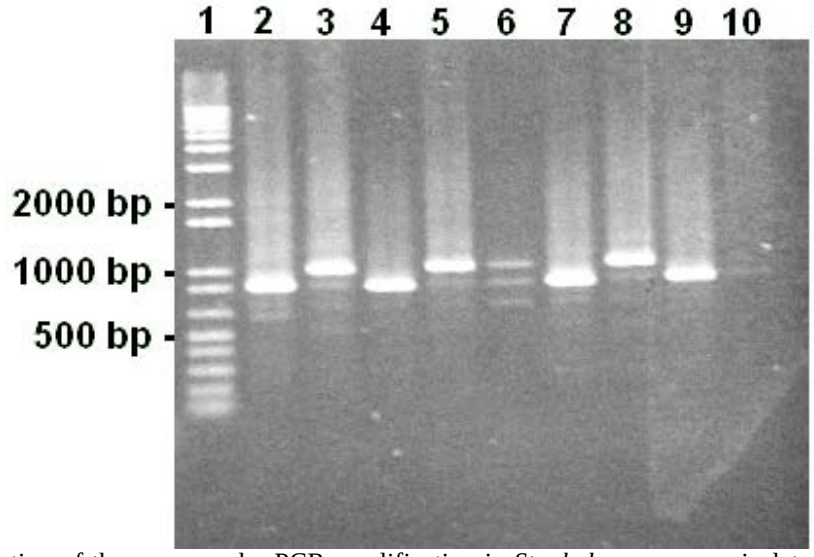

Figure 1. Detection of the *coa* gene by PCR amplification in *Staphylococcus* spp. isolated from Canatra cheese. Lanes: 1, Molecular DNA Ladder 1 Kb Plus (Invitrogen, USA); 2, positive control *coa+ sec+* (*S. aureus* FRI 363); 3, *S. aureus* UFMG-QJ1; 4, *S. aureus* UFMG-PJA; 5, *S. aureus* UFMG-QJ10; 6, *S. aureus* UFMG-QJ6; 7, *S. aureus* UFMG-QJ6B; 8, *S. aureus* UFMG-QJ9; 9, *S. aureus* UFMG-QJ5; 10, negative control (*S. epidermidis* ATCC12228).

None of the investigated *Staphylococcus* strains expressed enterotoxins SEA, SEB, SEC, and SED. These enterotoxin genes were not detected using the specific primers tested in this work (data not shown). Carmo et al. (2004) observed that *Staphylococcus* isolates from food poisoning outbreaks harbored genes for enterotoxins A, B, C, D, G, H, I, and J. However, Silva et al. (2005) found a lower frequency of enterotoxigenic *Staphylococcus* strains in milk from cows with mastitis. Among the 64 *S. aureus* strains isolated by these authors, only four strains showed co-amplification of the *sea* and *seb* genes and two had the *sec* gene. In the present study, no correlation was observed between the presence of the *coa* gene and the presence of the *sea*, *seb*, *sec*, and *sed* genes.

Enterotoxins SEA, SEC, and SED were not detected in any of the cheese samples collected during the 60-day ripening period (data not shown). According to Bergdoll (1989), certain conditions are required for enterotoxin production in food, such as enterotoxigenic *Staphylococcus* serotypes need to be present, the food needs to be a substrate for microbial growth, and the temperature and time must be suitable for toxin production. The results suggest that these conditions were not found during the elaboration of the Minas cheese produced in the region of Serra da Canastra.

CONCLUSIONS

The Minas cheese produced in the region of Serra da Canastra could be considered safe for consumption after the 15th day of production in two farms, and after the 30th day in one farm, according to the Brazilian legislation, only considering the staphylococcal counts and enterotoxin production data.

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