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ENTEROXIGENIC *STAPHYLOCOCCUS* SPP. AND OTHER MICROBIAL CONTAMINANTS DURING PRODUCTION OF CANASTRA CHEESE, BRAZIL

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ABSTRACT

Canastra cheese is produced from raw cow's milk, and it is made at the farmhouse level using artisanal procedures and natural starters. The aim of this work was to determine the main hygienic-sanitary indicators and enterotoxigenic staphylococcal strains present during the manufacturing of traditional cheese of Serra da Canastra region, Minas Gerais state, Brazil. Samples from 10 farms were studied, and they included: water employed in the process, raw milk, natural starters, cheese curd before salting and cheese after five days of ripening. All water samples exhibited faecal coliform contamination above the maximum acceptable value recommended by Brazilian standards. *Pseudomonas aeruginosa* and sulfite-reducing clostridia were also isolated from the water samples. In five samples of raw milk faecal coliform were above the limits allowed by the Brazilian legislation. The counts of *Staphylococcus* spp. in milk were between <2.0 to 4.9 log.cfu.ml^{-1}. The counts of microbiological indicators were higher in natural starters and curd. High levels of faecal and total coliform, as well as molds, were found in the cheese samples. In all cheeses analyzed *Staphylococcus* spp. were found in levels above 5.0 log.cfu.g^{-1}. The enterotoxins (SE) most frequently produced by S*taphylococcus* spp. strains were SEB and SEC. A high number of coagulase negative *Staphylococcus* strains were also enterotoxin producers. None of the samples contained *Salmonella* spp. or *Listeria* spp. These results point out a need for improvements in the production process of the artisanal cheese produced at Serra da Canastra in Brazil.

Key words: Artisanal cheese, coliforms, *Staphylococcus*, enterotoxin

INTRODUCTION

Serra da Canastra cheese is traditionally made with raw cow's milk employing natural starters (indigenous lactic acid bacteria) and commercial rennet. The cheeses produced in the region of Serra da Canastra have been manufactured in a traditional empirical manner for more than 200 years in Minas Gerais state, Brazil (25). The natural starter used in its production is composed by species of *Lactobacillus*, *Lactococcus* and *Streptococcus*, with counts of approximately 8 log cfu.ml^{-1}, and it is produced by dropping whey from previous cheese covered with salt (B. M. Borelli & C. A. Rosa, unpublished data). The production of Canastra cheese is about 375.5 tons per month, being the main economic activity of many families of the region (14). Nevertheless, there is no standardization of the manufacturing process, especially regarding time of coagulation, natural starter used, pressing, salt and humidity in the final product. Thus, it is possible to find Canastra cheeses in the market having different and characteristic flavors and aromas.

As Canastra cheese is an artisanal product, the production process does not follow safety standards regulary. Use of raw milk, for example, is a cause for worry because it constitutes an important source of pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes*, among others (6,16,20). The presence of *S. aureus* and the possibility of staphylococcal toxin production

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represent a potential risk for public health (3,4,17,22). Staphylococcal enterotoxins (SEs) are a family of nine thermostable, pepsin-resistant exoproteins forming a single chain with a molecular weight ranging from 26,000 to 29,600 Da (10,17). They are identifiable serologically and known as SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ. Most staphylococcal strains are able of producing one or more enterotoxins, which are the cause of the gastrointestinal symptoms, that include vomiting with or without diarrhea, observed during intoxications (17). *Staphylococcus* spp. coagulase negative strains that produce enterotoxins have been isolated, but there is very little information about their role in food poisoning (10). In Brazil, the commercialization of Canastra cheese produced from raw milk is common, although Brazilian food safety regulations for cheeses made from raw milk recommend that those products be made available only after ripening periods of at last 60 days (7).

There is little information available on the microbiological characteristics of artisanal Brazilian cheeses (13,23). The aims of this study were: (i) to determine counts of total and faecal coliforms, aerobic mesophilic bacteria, *Staphylococcus* spp. and *S. aureus*, molds; (ii); to establish the presence of *Salmonella* spp. and *Listeria* spp. and (iii) to investigate the evolution of enterotoxigenic Staphylococcal strains during the manufacturing of the artisanal cheese produced in region of Serra da Canastra, Brazil.

MATERIAL AND METHODS

Sample collection

Samples were obtained from ten different farms (A to J) in the city of São Roque de Minas, State of Minas Gerais, Brazil, in December 1999 and February 2000. Water, raw milk, natural starter, cheese curd before salting and cheese after five days of ripening at room temperature from each farm were sampled aseptically, and transported to the laboratory under refrigeration for microbiological analyses.

Microbiological analysis

Water samples

Water samples were surveyed for the presence of total and faecal coliforms, sulfite-reducing clostridia, enterococci and *Pseudomonas aeruginosa*, according to standard methods (11) and compared to safety standards of the Brazilian Health Legislation (8). Total and faecal coliform, enterococci and *P. aeruginosa* counts were determined by the most probable number (MPN) method and enumeration of sulfite-reducing clostridia was made in reinforced clostridial medium (11).

Milk, natural starter, curdle and cheese samples

Aliquots of 10 ml of milk and natural starter were diluted in 90 ml sterile 0.1% buffered peptone water before inoculation. For curdle and cheese 25g portions were homogenized with 225 ml of 0.1% buffered peptone water in a Stomacher 400 Lab Blender (London, UK) for 1 minute and decimal dilutions were prepared therefore using the same diluent. Aerobic mesophilic bacteria were determined on plate count agar (Difco Laboratories, Detroit, USA); occurrence of molds was determined on potato dextrose agar (Difco) acidified with 10 ml/l of 10% (w/v) sterile tartaric acid by pour plating. The plates were incubated at 30ºC for 48 h for mesophilic microorganisms, and 25ºC for five days for molds (11). All microbiological analyses were performed in triplicate.

Total and faecal coliform counts were determined by the Most Probable Number (MPN) method using a three-tube series. Total coliforms were enumerated in 2% bile brilliant green broth (Difco), incubated at 37ºC for 24-48 h; faecal coliforms were determined in EC broth (Difco) incubated at 44ºC for 24 h (11).

Salmonella detection was carried out after pre-enrichment in buffered peptone-water and enrichment in selenite cystine broth (Biobrás, MG, Brazil) and Rapapport-Vassiliads broth (Biobrás), incubated at 35ºC and 42ºC for 24h, respectively. Enrichment cultures were streaked onto *Salmonella-Shigella* agar (Biobrás) and Hektoen enteric agar (Biobrás). The plates were incubated at 35ºC for 48h (11). The typical colonies were identified by Triple Sugar Iron (TSI) agar (Oxoid, Ltd. Basingstoke, Hampshire, England), Lysine Iron Agar (LIA) (Oxoid) fermentation tests, urease test (Urea Broth, Oxoid) and serological tests such as polyvalent flagellar (H) and polyvalent somatic (O) tests (murex Salmonella polyvalent agglutinating sera) (11).

The presence of *Listeria* was established by homogenization of either 25g or 25ml of the sample with 225ml primary *Listeria* enrichment broth (LEB I, Merck, Darmstadt, Germany) in a Stomacher 400 Lab Blender. The enrichment broth was incubated at 35ºC for 48 h. LEB I cultures were transferred to secondary *Listeria* enrichment broth (LEB II, Merck) and incubated at 35ºC for 48 h. After incubation, LEB II cultures were streaked onto Palcam agar (Sigma, St. Louis, MO, USA) and Oxford agar (Sigma) and the plates were incubated at 35ºC for 48 h and analyzed for the presence of *Listeria* colonies (11). The purified isolates were identified by examination of TSAYE (tripticase soy agar plus 0.6% yeast extract) plates with oblique Henry illumination, Gram staining, examination of catalase activity, rotating or tumbling motility, hemolysis zone on blood agar, motility in SIM medium (hydrogen sulfide production, indole formation and motility) (Oxoid) for typical umbrella shape, and carbohydrate fermentation tests in Purple Carbohydrate Broth, and the CAMP test (11).

Staphylococcus spp. were counted on Baird-Parker agar (Biobrás) with added egg yolk tellurite, incubated at 37ºC for 48 h. After growth, *Staphylococcus* colonies were counted and classified as typical for *S. aureus* (jet black to dark gray, smooth, convex, entire margins with an opaque zone, clear halo beyond the opaque zone) and atypical (jet black to dark gray colonies,

entire margin without a halo). Ten colonies from each sample (5 typical and 5 atypical) were selected and transferred to individual tubes with nutrient agar (stock culture), and tested for coagulase, thermonuclease (TNase), anaerobic fermentation of glucose and mannitol, and production of hemolysin on sheep's blood agar (11).

Toxin production by *Staphylococcus* species

Strains of *Staphylococcus* spp. from the same sample and exhibiting similar physiological and biochemical profiles were pooled for testing. Individual pools comprised one to ten strains. Enterotoxin quantification was performed using the membrane-over-agar method with subsequent optimum-sensitivity-plating as described by Bergdoll (5).

RESULTS AND DISCUSSION

Water used in the manufacture of Canastra cheese exhibited high levels of contamination by coliform bacteria (Table 1). None of the water samples tested was in conformity with the standards set by the Brazilian's Ministry of Health (8), which defines the absence of faecal coliforms or *E. coli* in 100 ml of water as standard. Also, the presence of *P. aeruginosa* and sulfite reducing clostridia would offer risk to public health, probably leading to contamination of cheese, with consequent reduction of shelf life. Sulfite reducing clostridia are able to produce enzymes that cause late swelling in cheeses (12,19,21). *Pseudomonas aeruginosa* also produces thermoresistant proteases and lipases that could alter organoleptic properties of the final product. In addition, these bacteria represent a serious risk to public health since *P. aeruginosa* is frequently associated to infections in immunocompromised patients (15). Our results suggest that the water employed in cheese manufacturing could represent a source of contamination of the final product.

Microbiological analysis of raw milk revealed that 40% of samples did not exhibit detectable counts of faecal coliforms (less than 0.3 MPN.ml^{-1}) (Table 2). The other 60% of samples exhibited faecal coliform counts varying from 2.3 to 24.0 MPN.ml^{-1}. These counts were lower than those observed by Tornadijo *et al*. (24) in raw cow's milk. The counts of aerobic mesophilic bacteria and *Staphylococcus* spp. present in the raw milk ranged from 2.6 to 7.0 and <2.0 to 4.9 log cfu.ml^{-1}, respectively (Table 2). The counts of faecal coliforms, aerobic mesophilic bacteria and *Staphylococcus* spp. in milk are not related only to the poor hygienic conditions during milking; they are also associated to storage temperature of milk, without refrigeration before cheese making. Our results show that milk is a source of contamination of artisanal cheeses; thus, control measures in milking are needed for a better quality of the product.

High microbial counts for indicators of faecal contamination and *Staphylococcus aureus* were also detected in natural starter (Table 2). An association was found between contaminated natural starter added to raw milk and high counts of indicator bacteria in the curd. It can be concluded that the traditional use of natural starter in cheese manufacturing is a potential source of contamination of the Canastra cheese. An alternative to controlling the level of contamination would be the maintenance of the natural starter under refrigeration until use. Nevertheless, the use of natural starter should be maintained because it contains the lactic bacteria responsible for the production and ripening of Canastra cheese. Maintenance of microbial diversity present in the natural starter may, then, be what differentiates Canastra cheese from other manufactured cheeses, using similar processes.

In curd and cheese total and faecal coliforms reached values of about >11,000 MPN. g^{-1}, while mold counts were 4.2

Table 1. Microbial counts obtained of water from ten cheese producing farms in the region of the Serra da Canastra, MG, Brazil.

Farms	Total coliforms	Faecal coliforms	*Pseudomonas aeruginosa*	Enterococci	Sulfite reducing *Clostridium*
	(MPN/100 ml)[1]	(MPN/100 ml)	(MPN/100 ml)	(MPN/100 ml)	(MPN/100 ml)
A	> 16.0	> 16.0	5.1	<2.2	<2.2
B	> 16.0	> 16.0	> 16.0	<2.2	<2.2
C	> 16.0	> 16.0	9.2	<2.2	<2.2
D	> 16.0	> 16.0	16.0	<2.2	<2.2
E	> 16.0	> 16.0	>16.0	<2.2	<2.2
F	16.0	16.0	16.0	<2.2	2.2
G	> 16.0	2.2	>16.0	<2.2	5.1
H	>16.0	16.0	<2.2	<2.2	16.0
I	16.0	16.0	5.1	<2.2	5.1
J	>16.0	>16.0	>16.0	<2.2	2.2

[1] MPN: Most Probable Number.

Table 2. Microbial counts obtained for samples of raw milk, natural starter, curdle and Canastra cheese from ten farms in the region of the Serra da Canastra, MG, Brazil.

Microorganisms	Farms									
	A	B	C	D	E	F	G	H	I	J
Raw Milk										
Total coliform[a]	460	<0.3	<0.3	240	24	<0.3	2.3	2.3	9.3	460
Faecal coliform[a]	24	<0.3	<0.3	24	24	<0.3	<0.3	2.3	4.3	15
Staphylococcus spp.[b]	4.7	<2.0	<2.0	3.8	3.7	<2.0	4.3	3.9	4.9	3.7
Staphylococcus aureus[b]	3.6	<2.0	<2.0	<2.0	<2.0	<2.0	2.8	<2.0	4.6	<2.0
Mesophilic bacteria[b]	7.0	3.4	4.4	4.2	3.7	2.6	4.2	4.0	4.9	5.1
Natural starter										
Total coliform[a]	1100	1100	93	240	460	>1100	>1100	1100	240	150
Faecal coliform[a]	210	1100	93	21	150	240	1100	240	240	9
Staphylococcus spp.[b]	4.7	5.7	5.2	4.3	5.8	3.2	6.3	4.3	4.3	4.3
Staphylococcus aureus[b]	<2.0	5.7	5.1	4.2	<2.0	2.5	<2.0	3.1	2.1	<2.0
Moulds[b]	4.0	3.6	4.2	4.2	4.2	5.2	4.8	5.0	4.5	4.5
Curd										
Total coliform[a]	1500	23	460	>11000	>11000	>1100	>1100	>1100	>1100	>1100
Faecal coliform[a]	7	23	460	>11000	>11000	>1100	>1100	>1100	>1100	>1100
Staphylococcus spp.[b]	4.5	4.3	5.3	5.3	6.4	5.0	6.3	5.3	6.3	5.2
Staphylococcus aureus[b]	4.5	4.3	5.2	5.3	6.3	4.9	4.5	4.9	6.1	4.9
Moulds[b]	4.2	3.2	3.3	4.2	4.2	4.5	5.2	3.8	4.0	2.3
Cheese										
Total coliform[a]	>11000	1100	2400	>11000	>11000	>1100	>1100	240	>1100	>1100
Faecal coliform[a]	>11000	460	2400	>11000	>11000	>1100	150	240	>1100	>1100
Staphylococcus spp.[b]	5.2	6.3	6.3	6.2	6.3	6.3	6.3	6.3	6.3	6.3
Staphylococcus aureus[b]	<2.0	6.1	6.2	6.2	6.3	5.9	4.8	<2.0	6.0	<2.0
Moulds[b]	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2

[a] MPN.g^{-1} or ml^{-1} (Most probable number); [b] log cfu.g^{-1} or ml^{-1} (Colony forming units).

log cfu.g^{-1}, in most samples (Table 2). These results were similar to those found in other cheeses, such as Serrano (23) and Tetila (16). High levels of contamination with *Staphylococcus* spp. were also found in curd and cheese samples (Table 2). About 70% of the Canastra cheeses were contaminated with *S. aureus* with population counts varying from less than 4.8 log cfu.g^{-1} to 6.3 log cfu.g^{-1} (Table 2). Albenzio *et al*. (2) observed that the Italian Canestrato Pugliese cheeses produced with raw milk exhibited *Staphylococcus* counts around 4.1 log cfu.g^{-1}. *Staphylococcus aureus* is often found in raw milk and in cheese-making environment. This microorganism is salt-tolerant and has the ability to grow under very different conditions; low acid production may allow staphylococci to grow and produce enterotoxins (4,18). *Salmonella* spp. and *Listeria* spp. were not found in samples of raw milk, natural starter, curd or in cheese collected in this study.

Out of the 75 *Staphylococcus* spp. pools tested for the production of enterotoxin and TSST-1 toxin, 70 (93.3%) produced at least one of those toxins (Table 3). Cardoso *et al*. (9) found that 65% of 127 strains of *S. aureus* isolated from milk of cows suffering from mastitis produced either enterotoxin or TSST-1 toxin; the latter was most frequently produced (47% of positive samples). Carmo *et al*. (10), studying an outbreak of food poisoning caused by ingestion of raw milk and Minas cheese, isolated SEA, SEB and SEC from enterotoxigenic strains of *S. aureus*.

Although *S. aureus* is the most common staphylococcal species involved in outbreaks of food poisoning, two other coagulase positive species, *S. intermedius* and *S. hyicus*, have been cited as enterotoxigenic (1,4). In our study, *S. intermedius* and *S. hyicus* isolated from milk and curd have produced enterotoxin B and C. Most pools of *S. hyicus* producing enterotoxin were from milk, curd and cheese samples. Pools of

Table 3. Detection of toxin production by "pools" of *Staphylococcus* spp. from the different samples studied.

Species	Number of tested "pools" [a]	Toxin					Number of "pools" negative for toxin
		SEA	SEB	SEC	SED	TSST-1	
Milk	15						
Staphylococcus aureus	3	-	2	3	-	-	-
Staphylococcus coagulase negative	7	-	5	3	1	3	1
S. hyicus	5	-	4	2	1	1	-
Natural starter	15						
S. aureus	5	2	2	2	-	-	-
Staphylococcus coagulase negative	9	-	7	7	1	3	-
S. intermedius	1	-	1	1	-	-	-
Curd	26						
S. aureus	13	1	9	7	-	1	1
Staphylococcus coagulase negative	5	-	4	2	-	1	1
S. hyicus	7	1	5	6	-	3	1
S. intermedius	1	-	1	1	1	-	-
Cheese	19						
S. aureus	10	2	7	3	1	1	1
Staphylococcus coagulase negative	7	-	7	6	2	3	-
S. hyicus	2	-	1	1	2	2	-
Total	75	6	55	44	9	18	5

[a] Individual pools comprised one to ten strains of *Staphylococcus* spp. from the same sample and exhibiting similar physiological and biochemical profiles.

coagulase negative *Staphylococcus*, mainly from natural starter and cheese samples, were positive for production of enterotoxin B, C and D, and TSST-1 toxin. Carmo *et al*. (10) found that coagulase negative strains, from raw milk samples that were associated to food poisoning, produced enterotoxins C and D. These findings point to the need for research on toxin production by coagulase negative staphylococci even if there are no legal requirements for that. Detection of enterotoxigenic strains of coagulase negative *Staphylococcus* is of great importance for public health, although a Brazilian standard for those microorganisms has not yet been defined. The presence of enterotoxigenic Staphylococcal strain in milk and cheeses represents a risk to for consumers, even in low numbers (10,17). It is therefore imperative to ensure that milk used in the manufacturing is of the highest bacteriological quality. The use of pasteurized milk could be an alternative to improve the microbial quality of the Canastra cheese, associated to specific starter strain.

We conclude that sanitary measures are needed to improve the hygienic conditions during milking and manufacturing cheese in order to guarantee the quality of this highly popular cheese in Brazil. These measures must include a program of sanitary education for the milking personnel and cheese producers with emphasis on hygiene standards as well as technical and practical aspects of milking.

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RESUMO

Staphylococcus spp. enterotoxigênicos e outros contaminantes microbiológicos durante a produção de queijo Canastra, Brasil

O queijo Canastra é produzido a partir de leite cru, e é fabricado em fazendas utilizando procedimentos artesanais e culturas naturais de soro como iniciadoras. O objetivo deste trabalho foi determinar os principais indicadores higiênico-sanitário e as linhagens enterotoxigenicas de *Staphylococcus*

presentes durante a fabricação do queijo tradicional da região da Serra da Canastra, Minas Gerais, Brasil. Amostras provenientes de 10 fazendas foram estudadas, e estas incluíram: a água utilizada no processo, o leite cru, o soro iniciador, a coalhada antes da salga, e o queijo após cinco dias de cura. Todas as amostras de água apresentaram contaminação por coliformes fecais acima do valor máximo recomendado pelos padrões brasileiros. *Pseudomonas aeruginosa* e clostrídios sufito-redutores também foram isolados das amostras de água. Em cinco amostras de leite cru os coliformes fecais apresentaram-se acima dos limites permitidos pela legislação brasileira. As contagens de *Staphylococcus* spp. no leite variaram de <2.0 a 4.9 log.ufc.ml^{-1}. As contagens dos indicadores microbiológicos foram maiores no soro iniciador do que na massa coagulada. Níveis altos de contaminação por coliformes totais e fecais, como também bolores, foram observados nas amostras de queijo. Em todos os queijos estudados *Staphylococcus* spp. foram encontrados em níveis acima de 5.0 log.ufc.g^{-1}. As enterotoxinas mais freqüentes produzidas pelas linhagens de *Staphylococcus* spp. foram B e C. Um número elevado de linhagens de *Staphylococcus* coagulase-negativa foram também produtores de enterotoxinas. Em nenhuma das amostras foi isolada *Salmonella* spp. ou *Listeria* spp. Estes resultados mostram a necessidade de melhorias no processo de produção do queijo artesanal produzido na Serra da Canastra, Brasil.

Palavras-chave: Queijo artesanal, coliformes, *Staphylococcus*, enterotoxinas

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