

Research Paper

The influence of ripening period length and season on the microbiological parameters of a traditional Brazilian cheese

Valéria M. Cardoso^{1,2}, Ricardo S. Dias³, Barbara M. Soares¹, Letícia A. Clementino¹, Cristiano P. Araújo¹, Carlos A. Rosa^{1,4*}

¹Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

²Departamento de Farmácia, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil.

³Divisão de Vigilância Sanitária do Instituto Octávio Magalhães, Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil.

Submitted: February 03, 2012; Approved: September 10, 2012.

Abstract

The ripening process of Serro Minas cheese, one of the most popular cheeses produced with raw milk in Brazil, was studied over the course of 60 days of ripening during dry and rainy seasons. Brazilian legislation prohibits the production of cheese from raw milk unless it was submitted to a maturation period greater than 60 days. However Minas Serro cheese is sold within a few days of ripening. A total of 100 samples of Serro cheese were obtained from five farms; 50 samples were collected during the dry season (winter in Brazil) and 50 samples were collected during the rainy season (summer in Brazil). From each farm, ten cheeses were collected during each season after two days of ripening. Our results showed high levels of total and fecal coliforms at the beginning of the ripening period (approximately 4 Log MPN/g with 3 days of ripening) that decreased with 60 days of ripening reaching almost 1.5 Log MPN/g. Contamination by coagulase-positive staphylococci was reduced by the end of the ripening period. *Salmonella* spp. was not detected. The staphylococcal enterotoxins B and C were detected in 1% and 4% of the cheeses, respectively, after 30 days of ripening. These results suggest that the ripening process was not effective in eliminating staphylococcal enterotoxins from the cheese. However, none of the investigated strains of *Staphylococcus* spp. isolated from Serro cheese produced enterotoxins A, B, C or D. The high pathogen and coliform levels at the beginning of the ripening process for the cheese produced during both seasons indicate the need for improvement of the sanitation of the manufacturing conditions.

Key words: traditional Minas cheese, ripening, total and fecal coliforms, coagulase-positive staphylococci, staphylococcal enterotoxins.

Introduction

Traditional cheeses originate from a complex system which results in unique organoleptic characteristics, and they are characterized by strong links to their territory of origin (Lecitra, 2010). Traditional Minas cheese is one of the most popular cheese produced in Brazil, with a long history of manufacturing. Serro cheese is the original name of a variety of Minas cheese produced using raw cow's milk in a

region located in the central northeast of Minas Gerais state. It is a very popular cheese in Brazil, and it is considered a variety of semi-hard cheese because it has a typical and markedly acidic taste (Furtado *et al.*, 2003). Serro cheese has been made at the farm house level using traditional procedures for the last 200 years and has high consumption and appreciation levels in Brazil. The Minas Serro cheese producing-region is composed of ten small

cities, and the region produces about 3,106 tons of cheese per year.

Minas Serro cheese is traditionally made using raw cow's milk and by adding "pingo" (a natural fermentation starter) and industrial liquid rennet. Forty to 60 min after the addition of rennet and "pingo", the coagulum is cut and transferred to plastic molds, where the whey is removed by hand pressing. The cheese is covered with coarse salt on one side and remains that way for approximately seven hours. Next, the cheese is turned and salted on the other side. After another period of resting (approximately 12 hours), the cheese is turned again, and after 24 hours, it is removed from the mold and washed. "Pingo" is the whey that drops from the previous cheese covered with salt (Borelli *et al.*, 2006). Bacterial populations associated with "pingo" include *Lactobacillus*, *Lactococcus* and *Streptococcus* species (Borelli *et al.*, 2006, Lacerda *et al.*, 2011). This cheesemaking process takes place on farms in a room adjacent to the cow barn.

After three days of maturation at room temperature, the cheese is transported to a storage facility where it is weighed, washed again, dried in a drying chamber for one day and vacuum-packed in plastic bags. Afterward, it is refrigerated at approximately 10 °C and marketed under refrigeration.

Brazilian legislation (Brasil, 2000) prohibits the production of cheese manufactured with raw milk except for those types of cheese that have a maturation period greater than 60 days. Because Minas Serro cheese sells within a few days of ripening and because it is manufactured using raw milk, pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* spp. can contaminate the cheese. This contamination, specifically, the presence of coagulase-positive staphylococci and staphylococcal enterotoxins in the cheese, may present a potential health risk to consumers (Aarestrup *et al.*, 1995; Leloir *et al.*, 2003).

This study was performed to examine the populations of coagulase-positive *Staphylococcus*, both total and fecal coliforms, *E. coli* and *Salmonella* spp. in traditional Minas Serro cheese. The presence of staphylococcal enterotoxins (SE) A, B, C and D and of toxic shock syndrome toxin 1 (TSST-1) was also measured.

Materials and Methods

Sample collection

A total of 100 samples of Serro cheese were obtained from five farms; 50 samples were collected during the dry season (winter in Brazil) and 50 samples were collected during the rainy season (summer in Brazil). From each farm, ten cheeses were collected during each season after two days of ripening; they were then stored without packaging in a refrigerated room (around 10 °C) of a storage facility throughout the 60-day ripening period. The cheeses

were turned every day. The average temperature and the relative humidity (RH) of the refrigerated room during the ripening periods were 10.45 °C with 88.2% RH during the dry season and 11.51 °C with 81.32% RH during the rainy season. The cheeses were sampled after 3, 15, 30, 45 and 60 days of ripening. Out of the ten cheeses collected from each producer, two cheeses were sampled during each time of ripening. The samples were then transported to the laboratory under refrigeration for microbiological analyses. For the staphylococcal enterotoxins analyses, the samples were stored at -20 °C. The samples collected during the dry season were from cheeses manufactured in May and June of 2008, and the samples collected during the rainy season were from cheeses manufactured in January of 2009. The analyses were performed on cheeses ripened for 3, 15, 30, 45 and 60 days under refrigeration to verify the proper maturation period for this type of cheese. In addition, analyses were performed on cheeses manufactured during either the dry or rainy season to determine whether the microbiological parameters are influenced by the season in which the cheese is produced.

Microbiological analyses

Samples of 25 g each were homogenized with 225 mL of 0.1% peptone water in a Stomacher 400 lab blender (Seward, London, UK) for 1 min, and serial decimal dilutions were prepared using the same diluent. Total and fecal coliform bacteria were counted using the most probable number (MPN) technique. Lauryl sulfate tryptose and Brilliant Green broths (Difco, Detroit, USA) were used to determine the total coliform bacterial counts, and EC-MUG broth (Difco), incubated in a water bath at 45.5 °C, was used to determine the fecal coliform bacterial counts. For *E. coli* isolation, a loopful of culture from each gassing EC tube was streaked onto a Levine's eosin-methylene blue (L-EMB) agar plate and incubated for 18-24 h at 35 °C. Suspected *E. coli* colonies were transferred to PCA slants incubated for 18-24 h at 35 °C and were tested for the presence of indole production, Voges-Proskauer-reactive compounds, methyl red-reactive compounds, citrate, Gram stain and gas production from lactose. The MPN of *E. coli* was based on the proportion of EC tubes in three successive dilutions that contained *E. coli* (Downes and Ito, 2001).

Salmonella spp. detection was carried out after pre-enrichment in 1% buffered peptone water for 24 h at 37 °C and after enrichment in selenite cystine broth (Biobrás, MG, Brazil) and Rapaport-Vassiliadis broth (Biobrás) for 24 h at 35 °C and 42 °C, respectively. Enriched cultures were streaked onto *Salmonella*-*Shigella* agar (Biobrás) and Hecktoen enteric agar (Biobrás). The plates were incubated at 35 °C for 48 h. Typical-looking colonies were examined using the Triple Sugar Iron (TSI) agar (Oxoid, UK) or Lysine Iron Agar (LIA) (Oxoid) fermentation tests, urease tests (using Urea broth, Oxoid) and serological tests such as the polyvalent flagellar (H) and polyvalent somatic (O)

tests (using Murex Salmonella Polyvalent Agglutinating Sera) (Downes and Ito, 2001).

Staphylococcus spp. were counted on Baird-Parker agar (Biobrás) with added egg yolk tellurite after incubation at 37 °C for 48 h. After growth, *Staphylococcus* colonies were counted and classified as being either typical or atypical for *S. aureus*. Typical *S. aureus* colonies displayed the following phenotypes: jet black to dark grey, smooth, and convex with well-defined contours, off-white edges and an opaque zone. Atypical *S. aureus* colonies displayed different phenotypes: gray to dark gray and mucoid with the entire margin showing without a halo. Ten colonies from each sample, five typical and five atypical, were selected and transferred to tubes containing nutrient agar (stock culture); they were then tested for the presence of thermonuclease (TNase), catalase and coagulase for the biochemical identification of coagulase-positive *Staphylococcus* (Downes and Ito, 2001). Coagulase-negative colonies were further tested for furazolidone sensitivity to differentiate *Staphylococcus* spp. from *Micrococcus* spp. (Koneman *et al.*, 2001).

Detection of the coagulase gene in isolates of *Staphylococcus* spp.

Staphylococcus spp. that presented a negative phenotype for the coagulase test were next analyzed for the presence of the coagulase gene using a polymerase chain reaction (PCR). DNA extraction and PCR were performed according to the methodology described by Aarestrup *et al.* (1995). The coagulase gene-specific primers *Coa1* (5'-ACCACAAGGTACTGAATCAACG-3') and *Coa2* (5'-TGCTTTCGATTGTTTCGATGC-3') were used to amplify a fragment of approximately 987 bp. The amplicons were analyzed in a 1% agarose gel, stained with GelRed nucleic acid gel stain (Biotium, Uniscience, USA) and photographed.

Molecular identification of *S. aureus*

The isolates of *Staphylococcus* spp. that were positive for the phenotypic coagulase test or the presence of the coagulase gene were next analyzed for the presence of the *S. aureus* TNase gene and were consequently identified as *S. aureus*. For the confirmation of this species, the *nuc* gene of *S. aureus* species-specific oligonucleotides SANuc1 (5'-TGCTATGATTGTGGTAGCCATC-3') and SANuc2 (5'-TCTCTAGCAAGTCCCTTTTCCA-3') were used to amplify a fragment of 420 bp (Baron *et al.*, 2004). *S. aureus* ATCC 29213 was used as a positive control for the reaction. After amplification, the reactions were analyzed by electrophoresis through a 1% agarose gel, stained with GelRed nucleic acid gel stain and photographed.

Detection of the staphylococci enterotoxins

Enterotoxin extractions from Serro cheese samples were performed according to the procedures previously de-

scribed by Carmo (2001). The presence of enterotoxins in the extracts was analyzed in triplicate using the optimum-sensitivity-plating (OSP) method as described by Robbins *et al.* (1974). The extracts were tested for standard enterotoxins A, B, C, D and TSST-1. The limit of detection of the test is 0.5 µg of toxin per gram of food.

Toxin production by *Staphylococcus* species

Strains of *Staphylococcus* spp. exhibiting similar physiological and biochemical profiles and that did or did not present the *S. aureus* coagulase or TNase genes were pooled to test SEA, SEB, SEC, SED and TSST-1 production using the OSP method. Individual pools comprised one to five strains.

Statistical analyses

The data were submitted to descriptive statistics, a Normality test and Pearson Correlation using the program SAS Institute Inc. (SAS/STAT. version 8.0, SAS Institute Inc. Cary, NC, USA). An analysis of variance (ANOVA) and mean comparison using Students t test and Scott-Knott test was used to verify differences between seasons and ripening times, respectively, using the program SISVAR 2007 (SISVAR. version 5.0, DEX/UFLA, Lavras, MG, Brazil). The level of statistical significance was set at $p < 0.05$.

Results and Discussion

The number of total and fecal coliforms in cheese was high within a few days of ripening (approximately 4 Log MPN/g with 3 days of ripening) but then decreased throughout the ripening period reaching almost 1.5 Log MPN/g with 60 days of ripening (Table 1). This result was likely due to the reduction of moisture and inhibition by the native microbiota present in the cheese. It is well known that ripening acts as a natural selector, during which lactic acid bacteria normally inhibit pathogens (Nunez *et al.*, 1985). Leite *et al.* (2001) analyzed the contamination of Minas cheese and observed a decrease of approximately two Logs in the number of fecal coliform bacteria present on the thirty day of ripening. In this study, with 30 days of ripening, the cheese manufactured during the dry season had 2.3 Log MPN/g of total coliforms and 1.84 Log MPN/g of fecal coliforms. These values are lower than what is allowed by Brazilian law (3.69 Log MPN/g for total coliforms and 2.69 Log MPN/g for fecal coliforms) (Brasil, 1996). After 30 days of ripening, the cheese manufactured during the rainy season presented a number of total and fecal coliforms lower than what is allowed by law. The limits allowed by Brazilian law vary depending on the moisture content of the cheese. With 30 days of ripening, a significantly higher amount of total coliforms was observed in cheese produced during the rainy season (3.15 Log MPN/g) as compared with the dry season (2.30 Log MPN/g). The number of fecal coliforms was also significantly higher

Table 1 - Counts (mean \pm standard deviation) of total and fecal coliforms, *Escherichia coli*, *Staphylococcus* spp., and coagulase-positive *Staphylococcus* along cheese ripening times and seasons (dry and rainy) in traditional Serro Minas cheese, produced in Brazil.

Seasons	Days	Microbial counts				
		Total coliforms ¹	Fecal coliforms ¹	<i>Escherichia coli</i> ¹	<i>Staphylococcus</i> spp. ²	Coagulase positive <i>Staphylococcus</i> ²
Dry	3	3.99 \pm 0.69 ^{Aa}	3.80 \pm 0.84 ^{Aa}	1.99 \pm 0.81 ^{Ba}	6.24 \pm 0.97 ^{Bb}	5.41 \pm 1.46 ^{Aa}
	15	3.71 \pm 0.54 ^{Aa}	2.72 \pm 0.85 ^{Bb}	1.51 \pm 0.71 ^{Aa}	6.03 \pm 0.73 ^{Bb}	4.78 \pm 2.56 ^{Aa}
	30	2.30 \pm 0.76 ^{Bb}	1.84 \pm 0.53 ^{Bc}	1.41 \pm 0.79 ^{Aa}	6.37 \pm 0.61 ^{Ab}	4.10 \pm 2.90 ^{Aa}
	45	2.21 \pm 0.54 ^{Ab}	1.31 \pm 0.62 ^{Ac}	0.98 \pm 0.64 ^{Ab}	6.90 \pm 0.84 ^{Aa}	5.19 \pm 2.85 ^{Aa}
	60	1.39 \pm 0.43 ^{Ac}	1.05 \pm 0.49 ^{Bc}	0.79 \pm 0.28 ^{Ab}	6.95 \pm 0.62 ^{Aa}	1.32 \pm 2.79 ^{Ab}
Rainy	3	4.47 \pm 1.48 ^{Aa}	4.26 \pm 1.46 ^{Aa}	3.49 \pm 1.67 ^{Aa}	8.14 \pm 1.80 ^{Aa}	7.16 \pm 3.11 ^{Aa}
	15	4.06 \pm 0.90 ^{Aa}	3.75 \pm 0.84 ^{Aa}	0.93 \pm 1.06 ^{Ab}	7.16 \pm 0.92 ^{Ab}	7.10 \pm 0.96 ^{Aa}
	30	3.15 \pm 0.82 ^{Ab}	2.72 \pm 0.86 ^{Ab}	2.12 \pm 0.69 ^{Ab}	6.74 \pm 0.54 ^{Ab}	2.37 \pm 3.06 ^{Ab}
	45	2.07 \pm 0.85 ^{Ac}	1.81 \pm 0.94 ^{Ac}	1.72 \pm 0.99 ^{Ac}	6.74 \pm 0.52 ^{Ab}	3.99 \pm 3.48 ^{Ab}
	60	1.95 \pm 0.61 ^{Ac}	1.90 \pm 0.63 ^{Ac}	1.46 \pm 0.54 ^{Ac}	6.75 \pm 0.31 ^{Ab}	1.95 \pm 3.15 ^{Ab}

¹Log MPN/g (MPN, most probable number). Mean \pm SD (n = 10) of determinations of five batches.

²Log cfu/g (cfu, colony forming units). Mean \pm SD (n = 10) of determinations of five batches.

^{a,b,c}Within a column, different superscript lowercase letters denote significant differences (p < 0.05) amongst the different periods of storage for each studied season.

^{A,B}Within a column, different superscript uppercase letters denote significant differences (p < 0.05) amongst the different seasons for each studied period of storage.

with 15, 30 and 60 days of ripening for cheese produced during the rainy season (3.75 Log MPN/g, 2.72 Log MPN/g and 1.90 Log MPN/g, respectively) as compared with the dry season (2.72 Log MPN/g, 1.84 Log MPN/g and 1.05 Log MPN/g, respectively).

During the ripening process, a reduction in the amount of *E. coli* was also detected. The high numbers of coliforms and *E. coli* found at the beginning of ripening are indicative of poor sanitation conditions during milking because they are similar to those found in milk used for the production of other cheeses (Ordóñez and Burgos, 1977; Centeno *et al.*, 1994; Fontán *et al.*, 2001; Borelli *et al.*, 2006; Rosa *et al.*, 2008; Lecitra, 2010). The milk and “pingo” used in the manufacture of the cheese can possess a considerable amount of these microorganisms, as related by other authors who have also analyzed the raw materials used in the production of traditional Minas cheeses (Pinto, 2004; Borelli *et al.*, 2006; Martins, 2006; Lima *et al.*, 2008). The difference in the amount of *E. coli* present between the seasons was only statistically significant for the cheese that ripened for three days (Table 1); a greater number of *E. coli* was counted from the cheese manufactured during the rainy season (3.49 Log MPN/g) than the dry season (1.99 Log MPN/g). When studying the milk used for the manufacture of Serro cheese, Martins (2006) observed counts of *E. coli* slightly higher in the milk sampled during the rainy season. Caridi *et al.* (2003) also observed higher counts of *E. coli* in artisanal cheese manufactured at higher temperatures as those found during the rainy season in Brazil. According to Ferreira (2004), higher temperatures

directly increase the population of microbiota found in cheese, whether it is endogenous, a contaminant or added.

Salmonella spp. were not detected in the 100 samples analyzed. This result is similar to those found by Borelli *et al.* (2006) and Lima *et al.* (2008), who studied other varieties of traditional Minas cheeses.

High counts of *Staphylococcus* spp. were observed during the entire ripening period for the cheeses manufactured during both seasons (Table 1), but the highest counts were found in the cheese manufactured during the rainy season after three and 15 days of ripening (p < 0.05). *Staphylococci* contamination of food is generally associated with its handlers (Carvalho and Serafini, 1996; Normanno *et al.*, 2007). In addition, satisfactory microbiological conditions of raw materials, such as milk or “pingo”, are essential to obtain lower counts of *Staphylococcus* in cheese. High counts of *Staphylococcus* spp. have been found previously in milk (approximately 4.12 Log cfu/mL) and “pingo” (almost 3.60 Log cfu/mL) used in the manufacturing of traditional Minas cheeses (Borelli *et al.*, 2006; Martins, 2006).

In the present study, only after 45 days of ripening the cheeses made during both dry and rainy seasons have counts of coagulate-positive *Staphylococcus* under the limit allowed by Brazilian law (1000 cfu/g) (Table 1). There were no statistically significant differences (p < 0.05) between the seasons regarding the counts of coagulate-positive *Staphylococcus*. One hundred and forty-six isolates that presented a negative phenotype for coagulase and TNase were analyzed by PCR using primers to amplify the gene *Coa*, and four were positive for the presence of the coagulase gene (data not shown). Cremonesi *et al.* (2005)

suggest that the presence of the coagulase gene it is not necessarily associated with its phenotypic expression.

A decrease of almost 2 Log cfu/g in the amount of *S. aureus* was observed throughout the first 30 days of ripening for cheese produced during the rainy season ($p < 0.05$) (Table 2). Only after 15 days of ripening during the rainy season and 45 days during the dry season did the cheese contain an amount of *S. aureus* below the limit of 3 Log cfu/g allowed by Brazilian law. Sixty-eight isolates of *Staphylococcus* spp. that presented a positive phenotype for coagulase and TNase were analyzed by PCR using primers to amplify the gene *SAnuc* in order to confirm their identities as *S. aureus*, and 41 were positive for the presence of this gene. Approximately 60% of the isolates that displayed positive phenotypes for coagulase and TNase were confirmed as *S. aureus*. Some *SAnuc* amplicons determined by PCR are shown in Figure 1. The size of the PCR products obtained from isolates that were positive for the *SAnuc* gene is approximately 500 bp. Forty-nine isolates with phenotypes positive for coagulase and negative for TNase were also tested, among which 13 contained the *SAnuc* gene and were identified as *S. aureus*.

Staphylococcal food poisoning (SFP) has been demonstrated to be caused by the consumption of foods containing enterotoxins produced by staphylococci. Ingestion of food containing from 20 ng to $< 1 \mu\text{g}$ of SEs can cause SFP (Bergdoll, 1989). SEC and TSST-1 were detected in three and five samples, respectively, out of 50 samples from the cheeses manufactured during the dry season, in concentrations $> 0.5 \mu\text{g/g}$ of food. Two samples from the same producer with 30 days of ripening and one sample with 60 days of ripening contained SEC. Although the number of coagulase-positive *Staphylococcus* on the cheese with 60 days was under 3 Log cfu/g, SEC was found in one cheese sample. This enterotoxin was likely formed at the beginning of the ripening period when the quantity of coagulase-positive *Staphylococcus* in the cheese was high. This result suggests that the ripening process is not effective in eliminating staphylococcal enterotoxins from the

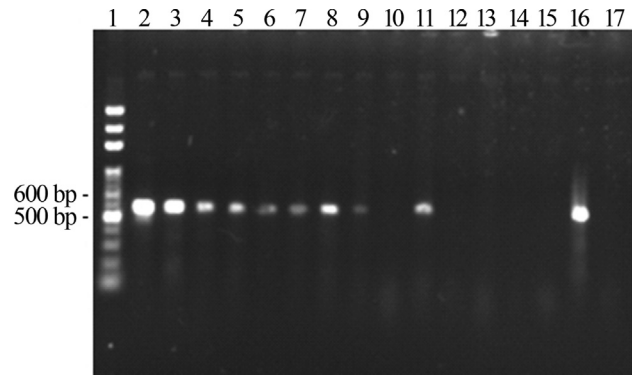


Figure 1 - PCR products of the *SAnuc* gene from *Staphylococcus* spp. strains isolated from the Serro Minas cheese that present positive phenotypes for coagulase and TNase. Lanes: 1: 100 bp DNA ladder; 2: *Staphylococcus aureus* ATCC 29213; 3, 4, 5, 6, 7, 8, 9, 11 and 16: *Staphylococcus* spp. isolates that contain the *SAnuc* gene; 10, 12, 13, 14, 15 and 16: *Staphylococcus* spp. isolates that did not contain the *SAnuc* gene.

cheese. Out of the 50 cheese samples obtained during the rainy season, one sample with 30 days of ripening contained SEB, SEC and TSST-1, and 16 samples, with 3 and 15 days of ripening, contained TSST-1 in concentrations $> 0.5 \mu\text{g/g}$ of food. Rosec *et al.* (1997) and Tamarapu *et al.* (2001) observed that 66% of samples from dairy products were positive for SEC, and it has been recognized as an important cause of SFP associated with the consumption of dairy products. Viana *et al.* (2009) also detected SEB and TSST-1 in samples of “Requeijão do Norte”, a traditional semi-hard cheese produced in Brazil.

During the test for the production of enterotoxins and TSST-1 by the isolates of *Staphylococcus* spp., none of the nine pools analyzed produced the enterotoxins A, B, C and D, although SEB and SEC were detected in one and four cheese samples, respectively. Nevertheless, the isolates of three pools (33.3% of the analyzed pools) produced TSST-1, which is consistent with the results indicating the presence of TSST-1 in the cheese samples. The TSST-1 detections indicate mastitis in cows that produce milk used to manufacture Serro cheese. Borelli *et al.* (2006) analyzed

Table 2 - Counts (log mean cfu/g \pm standard deviation) of *Staphylococcus aureus*, identified based on presence of the gene *Sanuc* along cheese ripening times and seasons (dry and rainy) in traditional Serro Cheese, produced in Brazil.

Seasons	Counts of <i>S. aureus</i> ¹				
	Days of ripening				
	3	15	30	45	60
Dry	5.13 \pm 2.30 ^{Aa}	4.61 \pm 2.48 ^{Aa}	3.03 \pm 3.20 ^{Ab}	4.75 \pm 3.37 ^{Aa}	1.26 \pm 2.66 ^{Ab}
Rainy	5.50 \pm 4.21 ^{Aa}	6.93 \pm 0.98 ^{Aa}	1.78 \pm 2.87 ^{Ab}	1.90 \pm 3.06 ^{Bb}	0.70 \pm 2.20 ^{Ab}

¹Log cfu/g (cfu, colony forming units).

Mean \pm SD (n = 10) of determinations of five batches.

^{a,b,c}Within a line, different superscript lowercase letters denote significant differences ($p < 0.05$) amongst the different periods of storage for each studied season.

^{A,B}Within a column, different superscript uppercase letters denote significant differences ($p < 0.05$) amongst the different seasons for each studied period of storage.

the production of enterotoxins in 19 pools of isolates of *Staphylococcus* spp. from samples of Canastra Minas cheese and detected the production of SEB, SEC and TSST-1 in 78.9%, 52.6% and 31.6% of the pools, respectively. In our study, the detection of TSST-1 was higher in cheeses made during the rainy season after three or 15 days of ripening, when the populations of *S. aureus* were high.

At the beginning of the cheese ripening process, the initial numbers of coliforms, *E. coli* and *Staphylococcus* spp. were very high, possibly because of the lower microbiological conditions of milk; the “pingo” may also have been the cause of high microorganism counts. The cheese produced during the rainy season had higher counts of *E. coli* and *Staphylococcus* spp. than the cheese produced during the dry season in the beginning of ripening, probably because of a higher number of these microorganisms in milk and pingo produced during that season. Therefore, the contamination of Serro cheeses may be caused by the contamination of raw materials used for their manufacturing, and these materials should be carefully controlled for quality to improve this traditional cheese.

Despite the finding that the investigated strains of *Staphylococcus* spp. isolated from Serro cheese did not produce enterotoxins, SEB and SEC were found in 4% of the analyzed cheese samples. Therefore, even though the cheese after at least 45 days of ripening meets the standards established by Brazilian law for coliforms and coagulase-positive *Staphylococcus*, the presence of SEC in the cheese after 60 days indicates a potential risk for the consumer's health.

Conclusions

The ripening process has marked effects on the microbiological parameters of traditional Minas Serro cheese. It was effective in reducing the contamination detected by the most important microbiological indicators for contamination of cheese according to Brazilian law, but ripening was not effective in eliminating staphylococcal enterotoxins from the cheese. Once formed, possibly when the counts of coagulase-positive *Staphylococcus* in the cheese were high, these enterotoxins remained in the cheese even after 60 days of ripening. The high pathogen and coliform levels at the beginning of the ripening process for the cheese produced during both seasons indicates the need for improvement of the sanitation of the manufacturing conditions. Also the high TSST-1 levels at the beginning of the ripening process for the cheese produced during rainy season indicates the need for improvement of the healthiness of herd.

Acknowledgments

We wish to thank the Cooperativa dos Produtores Rurais do Serro for taking samples and enabling the realization of this study. We also like to thank Fundação Ezequiel

Dias for the contribution on the performance of enterotoxin analyses. Financial support was received from Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

- Aarestrup FM, Dangler CA, Sordillo LM (1995) Prevalence of coagulase gene polymorphism in *Staphylococcus aureus* isolates causing bovine mastitis. *Can J Vet Res* 59:124-128.
- Baron F, Cochet MO, Pellerin JL, Zakour NB, Lebon A, Navarro A, Proudly I, Le Loir Y, Gautier M (2004) Development of a PCR test to differentiate between *Staphylococcus aureus* and *Staphylococcus intermedius*. *J Food Prot* 67:2302-2305.
- Bergdoll M S (1989) *Staphylococcus aureus*. In: Doyle, M. P. (Ed.). *Foodborne bacterial pathogens*. Marcel Dekker, New York, USA, 463-523.
- Borelli BM, Ferreira EG, Lacerda ICA, Santos DA, Carmo LS, Dias RS, Silva MCC, Rosa CA (2006) Enterotoxigenic *Staphylococcus* spp. and other microbial contaminants during production of Canastra cheese, Brazil. *Braz J Microbiol* 37:545-550.
- Brasil (1996) Regulamentos técnicos de identidade e qualidade dos produtos lácteos. Portaria no. 146, de 7 de março de 1996. Brasília: Diário Oficial da República Federativa do Brasil.
- Brasil (2000) Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Resolução no. 07, de 28 de novembro de 2000. Anexo I: Critérios de Funcionamento e de Controle da Produção de Queijarias, para Seu Relacionamento Junto ao Serviço de Inspeção Federal. Brasília: Diário Oficial da República Federativa do Brasil.
- Caridi A, Micari P, Caparra P, Cufari A, Sarullo V (2003) Ripening and seasonal changes in microbial groups and in physico-chemical properties of the ewes' cheese Pecorino del Poro. *Int Dairy J* 13:191-200.
- Carmo L S (2001) Produção e Purificação em grande escala das enterotoxinas estafilocócicas SEA, SEB, SEC2, SED e toxina TSST-1 para uso em ensaios imuno-enzimáticos. Belo Horizonte, MG, Brasil, 112 p (Thesis. Departamento de Microbiologia. UFMG).
- Carvalho CO, Serafini AB (1996) Grupos de microrganismos isolados de orofaringe, nasofaringe e das mãos de trabalhadores do restaurante da Universidade Federal de Goiás. *Hig Alim* 10:19-24.
- Centeno JA, Rodriguez-Otero JL, Cepeda A (1994) Microbiological study of Arzúa cheese (NW Spain) throughout cheesemaking and ripening. *J Food Safety* 14:229-241.
- Cremonesi P, Luzzana M, Brasca M, Morandi S, Lodi R, Vimercati C, Agnellini D, Caramenti G, Moroni P, Castiglioni B (2005) Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. *Mol Cel Probes* 19:299-305.
- Downes FP, Ito K (2001) *Compendium of Methods for the Microbiological Examination of Foods*. American Public Health Association, Washington, DC.
- Ferreira CLLF (2004) Fatores que afetam o crescimento de microrganismos em queijo. *Rev Leite Deriv* 76:91-96.

- Fontán MCG, Franco I, Prieto B, Tornadijo ME, Carballo J (2001) Microbiological changes in “San Simón” cheese throughout ripening and its relationship with physico-chemical parameters. *Food Microbiol* 18:25-33.
- Furtado MM, Mosquim MCAV, Fernandes AR, da Silva CAB (2003). Produção de queijo minas curado e meia-cura. *Projetos de Empreendimentos Agroindustriais: Produtos de Origem Animal*. Editora da Universidade Federal de Viçosa, Viçosa, MG.
- Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn Jr WC (2001) *Diagnóstico Microbiológico*. Texto e Atlas colorido. Editora Médica e Científica Ltda, Rio de Janeiro, RJ.
- Lacerda ICA, Gomes FCO, Borelli BM, Faria Jr. CLL, Franco GR, Mourão MM, Morais P B, Rosa CA (2011) Identification of the bacterial community responsible for traditional fermentation during sour cassava starch, cachaça and Minas cheese production using culture independent 16S rRNA gene sequence analysis. *Braz J Microbiol* 42:650-657.
- Leite RL, Carvalho EP, Moura CJ, Teixeira LAM, Pereira ML (2001) Estudo das condições higiênico-sanitárias de queijos “Minas frescal” e “Minas Padrão” elaborados a partir de leite proveniente de vacas com mastite sub-clínica. *Rev Inst Latic “Cândido Tostes”* 56:3-11.
- Leloir Y, Baron F, Gautier M (2003) *Staphylococcus aureus* and food poisoning. *Genet Mol Res* 2:63-79.
- Lecitra G (2010) World wide traditional cheeses: Banned for business? *Dairy Sci Technol* 90:357-374.
- Lima CDC, Cerqueira MMOP, Ferreira EG, Faria Jr CLL, Nelson DL, Carmo LS, Rosa CA (2008) Microbiological, physico-chemical and sensory evaluation of a traditional Brazilian cheese during the ripening process. *World J Microbiol Biotechnol* 24:2389-2395.
- Martins JM (2006) Características físico-químicas e microbiológicas durante a maturação do queijo Minas artesanal da região do Serro. Viçosa, MG, Brasil, 87 p. (Thesis, Departamento de Ciência e Tecnologia de Alimentos. UFV).
- Normanno G, La Salandra G, Dambrosio A, Quaglia NC, Corrente M, Parisi A, Santagada G, Firinu A, Crisetti E, Celano GV (2007) Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int J Food Microbiol* 115:290-296.
- Nunez M, Gaya P, Medina M (1985) Influence of manufacturing and ripening conditions on the survival of *Enterobacteriaceae* in Manchego cheese. *J. Dairy Sci.* 68:794-800.
- Ordóñez, J. A.; Burgos, J. (1977). Étude de La variété de fromage “Ulloa”. *Le Lait* 57:150-153.
- Pinto MS (2004) Diagnóstico socioeconômico, cultural e avaliação dos parâmetros físico-químicos e microbiológicos do queijo minas artesanal do Serro. Viçosa, MG, Brasil, 123 p. (M.Sc. Dissertation. Departamento de Ciência e Tecnologia de Alimentos. UFV).
- Robbins R, Gould S, Bergdoll MS (1974) Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl Microbiol* 28:946-950.
- Rosa TD, Wassermann GE, Souza CFV, Caron D, Carlini CG, Ayub MAZ (2008) Microbiological and physicochemical characteristics and aminopeptidase activities during ripening of Serrano cheese. *Int J Dairy Technol* 61:70-79.
- Rosec JP, Guiraud JP, Dalet C, Richard N (1997) Enterotoxin production by staphylococci isolated from foods in France. *Int J Food Microbiol* 35:213-221.
- Tamarapu S, Mckillip JL, Drake M (2001) Development of a multiplex polymerase chain reaction assay for detection and differentiation of *Staphylococcus aureus* in dairy products. *J Food Prot* 64:664-668.
- Viana FR, Oliveira AL, Carmo LS, Rosa CA (2009) Occurrence of coagulase-positive *Staphylococci*, microbial indicators and physical-chemical characteristics of traditional semihard cheese produced in Brazil. *Int J Dairy Technol* 62:372-377.