Lactic acid microbiota identification in water, raw milk, endogenous starter culture, and fresh Minas artisanal cheese from the Campo das Vertentes region of Brazil during the dry and rainy seasons

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ABSTRACT

Minas artisanal cheese, produced in the Campo das Vertentes region of Brazil, is made from raw milk and endogenous starter cultures. Although this cheese is of great historical and socioeconomic importance, little information is available about its microbiological and physical-chemical qualities, or about its beneficial microbiota. This work was aimed at evaluating the qualities of the cheese and the components used for its production, comparing samples collected during the dry and rainy seasons. We also conducted molecular identification and isolated 50 samples of lactic acid bacteria from cheese ($n = 21$), water ($n = 3$), raw milk ($n = 9$), and endogenous starter culture ($n = 17$). The microbiological quality of the cheese, water, raw milk, and endogenous starter culture was lower during the rainy period, given the higher counts of coagulase-positive *Staphylococcus* and total and thermotolerant coliforms. *Enterococcus faecalis* was the lactic acid bacteria isolated most frequently (42.86%) in cheese samples, followed by *Lactococcus lactis* (28.57%) and *Lactobacillus plantarum* (14.29%). *Lactobacillus brevis* (5.88%), *Enterococcus pseudoavium* (5.88%), *Enterococcus durans* (5.88%), and *Aerococcus viridans* (5.88%) were isolated from endogenous starter cultures and are described for the first time in the literature. The lactic acid bacteria identified in the analyzed cheeses may inhibit undesirable microbiota and contribute to the safety and flavor of the cheese, but this needs to be evaluated in future research.

Key words: artisanal cheese, 16S rRNA sequencing, cheese composition, microbiology

INTRODUCTION

The state of Minas Gerais is the greatest milk producer in Brazil, producing about 8.7 billion L per year, or 27.1% of the national total (Instituto Brasileiro de Geografia e Estatística, 2011). Minas Gerais is also Brazil's largest producer of cheese, including the Minas artisanal variety, which is recognized throughout the country (Martins et al., 2015). Minas artisanal cheesemaking has been designated as an intangible cultural heritage (Instituto Do Patrimônio Histórico e Artístico Nacional, 2008), and Minas cheeses are produced only on farms from certified regions using raw milk, obtained from zoonoses-free herds, with added endogenous starter cultures and clotting agents, under good manufacturing practices. The endogenous starter culture comes from the whey drained from previously made cheeses, which is collected and used for cheesemaking on the next day.

Some aspects of Minas artisanal cheese production—including turning cheeses during brining and ripening, as well as rinsing—are critical to its quality but mean that the product is frequently manipulated. These steps must be carried out with caution to avoid bacterial contamination. Moreover, cheeses must be ripened to reach quality standards that make them safe to consume, but the majority of Minas artisanal cheeses are sold fresh and in some cases produced without starter endogenous cultures.

Lactic acid bacteria (**LAB**), originating mainly from raw milk and endogenous starter cultures, are essential to the ripening and the safety of Minas artisanal cheese. They synthesize many antimicrobial compounds, including organic acids (mainly lactic acid), hydrogen peroxide, and bacteriocins, which may inhibit spoiling and the growth of pathogenic bacteria. They also contribute to cheese flavor and may play a probiotic role.

In 2009, the Campo das Vertentes region of Minas Gerais received official identification as a Minas artisanal cheese producer (Minas Gerais, 2009), taking

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into consideration edaphoclimatic, agroecological, and sociocultural studies. Since then, a growing number of producers have sought certification of their cheeses from the state inspection service.

Although there is a long tradition of Minas artisanal cheese production from the Campo das Vertentes region, little information is available about the microbiological and physical-chemical qualities of the cheeses; the raw milk and endogenous starter cultures; or the LAB present in the cheeses. The present work was aimed at evaluating the qualities of fresh Minas artisanal cheese from Campo das Vertentes, comparing samples collected in the dry and rainy seasons. The study also aimed at identifying the LAB present in samples of cheese, water, raw milk, and endogenous starter culture, using molecular approaches.

MATERIALS AND METHODS

Sampling

We selected 6 Minas artisanal cheese producers who were not certified by the state inspection service from Campo das Vertentes because they used endogenous starter culture in their cheesemaking. We collected fresh cheeses sampled on the day after production, raw milk, endogenous starter culture, and water used for cheesemaking at the farms in December 2013 and April 2014 (rainy season) and in July 2014 (dry season). We collected 12 samples of cheeses and their components during the experimental period.

Microbiological Analyses

All microbiological analyses were carried out following the recommendations of American Public Health Association (Andrews et al., 2001; Kornacki and Johnson, 2001; Lancette and Tatini, 2001; Morton, 2001), except for mold and yeast counts, which were performed according to the *Bacteriological Analytical Manual* (Tournas et al., 2001).

Water samples were analyzed for the most probable number (MPN) of total and thermotolerant coliforms at 30°C and 45°C, respectively (American Public Health Association, 2005), and for aerobic mesophilic microorganism counts (Morton, 2001). Samples of raw milk, endogenous starter culture, and fresh cheese were analyzed for the MPN of total and thermotolerant coliforms (Kornacki and Johnson, 2001); counts of *Staphylococcus* spp., coagulase-positive *Staphylococcus* (Lancette and Tatini, 2001), and molds and yeasts (Tournas et al., 2001); and the presence of *Salmonella* spp. (Andrews et al., 2001).

Physicochemical Analyses

Water samples were analyzed in triplicate for hardness, pH, and residual chlorine (American Public Health Association, 2005).

Raw milk samples were analyzed for percentage of fat, protein, lactose, and total solids using the Bentley CombiSystem 2300 (Bentley Instruments Inc., Chaska, MN) and based on the absorption of infrared wavelengths (IDF, 2000). Using the same equipment, SCC was determined by flow cytometry (IDF, 1995). Total bacterial count was also determined by flow cytometry (Suhren and Walte, 2000) using the BactoCount IBC (Bentley Instruments Inc.).

Fresh cheese samples were analyzed for percentage of total solids and moisture using the gravimetry method (IDF, 1982); fat content using the Gerber-Van Gulik butyrometer method (IDF, 2008); nitrogen compounds using the Kjeldahl method (IDF, 1993); pH (British Standards, 1976); and titratable acidity (Merck, 1993). Total protein content was obtained by multiplying total nitrogen by 6.38. All physical-chemical analyses were carried out in triplicate.

Molecular Identification of Lactic Acid Bacteria

The molecular identification of LAB was carried out from material obtained from all kinds of collected samples. Decimal dilutions were prepared, and 0.1 mL of each sample was spread on the surface of MRS and M17 media (both from Difco Laboratories Inc., Detroit, MI). Plates were incubated under aerobiosis at 37°C for 48 h (IDF, 1988; Resende et al., 2011). Isolation, enumeration, gram staining, catalase testing, and purification of LAB were performed according to MacFaddin (1980) and IDF (1988). After growth, colonies were enumerated and distinct gram-positive and catalase-negative morphologic types were submitted to molecular identification.

For DNA extraction, the selected bacteria were grown in the same isolation broth under the same conditions of time and temperature, followed by centrifugation for 15 min at $4,000 \times g$ at room temperature. Pellets were treated with 5 M lithium chloride and incubated at room temperature under agitation for 90 min. Then, they were centrifuged and suspended in 1 mL of a protoplasts buffer (50 mM Tris HCl; pH 8.0, 10 mM EDTA, 10 mg/mL of lysozyme, and 25 mM sucrose) and incubated at 37°C under agitation for 1 h (Sandes et al., 2014). The DNA was obtained using the Kit Wizard SV Genomic DNA Purification System (Promega Corp., Madison, WI), according to manufacturer instructions.

The DNA samples were submitted to PCR using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), as described by Lane (1991). Amplification was performed as follows, according to Moreira et al. (2005): 1 cycle of 95°C for 2 min; 35 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min; and 1 last cycle of 72°C for 5 min. The obtained PCR products were sequenced using the Sanger method with the MegaBACE 1000 automated sequencer (GE Healthcare, Piscataway, NJ), according to Reysenbach et al. (2000). Results were submitted to the BLAST algorithm on the National Center of Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

We differentiated between *Lactobacillus* (*Lb.*) *plantarum*, *Lb. paraplantarum*, and *Lb. pentosus* using multiplex-PCR with specific primers targeted at the amplification of the *recA* gene, according to Torriani et al. (2001). We differentiated between *Lb. casei*, *Lb. paracasei*, and *Lb. rhamnosus* using PCR with primers specific to each of the species and targeting the V1 region of the *16S* rRNA gene, as described by Ward and Timmins (1999).

Statistical Analyses

All experimental data are reported as means, CV, and minimum and maximum values. Normality was confirmed using the Shapiro Wilk method. All statistical analyses were processed using the 2008 version of InfoStat/Professional (Grupo InfoStat, Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Córdoba, Argentina). Nonparametric variables were analyzed using the Friedman test. Parametric variables were analyzed using ANOVA, and significance was assessed using Fisher's least significant difference (LSD) test. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Microbiological and Physicochemical Quality of Water

Water collected from cheesemaking facilities during the dry and rainy seasons did not reach the potability standard of the absence of total and thermotolerant coliforms (WHO, 2011a). It was considered inadequate for cheesemaking (Table 1). The mean count of mesophilic aerobic microorganisms in the dry season was lower than in the rainy season ($P < 0.05$; Table 1). Contamination in the latter may have been associated with higher temperatures and humidity (Instituto Nacional de Meteorologia,

Table 1. Microbiological and physicochemical analyses of water collected at Minas artisanal cheesemaking facilities in Campo das Vertentes, Minas Gerais, Brazil, during dry and rainy seasons

Item ¹	Dry				Rainy			
	Mean	CV	Minimum	Maximum	Mean	CV	Minimum	Maximum
Total coliforms (MPN/100 mL)	43	227.66	<3	240	186	240.99	<3	>1,100
Thermotolerant coliforms (MPN/100 mL)	4	62.76	<3	9.2	186	240.99	<3	>1,100
Aerobic mesophilic (cfu/mL)	6 × 10 ^{1b}	173.75	<1	2.6 × 10 ²	5.3 × 10 ^{2a}	209.41	<1	2.8 × 10 ³
LAB MRS (cfu/mL)	3 × 10 ¹	147.57	<1 × 10 ¹	1.2 × 10 ²	1.7 × 10 ¹	97.98	<1 × 10 ¹	5 × 10 ¹
LAB M17 (cfu/mL)	8.2 × 10 ²	194.21	<1 × 10 ¹	4 × 10 ³	1.5 × 10 ²	228.06	<1 × 10 ¹	8.2 × 10 ²
pH	7.06 ^a	2.57	6.82	7.28	6.05 ^b	10.13	5.3	6.76
Hardness (mg/L)	12.78	84.38	1.5	28.6	7.83	65.8	4	18
Residual chlorine (μL/L)	4.43 ^a	65.53	0.71	6.74	2.66 ^b	72.91	0.35	5.32

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹MPN = most probable number; LAB MRS = count of lactic acid bacteria on de Man, Rogosa, and Sharpe; LAB M17 = count of lactic acid bacteria on M17 agar.

2013a,b, 2014a,b) data. Such climatic factors enhance the growth of bacteria, even in water samples.

Water pH mean values in the rainy and dry seasons were different, at 6.05 and 7.06 (Table 1), respectively ($P < 0.05$), but both were adequate for cheesemaking, which requires a pH of 6.0 to 9.5 (Brasil, 2012). These findings favored the efficiency of the cleaning and disinfection procedures at the cheesemaking facilities, and may contribute to a longer lifespan for the cheesemaking equipment.

The Brazilian Ministry of Health has determined that the water for human consumption must have a maximum hardness of 500 mg/L of calcium carbonate (Brasil, 2012). In both seasons, water quality met that standard (Table 1); in fact, water samples collected in both seasons were very soft (WHO, 2011b).

Residual chlorine concentration in water samples was higher in the dry season than in the rainy season ($P < 0.05$; Table 1). This difference may have been because most farmers chlorinated water manually directly at the reservoir without taking water flow rate into account. Adding the same amount of chlorine regardless of season would increase the chlorine concentration in the dry season because water flow rates are lower than in the rainy season. High chlorine concentrations in the dry season are undesirable in food processing, causing abnormal flavors and leading to possible consumer health concerns because of the toxic effects of excessive chlorine intake.

On the other hand, the minimum values of chlorine recorded in water samples in the dry and rainy seasons, 0.75 and 0.35 ppm (Table 1), respectively, highlighted the inefficiency of chlorination in some of the studied farms and raised concerns about cleaning and disinfection procedures (WHO, 2011a), possibly compromising cheese quality.

Microbiological Quality of Endogenous Starter Cultures

Endogenous starter cultures showed high counts of all microorganisms of interest, in line with results from previously published papers (Borelli et al., 2006; Lima et al., 2009; Dores et al., 2013). Endogenous starter cultures with high MPN of coliforms and high counts of *Staphylococcus* spp., molds, and yeasts (Table 2) represent a source of contamination. On the other hand, high LAB counts may play an intrinsic protective role against the undesirable microorganisms, as well as contributing to the sensory profile of the cheese.

The higher mean MPN of thermotolerant coliforms in endogenous starter cultures samples during the rainy season ($P < 0.05$; Table 2) may be explained by climatic conditions, which were favorable to microbial

Table 2. Microbiological analyses of endogenous starter culture collected at Minas artisanal cheesemaking facilities in Campo das Vertentes, Minas Gerais, Brazil, during dry and rainy seasons

Item ¹	Dry				Rainy			
	Mean	CV	Minimum	Maximum	Mean	CV	Minimum	Maximum
Total coliforms (MPN/mL)	742	75.95	120	1,500	6,766	71.79	93	>11,000
Thermotolerant coliforms (MPN/mL)	3.2 ^b	9.68	<3	3.6	2,854 ^a	145.9	93	>11,000
Coagulase-positive <i>Staphylococcus</i> (cfu/mL)	<1 × 10 ^{3b}	0	<1 × 10 ³	<1 × 10 ³	2.4 × 10 ^{5a}	182.47	<1 × 10 ³	1.1 × 10 ⁶
<i>Staphylococcus</i> spp. (cfu/mL)	3.5 × 10 ⁴	109.18	<1 × 10 ³	1 × 10 ⁵	2.1 × 10 ⁶	135.54	<1 × 10 ³	7.1 × 10 ⁶
Molds and yeasts (cfu/mL)	9.6 × 10 ⁵	69.03	<1 × 10 ²	1.5 × 10 ⁶	5.1 × 10 ⁵	207.5	<1 × 10 ²	2.6 × 10 ⁶
LAB MRS (cfu/mL)	3.6 × 10 ⁷	147.96	3.3 × 10 ⁵	1.4 × 10 ⁸	9 × 10 ⁷	139.88	1.5 × 10 ⁶	2.6 × 10 ⁸
LAB M17 (cfu/mL)	6.5 × 10 ⁷	136.49	3.3 × 10 ⁵	2.2 × 10 ⁸	2.3 × 10 ⁸	136.77	1.1 × 10 ⁶	7.2 × 10 ⁸
<i>Salmonella</i> spp. (presence/25 mL)	—	—	—	—	—	—	—	—

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹MPN = most probable number; LAB MRS = count of lactic acid bacteria on de Man, Rogosa, and Sharpe; LAB M17 = count of lactic acid bacteria on M17 agar.

growth. Endogenous starter cultures are kept without cooling until they are used in the next day's cheese-making. Deficiencies in the cleaning and disinfection of cheesemaking utensils may also have played a role. Once they are present in high numbers, as seen in Table 2, these bacteria quickly cause the food to deteriorate along with the presence of gases, as lactose is metabolized to carbon dioxide in the fermentation process. Furthermore, some microorganisms in this group have pathogenic potential (Jay et al., 2005).

We observed no differences in MPN of total coliforms during the dry and rainy seasons (Table 2). Borelli et al. (2006) have reported that the MPN of total coliforms varied from 93 to >1,100/mL in endogenous starter cultures of Minas artisanal cheeses produced in the Serra da Canastra region.

We observed higher counts of coagulase-positive *Staphylococcus* in endogenous starter cultures collected in the rainy season ($P < 0.05$; Table 2). This may be for reasons similar to those for the high MPN of coliform bacteria described above, associated with the manipulation of endogenous starter culture, because 30 to 50% of humans are asymptomatic carriers of coagulase-positive *Staphylococcus* (Stamford et al., 2006). Another factor may have been the high incidence of mastitis in Brazilian dairy herds (Costa et al., 2012).

Counts of molds and yeasts in endogenous starter cultures from the present study were similar to those recorded using the same materials and methods for Minas artisanal cheese production in Serra do Salitre; values ranged from 3×10^1 to 2.7×10^5 cfu/mL (Lima et al., 2009).

Microbiological and Physicochemical Quality of Raw Milk

Milk fat percent content may vary from 2 to 4 percentage points. Factors such as genetics, season, ratio of roughage to concentrate, fiber intake, type of concentrate fed, and fat and additive feeding may influence fat content (Lerch et al., 2015). The lower mean fat content recorded in samples of raw milk collected during the dry season ($P < 0.05$; Table 3) may be explained by cows' higher concentrate intake because roughage is less available than in the rainy season. A diet higher in concentrate increases acid production in the rumen, lowering the pH and compromising fiber digestion, so that the proportion of acetic acid is decreased and the proportion of propionic acid is increased. Acetic acid is a main precursor for milk fat, so its decrease is directly related to lower fat content in milk (Guo et al., 2013).

Protein and lactose content in milk are less influenced by extrinsic factors (Wattiaux, 2012), and we also observed an absence of variation in these param-

Table 3. Microbiological and physicochemical analyses of raw milk collected at Minas artisanal cheesemaking facilities in Campo das Vertentes, Minas Gerais, Brazil, during dry and rainy seasons

Item ¹	Dry				Rainy			
	Mean	CV	Minimum	Maximum	Mean	CV	Minimum	Maximum
Total coliforms (MPN/mL)	61 ^b	145.91	<3	240	3,742 ^a	150.29	23	>11,000
Thermotolerant coliforms (MPN/mL)	9 ^b	136.66	<3	35	81 ^a	181.8	<3	380
Coagulase-positive <i>Staphylococcus</i> (cfu/mL)	7.8×10^3	123.99	$<1 \times 10^3$	2.5×10^4	7.1×10^3	208.13	$<1 \times 10^3$	3.7×10^4
<i>Staphylococcus</i> spp. (cfu/mL)	2.5×10^4	195.77	$<1 \times 10^3$	1.3×10^5	3.9×10^5	198.9	$<1 \times 10^3$	2×10^6
Molds and yeasts (cfu/mL)	5.5×10^5	229.53	$<1 \times 10^2$	3.1×10^6	1.6×10^4	184.01	$<1 \times 10^2$	7.3×10^4
LAB MRS (cfu/mL)	4.4×10^6	244.64	$<1 \times 10^3$	2.6×10^7	4×10^6	192.32	1.7×10^4	2×10^7
LAB M17 (cfu/mL)	3.4×10^6	240.73	$<1 \times 10^3$	2×10^7	7.3×10^6	219.65	1.8×10^4	4×10^7
<i>Salmonella</i> spp. (presence/25 mL)	—	—	—	—	—	—	—	—
SCC (cells/mL)	2.4×10^5	55.22	1.8×10^4	3.9×10^5	2.5×10^5	58.81	5×10^4	4.8×10^5
Total bacterial count (cfu/mL)	9.7×10^5	234.4	6×10^3	5.6×10^6	3.3×10^6	84.89	2×10^3	5.6×10^6
Lactose (%)	4.56	2.98	4.38	4.79	4.46	3.36	4.27	4.72
Protein (%)	3.37	10.11	3.05	3.94	3.51	8	3.3	4.03
Fat (%)	2.96 ^b	34.3	0.95	3.79	4.44 ^a	33.72	2.01	6.49
Total solids (%)	12.37	13.09	10.25	14.99	13.41	10.58	11.09	15.2

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹MPN = most probable number; LAB MRS = count of lactic acid bacteria on de Man, Rogosa, and Sharpe; LAB M17 = count of lactic acid bacteria on M17 agar.

eters, based on mean values from the different seasons and a low CV (Table 3). Milk TS content is normally influenced by the factors that alter fat content, and we observed this trend (Table 3): TS were numerically higher in the dry season, but this difference was not significant.

Another parameter that influences milk quality for cheesemaking is total bacterial count. We did not observe a statistical difference between seasons for milk total bacterial count. Souza et al. (2003) also observed no seasonal difference in mean total bacterial count of the raw milk used for Serrano artisanal cheesemaking. The mean total bacterial count was 1.2×10^6 cfu/mL in the summer and 5×10^5 cfu/mL in the winter.

We observed higher counts of total and thermotolerant coliforms and high CV in raw milk sampled during the rainy season ($P < 0.05$; Table 3), similar to what was described by Martins et al. (2015) in the Serro region. These findings were likely caused by environmental conditions that favored the growth of that group of bacteria. Inadequate cleaning and disinfection of the milking equipment and of the environment in which the animals were raised may also have been a factor. As well, contamination during and after milking, during milk transportation to the cheesemaking facility, and inside the facility, may have increased coliform MPN in raw milk. Environmental mastitis in dairy cattle also could explain the increase in coliform MPN, which are generally not associated with increased SCC in bulk tank milk, as observed in the present study (Table 3).

We observed the application of measures to reduce the presence of *Staphylococcus* spp. in milk at some of the farms, such as pre- and postdipping of the teat, milking management (order of animals for milking), dry cow therapy, and culling of chronically infected animals, but *Staphylococcus* counts were high in both seasons. It is evident that another reservoir of the bacterium existed in the milking production system, allowing for new infections, such as the cow itself (skin and teat lesions), the milkers (contaminated hands), or the milking equipment (biofilms).

Counts of LAB in raw milk were high in both seasons (Table 3). This is desirable for Minas artisanal cheesemaking, because LAB are responsible for flavor, and likely for inhibiting pathogens. In a study of raw milk used for production of Zlatař, an artisanal Slovakian cheese, LAB reached mean values similar to those recorded in the present study (Veljovic et al., 2007).

Counts of molds and yeasts in the present study were opposite to those observed in raw milk used for Casin artisanal cheese from the Asturias region of Spain; in that study, no molds and yeasts were found (Alegría et al., 2009).

Microbiological and Physicochemical Quality of Fresh Minas Artisanal Cheese

Mean total and thermotolerant coliform counts were higher in cheeses collected in the rainy season than in the dry season ($P < 0.05$; Table 4). This result was expected, because higher counts of those bacteria were found in both raw milk and endogenous starter cultures during the rainy season (Tables 2 and 3). High MPN of total coliforms are responsible for cheese deterioration, causing early blowing. Thermotolerant coliforms cause the cheese to deteriorate and include pathogenic samples that are harmful to humans and other animals (Jay et al., 2005).

The mean count of coagulase-positive *Staphylococcus* was higher in cheeses collected during the rainy season than in those collected during the dry season ($P < 0.05$; Table 4). Most of that difference may be attributed to the use of an endogenous starter culture that was contaminated with the same microorganism in the same season ($P < 0.05$; Table 2). The presence of this bacterium in counts higher than 10^4 cfu/g in all analyzed cheeses from both seasons is alarming; it may produce enterotoxins and cause food poisoning (Jöhler et al., 2015).

The mean count for LAB on M17 agar was higher in cheeses sampled in the dry season than in the rainy season ($P < 0.05$; Table 4). This may have been because a higher amount of starter endogenous culture was used for cheesemaking in the dry season. Most cheese producers report using this procedure to maintain the milk clotting process when room temperature is lower. Another explanation could be the lower coliform bacteria counts for the same period ($P < 0.05$; Table 4); coliform bacteria can ferment lactose and compete with LAB for nutrients.

The high mean counts of molds and yeasts recorded in cheeses (Table 4) point to inadequate disinfection of the utensils used for cheesemaking and the use of contaminated raw material. These microorganisms are undesirable because they may synthesize mycotoxins, which may be involved in food poisoning (Jay et al., 2005).

We detected no *Salmonella* spp. in the analyzed cheeses. The absence of that bacterium in the raw materials used for cheesemaking (Tables 2 and 3), care in cheese processing and handling, and preventive methods used to avoid cross-contamination, as well as possible inhibition by LAB, may explain these results.

We were unable to verify any statistical difference in mean moisture content and TS in the cheeses sampled in either season. This was because of the short time cheeses stayed on the farms under the environmental

Table 4. Microbiological and physical-chemical analyses of fresh cheese collected at Minas artisanal cheesemaking facilities in Campo das Vertentes, Minas Gerais, Brazil, during dry and rainy seasons

Item ¹	Dry				Rainy			
	Mean	CV	Minimum	Maximum	Mean	CV	Minimum	Maximum
Total coliforms (MPN/g)	2,203 ^b	196.19	120	> 11,000	9,417 ^a	41.19	1,500	> 11,000
Thermotolerant coliforms (MPN/g)	264 ^b	160.98	<3	1,100	5,645 ^a	103.92	92	> 11,000
Coagulase-positive <i>Staphylococcus</i> (cfu/g)	3.3 × 10 ^{4b}	159.29	<1 × 10 ³	1.3 × 10 ⁵	2 × 10 ^{7a}	154.68	<1 × 10 ³	7.2 × 10 ⁷
<i>Staphylococcus</i> spp. (cfu/g)	1.1 × 10 ⁶	135.1	4.7 × 10 ⁴	3.9 × 10 ⁶	3.5 × 10 ⁷	125.1	4.8 × 10 ⁴	1.1 × 10 ⁸
Molds and yeasts (cfu/g)	1.1 × 10 ^{7a}	71.08	1.3 × 10 ⁵	2.1 × 10 ⁷	1.4 × 10 ^{6b}	239.92	<1 × 10 ³	8.4 × 10 ⁶
LAB MRS (cfu/g)	5.1 × 10 ⁸	138.17	1.6 × 10 ⁶	1.7 × 10 ⁹	3.2 × 10 ⁸	137.35	5.1 × 10 ⁷	1.1 × 10 ⁹
LAB M17 (cfu/g)	6.4 × 10 ^{8a}	108.29	1 × 10 ⁷	1.6 × 10 ⁹	5.4 × 10 ^{7b}	145.63	6.1 × 10 ⁶	2 × 10 ⁸
<i>Salmonella</i> spp. (presence/25 g)	—	—	—	—	—	—	—	—
Moisture (%)	55.97	10	45.04	60.7	49.35	10.61	42.08	56.16
Total solids (%)	44.03	12.71	39.3	54.96	50.65	10.34	43.84	57.92
Protein (%)	17.54	15.03	14.06	20.35	19.86	13.17	16.52	24.58
Fat (%)	28.28	18.4	20.67	37	29.06	26.74	17.33	40
Titratable acidity (% of lactic acid)	0.15 ^a	47.04	0.04	0.24	0.08 ^b	71.46	0.03	0.17
pH	5.53	8.75	5.13	6.48	5.44	7.57	4.84	5.86

^{a,b}Means in the same row with different superscripts differ ($P < 0.05$).

¹MPN = most probable number; LAB MRS = count of lactic acid bacteria on de Man, Rogosa, and Sharpe; LAB M17 = count of lactic acid bacteria on M17 agar.

conditions: samples were collected on the day after production. Season did not influence moisture content, demonstrating that cheesemaking was performed in a similar way on the different farms, even in different seasons. This finding may be corroborated by low CV values for moisture reported in the dry and rainy seasons (Table 4).

Protein content results similar to those of the present study have been reported for Minas artisanal cheeses produced in the Serro, Serra da Canastra, and Cerrado regions, showing mean values of 14.08, 18.51, and 14.55%, respectively (Oliveira et al., 2013). In the present study, the mean milk fat percentage was higher in raw milk sampled during the rainy season ($P < 0.05$; Table 2), but the mean milk fat percentage was not statistically different in cheeses in the different seasons (Table 4). Cheese fat content depends on milk fat content, but it may be influenced by other factors, such as the moisture content of the cheese, the time of aging, and the presence of lipolytic microorganisms.

The mean titratable acidity of the cheeses produced in the dry season was higher than those recorded in the rainy season ($P < 0.05$; Table 4), similar to results described by Martins et al. (2015). This finding may be explained by the higher LAB counts in the dry season ($P < 0.05$). During lactose formation, LAB produce lactic acid as a major metabolic compound. The higher titratable acidity in cheese partially justifies the lower counts ($P < 0.05$) of coagulase-positive *Staphylococcus* and total and thermotolerant coliforms, as well as the absence of *Salmonella* spp. in the cheese samples from the dry season. Higher acidity favors the development of molds and yeasts (Jay et al., 2005), and these occurred in higher mean counts in samples from the dry season ($P < 0.05$; Table 4). However, we did not observe statistical differences between the mean pH values of the analyzed cheeses (Table 4), corroborating the lack of direct relation between titratable acidity and pH (Scott, 2002).

Molecular Identification of LAB in Water, Endogenous Starter Cultures, Raw Milk, and Fresh Minas Artisanal Cheeses

We identified 50 samples of LAB at the molecular level by the amplification and sequencing of the *16S* rRNA gene (fragments of approximately 1,500 bp) with a similarity threshold higher than 97%. We isolated 3 samples from water (6%), 9 from raw milk (18%), 17 from endogenous starter culture (34%), and 21 from fresh cheese (42%; Table 5).

From the identified samples, *Enterococcus faecalis* was the LAB most frequently isolated from raw milk (66.67%), endogenous starter culture (41.18%), and

fresh cheeses (42.86%) during the dry or rainy seasons. Similar results were recorded by Andrighetto et al. (2001) for Asiago, Montasio, Monte Veronese, and Fontina, all artisanal cheeses produced in Italy.

Enterococcus durans has been described in milk and dairy products (Ercan et al., 2014), but we isolated it from the endogenous starter culture, along with *Enterococcus pseudoavium*. These are the first reports in the literature for both of these LAB.

Generally, the presence of *Enterococcus* spp. in foods is of concern, because some types may be pathogenic to human, causing cardiovascular, urinary tract, or nervous system infections (Murray, 1990). A lack of knowledge about the virulence and transmission of resistance to important antimicrobials such as vancomycin inhibits the use of *Enterococcus* spp. in the food industry, especially if the focus is the elaboration of foods carrying probiotics (Hammad et al., 2015). On the other hand, the presence of these bacteria in cheeses is related to flavoring compounds that contribute to sensory properties (Kirmaci et al., 2014). Furthermore, *Enterococcus* spp. may be producers of bacteriocins, which are responsible for inhibiting the growth of pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Silvetti et al., 2014).

Lactococcus lactis was isolated from water (100%), raw milk (11.11%), and fresh cheeses (28.57%) in the dry or rainy seasons. This bacterium is commonly used in cheese starter cultures and efficiently converts lactose to lactic acid. This LAB is dominant in fresh cheese, as identified in the present study, and it acts as a starter for the acidifying process. Its presence is reduced as the cheeses age (Fontán et al., 2001).

We isolated *Lb. plantarum* only in the rainy season from raw milk (22.22%), endogenous starter culture (17.65%), and fresh cheeses (14.29%). In a study carried out in the Serra da Canastra region, *Lb. plantarum* was the most isolated LAB in artisanal Minas cheese (Resende et al., 2011).

One sample of *Lb. rhamnosus* (4.76%) and another of *Lc. garvieae* (4.76%) were isolated from cheeses produced in the rainy season. Likewise, 1 sample of *Lb. paracasei* (5.88%) and another of *Lb. brevis* (5.88%) were isolated from endogenous starter culture, also in the rainy season. At least 1 of these LAB species has been isolated in artisanal cheese or raw milk in different countries around the world (Coppola et al., 2006; Terzic-Vidojevic et al., 2009; Resende et al., 2011; Feutry et al., 2012). Although *Lb. brevis* has been isolated from samples of Minas artisanal cheeses, the literature contains no reports on its isolation from the endogenous starter culture used for cheesemaking. *Lactobacillus rhamnosus*, *Lb. paracasei*, and *Lb. brevis* are desirable

for the elaboration of artisanal cheeses, either for the development of desirable sensory characteristics or as probiotics (Phillips et al., 2006; Zahedi et al., 2011).

Aerococcus viridans (5.88%) was isolated from 1 sample of endogenous starter culture, only in the dry season. The literature contains no reports of that LAB in endogenous starter cultures used for the elaboration of artisanal cheeses. Although studies of its beneficial effects in foods are scarce, some reports point to its probiotic potential, but testing in vivo is needed (Hamed and Elattar, 2013; Ramirez-Chavarin et al., 2013).

Two samples of *Leuconostoc mesenteroides* (11.76%) were isolated from endogenous starter cultures in the dry season. This bacterium has been found in endogenous starter cultures used for Mozzarella (Coppola et al., 1988) and for Minas artisanal production in the Serro region (Chaves et al., 1995). *Leuconostoc mesenteroides* has been used as a starter culture in cheeses to produce desirable sensory effects in the final product, some of which come from compounds formed during citrate metabolism, such as acetaldehyde, diacetyl, and acetoin. Furthermore, this LAB is responsible for the production of dextran from sucrose, as well as the synthesis of lipolytic and proteolytic enzymes that aid

Table 5. Molecular identification of lactic acid bacteria isolated from samples of water, raw milk, endogenous starter cultures, and fresh Minas artisanal cheeses collected at Minas artisanal cheesemaking facilities in Campo das Vertentes, Minas Gerais, Brazil, during dry and rainy seasons

Microorganism	Dry	Rainy	Total
Water			
<i>Lactococcus lactis</i>	3	—	3
Total	3	—	3
Raw milk			
<i>Enterococcus faecalis</i>	6	—	6
<i>Lactococcus lactis</i>	1	—	1
<i>Lactobacillus plantarum</i>	—	2	2
Total	7	2	9
Endogenous starter culture			
<i>Aerococcus viridans</i>	1	—	1
<i>Lactobacillus plantarum</i>	—	3	3
<i>Enterococcus faecalis</i>	4	3	7
<i>Enterococcus pseudoavium</i>	1	—	1
<i>Lactobacillus brevis</i>	—	1	1
<i>Enterococcus durans</i>	—	1	1
<i>Lactobacillus paracasei</i>	—	1	1
<i>Leuconostoc mesenteroides</i>	2	—	2
Total	8	9	17
Fresh Minas artisanal cheese			
<i>Lactococcus lactis</i>	5	1	6
<i>Lactobacillus plantarum</i>	—	3	3
<i>Enterococcus faecalis</i>	5	4	9
<i>Lactococcus garvieae</i>	—	1	1
<i>Enterococcus faecium</i>	—	1	1
<i>Lactobacillus rhamnosus</i>	—	1	1
Total	10	11	21
Overall total	28	22	50

in the development of flavor, texture and color (Ni-eto Arribas et al., 2010). As well as its technological properties, some samples of *Leuconostoc mesenteroides* possess in vitro probiotic potential, including biliary salts deconjugation, self-aggregation, adhesion properties, β -galactosidase activity, and sensitivity to antimicrobials that are important for human and veterinary medicine (Abriouel et al., 2008; Paula et al., 2014).

CONCLUSIONS

The microbiological quality of the water, endogenous starter culture, raw milk, and fresh Minas artisanal cheese from the Campo das Vertentes region was influenced by season (dry or rainy). Fresh cheeses produced in the rainy season had worse microbiological quality, highlighting the importance of selling those cheeses only after aging. We isolated LAB from all studied samples, regardless of season. *Enterococcus faecalis* was isolated with higher frequency in samples of raw milk, endogenous starter culture, and fresh cheeses, followed by *Lc. lactis* and *Lb. plantarum*. *Lactobacillus paracasei*, *Lb. brevis*, *Enterococcus pseudoavium*, and *Aerococcus viridans* were isolated from endogenous starter culture, all of these described in the literature for the first time. The microbiological quality of the analyzed cheeses and components was compromised by the presence of undesirable microorganisms in both seasons. The LAB present in high counts may inhibit undesirable microbiota as well as contributing to flavor, but this must be evaluated in future research.

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