Isolation and identification of lactic acid bacteria from Brazilian Minas artisanal cheese

L. M. P. Luiz, R. D. Castro, S. H. C. Sandes, J. G. Silva, L. G. Oliveira, G. A. Sales, A. C. Nunes and M. R. Souza

Departamento de Tecnologia e Inspeção de Produtos de Origem Animal, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

ABSTRACT

The microbiota of lactic acid bacteria (LAB) from Minas artisanal cheese was evaluated. Samples of Minas artisanal cheese were collected at the farms in the region of Araxá, Brazil, over two seasons. Molecular approaches were used to assess the biodiversity of the isolates. The median value of the counts of LAB ranged from 6.4 to 9.5 log units in cheeses throughout the ripening period, in both seasons. The genera of Lactobacillus, Enterococcus, Pediococcus, and Lactococcus were found. L. plantarum and L. rhamnosus were the prevailing species during both seasons, suggesting they may play a role in the development of the cheese attributes. Enterococcus rivorum was identified in one sample and it was probably the first description in cheeses. This is an important report describing the varieties of LAB in this cheese and the identification of its lactic communities could be a source of strains with specific properties.

INTRODUCTION

Minas artisanal cheese is one of the most popular cheeses in Brazil. It is produced in the state of Minas Gerais, which is referred to as a major producer of artisanal cheese made from raw milk, in Brazil (Martins et al., 2015). Minas artisanal cheeses are produced only on farms from certified regions, using whole raw milk and endogenous culture starter for its production. The region of Araxá is one of these regions and has produced Minas artisanal cheese for more than a century (Dores, Nobrega, & Ferreira, 2013). This endogenous culture is obtained from cheese whey, which is derived from the cheese made previously, and is added to the raw milk in concentrations between 0.1% and 2%. This volume varies according to the cheesemakers or to the season. Minas artisanal cheese has a semi-hard texture of raw paste, a cylindrical shape, and it can weigh from 1.0 to 1.2 kg. It is also characterized by an ivory-white color crust and acid flavor. The fresh Minas artisanal cheese has about 56% of moisture, 25% of fat, 43% of dry matter and pH of 4.9. The importance of raw milk and natural endogenous starters as a source of bacterial strains harboring genetic diversity has been described in traditional cheeses (Tormo, Lekhal, & Roques, 2015). In addition to inhibiting pathogenic bacteria, the origin and authenticity of artisanal cheeses is also guaranteed.

However, there is a lack of information regarding the microorganisms present in Brazilian artisanal cheeses, especially with respect to lactic acid bacteria (LAB). The study of Arcuri, Sheikha, Rychlik, Isabelle, and Monet (2013) seems to be the first research about the identification of bacterial communities and origin in Minas artisanal cheese from Araxá region. Thus, more researches are needed to know the LAB diversity in that cheese. The knowledge of the LAB, mainly lactobacilli, present in this cheese would be of great interest, to acquire a real picture of its sanitary and sensory properties. The heterofermentative species of Lactobacillus can play an important role during the ripening of artisanal cheeses. They can contribute to the flavor and texture of the cheese (Milesi, Wolf, Bergamini, & Hynes, 2010), besides being health promoters, with probiotic features (Caggia, De Angelis, Pitino, Pino, & Randazzo, 2015).
Prior to this study, no information was available on the diversity of microbiota in Minas artisanal cheese from the region of Araxá. Thus, the aim of this study was to identify the microbiota of LAB in cheeses during ripening up to 57 days, over two seasons, in order to contribute to the preservation of the Brazilian dairy heritage.

Materials and methods

Sampling

A total of 84 samples of Minas artisanal cheeses from the region of Araxá was collected from six farms. The cheese samples originated from the same batch, from farms at 1, 7, 14, 21, 28, 42, and 57 days of ripening, totaling 42 samples per each season – rainy (from March to May) and dry (from August to October) seasons. Samples of raw milk and endogenous culture used to manufacture cheeses were collected from each farm. Absolute humidity is the main difference observed in the region throughout the year, thus the terms ‘dry’ and ‘rain’ are used to characterize the seasons. Ripening occurred at room temperature, which varied from 18°C to 30°C and from 15°C to 32°C in the rainy and dry seasons, respectively. The mean relative humidity was higher than 80% in the rainy season and lower than 60% in the dry season. Cheese samples were transported under refrigerated conditions to the laboratory.

Enumeration and isolation of LAB

A sample of 25 g of each cheese was taken aseptically and added to 225 mL of sterile peptone water (Himedia Laboratories, Sydney, Australia) and homogenized for 2 min using a stomacher (Boitton, MK 1204, Brazil). Serial dilutions of the samples were then surface plated on MRS and M17 agars (Difco Laboratories Inc., Detroit, MI, USA) and incubated at 37°C for 48 h, under aerobic condition (IDF, 1988). From each count plate, three to four Gram-positive and catalase-negative colonies, representing LAB characteristic morphology type, were selected and purified by streaking and growth on the same medium. The isolates were subcultured in an MRS or M17 medium broth, supplemented with 15% (v/v) glycerol as a cryoprotectant, and stored at −20°C.

The samples of raw milk and endogenous culture were also submitted to the determination of LAB, as previously described.

Identification of LAB

Colonies of LAB, which showed a distinct morphology in the isolation medium, were submitted for molecular identification by 16S rRNA gene sequencing and analysis. Briefly, the genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer’s instructions. Next, the full length of the 16S rRNA locus was amplified using 27F and 1492R primers (Lane, 1991). Nucleic acid sequencing of the 16S rRNA gene was performed using an ABI3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) with the Big Dye Terminator v3.1 (Life Technologies, Carlsbad, CA, USA) as an automatic sequencing system. The obtained sequences were analyzed using the Seqmatch algorithm (RDP – Ribosomal Database Project). Isolates with a similarity threshold higher than 97% were considered in this study.

When necessary, a multiplex PCR with rec-A gene-derived primers was performed according to Torriani, Felis, and Dellaglio (2001), to differentiate the LAB belonging to the L. plantarum group. The rec-A locus was amplified using paraF, pentF, pREV, and planF primers and PCR Pre-Mix Pht (Phoneutria Biotecnologia, Belo Horizonte, Brazil). For LAB belonging to the Lactobacillus casei group, an approach described by Ward and Timmins (1999) was used. The V1 region of the 16S rRNA gene was amplified using a Y2 non-specific primer and casei-, para- or rham-specific primers, and PCR Pre-Mix Pht (Phoneutria Biotecnologia, Belo Horizonte, Brazil).

Statistical analyses

The experiment was designed in a factorial arrangement of $6 \times 7 \times 2$. Linear regression was used to evaluate the mean counts of LAB throughout the ripening times and seasons of the year, using the InfoStat/Professional Program, version 2008. The means were compared by the Tukey test with levels of significance set at $p < 0.05$.

Results and discussion

Microbiological counts and basic characterization of isolates

The mean values of LAB in the Minas artisanal cheeses throughout the ripening period in rainy and dry seasons are given in Table 1. Although the relative humidity is higher in the rainy period, no difference between seasons was observed for LAB counts. High counts of LAB were detected in the cheeses, irrespective of the season. It could be assumed that these LAB originated from raw milk and the endogenous culture, as they presented high counts of LAB at the time of cheese manufacture, 6.4 log units for raw milk (both seasons) and 7.0 and 8.0 for endogenous culture (rainy and dry seasons, respectively). Delcenserie et al. (2014) reported that the average count of LAB in a Belgian protected designation of origin cheese was 7.5 log units when made with raw milk. The ripening time only influenced the mean counts of LAB in the M17 medium during the dry season (Table 1). It may be explained by the higher pH of that culture medium than MRS, allowing a wider variation of

| Ripening days | MRS | M17 | MRS | M17 |
|---------------|-----|-----|-----|-----|
| 1             | 8.1 | 8.5 | 8.6 | 9.3* |
| 7             | 7.8 | 7.1 | 7.4 | 7.6* |
| 14            | 7.8 | 7.9 | 7.1 | 9.5* |
| 21            | 7.5 | 7.1 | 7.6 | 7.0* |
| 28            | 7.3 | 7.0 | 7.5 | 6.8* |
| 42            | 7.0 | 7.0 | 7.9 | 6.9* |
| 57            | 6.9 | 6.4 | 6.8 | 6.8* |

*Means followed by distinct letters in the same column are different by the Tukey test ($P < 0.05$).

*Los promedios seguidos de distintas letras en la misma columna son diferentes mediante el test de Tukey ($P < 0.05$).

Table 1. Mean values of LAB population from Minas artisanal cheeses during 57 days of ripening over two seasons.
Table 2: Isolates of LAB from Minas artisanal cheeses during 57 days of ripening over two seasons.

| Isolates            | Season | Ripening days | No. of isolates |
|---------------------|--------|---------------|-----------------|
| Lactobacillus sp.   | Rainy 1| 7–14–21–28–57 | 1–1–1           |
| Lacticoccus sp.     | Dry    | 7–14–21–28–57 | 4–2–1–1–1       |
| L. plantarum        | Dry    | 21–57         | 1–3             |
| L. brevis           | Rainy 1| 21–57         | 1–3             |
| L. rhamnosus        | Dry    | 21–7–21       | 1–1              |
| L. rhamnosus        | Dry    | 21–28–42–57   | 1–1–1–2–2       |
| E. casei            | Rainy 1| 42            | 1               |
| E. casei            | Dry    | 28            | 1               |
| Enterococcus sp.    | Rainy 1| 1             | 1               |
| Enterococcus sp.    | Dry    | 1–14–21–28–57 | 1–1–1–2–2       |
| E. faecalis         | Dry    | 1–7           | 2–1             |
| E. rivorum          | Dry    | 1             | 1               |
| Lactococcus lactis  | Rainy 1| 1             | 1               |
| Pediococcus sp.     | Rainy 1| 42            | 1               |

bacterial growth. Renye, Somkuti, Van Hekken, and Prietot (2011) demonstrated that M17 medium was able to support the growth of other bacterial species than LAB.

Identification of LAB

From 263 isolates, a total of 50 morphologically distinct, Gram-positive and catalase-negative strains, were identified at the genus and species level (Table 2).

Four different genera were found in cheeses: Lactobacillus, Enterococcus, Pediococcus, and Lactococcus. An important rate of LAB isolates were identified as Lactobacillus spp. (72%), present in the two seasons and during all stages of cheese ripening. This result is in accord with Renye et al. (2011), since they isolated Lactobacillus species from all samples of raw milk Chihuahua cheeses analyzed. The Lactobacilli constitute a major group of LAB and comprise a wide range of niches. They play an important role during ripening of industrial and artisanal cheeses, as they produce volatile flavor compounds that contribute to their sensory profile (Sgarbi et al., 2013). Among the identified isolates, the most frequent LAB species recovered from cheese in the rainy season belonged to the species L. plantarum and L. brevis, while in the dry season, the most frequent LAB belonged to the L. rhamnosus species. L. rhamnosus, L. plantarum, and L. casei are desirable species in cheese production, as they are related to the development of unique sensory characteristics, and also may have probiotic potential (Huang et al., 2015).

The Enterococcus spp. are mostly found in high amounts in the dry season. These microorganisms are generally isolated from raw milk cheeses, and have been discussed as possible adjunct cultures because of their proteolytic and lipolytic activities (Reny et al., 2011). Moreover, Silvetti, Morandi, and Brasca (2013) reported the bio-preservation potential of E. faecalis isolated from Italian traditional cheeses and its antagonistic effects against well-recognized pathogens. However, for many authors, the presence of Enterococci is an evidence of possible fecal contamination, and therefore, a risk to consumers, especially because of the emergence of antibiotic-resistant strains (Hammet, Hassan, & Shimamoto, 2015). In a study carried out in the Campo das Vertentes region, species of Enterococcus were the most isolated LAB in Minas artisanal cheese (Castro et al., 2016). The authors used the same LAB protocol identification we used in this study. E. rivorum, a novel species of Enterococcus, was first isolated from the water of pristine brooks in Finland (Niemi et al., 2012). This strain was found in one sample of cheese in the dry season (99% of identity of closest related species in GenBank). This is probably the first identification of E. rivorum in traditional cheeses. The presence of this species in fermented products is not yet documented and its role in cheeses should be further studied. E. rivorum strains are closely related to the E. faecalis group (Niemi et al., 2012).

In this study, one strong acidifying bacterial species was identified at the beginning of ripening – Lactococcus lactis. Also, one sample of the Pediococcus sp. was also identified in cheeses at 42 days of ripening, both in the rainy season.

Our results indicated that diverse bacterial species arise in Minas artisanal cheese from Araxá, with a possible dominance of members of the Lactobacillus genus. Differently, in the Canastra region, Lacerda et al. (2011) found predominantly Lactococcus lactis at 7 days of ripening while at 60 days, Streptococcus salivarius was the most prevalent in Minas artisanal cheese. Arcuri et al. (2013) identified by PCR-DGGE bacterial communities in two samples of Minas artisanal cheese of Araxá region, collected at the market. The authors found mainly Streptococcus spp. in the cheese microbiota, but stated that more studies with a larger number of samples would be necessary to better understand this cheese microbiota. Lactobacillus and Enterococcus species may serve as non starter LAB for development of the sensorial characteristics, and could contribute with the health safety of the products. Molecular studies of these artisanal cheeses may reveal a complex microbiota, including novel species, such as E. rivorum. Identification of indigenous LAB strains during the ripening of Minas artisanal cheese is the first step in understanding the specific microbial ecosystem of the artisanal cheeses and their role in cheese making as well as their potential probiotic activities. All isolates from this study were deposited in a culture collection which may be researched in future studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was funded by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

ORCID

L. M. P. Luiz, https://orcid.org/0000-0003-1304-9420

References

Arcuri, E.F., Sheikha, A.F., Rychlik, T., Isabelle, P.M., & Monet, D. (2013). Determination of cheese origin by using 16S rDNA fingerprinting of bacteria communities by PCR-DGGE: Preliminary application to traditional Minas cheese. Food Control, 30, 1–6. doi:10.1016/j.foodcont.2012.07.007

Caggia, C., De Angelis, M., Pitino, I., Pino, A., & Randazzo, C.L. (2013). Probiotic features of Lactobacillus strains isolated from Ragusano and Pecorino Siciliano cheeses. Food Microbiology, 30, 109–117. doi:10.1016/j.fm.2013.03.010

Castro, R.D., Oliveira, L.G., Sant’Ana, F.M., Luiz, L.M.P., Sandes, S.H.C., Silva, C.I. … Souza, M.R. (2016). Lactic acid microbiota identification
in water, raw milk, endogenous starter culture, and fresh Minas artisinal cheese from the Campo das Vertentes region of Brazil during dry and rainy seasons. Journal of Dairy Science, 99, 1–11. doi:10.3168/jds.2015-10579

Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S. ... Daube, G. (2014). Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. Journal of Dairy Science, 97, 6046–6056. doi:10.3168/jds.2014-8225

Dores, M.T., Nobrega, J.E., & Ferreira, C.L.L.F. (2013). Room temperature aging to guarantee microbiological safety of Brazilian artisan Canastra cheese. Food Science and Technology, 33, 180–185.

Hammet, A.M., Hassan, H.A., & Shimamoto, T. (2015). Prevalence, anti-biotic resistance and virulence of Enterococcus spp. in Egyptian fresh raw milk cheese. Food Control, 50, 815–820. doi:10.1016/j.foodcont.2014.10.020

Huang, R., Tao, X., Wan, C., Li, S., Xu, H., Xu, F. ... Wei, H. (2015). In vitro probiotic characteristics of Lactobacillus plantarum ZDY 2013 and its modulatory effect on gut microbiota of mice. Journal of Dairy Science, 98, 1–12. doi:10.3168/jds.2014-9153

IDF (International Dairy Federation). (1988). Yogurt: Enumeration of characteristics microorganisms colony count technique at 37 °C. IDF Standard 117A. International Dairy Federal, 1–5. Brussels, Belgium.

Lacerda, I.C.A., Gomes, F.C.O., Borelli, B.M., Faria, C.L.L., Jr., Franco, G.R., Mourão, M.M. ... Rosa, C.A. (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. Brazilian Journal of Microbiology, 42, 650–657. doi:10.1590/S1517-83822011000200029

Lane, D.J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt & M. Goodfellow (Eds.), Nucleic acid techniques in bacterial systematics (pp. 115–175). Chichester: Wiley.

Martins, J.M., Galinari, E., Filho, N.J.P., Ribeiro, J.J., Jr, Furtado, M.M., & Ferreira, C.L.L.F. (2015). Determining the minimum ripening time of artisanal Minas cheese, a traditional Brazilian cheese. Brazilian Journal of Microbiology, 46, 219–230. doi:10.1590/S1517-8382461201310003

Milesi, M.M., Wolf, I.V., Bergamini, C.V., & Hynes, E.R. (2010). Two strains of nonstarter lactobacilli increased the production of flavor compounds in soft cheeses. Journal of Dairy Science, 93, 5020–5031. doi:10.3168/jds.2009-3043

Niemi, R.M., Ollinkangas, T., Paulin, L., Svec, P., Vandamme, P., Karkman, A. ... Lindstrom, K. (2012). Enterococcus rivosar sp. nov., from water of pristine brooks. International Journal of Systematic Evolutionary Microbiology, 62, 2169–2173. doi:10.1099/ijs.0.038257-0

Renye, J.A., Jr., Somkuti, G.A., Van Heekten, D.L., & Prietot, G.V.M. (2011). Short communication: Characterization of microflora in Mexican Chihuahua cheese. Journal of Dairy Science, 94, 3311–3315. doi:10.3168/jds.2011-4177

Sgarbi, E., Lazzi, C., Tabanelli, G., Gatti, M., Neviani, E., & Garini, F. (2013). Nonstarter lactic acid bacteria volatilomes produced using cheese components. Journal of Dairy Science, 7, 4223–4234. doi:10.3168/jds.2012-6472

Silvetti, T., Morandi, S., & Brasca, M. (2013). Biopreservation potential of Enterococcus faecalis isolated from Italian traditional raw milk cheeses. CyTA-Journal of Food, 3, 201–217.

Torno, H., Lekhal, D.A.H., & Roques, C. (2015). Phenotypic and genotypic characterization of lactic acid bacteria isolated from raw goat milk and effect of farming practices on the dominant species of lactic acid bacteria. International Journal of Food Microbiology, 210, 9–15. doi:10.1016/j.ijfoodmicro.2015.02.002

Torriani, S., Felis, G.E., & Dellaglio, F. (2001). Differentiation of Lactobacillus plantarum, L. pentosus, and L. paraplantarum by recA Gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Applied Environmental Microbiology, 67, 3450–3454. doi:10.1128/AEM.67.8.3450-3454.2001

Ward, L.J.H., & Timmins, M.J. (1999). Differentiation of Lactobacillus casei, Lactobacillus paracasei and Lactobacillus rhamnosus by polymerase chain reaction. Letters in Applied Microbiology, 29, 90–92. doi:10.1046/j.1365-2672.1999.00586.x