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Further culture-independent characterization of the lactic microbiota of Serro artisanal cheese

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Abstract

This study aimed to provide a further characterization of the lactic microbiota present in Minas artisanal cheese (MAC) from the Serro region by using culture-independent methods, as a complementary analysis of a previous study. The total DNA extracted from MAC samples ($n = 55$) was subjected to repetitive extragenic palindromic-PCR (rep-PCR) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE). Rep-PCR analysis showed that core microbiota of Serro MAC was closely related, independent of the production town, farm size, or time of production. The sequencing of PCR-DGGE bands identified the prevalence of *Lactococcus lactis* in all samples, and *Streptococcus salivarius* was also identified. Thus, we conclude that when more accurate methods are unavailable, rep-PCR can be used as a culture-independent method to demonstrate if the microbiota is closely related or not among the samples. PCR-DGGE results also matched to the main findings of high-throughput sequencing, previously presented, confirming its confidence to detect the main microbial groups present in the raw milk cheeses.

Keywords Artisanal cheese · Lactic microbiota · Rep-PCR · PCR-DGGE

Introduction

Recently, a study conducted by our research group have characterized by culture-dependent and culture-independent methods the microbiota signature of Minas artisanal cheeses (MAC) from Serro region, a traditional artisanal cheese-producing region in the state of Minas Gerais, Brazil [1]. The cheese samples were subjected to lactic acid bacteria (LAB) enumeration, and most of the obtained isolates were identified as lactobacilli; producer city, farm size, starter culture, and time of production had no influence on LAB counts and species distribution ($P > 0.05$) [1]. The total DNA from cheese samples was then subjected to high-throughput 16S rRNA sequencing, targeting V3–V4 region of the 16S rRNA, and the results revealed *Lactococcus lactis* as the predominant species in all samples, with no differences detected by alpha-diversity and beta-diversity analysis [1].

Minas artisanal cheeses are produced using raw milk, and the presence of LAB in this product is extremely desirable, once these microbial group confers typical sensory characteristics [2]. These cheeses are composed by complex ecosystems susceptible to abiotic and environmental factors, and changes in these factors have a direct influence within the microbiome community, affecting the organoleptic properties as well as the safety of the end products [3, 4].

The assessment of the microbial diversity of artisanal cheeses is very important to ensure their quality and safety, and that can be performed by culture-dependent and culture-independent methods. Culture-dependent methods are more laborious, consisting of homogenization of the sample, followed by dilution and plating in culture medium, selection of colonies, and phenotypic characterization [5, 6]. These methods have some limitations, since it is difficult to simulate the necessary nutrients and the conditions for many

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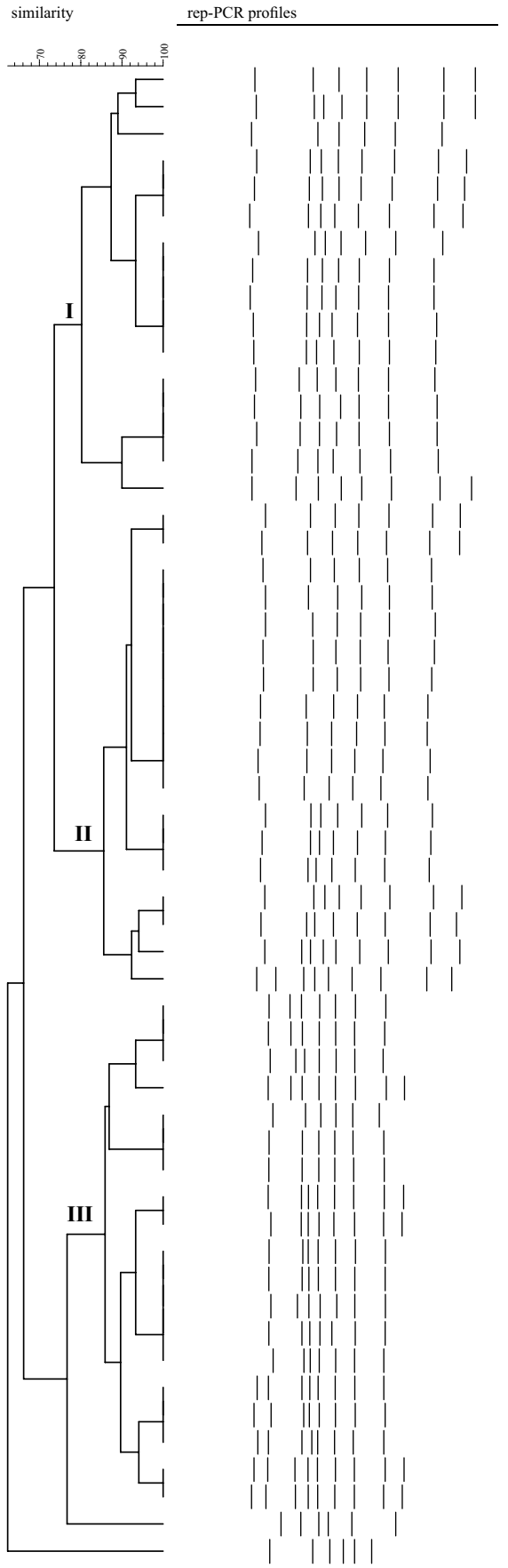

Cheese sample	Starter	Producer city	Farm size	Time of production
S34	Rala	Serra Azul de Minas	Large	21
S35	Rala	Serro	Medium	21
S33	Rala	Serro	Medium	21
S20	Rala	Santo Antônio do Ita.	Large	5
S21	Pingo	Serro	Small	5
S22	Rala	Serra Azul de Minas	Large	12
S19	Rala	Santo Antônio do Ita.	Large	12
S23	Rala	Sabinópolis	Large	19
S24	Rala	Sabinópolis	Medium	5
S26	Rala	Alvorada de Minas	Medium	17
S27	Pingo	Santo Antônio do Ita.	Medium	13
S25	Rala	Serro	Small	5
S28	Rala	Santo Antônio do Ita.	Medium	13
S29	Rala	Santo Antônio do Ita.	Medium	13
S31	Rala	Sabinópolis	Large	21
S32	Rala	Serro	Medium	10
S43	Rala	Serro	Medium	8
S45	Rala	Alvorada de Minas	Medium	8
S30	Rala	Serra Azul de Minas	Large	6
S40	Rala	Serro	Medium	8
S41	Rala	Alvorada de Minas	Small	5
S42	Rala	Alvorada de Minas	Medium	5
S44	Rala	Alvorada de Minas	Medium	8
S49	Rala	Serro	Medium	6
S50	Rala	Serro	Medium	6
S51	Rala	Serro	Small	6
S52	Rala	Serro	Small	10
S38	Rala	Santo Antônio do Ita.	Large	8
S47	Rala	Serra Azul de Minas	Large	16
S48	Rala	Serro	Medium	10
S37	Pingo	Serro	Medium	8
S46	Rala	Serro	Medium	6
S39	Rala	Serro	Medium	8
S53	Rala	Serro	Large	6
S13	Rala	Sabinópolis	Large	12
S9	Rala	Serro	Medium	9
S3	Rala	Serro	Medium	5
S12	Rala	Serra Azul de Minas	Large	9
S54	Pingo	Serro	Medium	6
S6	Rala	Santo Antônio do Ita.	Large	9
S7	Rala	Sabinópolis	Large	9
S17	Rala	Santo Antônio do Ita.	Small	15
S4	Pingo	Serro	Medium	5
S10	Rala	Serro	Small	12
S11	Pingo	Santo Antônio do Ita.	Medium	16
S15	Rala	Serro	Small	5
S16	Pingo	Sabinópolis	Small	12
S5	Rala	Serro	Small	5
S18	Rala	Alvorada de Minas	Small	5
S2	Rala	Alvorada de Minas	Medium	5
S36	Rala	Serro	Small	5
S14	Rala	Serro	Medium	5
S8	Rala	Serro	Medium	9
S55	Rala	Serro	Large	6
S1	Rala	Alvorada de Minas	Medium	5

◂**Fig. 1** Cluster analysis of rep-PCR fingerprints obtained from Serro Minas artisanal cheese samples with different production characteristics (city; farm size—based on daily milk production—small, lower than 150 L; medium, 150–200 L; large, higher than 200 L; starter culture; and time of production, days). The similarities between the profiles were calculated by using Dice coefficient, and the dendrogram were constructed using unweighted pair group method with arithmetic mean (UPGMA), with tolerance of 1.5% and optimization of 5%

genera of bacteria to grow in vitro. On the other hand, the culture-independent methods are based on the analysis of genetic material extracted directly from the cheese. These methods have advantages for detection and differentiation among species and for identification of unculturable microbes [6, 7].

Considering the advantages and limitations of culture-dependent and culture-independent methods, combined approaches are the best way to characterize the microbial diversity of artisanal cheeses. Since there are many protocols available with this purpose, herein we continued a previous study of our group related to the characterization of the lactic microbiota of MAC from Serro region [1], by using further culture-independent methodologies.

Material and methods

The same Serro MAC samples ($n = 55$) obtained by Nero et al. [1] were considered in the present study, as well as the procedures for total DNA extraction. Samples were obtained from producers of different cities located in the Serro region, with distinct production characteristics as detailed previously [1].

Rep-PCR reactions of total DNA were performed according to Gevers et al. [8], using single primer $(GTG)_5$ (5′-GTG GTG GTG GTG GTG-3′). PCR reactions contained 12.5 µL of GoTaq Master Mix 2X (Promega Corp., Madison, WI, USA), 1 mL of 50 pMol primer, 2 µL of DNA extracted from each cheese, and ultra-pure water (Promega) to complete a final volume of 25 µL. The PCR conditions were 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 40 °C for 45 s, 65 °C for 8 min, and the final extension at 65 °C for 16 min. The PCR products were electrophoresed on agarose gels (1.5% w/v) in TBE buffer (0.5 × Tris/Borate/EDTA) at constant voltage (90 V) for 3 h. 1 kb DNA ladder (Sigma-Aldrich, St. Louis, MO, USA) was used as a molecular size marker. The obtained fingerprints were analyzed using BioNumerics 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarities between the profiles were calculated using the Dice coefficient and UPGMA method (tolerance 1.5%, optimization 5%).

To perform PCR-DGGE, the V3 variable region of the bacterial 16S rRNA gene was amplified according to Ampe et al. [9] by using primers 338F (5′-AC TCC TAC GGG AGG CAG CAG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′). A 40 bp GC-clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG-3′) was attached to the 5′ end of the forward primer for DGGE analysis in order to ensure that the fragment of DNA remains partially double stranded and that the region screened is in the lowest melting domain, generating amplicons with a size of approximately 236 bp [10].

PCR reaction was performed in a final volume of 50 µL containing 1 × GoTaq® Flexi Reaction buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1.25 U Taq pol DNA polymerase (Cellco Biotec, São Carlos, SP, Brazil), 2 µL of total DNA of cheese, and ultra-pure PCR water. PCR conditions were 10 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 42 °C, 2 min at 72 °C, and a final extension of 7 min at 72 °C. The PCR products were electrophoresed on agarose gels (1.5% w/v) in TBE buffer and stained with ethidium bromide.

The DCode™ universal mutation detection system (Bio-Rad Lab., Hercules, CA, USA) was used for DGGE analysis. Electrophoresis was performed in a 0.8-mm-thick polyacrylamide gel (8% w/v) acrylamide:bisacrylamide (37.5:1) with a denaturing gradient of urea and formamide ranging from 25 to 60% in 1 × TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8) at constant voltage of 120 V for 6 h at 60 °C, stained (30 min) in 1 × TAE containing 1 × SYBR GOLD (Sigma-Aldrich). Ladders were included as reference in gels (1 kb, Kasvi, São José dos Pinhais, PR, Brazil). Gels were photographed and analyzed on a UV transilluminator with the photo documentation imaging system.

Representative bands of polyacrylamide gels were collected using sterile pipette tips, and their DNA eluted overnight in 50 µL of nuclease-free water at 4 °C. The DNA was then re-amplified using the same primer pair but without the GC-clamp by using the conditions described above. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced on an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Sequences were compared to those deposited in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/genbank), using the Basic Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) software.

Results and discussion

In the previous study carried out by Nero et al. [1], lactobacilli were the predominant LAB identified by conventional plating and sequencing of amplified 16S rRNA. On the other hand, when the total DNA extracted from cheese samples was subjected to high-throughput 16S rRNA sequencing,

Lactococcus lactis was clearly the dominant species. Considering the obtained data, further analysis was carried out to characterize the lactic microbiota of the same Serro MAC samples by alternative culture-independent methods. Then, results of these alternative approaches were compared to those presented by Nero et al. [1].

The rep-PCR profiles, cheese origin, and their production characteristics are presented in the Fig. 1. Considering a coefficient of similarity of 80%, three main clusters were obtained in the generated dendrogram. Rep-PCR is a subtyping method usually employed to differentiate LAB by grouping clusters of genetic-related isolates for subsequent selection of isolates and identification by sequencing [11]. However, this method may have other application, as to demonstrate the total microbiota of artisanal cheeses by grouping them based on their different geographical origins [12]. Another study conducted by Perin et al. [13] has demonstrated that rep-PCR can provide a clear evidence of the in situ interference caused by adding a bacteriocinogenic *L. lactis* subsp. *lactis* strain in Minas cheese produced with raw goat milk.

Interesting, in this study, the application of rep-PCR as culture-independent method suggested that the core microbiota of Serro MAC can be closely related independent of the production town, farm size, or maturation time (Fig. 1). This result makes sense once the towns where samples were collected compose a micro-region with particular climate and soil characteristics (terroirs), influencing the selection of cheese microbiota. Despite microbial groups present in each sample cannot be identified by rep-PCR, this method provided evidence that factors related to the production of Serro MAC did not have a major interference in the microbiota of the final products, as demonstrated in the first study by Nero et al. [1].

PCR-DGGE confirmed that LAB profile was similar in all 55 cheese samples, regardless of production characteristics. Based on denaturation profiles, 3 to 6 bands were observed as specific to all samples, and 14 bands were selected for sequencing and identification (Fig. 2). The sequencing of bands indicated the prevalence of *L. lactis* in all samples. In addition, *L. lactis* subsp. lactis, *L. lactis* sp., *Streptococcus salivarius*, and *Streptococcus* sp. were identified (Table 1). More than one genus was identified in bands 4 and 6, indicating the same denaturation profile (Table 1).

PCR-DGGE proved to be useful for identification of the main species which compose the cheese microbiota, and our results agree to those observed in other studies [1, 2], where16S rRNA high-throughput sequencing was employed to study microbial signature of Serro MAC. However, PCR-DGGE was clearly much more limited when compared to high-throughput sequencing to study microbial diversity in complex food matrix.

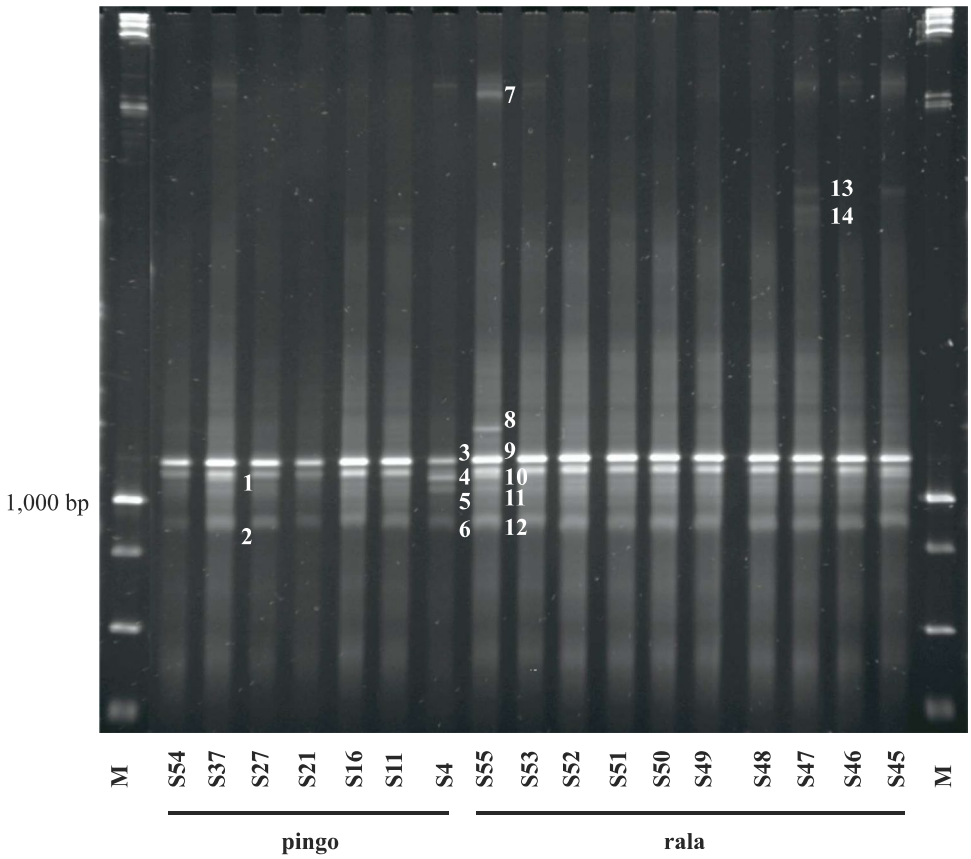

Fig. 2 PCR-DGGE profiles of the bacterial ecology of Serro Minas artisanal cheese samples (17 out of 55) with different production characteristics (city; farm size—based on daily milk production—small, lower than 150 L; medium, 150–200 L; large, higher than 200 L; starter culture; and time of production, days). The lines indicate the identification of the 17 samples (S54, S37, S27, S21, S16, S11, S4, S55, S53, S52, S51, S50, S49, S48, S47, S46, and S45) and ladder markers of 1 kb (M). The numbers indicate the bands subjected to sequencing for molecular identification (results reported in Table 1)

Table 1 Identification of bacterial species present in Serro Minas artisanal cheese samples based on sequencing of PCR-DGGE bands and BLAST sequence comparison in GenBank

Band[a]	Closest relative	% of identity[b]	GenBank accession no
1	*Lactococcus lactis*	99	MT645510.1
2	*Lactococcus lactis*	98	MT645510.1
3	*Lactococcus lactis*	98	MT645510.1
4	*Lactococcus* sp.	97	KJ804064.1
	Streptococcus sp.	96	MW045815.1
5	*Streptococcus salivarius*	96	MT512105.1
6	*Streptococcus* sp.	96	MW045807.1
	Lactococcus lactis subsp. lactis	94	JQ973604.1
7	*Streptococcus* sp.	97	MT512026.1
8	*Lactococcus lactis*	96	MT645510.1
9	*Lactococcus lactis*	99	MT645510.1
10	*Lactococcus lactis*	99	MT645510.1
11	*Lactococcus lactis*	99	MT645510.1
12	*Lactococcus lactis*	99	MT645510.1
13	*Lactococcus lactis*	96	JQ754459.1
14	*Lactococcus lactis*	96	MT115989.1

[a]The numbers correspond to the band numbers in Fig. 2

[b]Percentage of similarity between the sequences obtained from the PCR-DGGE band and the sequence of the closest species in the GenBank database

Arcuri et al. [10] analyzed samples of MAC from Serro, Araxá, Cerrado, and Serra da Canastra regions by PCR-DGGE. The samples of Serro MAC also presented low microbial diversity and showed 4 or 5 bands as specific. One specific band was the most prominent, and it was present in all MAC samples, corresponding to *Streptococcus thermophilus* after sequencing; bands correspondent to *L. lactis* were also observed in nearly all MAC samples. However, this study included a very limited sampling. Van Hoorde et al. [14] evaluated the LAB diversity of Flemish artisan raw milk Gouda-type cheese by conventional culturing and PCR-DGGE. As results, lactobacilli were predominant by culture-dependent methodology, as well as by PCR-DGGE analysis of culturable fractions. However, *L. lactis* was predominant (present in all profiles) when PCR-DGGE was done using the total DNA extracted direct from cheeses. The authors emphasize the use of an integrated approach between culture-dependent and culture-independent techniques to cover as much as possible the taxonomic spectrum of LAB present in raw milk cheeses.

L. lactis is a highly relevant LAB for the dairy industry due its ability to acidify and grow at different concentrations of NaCl. This species has the ability to colonize and to adapt at different niches, being found naturally in a wide variety of environments, such as vegetables (grasses and silages) and animals, mainly in raw milk from different animals [15], explaining their prevalence in artisanal cheeses. However, our results suggests that *L. lactis* population may be underestimated by culture-dependent methods, as stated previously due to different factors related to laboratory conditions of cultivation and the viability of microorganisms conferred by adverse environmental conditions, such as effects of fermentation, nutrient depletion, and pH variation [16]. These factors can induce the microbial cells in a stressed state characterized by the inability to produce colonies on culture media even if they are still able to perform metabolic activity [16, 17].

Considering the observed results, culture-dependent methods do not fully represent the community and the actual microbial diversity present in raw milk cheeses. The conventional protocols used for isolation, identification, and characterization of LAB will always be relevant, especially for the selection of beneficial microbes that can be applied in the food industry. To overcome such limitation in studies focused on microbial diversity, nowadays the high-throughput sequencing is considered an excellent method, being applied to different food matrix [4, 18, 19]. However, it must be considered that by using this method, DNA of all microorganism present, including potentially dead, lysed, and in non-cultivable viable bacteria can be amplified [12, 20, 21].

Combining culture-dependent and culture-independent approaches is always the best for obtaining a complete view of the existing microbial ecosystem in complex food matrix. Among culture-independent methods, high-throughput sequencing emerged as a very powerful tool to study microbial diversity in fermented foods. But it is well know that the costs of high-throughput sequencing and technical support for data analysis can still be limiting factors. Considering the results presented by Nero et al. [1] and by this complementary study, we concluded that application of rep-PCR as culture-independent method in raw milk cheeses may provide evidence of factors related to the production when

other methods are unavailable. In addition, the results of PCR-DGGE matched to the main findings of high-throughput sequencing, confirming its confidence to detect the main microbial groups present in the raw milk cheeses.

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Author contribution Leticia R. Ferreira: methodology, investigation, and writing (original draft and review). Milimani Andretta and Thaiza T. Almeida: methodology and investigation. Luana M. Perin and Anderson C. Camargo: supervising, methodology, formal analysis, and writing (review and editing). Antonio F. Carvalho and Luís A. Nero: conceptualization, validation, formal analysis, resources, data curation, writing (review and editing), supervision, project administration, and funding acquisition.

Declarations

Competing interests The authors declare no competing interests.

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