



Fingerprinting of Lab Isolated From Silage, Water, Raw Milk, Starter Culture and Minas Artisanal Cheese from the Campo Das Vertentes Region, Brazil



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Abstract

The aim of this study was to establish phylogenetic relations of LAB isolated from water, silage, milk, endogenous starter culture and Minas artisanal cheese from Campo das Vertentes region. Eighty-eight Gram positive, catalase negative samples were submitted to 16S-23S rDNA reaction, identifying 36 samples as belonging to *Lactobacillus*/*Pediococcus* genus. The 16S rRNA sequencing identified five species: *Lactobacillus plantarum*, *L. paracasei*, *L. brevis*, *L. rhamnosus* and *Pediococcus acidilactici*. The rep-PCR (GTG)₅ fingerprinting showed predominance of *L. plantarum* (75.7%) in all sources, followed by *L. brevis* (9.1%), *L. paracasei* (6.1%), *P. acidilactici* (6.1%) and *L. rhamnosus* (3%).

Keywords : Milk analysis; Cattle; Reproduction; Urea nitrogen

Abbreviations : CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; FAPEMIG: Fundação de Amparo à Pesquisa de Minas Gerais; LAB: Lactic acid bacteria; ITS: Internal transcript spacer; UPGMA: Unweighted Pair Group with Arithmetic Mean

Introduction

Minas Gerais State is the largest cheese producer in Brazil. There are some traditional regions that produce Minas artisanal cheese, such as Campo das Vertentes. Lactic acid bacteria (LAB) are commonly found in milk and endogenous starter culture used for this type of cheese making [1]. Those LAB are partially responsible for the development of typical sensory characteristics and may act like natural inhibitors of undesirable microorganisms in cheeses.

Material and Methods

Sample acquisition

Samples of silage, water, raw milk, endogenous starter culture and Minas artisanal cheeses (at 0, 7, 14, 21, 28 and 60 days of ripening) were collected from six dairy farms - three certified dairy farms (A, B, C) and three non-certified dairy farms (D, E, F) located in the Campo das Vertentes region.

Isolation, gram staining and catalase test

Each sample was diluted and spread onto M17 and MRS media (Difco Laboratories, Detroit, Michigan, USA). Plates were incubated under aerobiosis at 37 °C for 48h [2,3]. Isolation, Gram staining and catalase test were performed according to Mac Faddin [4] and Standard I D F [2].

DNA extraction

Pre-treatment of bacterial cells was performed according to Castro et al. [1]. The total DNA from each sample was extracted with the Wizard SV Genomic DNA Purification System (Promega Corporation, Madison, Wisconsin, USA), following manufacturer's instructions.

16S-23S ITS amplification

Genus identification was performed by amplification of the intergenic spacer region from 16S-23S genes. Internal transcript spacer 1 (ITS 1) was assessed by primers 16-1 and 23-1B. Methodology was performed according to Tilsala, Timisjarvi e Alatossava [5].

(GTG)₅ rep-PCR finger printing

Genotypic differentiation amongst samples was performed in accordance to Gevers, Huys and Swings (2001). rep-PCR was performed using the (GTG)₅ PCR as primer. DNA band profiles obtained by the PCR reaction were analyzed in the BioNumerics v6.5 (Applied Maths, Kortrijk, Belgium). Genetic similarity values were calculated by Pearson correlation and used to build a dendrogram by UPGMA (Unweighted Pair Group with Arithmetic Mean).

16S rRNA sequencing

Samples were identified by the 16s rRNA gene sequencing using Sanger's method with automated Mega BACE 1000 sequencer (Amersham Biosciences-GE Healthcare, Little Chalfont, Buckinghamshire, UK) at Myleus Biotechnology Facilities (Myleus, Belo Horizonte, Minas Gerais, Brazil). Primers 27F and 1492R were used, according to Reysenbach, Longnecker and Kirshtein. The sequencing results were analysed using the Seq Scanner Software (Applied Biosystems, Foster City, California, USA) and the results were compared with database from NCBI and RDP with minimal percentage of 98% of similarity established for each identification sequence.

Results

From a total of 230 samples from silage, water, raw milk, endogenous starter culture and cheese, 88 Gram positive, catalase negative rods/cocci samples were pre-selected and then submitted to 16S-23S ITS amplification. The 16S-23S ITS showed 11 samples belonging to genus *Streptococcus*/*Lactococcus* (12.5%), 42 belonging to *Enterococcus* (47.7%) and 35 belonging to *Lactobacillus*/*Pediococcus* (39.7%). Genus distinction was performed based on methodology described by Nour 1998, Dobson et al. and Magalhães, Floresta and Moraes. Samples showing three different bands were submitted to rep-PCR (GTG)₅ reaction, and the results were expressed in the dendrogram shown in Figure 1. Samples were clustered in accordance to the results of Pearson correlation. One sample of each clustered group with similarity values over 90% was selected for 16S rRNA sequencing, in accordance to methodology described by Van Hoorde, Vandamme and Huys [6].

Discussion

LAB samples associated with raw milk are crucial elements that influence the quality, flavor, peculiarity and authenticity of cheeses. Several factors such as cattle feeding, milk composition, good manufacturing practices and environmental characteristics of the region may determine the diversity and dynamics of microbial communities associated with artisanal cheeses, as well as sensorial properties and the quality of the final product. As physical-chemical characteristics of the cheese are directly linked to the LAB activity, it is more than necessary to taxonomically establish lineages and bacterial groups that could potentially contribute to enhance cheese sensorial aspects [6-8].

In the present study, *L. plantarum*, *L. brevis*, *L. rhamnosus*, *L. paracasei* e *P. acidilactici* were present in different sources and dairy farms. The *L. plantarum* specie was dominant alongside the entire fingerprinting analysis. In a study performed by Van Hoorde, Vandamme e Huys, *L. paracasei*, *Lactococcus lactis* subsp. *lactis*, *L. plantarum*, *Pediococcus pentosaceus*, *L. brevis*, *L. rhamnosus*, *L. curvatus*, *Enterococcus faecalis*, *Enterococcus durans* L. *perolens* were identified by rep-PCR-(GTG)₅ and 16S rRNA sequencing in traditional raw milk cheeses Bellie and Dulsels. Pogacic et al. identified *Lactobacillus rhamnosus*,

Lactobacillus casei, *Lactobacillus paracasei*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus* e *Pediococcus acidilactici* as predominant microbiota in Grana Padano cheese, also made with raw milk and endogenous starter culture, as predominant microbiota. As shown in Table 1, *L. plantarum* was identified in every source, except cheese with 28 days of ripening and silage, although it was the only species found in milk. This endorses the idea that *L. plantarum* is a predominant LAB in the region.

Regarding the Minas artisanal cheese, Borelli et al. found *L. plantarum*, *L. casei*, *L. brevis*, *L. rhamnosus*, *Enterococcus faecalis*, *P. acidilactici* and *Weissella paramesenteroides* throughout ripening time in Minas artisanal cheeses from Serra da Canastra region. Assessing LAB profile via DGGE in Minas artisanal cheeses from four distinct regions (Serra, Cerrado, Serra da Canastra and Araxá), Arcuri et al. [9] found *Streptococcus salivarius*, *Streptococcus thermophilus*, *L. plantarum* and *Lactococcus lactis* as predominant microorganisms. At 65% of similarity, DGGE clustering showed a clear distinction between cheese LAB samples from different regions, although detecting low diversity regarding species-level. In the present study, low diversity regarding LAB samples was observed, but with a clear distinction in two major clusters at a higher similarity value (90%), indicating greater divergences from subsequent lineages or samples.

LAB samples such as *L. plantarum* from certified dairy farms (A, B and C) were detected in the two major clusters, while non-certified dairy farms (D, E and F) showed samples restricted in a unique cluster. Even if the second major cluster showed higher diversity for species, much of this diversity derived of samples originated from certified dairy-farms. This indicates that these dairy farms may develop greater bacterial diversity regarding the environment in which the artisanal cheeses are produced, probably due to better manufacturer proceedings.

Pediococcus acidilactici was isolated from cheeses originated from dairy farm C and dairy farm B, both presenting isolated clusters allocated in the second major cluster. Isolation and molecular identification of *P. acidilactici* in Minas artisanal cheeses was not yet related, although many studies had described the presence of this species in artisanal cheeses. Settanni and Moschetti [10] described interesting data regarding detection of *L. paracasei*, *L. rhamnosus* and *P. acidilactici* and their role as non-starter LAB (NSLAB), developing in advanced stages of cheese ripening. Thus, silage could possibly be acting as an NSLAB inoculator agent, which can be demonstrated with samples of silage and cheese with 28 days of ripening from dairy farm C, where *L. paracasei* was isolated. Although, more studies are necessary regarding the dynamic in microbial populations within these cheeses. According to Pogacic et al. [11], these NSLAB may represent only a small part of microbial populations in environments with high diversity such as milk and cheese, being hard to establish solid evidence that extrapolates the concept of microbial community of these microorganisms.

Samples of *L. plantarum*, *L. paracasei* and *L. brevis* were detected in water, milk, endogenous starter culture and, specifically *L. plantarum*, throughout many ripening stages in different dairy farms. This can be explained due to their role as NSLAB, developing in later stages of ripening regarding higher levels of proteolysis and development of flavor, as well as reducing pathogenic or spoiling microorganisms in cheeses [12-19].

Detection of *L. plantarum* in endogenous starter culture can be compared to data from Poveda, Cabezas and Mc Sweeney, which consisted in inoculation of a starter culture of *L. plantarum* in Manchego cheeses. It was observed a higher concentrations of free amino acids in cheese curd with *L. plantarum* in contrast of cheeses made with a commercial starter culture. Terzic-

Vidojevic et al. identified that *L. plantarum* samples from raw milk artisanal goat cheese from Serbian showed important features in physical-chemical properties such as production of EPS and higher proteolytic activity, implying that this microorganism could be applied in starter cultures for cheese manufacturing.

Conclusion

Lactobacillus plantarum was the most abundant amongst other LAB, showing high similarity phylogenetically, despite its source. Certified dairy-farms showed high LAB diversity, probably due to good manufacturer proceedings. Water, silage and endogenous starter culture could act as disseminating agents in the environment and cheese, enhancing flavor sensorial aspects in the final product (Table 1).

Table 1: Distribution of LAB samples isolated from silage, water, endogenous starter culture, milk and cheeses from Campo das Vertentes region.

Source	Species				
	<i>L. Plantarum</i>	<i>L. paracasei</i>	<i>L. brevis</i>	<i>L. rhamnosus</i>	<i>P. acidilactici</i>
Cheese 0 d	D; F; G		C		
Cheese 7d	A; C				
Cheese 14d	A;B;C		A		
Cheese 21d	A;C;E				
Cheese 28d		C			
Cheese 60d	A;B;D				
Starter culture	E;F				
Silage				B	B
Water	D		D		
Milk	D				

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