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Bacterial ecology of artisanal Minas cheeses assessed by culture-dependent and -independent methods

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ABSTRACT

Artisanal Minas cheese is produced in Minas Gerais state, Brazil and its varieties are named according to their geographical origin (Serro, Canastra, Serra do Salitre, Araxá and Campo das Vertentes). The cheese is produced with raw cow's milk and the whey from the previous cheese production ("pingo"). The high economic and cultural importance of artisanal cheese in Brazil justifies the efforts to ensure its safety, quality and provenance. This study aimed to characterize the microbial diversity composition, and geographical distribution of artisanal Minas cheese, focusing on the characterization of its autochthonous lactic acid bacteria (LAB) microbiota. Artisanal Minas cheese varieties from Serro, Canastra, Serra do Salitre, Araxá and Campo das Vertentes were analyzed by culture-dependent (culturing and LAB sequencing) and -independent (repetitive extragenic palindromic-PCR (rep-PCR) and length heterogeneity-PCR, LH-PCR) methods to characterize the microbiota. The microbial counts were variable between cheese samples, and some samples presented high number of coagulase positive bacteria and coliforms that may be associated with hygienic issues. In all samples was observed a prevalence of LAB. 16S rRNA sequencing and rep-PCR of the LAB strains identified four genus (*Lactobacillus*, *Lactococcus*, *Enterococcus* and *Weissella*), ten species and more than one strain per species. *Lactobacillus* was the most prevalent genera in all the cheeses. LH-PCR revealed a further six genera and ten species that were not identified by culturing, highlighting the importance of combining both culture-dependent and -independent methods to fully characterize microbiota diversity. Principal component analysis of the LH-PCR data and cluster analysis of rep-PCR data revealed that the artisanal Minas cheese microbiota was influenced not only by their geographical origin but also by the cheese farm. The lack of standardization in the milking and cheese manufacturing procedures between artisanal cheese farms could explain the microbial diversity.

1. Introduction

Artisanal Minas cheese in Minas Gerais state, Brazil is manufactured using a traditional empirical approach. The artisanal cheese is produced using raw milk, with the addition of "pingo" (the natural lactic starter culture) obtained from the whey of the previous cheese production, and chymosin, then ripened for 5–10 days under the environmental conditions of the cheese farm. Despite involving similar production steps, the sensory characteristics of each variety are influenced by the microbiota diversity, which is a result of the environmental characteristics of each region (EMATER, 2016).

The production of artisanal Minas cheese began to be regulated in 2002 with a specific state legislation (Minas Gerais, 2002) and later recognized as an immaterial heritage of Minas Gerais state by IPHAN (National Institute of Historical and Cultural Heritage, 2008). Considering these guidelines, artisanal cheeses are named according to the region in which they are produced, such as Serro, Canastra, Serra do Salitre, Araxá and Campos das Vertentes, among others. These cheeses are simple and inexpensive to produce. They are highly appreciated by consumers, and have an important social, economic and cultural significance, hence the interest in preserving

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artisanal Minas cheese production in Brazil. Most studies related to artisanal Minas cheese are either focused on its technological and sensory characteristics or on the occurrence of specific microorganisms or pathogens (Brito et al., 2008; Nogueira et al., 2005; Pinto et al., 2009; Sant'Ana et al., 2013). To our knowledge, only one study has investigated the bacterial ecology of Minas cheese. In that study, the culture-independent method, denaturing gradient gel electrophoresis-polymerase chain reaction (DGGE-PCR), was applied (Arcuri et al., 2013). Microbial ecology characterization of complex food matrices, such as raw milk cheeses, provides important knowledge regarding its safety, the interference of production technology and its geographic origin distinction.

Microbiota characterization using culture-dependent methods may not reveal the entire microbial diversity of complex environments. Stressed and injured cells are not identified and small microbial populations are often inhibited by those numerically more abundant (Cocolin et al., 2007). Advanced detection and identification of microorganisms in complex food matrices are achieved by culture-independent methods based on molecular biology techniques, such as length heterogeneity analysis of PCR-amplified genes (LH-PCR) and PCR amplification of repetitive extragenic palindromic sequences (rep-PCR). LH-PCR has proved suitable for profiling microbial communities in grapevine leaves (Bulgari et al., 2009), soil (Moreno et al., 2011) and cheese (Bulgari et al., 2009; Gatti et al., 2008; Lazzi et al., 2004; Moreno et al., 2011; Pogačić et al., 2013). Within the complex bacterial community of traditional raw milk cheeses, lactic acid bacteria (LAB) have a crucial role in cheese making and ripening, impacting cheese texture, flavor and aroma (Montel et al., 2014; Morandi et al., 2011). They also contribute to the microbial stability of the final product by inhibiting the development of spoilage and pathogenic microorganisms (Cotter et al., 2005; Perin et al., 2015; Picon et al., 2015).

The present study aimed to use culture-dependent and -independent methods to characterize the microbial diversity of five artisanal Minas cheese varieties (Serro, Canastra, Serra do Salitre, Araxá and Campo das Vertentes), focusing the characterization of their autochthonous lactic acid bacteria microbiota.

2. Material and methods

2.1. Minas cheese samples

Two to four samples of artisanal Minas cheeses from Serro, Serra do Salitre, Araxá, Canastra and Campo das Vertentes (Fig. 1) were purchased from local retail stores within the state of Minas Gerais, Brazil. The samples were stored at 4 °C, until analysis.

2.2. Culture-dependent methods

2.2.1. Microbial enumeration and lactic acid bacteria isolation

Portions (25 g) of each cheese were homogenized in 225 mL of 0.1% saline peptone solution (pH 7.0) using a Stomacher® 400 Circulator (Seward Ltd., Worthing, England) for 5 min, and then plated onto selective media for enumeration of the following microbial groups: aerobic mesophilic bacteria on Petrifilm™ AC (3M, St. Paul, MN, USA) at 35 °C for 48 h; coliforms and *Escherichia coli* on Petrifilm™ EC (3M) at 35 °C for 48 h; LAB on de Man, Rogosa and Sharpe agar (MRS, Oxoid Ltd., Basingstoke, England) at 35 °C for 48 h; and coagulase-positive cocci on fibrinogen rabbit plasma agar (bioMérieux, Marcy l'Étoile, France) at 35 °C for 48 h. The results were expressed as log CFU/g.

Based on colonies morphology (shape, size and colors), representative colonies were selected from each sample (about 10% of the observed count) on the MRS plates and assessed by Gram stain and catalase test. A total of 125 isolates were kept in MRS broth (Oxoid), containing 20% (v/v) glycerol, at - 80 °C, prior to fingerprinting and identification, as described in section 2.2.2.

2.2.2. LAB fingerprinting and identification

LAB isolates were cultured in MRS broth (Oxoid) and incubated at 35 °C for 12 h. The cultures (1 mL) were then centrifuged (10,000 × *g*, 5 min) and their DNA isolated using the Genomic Wizard DNA Purification Kit (Promega Corp., Madison, WI, USA).

rep-PCR was performed using DNA extracted from the 125 LAB strains. The PCR reactions were performed according to a modified procedure of Gevers et al. (2001), using a single primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′). The PCR reactions contained 10 μL of Go Taq Master Mix 2x (Promega), 50 pMol of the primer, 2 μL of DNA (50 ng/μL) and ultra-pure PCR water (Promega) to a final volume of 20 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 final extension at 65 °C for 16 min. The PCR products were electrophoresed on agarose gels (2% w/v) in 1 × tris/borate/EDTA buffer (TBE) at constant voltage (95 V) for 3 h. A 1 kb DNA ladder (Sigma-Aldrich) was used as a molecular size marker. Fingerprints were compared by cluster analysis using BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between the strains profiles were calculated using the Dice correlation coefficient and dendrograms constructed by cluster analysis (unweighted pair group method with arithmetic mean, UPGMA).

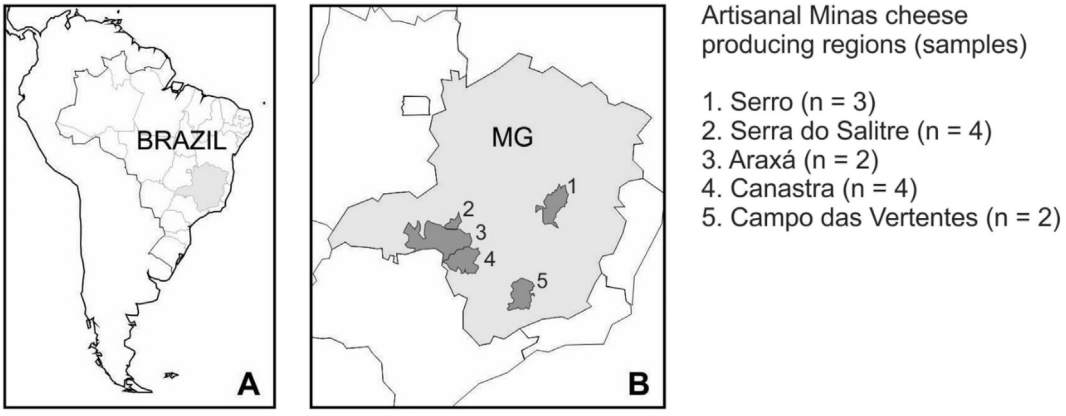

Fig. 1. A. Localization of Minas Gerais state in Brazil and South America (grey area). B. Localization of artisanal Minas cheese regions in Minas Gerais state, and number of samples collected for the present study. 1: Serro, 2: Serra do Salitre, 3: Araxá, 4: Canastra, 5: Campo das Vertentes.