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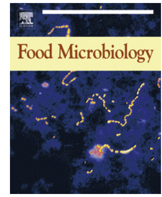

Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese

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ABSTRACT

A total of 114 lactic acid bacteria were isolated at one and 21 days of ripening from a traditional raw cow's milk cheese without the addition of starter culture, produced by three artisanal cheese-makers in Azores Island (Pico, Portugal). Identification to species and strain level was accomplished by16S rRNA gene and PFGE analysis. Carbohydrate utilization profiles were obtained with the relevant API kits. Isolates were evaluated according to safety and technological criteria. The most frequently observed genus identified by 16S rRNA sequencing analysis was *Enterococcus*, whereas API system mostly identified *Lactobacillus*. The highest percentages of antibiotic resistance were to nalidixic acid (95%), and aminoglycosides (64–87%). All isolates were sensitive to several beta-lactam antibiotics and negative for histamine and DNase production. Gelatinase activity was detected in 49.1% of isolates, 43% were able to degrade casein and 93% were α-hemolytic. Most enterococci presented virulence genes, such as *gelE*, *asaI*, *ace*. Diacetyl production was found to be species dependent and one strain (*Leu. citreum*) produced exopolysaccharides. Selected strains were further studied for technological application and were found to be slow acid producers in milk and experimental cheeses, a desirable trait for adjunct cultures. Two strains were selected on the basis of technological and safety application as adjunct cultures in cheese production and presented the best cheese aroma and flavor in consumer preference tests. This is the first effort to characterize Pico cheese LAB isolates for potential application as adjunct cultures; the results suggest the potential of two strains to improve the quality of this traditional raw milk product.

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1. Introduction

Due to their unique taste, artisanal cheeses produced through spontaneous fermentation of unpasteurized milk receive a great deal of attention from consumers around the world. Pico cheese is an artisanal semi-hard cow's milk cheese produced in Azores Island (Pico, Portugal) and is made on small scale using traditional practices involving the addition of animal rennet and salt to raw milk and no starter addition. It has a very flat cylindrical shape (16–17 cm wide and 2–3 cm height), a distinct mild flavour and a very short maturation time (21 days). Pico cheese has its place in the niche products that have been recognized by the European Community with the attribution of the Protected Denomination Origin status (European Union, 2006).

In the traditional Pico PDO all the phases of processing are manual. The manufacturing process starts with filtration of raw cow milk through a cheesecloth and addition of calf rennet. Coagulation is allowed to proceed for 45–60 min at 26–30 °C after which the coagulum is cut, the whey is drained off and the curd is wrapped and manually pressed from both sides, in order to produce a smooth appearance. Cheese salting is done by adding salt to each face of the cheese. On the following day after manufacture, cheeses are placed in cold storage at 10–14 °C and relative humidity of 80–85%, for ripening during 20 days.

The lactic acid bacteria (LAB) belong to a large family of fermentative Gram-positive, non-sporulating, micro-aerophilic bacteria that produce lactic acid as the main fermentation product from glucose, and they occur naturally as indigenous microbiota in raw milk. Their presence contributes to the large differences in the organoleptic, biochemical and flavour characteristics of

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artisanal dairy products (Bao et al., 2012; Colombo et al., 2009; Moraes et al., 2013; Randazzo et al., 2006). There have been many reports describing the microbiota of different raw milk cheeses made by artisanal manufacturing (Casalta et al., 2009; Ostlie et al., 2005; Ouadghiri et al., 2005). Lactobacilli generally dominate the non-starter lactic acid bacteria (NSLAB) population in most cheeses (Bouton et al., 1998; Fitzsimons et al., 1999; Swearingen et al., 2001). However, *Enterococcus faecalis* was found to be the predominant species detected in some artisanal cheeses (Franz et al., 1999; Giraffa et al., 1997; Nieto-Arribas et al., 2011). Other LAB species, *Lactococcus lactis, Enterococcus faecium, Leuconostoc mesenteroides* subsp. *mesenteroides and Leu. mesenteroides* subsp. *dextranicum*, have also been found in the microbiota of artisanal raw ewe's milk cheeses (Manolopoulou et al., 2003; Nieto-Arribas et al., 2010; Terzic-Vidojevic et al., 2009).

To analyze and rapidly identify microbial communities, conventional phenotypic, biochemical and physiological tests have been used on cheese isolates (Fortina et al., 2003; Moraes et al., 2013). Although these conventional methods have proven to be useful and an indispensable tool for LAB characterization, they are not fully reliable due to the similar nutritional and growth requirements of LAB (Colombo et al., 2009; Fortina et al., 2003; Ostlie et al., 2005). In addition, phenotypic methods are limited in terms of both discriminating ability and accuracy, so distinguishing strains always requires DNA-based techniques. Identification by the 16S rDNA gene sequence analyses has proven useful for differentiating a wide range of LAB at species level (Bao et al., 2012; Colombo et al., 2009; Fortina et al., 2003; Moraes et al., 2013). Moreover, the combination of phenotypic and molecular methods has become the preferred approach for determining and analyzing the species composition of targeted microbial communities (Kesmen et al., 2012; Ostlie et al., 2005).

Numerous studies have shown that autochthonous microorganisms in fermented foods may improve safety, technological and sensory properties, and also shelf-life of dairy products (Abriouel et al., 2008; Bao et al., 2012; Moraes et al., 2013). However, the demand of the dairy industry for more uniform and standardized products led to a growing concern for the loss of typical indigenous microbial populations present in artisanal cheeses. While artisanal Pico cheese has important organoleptic features that must be preserved, limited data exist on the composition of this cheese microbiota (Riquelme et al., 2015).

The microbiological characterization of isolated LAB from Pico cheese constitutes an important step towards the preservation of the autochthonous bacterial heritage that provides specific and unique characteristics to this cheese. In addition, safety assessments with regard to virulence traits and antibiotic resistance remain essential in the selection of LAB for application in food production. Therefore, the objective of present study was to identify and characterize LAB isolated from Pico cheese in order to select appropriate candidates for use as adjunct cultures. Strains that were considered safe after this first screening and exhibited good technology characteristics were further investigated for acid production, growth dynamics in the cheese environment and desirable sensory characteristics in experimental fresh cheeses.

2. Materials and methods

2.1. Isolation of LAB

A total of 12 artisanal Pico cheeses from two production batches, three artisanal cheese-makers and two ripening days (1 and 21) were collected from the processing plants, during the summer season. Cheese samples (25 g) were diluted in 225 ml of buffered peptone water (Biokar, Beauvais, France) and homogenized with a Stomacher Lab-Blender 400 (Seward Medical, London, UK). Serial dilutions in sterile peptone water were prepared and plated in duplicate on the adequate media for the isolation of ten colonies from each countable dilution. The media used to obtain pure cultures were as follows: presumptive enterococci - KF Agar (Biokar, Beauvais, France), aerobic incubation at 43 °C, for 48 h; presumptive leuconostocs - MSE Agar (Biokar), aerobic incubation at 21 °C, for 4 days; presumptive lactococci - M17 Agar (Biokar), aerobic incubation at 30 °C, 72 h; and presumptive lactobacilli - Rogosa Agar (Merck), under anaerobic conditions using an AnaeroGen kit (Oxoid, Milan, Italy), at 30 °C, for 5 days.

All the isolates matching the basic traits of the LAB group, non-spore forming, Gram-positive, catalase and oxidase-negative, were considered for identification. The isolates were routinely propagated in MRS broth (AES, Bruz, France), under aerobic conditions and stored at −20 °C and −80 °C in liquid medium (MRS broth) supplemented with 30% (vol/vol) glycerol as a cryoprotectant.

2.2. Identification of isolates

2.2.1. Phenotypic identification

Overnight cultures of isolates grown on MRS agar were submitted to Gram staining and tested for catalase production and oxidase activity as described by Kozaki et al. (1992). All the isolates matched to the basic traits of the LAB group, non-spore forming, Gram-positive, catalase and oxidase-negative were considered for identification. The phenotypic characteristics of 114 isolates were studied using the API 50 CHL (75 isolates from MSE, M17 and Rogosa Agar) and API 20 Strep (39 isolates from KF Agar) kits (BioMérieux, Marcy-l'Etoile, France), following manufacturer's instructions.

2.2.2. PCR amplification and sequence of 16S rRNA gene

Single colonies were sub-cultured, under aerobic conditions, for 24 h in MRS broth at 30 °C and aliquots of 1 mL were transferred to microcentrifuge tubes and centrifuged at 14,000 *g* for 2 min. The cell pellet was submitted to total genomic DNA extraction using the UltraClean® Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the supplier's specifications, and stored at −20 °C. Fragments of 1363–1388 bp of the eubacterial 16S rRNA sequence coding region were amplified by polymerase chain reaction (PCR) using the universal bacterial primers 46F (5′-GCYTAAYACATGCAAGTCG-3′) and 1409R (5′-GTGACGGGCRGTGTGTRCAA-3′) (Northup et al., 2010) for LAB isolates and positive control *Escherichia coli* ATCC 25922. The PCR reaction was performed in a final 20 μl reaction volume containing 1 × Taq Polymerase reaction buffer, 1.8 mM of $MgCl_2$, 0.25 mM of dNTP (Applied Biosystem, Foster City, Ca., USA), 0.38 pmol of each primer, 1U *Taq* DNA polimerase (Fermentas, Life Technologies, Waltham, MA) and 25 ng of DNA template. The amplification program consisted of an initial denaturing step at 94 °C for 3 min followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and elongation at 72 °C for 90 s and one cycle of final extension at 72 °C for 10 min, performed in a thermocycler (TProfessional, Biometra, Germany). The 16S rDNA amplicons were analyzed by electrophoresis on 1.5% (wt/vol) agarose gel with 5 μl of SYBR® Safe DNA gel stain (Invitrogen, Life technologies, USA) at 120 V for 45 min in 1 × TAE buffer (2 mol/L Tris base, 1 mol/L acetic acid, 0.05 mol/L EDTA pH 8.0) and visualized by UV light. The molecular marker used was Gene Ruler™ 1 kb plus DNA Ladder (Fermentas, Life Sciences).

The PCR products were purified by gel filtration and sequenced with both primers *Foward* (46F) and *Reverse* (1409R) on an ABI PRISM® 3730XL Sequencer (Applied Biosystems, Foster City, USA) by StabVida (Investigação e Serviços em Ciências Biológicas, Lda, Portugal).

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