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Biodiversity of dairy *Propionibacterium* isolated from dairy farms in Minas Gerais, Brazil

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ABSTRACT

Dairy propionibacteria are used as ripening cultures for the production of Swiss-type cheeses, and some strains have potential for use as probiotics. This study investigated the biodiversity of wild dairy *Propionibacteria* isolates in dairy farms that produce Swiss-type cheeses in Minas Gerais State, Brazil. RAPD and PFGE were used for molecular typing of strains and MLST was applied for phylogenetic analysis of strains of *Propionibacterium freudenreichii*. The results showed considerable genetic diversity of the wild dairy propionibacteria, since three of the main species were observed to be randomly distributed among the samples collected from different farms in different biotopes (raw milk, sillage, soil and pasture). Isolates from different farms showed distinct genetic profiles, suggesting that each location represented a specific niche. Furthermore, the STs identified for the strains of *P. freudenreichii* by MLST were not related to any specific origin. The environment of dairy farms and milk production proved to be a reservoir for *Propionibacterium* strains, which are important for future use as possible starter cultures or probiotics, as well as in the study of prevention of cheese defects.

1. Introduction

The genus *Propionibacterium* is divided into two groups, namely "cutaneous" *Propionibacterium*, primarily isolated from human skin and commonly associated with infections, and "classical" or "dairy" *Propionibacterium*, isolated from dairy products and particularly relevant for food industries (Cummins and Johnson, 1986; McDowell et al., 2012). However, both groups can be found in other biotopes for example, classical propionibacteria have been isolated also from silages and vegetables (Mantere-Alhonen and Ryhänen, 1994), ruminal content and feces of cows and calves (Rinta-Koski et al., 2001) and the intestine of pigs and laying hens (Argañaraz-Martínez et al., 2013), whereas cutaneous propionibacteria have been isolated also from the intestine of humans, chicken and pigs (Argañaraz-Martínez et al., 2013; Cummins and Johnson, 1986; Macfarlane et al., 1988). *Propionibacterium freudenreichii* was the first propionic acid-producing bacteria isolated from Emmenthal cheese (Von Freudenreich and Orla-Jensen, 1906). Later, *Propionibacterium acidipropionici*, *Propionibacterium jensenii*, and *Propionibacterium thoenii* were classified as dairy species after having been isolated from milk and dairy products, as well as from soil, silage, and dairy plants (Cummins and Johnson, 1992). However in the last years, the new species *Propionibacterium* (Kusano et al., 1997) and *Propionibacterium microaerophilum* (Koussémon et al., 2001) were identified. Although they do not fit exactly to dairy biotopes, based on homology studies they were allocated into the classical group (Rossi et al., 2006).

The dairy propionibacteria, particularly *P. freudenreichii*, have an important role in the Swiss cheese production process, as their metabolism positively contributes to the flavor and development of the typical holes during ripening (Falentin et al., 2010; Langsrud and Reinbold, 1973). Furthermore, *Propionibacterium* spp. are able to produce a wide variety of biological compounds that enhance the human health like folic acid, proline, conjugated linoleic acid (CLA) and vitamin B12 (Hugenholtz et al., 2002; Iida et al., 2007), and synthesize several different bioprotective compounds such as bacteriocins or antifungal compounds (Ho et al., 2009; Lind et al., 2007). Some strains produce bifidogenic compounds and present the ability to survive and maintain activity during passage of the digestive tract, and have thus relevance for potential use as probiotic cultures (Cousin et al., 2010; Hervé et al., 2007; Uchida et al., 2011). However, *P. jensenii* and *P. thoenii* are commonly associated with problems in cheese production, such as formation of red or brown spots, pigmentation, or excessive production of gas (CO_2) (Carcano

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et al., 1995; de Carvalho et al., 1995; Fessler et al., 1999). The level of *P. freudenreichii* added to the cheese was also related to the formation of brown spots (Baer et al., 1993).

Propionibacteria have low nutritional requirements and maintain their viability under various environmental conditions (Thierry et al., 2011). At the moment, genetic information related to the ecologic adaption capacity of dairy propionic bacteria is largely unknown and only few studies have reported the biodiversity in different surroundings (niches). Some studies have shown that the genus is homogenous and that *P. freudenreichii* forms an isolated cluster among the dairy species (Britz and Riedel, 1991; Charfreitag and Stackebrandt, 1989). Molecular typing has been used for the identification and differentiation of bacteria, as well as in research on the different aspects related to microbial adaptation in different environments. The characterization of dairy *Propionibacterium* strains by molecular typing allows the traceability of strains with industrially useful properties such as the production of aroma or functional compounds. Some genotypic methods, such as random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE), have been used to study the biodiversity of microorganisms (Kaufmann, 1998; Perry et al., 2003; Rossetti and Giraffa, 2005). The multilocus sequence typing (MLST) technique has been used in studies where information about the historic evolution is considered necessary (Maiden et al., 1998).

The present study aimed to characterize the biodiversity of *Propionibacterium* in Brazilian dairy farms that produce Swiss-type cheeses, in order to check the main origin of these microorganisms and their diversity at species and strain level.

2. Material and methods

2.1. Reference strains

Five reference strains of *Propionibacterium* spp. (*Propionibacterium* spp. [CIRM-BIA1426], *P. freudenreichii* [CIRM-BIA703^T], *P. jensenii* [CIRM-BIA39^T], *P. thoenii* [CIRM-BIA1792], and *Propionibacterium acidipropionici* [CIRM-BIA38^T]) were obtained from the International Centre for Microbial Resources collection of bacteria (CIRM-BIA, UMR1253, INRA, Rennes, France). These strains were used as positive controls for the identification of isolates by polymerase chain reaction (PCR).

In addition, 12 strains from the CIRM-BIA, present in the MLST scheme for *P. freudenreichii* (Dalmasso et al., 2011), were used for the molecular characterization by PFGE and MLST.

2.2. Collection of Propionibacterium strains from Brazilian food systems

Samples of raw milk (n = 5), soil (n = 5), silage (n = 4), and pasture (n = 4) were aseptically collected in five dairy farms in Campo das Vertentes (Minas Gerais, Brazil) and kept under refrigeration until analysis. The number of samples and the geographical position of the farms were decided together with the Association of Dairy Farms and Dairy Industries from Campos das Vertentes MG — EMATER-MG. The raw milk samples were diluted in 0.85% NaCl (w/v). For solid samples, 25 g of each sample was obtained with a sterile knife and transferred to individual sterile bags containing 225 mL of 0.85% NaCl (w/v), homogenized, and diluted in the same solution. From each sample, the dilutions were pour-plated in duplicate in lithium glycerol (LG) agar, as described by Madec et al. (1994), containing a mixture of commercial antibiotics (Pal-propiobac™, Laboratoire Standa, Caen, France). The plates were incubated at 30 °C for 6 days under anaerobic conditions (Anaerocult, Merck KGaA, Darmstadt, Germany). After incubation, 10% of colonies were randomly picked from the highest dilutions and submitted to microscopy observation (Nikon optiphot phase contrast equipment) in order to discard isolates for which the morphological criteria were not consistent with propionibacteria (nonmotile, nonsporeforming and rod-shaped or branched, singularly, in pairs, or in groups). Isolates were then purified by streaking (three successive times) on yeast extract lactate (YEL, Oxoid Ltd., Basingstoke, England) plates and incubated at 30 °C for 5 days under anaerobic conditions. Purified isolates were stored at −80 °C in isolation broth media (de Man Rogosa and Sharpe [MRS] or Brain and Heart Infusion added to yeast extract [BHI-YE], Oxoid) supplemented with 30% (v/v) glycerol. Prior to molecular analysis, the isolated colonies were transferred in YEL broth and incubated at 30 °C for 2 days.

2.3. Molecular identification of Propionibacterium spp.

The purified isolates were subjected to DNA extraction using the DNeasy Kit (QIAGEN, Courtaboeuf, France). The identification of isolates of *Propionibacterium* spp. was performed by genus-specific and species-specific PCR according to Tilsala-Timisjärvi and Alatossava (2001).

2.4. RAPD genotyping of Propionibacterium strains

The oligonucleotide primer M13 (5′GAGGGTGGCGGTTCT-3′) was used for RAPD analysis. The PCR reactions contained 2.5 μL of Taq buffer (with 2 mM $MgCl_2$), 0.5 μL of Taq DNA polymerase (Qbiogene, Burlingame, California, USA), 0.51 μL of primer (100 μM), 1.0 μL of dNTP, 1.5 μL of DNA (at a minimal concentration of 25 ng/μL), and PCR water (Sigma, St. Louis, USA) to a final volume of 50 μL. The PCR conditions were: initial denaturation at 94 °C for 2 min; 40 cycles at 94 °C for 1 min, 42 °C for 20 s, and 72 °C for 2 min with a final extension step at 72 °C for 10 min.

Gels were stained in GelRed (3× in 0.1 M NaCl solution) (FluoProbes, Interchim, France) and were scanned for analysis using BioNumerics, version 4.1 (Applied Maths, Kortrijk, Belgium). Comparisons between the normalized band profiles were made using the Dice similarity coefficient (1%). The compiled matrix was used for cluster analysis using the arithmetic average (UPGMA) clustering algorithm.

2.5. Characterization of Propionibacterium strains by PFGE

Propionibacterium strains were grown to an optical density (OD_{650}) of 0.3 in YEL broth (Malik et al., 1968). Cells were harvested by centrifugation (3500 ×g, 10 min) from 10 mL of culture, the supernatant was discarded and TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 10 mg/mL of lysozyme (Sigma, St. Louis, MO, USA) was added to the pellet. After incubation at 37 °C for 1 h, the suspension was heated at 50 °C, and 700 μL of 0.5% (w/v) ultrapure agarose (Gibco-BRL, paisley, UK) in 125 mM EDTA (pH 7.0) at the same temperature was added before solidifying the suspension in molds. After 15 min at 4 °C the solidified agarose block was transferred into a solution of deproteinising buffer (10 mM Tris-base, 100 mM EDTA, pH 8.0, 10% SDS, 20 mg/mL Proteinase K Qiagen) for 2 h at 55 °C. The agarose blocks were washed in water for 10 min and four times in 20 mM of TE buffer (pH 8.0) for 10 min per wash. The agarose blocks were conserved at 4 °C in TE Buffer (pH 8) until digestion.

For DNA digestion, the agarose blocks were equilibrated for 1 h at 4 °C in restriction buffer (Cut Smart™ buffer, New England Biolabs, Evry Cedex, France). Restriction enzyme digestion with 20 units of enzyme *Xba*I (Bioloabs) was performed at 37 °C for 4 h. Electrophoresis was carried out in 0.5 × TBE buffer (45 mM L^{-1}, 45 mM boric acid, 1 mM EDTA, pH 8, Sigma) in a 1% (w/v) agarose gel (PFGE certified agarose, Bio-Rad) with a pulse time of 2 to 20 s, voltage of 6 V/cm, for 20 h at 14 °C, using a CHEF DR II apparatus (Bio-Rad, Hercules, CA, USA). The gel was stained with GelRed (3× in 0.1 M NaCl solution) (Fluoprobes, interchim, Montluçon, France) and visualized under UV light. Photographs of PFGE gels were scanned, and the band profiles were analyzed using BioNumerics, version 4.1 (Applied Maths, Kortrijk, Belgium). Comparisons between the normalized band profiles were made using the Dice similarity coefficient (1%). The compiled matrix was used