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Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains

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ABSTRACT

A total of 34 lactic acid bacteria isolates from 4 different Brazilian kefir grains were identified and characterized among a group of 150 isolates, using the ability to tolerate acidic pH and resistance to bile salts as restrictive criteria for probiotic potential. All isolates were identified by amplified ribosomal DNA restriction analysis and 16S rDNA sequencing of representative amplicons. Eighteen isolates belonged to the species *Leuconostoc mesenteroides*, 11 to *Lactococcus lactis* (of which 8 belonged to subspecies *cremoris* and 3 to subspecies *lactis*), and 5 to *Lactobacillus paracasei*. To exclude replicates, a molecular typing analysis was performed by combining repetitive extragenic palindromic-PCR and random amplification of polymorphic DNA techniques. Considering a threshold of 90% similarity, 32 different strains were considered. All strains showed some antagonistic activity against 4 model food pathogens. In addition, 3 *Lc. lactis* strains and 1 *Lb. paracasei* produced bacteriocin-like inhibitory substances against at least 2 indicator organisms. Moreover, 1 *Lc. lactis* and 2 *Lb. paracasei* presented good total antioxidative activity. None of these strains showed undesirable enzymatic or hemolytic activities, while proving susceptible or intrinsically resistant to a series of clinically relevant antibiotics. The *Lb. paracasei* strain MRS59 showed a level of adhesion to human Caco-2 epithelial cells comparable with that observed for *Lactobacillus rhamnosus* GG. Taken together, these properties allow the MRS59 strain to be considered a promising probiotic candidate.
Key words: kefir grain, lactic acid bacteria, lactobacilli, probiotic property

INTRODUCTION

Kefir is a fermented milk product originating from the Northern Caucasus. The name kefir is derived from the Turkish language word keyif, meaning "good feeling" for the feelings experienced after drinking it (Leite et al., 2013b). The fermented beverage is acidic, viscous, slightly carbonated, and presents small amounts of alcohol (Leite et al., 2013a). Traditionally, kefir is made by using kefir grains as a starter (Leite et al., 2013a,b). Kefir grains are white to yellowish, cauliflower-like grains, 0.3 to 3.5 cm in diameter, with a slimy but firm texture. The grains are composed of an inert matrix made up of polysaccharides and proteins. The matrix is densely populated by lactic acid bacteria (**LAB**) species, acetic acid bacteria, and yeasts (Leite et al., 2012, 2013b).

Kefir beverage has a long tradition of consumption in Eastern Europe, and it is now spreading around the world due to its potential health-associated properties. Although some of the reported health benefits do not yet have well-documented scientific evidence or clinical demonstration, several in vitro and animal studies have associated kefir beverage with alleviation of lactose intolerance (Hertzler and Clancy, 2003), immunomodulation (Hong et al., 2009), antimicrobial activity against pathogenic microorganisms (Chifiriuc et al., 2011), and balance of the intestinal microbiota (Urdaneta et al., 2007). Traditionally, the functional properties of kefir have been attributed mainly to its bioactive peptide content and to kefiran, its main soluble exopolysaccharide (Santos et al., 2003; Rodrigues et al., 2005). However, the potential beneficial effects might also be mediated by the undefined microbial composition of this fermented milk or by the secondary metabolites (Nielsen et al., 2014).

Although a reasonable number of well-characterized probiotic strains are commercially available around the world, screening for novel strains is still of great interest from an industrial point of view (Vinderola et al., 2008; Ayeni et al., 2011). Additionally, strains ex-

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pressing unique and particular characteristics that may enable health benefits may arise in the characterization of natural fermented dairy products such as kefir. This traditional product might be an interesting source of LAB strain with specific functional properties. Even though many authors advocate the importance of human origin as a selective criterion for the search of probiotic strains, an expert panel proposed by FAO/WHO (2006) suggested that the probiotic activity is more important than the source of the microorganism. In fact, previous reports described the evaluation and selection of kefir LAB isolates for potential use as probiotics (Golowczyc et al., 2008; Zheng et al., 2013).

The aim of the present study was to identify and characterize LAB strains isolated from traditional kefir grains, displaying in vitro properties related to their probiotic potential, according the guidelines recommended by FAO/WHO (2006). After a complete characterization and the corresponding in vivo trials, these strains could be ultimately included as probiotics in functional foods.

MATERIALS AND METHODS

Isolation of Bacteria from Kefir Grains

The LAB were isolated by dilution and plating from 4 kefir grains collected in different regions of Brazil. Briefly, 10 g of each kefir sample was homogenized in 90 mL of sodium citrate (2%). Serial decimal dilutions were obtained and plated on lactobacilli de Man, Rogosa, Sharpe (**MRS**) and M17 agar media (Difco, Sparks, MD) supplemented with 200 μg/mL of cycloheximide (Sigma-Aldrich, St. Louis, MO), and incubated in aerobic and anaerobic (Gaspak EZ, Difco) conditions at 30°C for 72 h. Representative colonies of all morphologies were taken randomly and purified on the same media by subculturing. Gram-positive, catalase-negative isolates were considered as presumptive LAB, which were stored in 15% glycerol at −80°C.

For all subsequent assays, LAB were activated in the corresponding media at 30°C for 18 to 24 h, and subcultured in the same conditions.

Tolerance of the Isolates at Low pH

The ability of the isolates to tolerate low pH was assayed as described by Nishida et al. (2008) in MRS broth supplemented with 0.2% sodium thioglycolate (**MRS-THIO**). In short, overnight cultures were harvested by centrifugation and cells were suspended in PBS (pH 6.5) to obtain an optical density at 600 nm ($\mathbf{OD_{600}}$) = 0.5. Cell suspensions ($\approx 10^7$ to 10^9 cfu/mL) were 10-fold diluted with MRS medium, adjusted to pH 3.0 with HCl, and incubated at 37°C for 3 h. The pH tolerance of the cells was determined by enumerating the viable cells on MRS agar plates. Nontreated cultures used as controls were suspended in conventional, nonacidified MRS (pH 6.5).

Bile Tolerance of the Isolates

Tolerance to bovine bile (Oxgall, Difco) was assayed by growing the isolates in agar plates and broth, following the procedure reported by Delgado et al. (2007) and Guo et al. (2009), respectively. The growth rate of the strains was estimated in MRS-THIO broth in the absence (control) and in the presence (test) of 0.3% Oxgall. Overnight cultures were inoculated (1%) into the liquid medium, and cultured at 37°C for up to 9 h. Absorbance at 620 nm was measured every hour. The effect of the bile salts was scored as the time difference required to increase by 0.3 units the absorbance of the culture at 620 nm (OD_{620}) in MRS-THIO broth with and without 0.3% bile salts. The growth delay (hours) between the culture media was considered as the lag time (**LT**).

Additionally, tolerance of strains to different concentrations of bile salts was assayed by a plate assay. Individual colonies growing in MRS agar plates were suspended in 2 to 5 mL of sterile saline solution 0.85% at a density corresponding to McFarland standard 1. Aliquots of the suspensions (10 μL) were spotted onto bile-containing 0.3, 0.5, 1, and 2% (wt/vol) agar plates. The plates were incubated at 37°C under anaerobic conditions and growth was recorded after 24 to 48 h. A plate without bile was used as positive control. The experiments were performed in duplicate.

Identification of LAB Isolates

Total genomic DNA of the isolates was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich), following the manufacturer's recommendations. Purified DNA was used as a template to amplify a segment of the 16S rRNA gene by the PCR technique using the universal prokaryotic primers S-D-Bact-0008-a-S-20 (27F; 5′-AGAGTTTGATCCTGGCTCAG-3′) and S-*-Univ-1492R-b-A-21 (1492R; 5′-GGTTACCTTGTTACGACTT-3′). For the amplified ribosomal DNA restriction analysis (**ARDRA**), amplicons were purified through GenElute PCR Clean-Up columns (Sigma-Aldrich), digested with *Hae*III and *Hha*I restriction enzymes (Invitrogen, Paisley, UK) and electrophoresed in agarose gels. Gels were stained with ethidium bromide (0.5 mg/mL) and photographed under UV light. Representative amplicons of the different ARDRA profiles were sequenced. Sequencing