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A comprehensive approach to determine the probiotic potential of human-derived *Lactobacillus* for industrial use

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ABSTRACT

Specific strains should only be regarded as probiotics if they fulfill certain safety, technological and functional criteria. The aim of this work was to study, from a comprehensive point of view (*in vitro* and *in vivo* tests), three *Lactobacillus* strains (*Lactobacillus paracasei* JP1, *Lactobacillus rhamnosus* 64 and *Lactobacillus gasseri* 37) isolated from feces of local newborns, determining some parameters of technological, biological and functional relevance. All strains were able to adequately grow in different economic culture media (cheese whey, buttermilk and milk), which were also suitable as cryoprotectants. As selective media, LP-MRS was more effective than B-MRS for the enumeration of all strains. The strains were resistant to different technological (frozen storage, high salt content) and biological (simulated gastrointestinal digestion after refrigerated storage in acidified milk, bile exposure) challenges. *L. rhamnosus* 64 and *L. gasseri* 37, in particular, were sensible to chloramphenicol, erythromycin, streptomycin, tetracycline and vancomycin, increased the phagocytic activity of peritoneal macrophage and induced the proliferation of IgA producing cells in small intestine when administered to mice. Even when clinical trails are still needed, both strains fulfilled the main criteria proposed by FAO/WHO to consider them as potential probiotics for the formulation of new foods.

1. Introduction

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a benefit on the host (FAO/WHO, 2002). Numerous microorganisms are currently used as human probiotics; among them *Lactobacillus* and *Bifidobacterium* constitute the most frequently used genera. Even though there has been a long history of safe consumption of lactobacilli in traditional foods, the probiotic strains can only be utilized if they fulfill certain criteria related to safety, technological and functional aspects (Reid, 2005; Vankerckhoven et al., 2008).

Although there are no analytical tools to determine the environmental source of a strain after its primary isolation, it frequently represents the initial factor to be considered. In general, the selection of strains from appropriate sources depends on the targeted population, such as neonates, children, pregnant women or elderly, whose microbiota may differ from that of healthy adults (O'Toole and Claesson, 2010). Isolation from the intestinal tract of healthy individuals is generally recommended for probiotic use in humans (FAO/WHO, 2002; Reid, 2005). Following isolation, the next step is the identification at genus and species level using internationally accepted methodologies (in particular, sequencing of DNA encoding 16S rRNA) (Reid, 2005; Vankerckhoven et al., 2008).

Even when there is a great number of strains being used as probiotics in different food matrixes, the majority of the studies are based on functional properties (Szajewska et al., 2001; Kirjavainen et al., 2003; Reid et al., 2003; Santosa et al., 2006; Ezendam and van Loveren, 2006) and less information is available concerning their capacity to withstand stresses related to food processing. Among technological criteria, strain viability and maintenance of desirable characteristics during product manufacture and storage is a requirement for probiotic strains to assure a beneficial effect on the consumer (Champagne et al., 2011). In this regard, before reaching the intestinal environment, probiotic strains must overcome several technological and biological barriers (low pH in the stomach, bile salts) (Collins et al., 1998). Safety assessment is also a required step in the selection and evaluation of probiotics. Few probiotic strains have been specifically tested for safety based on the long history of safe consumption of lactic acid bacteria. The FAO/WHO guidelines recommend the determination of antibiotic resistance patterns as one of the most important safety test (FAO/WHO, 2002).

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In spite of the value of *in vitro* tests for an initial screening of potential probiotic strains, they are insufficient to assert a strain as probiotic. *In vivo* trials (at least using an animal model) are strongly suggested by the FAO/WHO to prove that the probiotic confers a significant improvement in health. These assays generally include evaluations of the capacity to stimulate the host immune system, so as to prevent enteric infections or other gut-associated pathologies.

Even when a high number of probiotic strains is now available for commercial use around the world, the isolation and characterization of new strains is still desirable, mainly in developing countries. The aim of this work was to characterize, from a comprehensive point of view (by *in vitro* and *in vivo* tests), three *Lactobacillus* strains isolated from local newborns, determining some parameters of technological, biological and functional relevance for their potential addition to new functional products.

2. Material and methods

2.1. Strains and culture conditions

The *Lactobacillus* strains tested in this work were isolated in a previous study (Vinderola et al., 2008) from feces of three newborns from Santa Fe, Argentina, frozen stored (−80 °C) in MRS broth (Biokar, Beauvais, France) supplemented with 15% (v/v) glycerol, and kept at the INLAIN culture collection. They were preliminarily identified by RAPD-PCR techniques as *Lactobacillus paracasei* JP1, *Lactobacillus rhamnosus* 64 and *Lactobacillus gasseri* 37 and selected, based on preliminary characterization, as potential probiotic strains. For RAPD assays, *L. paracasei* DN114001 (Danone), *L. rhamnosus* GG (Valio) and *L. gasseri* ATCC 33323 were used as reference strains. All strains were cultured in MRS broth (37 °C, 16 h, aerobic incubation).

2.1.1. Identification of isolates

Total DNA of isolates was obtained from overnight cultures by using the GenElute Bacterial Genomic DNA kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Purified DNA samples were stored at −20 °C until use. To determine the bacterial species, the identity of isolates was analysed by amplifying, sequencing and comparing a 1500 bp fragment within their 16S rRNA gene (Edwards et al., 1989). All PCR reactions were performed using 1 μL of diluted (1:50) DNA as template, 2.5 U Taq DNA polymerase (GE Healthcare, Little Chalfont, United Kingdom), 200 nM dNTPs (GE Healthcare) and 400 nM each primer (Sigma-Genosys, The Woodlands, TX, USA) in a final volume of 50 μL. Amplifications were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA USA) under the following conditions: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 2 min at 51 °C and 2 min at 72 °C, and a final step of 7 min at 72 °C. The PCR products were separated on 0.8% (w/v) agarose gels in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) buffer, stained with GelRed (Biotium, Hayward, CA, USA) and visualized under UV light (Sambrook and Russell, 2001). Amplicons were purified with MicroSpin Columns (GE Healthcare) and their nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity of strains at species level was checked by nucleotide–nucleotide BLAST of the NCBI database (www.ncbi.nlm.nhi.gov/blast).

2.1.2. RAPD analysis

Genotypic diversity among strains was analysed by RAPD-PCR using two single arbitrary primers, B07 (also named P2, Binetti et al., 2007) and M13 (Huey and Hall, 1989), in independent reactions. Amplifications were carried out under the following conditions: an initial denaturation step of 5 min at 94 °C, followed by 30 (B07) or 35 (M13) cycles of 1 min at 94 °C, 2 min at 36 °C (B07) or 20 s at 45 °C (M13), 2 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR reactions were performed in a total volume of 25 μL with 1 μL template diluted DNA, 500 nM (B07) or 200 nM (M13) primer (Sigma-Genosys), 2.5 U Taq Polymerase (GE Healthcare) and 200 nM of each dNTP (GE Healthcare). A negative control (without template) was included in all reactions. Amplification products were analysed by electrophoresis in 1.0% (w/v) agarose gels in TBE buffer, following standard protocols.

2.1.3. Growth in different culture media

The culture media used in this assay were skim milk (10% w/v) (San Regim, Buenos Aires, Argentina), cheese whey (5% w/v) and buttermilk (7.8% w/v), all supplemented with 0.3% (w/v) of yeast extract (Burns et al., 2008a). Dried cheese whey and buttermilk were provided from a local dairy industry and reconstituted to give a final lactose concentration of 4.8% (w/v). MRS broth was included as reference medium. Overnight cultures (MRS broth, 16 h, 37 °C) of the strains were centrifuged (8000 *g*, 15 min, 4 °C), washed twice with PBS (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.0) solution, resuspended in the same volume of PBS and inoculated (2% v/v) in the corresponding culture media, being finally incubated in a water bath for 24 h at 37 °C. Colony counts on MRS agar (37 °C, 48 h) were carried out periodically.

2.1.4. Growth in selective/differential culture media

Cell counts of the strains under study were performed in selective culture media and compared to counts obtained in MRS agar. Bile MRS agar (B-MRS) and lithium chloride sodium propionate MRS agar (LP-MRS) were used as selective culture media according to previous studies (Vinderola and Reinheimer, 2000). Bovine bile (0.15% w/v, B-MRS) and lithium chloride (0.2% w/v) plus sodium propionate (0.3% w/v, LP-MRS) were suggested as adequate to inhibit the growth of the starter microbial population which were present in fermented dairy products carrying probiotic bacteria. In some commercial products, however, these concentrations are not enough to accomplish this aim, higher amounts of bile salts, lithium chloride and sodium propionate being needed (personal observations at the INLAIN). For this reason, the viability of overnight cultures (MRS broth, 37 °C) was determined in B-MRS containing increasing amounts of bovine bile (0.15, 0.2, 0.25 and 0.3% w/v) and LP-MRS with increasing concentrations of the selective agents (0.2, 0.25, 0.3 and 0.4% w/w lithium chloride; 0.3, 0.4, 0.45 and 0.6% w/v sodium propionate). MRS agar was used as control medium. Plates were incubated at 37 °C (48 h, aerobic incubation).

2.2. Resistance to technological challenges

2.2.1. Resistance to frozen storage

Overnight cultures in MRS broth were centrifuged (8000 *g*, 15 min, 5 °C), washed twice with PBS (pH 7.0) buffer and adjusted to ca. 10^9 CFU/mL. Cell suspensions were centrifuged (8000 *g*, 10 min, 5 °C) and resuspended in skim milk (10% w/v), buttermilk (10% w/v) and cheese whey (10% w/v). MRS broth supplemented with 15% (v/v) of glycerol as cryoprotectant was used as control medium. Cell suspensions were then frozen stored at −20 and −70 °C for 12 months. At the beginning (day 0) and every 30 days for 12 months, cell counts (MRS Agar, 37 °C, 48 h, aerobic incubation) were performed.

2.2.2. Tolerance to salts

Overnight cultures were inoculated (2% w/v) in MRS broth added with NaCl and with KCl (both 1 and 2% w/v) according to Reinheimer et al. (1997). MRS broth was used as control medium.