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Technological and probiotic selection criteria of a bile-adapted *Bifidobacterium animalis* subsp. *lactis* strain

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ABSTRACT

The technological and probiotic properties of the bile-adapted strain *Bifidobacterium animalis* subsp. *lactis* 4549dOx have been studied in comparison with its parental strain IPLA4549. The survival of the bile-adapted derivative to the simulated gastrointestinal transit was significantly higher than that of the parental strain, except in the presence of 10% skimmed milk. The adherence of strain 4549dOx to the intestinal cell line HT29-MTX almost duplicated that of strain IPLA4549. However, both strains had similar behaviour throughout the shelf-life of fermented milk. In conclusion, the bile-adapted strain 4549dOx presented advantages for its inclusion in dairy probiotic foods.

1. Introduction

The term probiotic refers to live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2006), their target usually being the human gastrointestinal tract (GIT). Probiotic *Bifidobacterium* strains are usually implemented as active cultures in fermented dairy products (Jayamanne & Adams, 2006) and, after being ingested, they must overcome the biological barriers of the GIT to transiently colonize the intestinal mucosa and exert their beneficial effects.

We have obtained several bile-adapted bifidobacteria strains by progressive subcultures in gradually increasing bovine bile salt concentrations (Noriega, Gueimonde, Sánchez, Margolles, & de los Reyes-Gavilán, 2004). A significant part of our work has been focused on *Bifidobacterium animalis* subsp. *lactis* IPLA4549 and its derivative strain *B. animalis* subsp. *lactis* 4549dOx. Strain 4549dOx, displayed enhanced resistance to the GIT conditions (low pH in the stomach, and high bile salt concentration), mainly linked to an increase in the membrane-bound F_1F_0-ATPase activity, as well as to changes on the glycolytic flux (Sánchez et al., 2007; Sánchez, de los Reyes-Gavilán, & Margolles, 2006; Sánchez, Ruiz, de los Reyes-Gavilán, & Margolles, 2008). *B. animalis* subsp. *lactis* is the most common *Bifidobacterium* species included in fermented dairy products, due to its good tolerance to both acidic and oxidative stress (Gueimonde, Noriega, Margolles, de los Reyes-Gavilán, & Salminen, 2005; Masco, Crockaert, van Hoorde, Swings, & Huys, 2007; Vernazza, Gibson, & Rastall, 2006), being able to maintain higher viability during product storage than other species (Jayamanne & Adams, 2006).

In the present study, we aimed to characterise the technological and some probiotic capabilities of the strains *B. animalis* subsp. *lactis* IPLA4549 and its bile-adapted derivative 4549dOx (Ruas-Madiedo, Hernández-Barranco, Margolles, & de los Reyes-Gavilán, 2005). The study was carried out in order to assess whether the acquisition of the bile resistance phenotype might confer additional advantages for the inclusion of bile-adapted strains in fermented dairy products.

2. Material and methods

2.1. Bifidobacteria strains

The parental strain *B. animalis* subsp. *lactis* IPLA4549 and its bile-adapted derivative strain 4549dOx were used in this study. Bifidobacteria were cultured in MRSC [MRS (Biokar Diagnostics, Beauvais, France) with 0.05% (w/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA)] and incubated for 24 h at 37 °C in the anaerobic chamber MG500 (Don Whitley Scientific, West Yorkshire, UK) under 10% (v/v) H_2, 10% CO_2 and 80% N_2. Cultures were collected by centrifugation (10,000 × *g*, 20 min, 5 °C), washed twice with PBS solution and resuspended (10-fold concentration) in sterile (11%, w/v) reconstituted skimmed milk (Difco®, Becton Dickinson, Franklin

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Lakes, NJ, USA). Finally, bacterial suspensions were lyophilised (Freezemobile 12EL, Virtis, Gardiner, NY, USA) for 24 h.

2.2. Manufacture of fermented milk

Two yoghurt starters were used for the manufacture of fermented milk: Fermovac® 1000 V9 (containing *Streptococcus thermophilus*) and Fermovac® 1000 MSK R1 (containing *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) from Danisco (Copenhagen, Denmark). Both starters were separately propagated (1% w/v) in skimmed milk overnight at 37 °C. Cultures were then mixed in equal amounts and used to inoculate (2% v/v) whole milk supplemented with 1% skimmed milk powder previously pasteurised at 85 °C for 30 min. When appropriate, lyophilised bifidobacteria strains were added to a final concentration of 10^8 cfu mL^{-1} just after the inoculation with the yoghurt starter mixture. Milk fermentations were carried out in a water bath at 42 °C until they reached a pH of 5.0 ± 0.1 and, afterwards, each fermented milk type was separated in several aliquots which were stored at 4 °C for 24 days. Three types of fermented milk were manufactured in triplicate: control (containing the yoghurt starter mixture), IPLA4549 (containing the yoghurt starter mixture and *B. animalis* IPLA4549) and 4549dOx (containing the yoghurt starter mixture and *B. animalis* 4549dOx). Several samples were collected at different points: after milk inoculation (0 h), after 1 h of fermentation, at the final fermentation (FF) time, and after 2, 10, 18 and 24 days of refrigeration.

Bacterial counts and pH were determined at each sampling point using different growth media and culturing conditions. After serial dilutions of samples in Ringer's solution (Merck, Darmstadt, Germany), *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were enumerated by spread plating on agar-M17 (Oxoid Ltd., Basingstoke, Hampshire, UK) or agar-MRS, respectively. All plates were incubated at 37 °C for 48 h. *Bifidobacterium* strains were counted in the same way in agar-MRSC-LP [MRSC supplemented with 0.2% (w/v) lithium chloride and 0.3% sodium propionate (Sigma)] after growing in an anaerobic chamber at 37 °C for 72 h. The residual lactose and the accumulated galactose, as well as the production of acetic and lactic acids were quantified by HPLC and the production of volatile compounds by means of GC–MS as previously reported (Salazar et al., 2009). Results were expressed as mM for sugar and organic acids and in μM for volatile compounds.

2.3. Resistance to the chemically simulated gastrointestinal transit

The survival of the bifidobacteria strains during the passage through the gastrointestinal tract (GIT) was studied in an in vitro model that chemically simulates the physiological conditions, which had been modified from that previously described (Fernández, Boris, & Barbes, 2003). The following components were used: (i) simulated gastric juice (GJ) containing 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 0.3% pepsin (Sigma) adjusted with HCl to pH 2.0 or to pH 3.0, (ii) simulated duodenal juice (DJ) containing 1% bovine bile (Sigma) adjusted with 10 M NaOH to pH 8.0, and (iii) simulated intestinal juice (IJ) containing 0.3% bovine bile, 0.1% pancreatin (Pancreas acetone powder porcine, Type I, Sigma, Catalogue number P4251) pH 8.0. To simulate the GIT transit conditions, bifidobacteria from 24 h MRSC-grown cultures of the parental and bile-adapted derivative were harvested by centrifugation (10,000 × *g*, 15 min, 5 °C), washed twice with 0.85% NaCl and resuspended in three types of GJ: pH 2.0, pH 3.0 and pH 2.0 containing 10% w/v skimmed milk powder. The three bacterial suspensions of each strain were then incubated for 180 min at 37 °C with stirring (200 rpm). Afterwards, samples were centrifuged (10,000 × *g*, 15 min), cells were resuspended in DJ and incubated for 10 min at 37 °C in an anaerobic chamber. After this step, cells were again centrifuged, each resuspended in IJ and incubated for 180 min at 37 °C in anaerobic conditions. Viable cell counts were obtained from the initial cultures, after 90 and 180 min of GJ challenge, after 10 min of DJ challenge, and after 90 and 180 min of IJ challenge. Results were expressed as Log cfu mL^{-1} and the percentage of survival was calculated from the viable counts recovered after the complete chemically simulated GIT transit with respect to the initial counts (% cfu recovered bacteria/cfu initial bacteria). Experiments were carried out in triplicate.

2.4. Adhesion to the HT29-MTX cell line

The adhesion capability of the strains was assessed with the epithelial intestinal cell line HT29-MTX, which was kindly supplied by Dr. T. Lesuffleur (INSERM U843, Paris, France). This cell line is able to constitutively produce mucin (Lesuffleur, Barbat, Dussaulx, & Zweibaum, 1990). The cell line was maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated bovine foetal serum and a mixture of antibiotics to give a final concentration of 50 μg mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 50 μg mL^{-1} gentamicin and 1.25 μg mL^{-1} amphotericin B. All media and reagents were purchased from Sigma and incubations took place at 37 °C, 5% CO_2 in a SL Waterjacked CO_2 Incubator (Sheldon Mfg. Inc., Cornelius, Oregon, USA). Culture media were changed every two days and the cell lines were trypsinized with 0.25% trypsin–EDTA solution (Sigma) following standard procedures (Sánchez et al., 2009). For experiments, 10^5 cells mL^{-1} were seed in 24-well plates and incubated to confluence (reaching about 10^7 cells mL^{-1}) for 13 ± 1 days.

Parental and bile-adapted derivative strains and the reference strain *B. animalis* subsp. *lactis* Bb12 were grown in MRSC for 24 h. Cultures were harvested by centrifugation, washed twice with Dulbecco's PBS buffer (Sigma) and resuspended in DMEM without antibiotics at a concentration of about 10^8 cfu mL^{-1}. HT29-MTX monolayers were washed twice with Dulbecco's PBS to remove the antibiotics and then bacterial suspensions were added in a ratio of epithelial cells:bacteria of 1:10. Plates were incubated for 1 h at 37 °C, 5% CO_2 in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany). After the incubation period, supernatants were removed and wells were gently washed three times with Dulbecco's PBS buffer to remove the non-attached bacteria. Afterwards, the monolayers were trypsinized and bacterial counts were carried out in agar-MRSC to determine the number of adhered bacteria. Results were expressed as the percentage of bacteria adhered with respect to the amount of bacteria added (% cfu bacteria adhered/cfu bacteria added). Experiments were carried out in duplicate (using two HT29-MTX plates) and in each plate the strains were also tested in duplicate.

2.5. Statistical analysis

Data were statistically analysed using the SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA) by means of one-way ANOVA tests independently performed at each sampling point. When needed, the mean comparison LSD (least-significant difference, $p < 0.05$) test was used to assess differences among fermented milks or strains.

3. Results and discussion

3.1. Growth and metabolic activity in milk

The pH and bacterial dynamics of milk fermented with a starter mixture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*