

Probiotic, technological, and health-related characteristics of lactobacilli isolated from breast milk

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Abstract

Aims: Isolation and characterization of lactobacilli from human milk and determination of their probiotic, technological, and *in vitro* health-promoting properties with a view to their potential use in food fermentation.

Methods and Results: Seven lactobacilli isolates were obtained from human milk and identified as *Lactocaseibacillus paracasei* (isolates BM1–BM6) and *Lactobacillus gasseri* (BM7). The isolates were examined *in vitro* for their technological, probiotic, and health-promoting potential. Overall, all isolates showed important technological properties based on the ability to grow in milk whey, a high to moderate acidification capacity and the absence of undesirable enzymatic activities. *Lactocaseibacillus gasseri* (BM7) differed from the *L. paracasei* isolates by the absence of several glycosidases and the inability to ferment lactose. Isolates *L. paracasei* BM3 and BM5 produced exopolysaccharides (EPS) from lactose. All isolates showed probiotic potential as they were tolerant to simulated gastrointestinal conditions, had high cell surface hydrophobicity, had not acquired resistance to relevant antibiotics and had no virulence characteristics. All *L. paracasei* showed high antimicrobial activity against various pathogenic bacteria and fungi, while *L. gasseri* showed a narrower spectrum of antimicrobial activity. All isolates showed health-promoting potential *in vitro*, as evidenced by high cholesterol-lowering activity, high ACE inhibitory activity and marked antioxidant activity.

Conclusions: All strains showed excellent probiotic and technological properties for use in lactic ferments.

Significance and impact of the study

Lactobacilli are an important part of the microbial population in human milk. A more detailed characterization of these bacteria can contribute to the understanding of their functional and health importance for infants and to the selection of new probiotic strains for the dairy industry.

Keywords: Human milk, lactobacilli, *Lactocaseibacillus paracasei*, *Lactobacillus gasseri*, probiotics

Introduction

Numerous species of bacteria live in the gastrointestinal tract, forming the intestinal microbiota and living in a reciprocal relationship with the host and its respective immune system (Maynard et al. 2012, Alou et al. 2016, Zheng et al. 2020). In recent years, the importance of the intestinal microbiota for human health has emerged. Therefore, the knowledge of the benefits of the intestinal microbiota for many physiological processes of the host opened new opportunities for the use of certain bacterial strains as probiotics.

Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2001, Hill et al. 2014). Many of the probiotic organisms belong to the lactic acid bacteria group (LAB), with lactobacilli and *Bifidobacterium* being the most common (Fooks and Gibson 2002, Vlasova et al. 2016). Thus, LAB and *Bifidobacterium* are important members of the gut microbiota and their beneficial effects such as inhibiting the growth of potentially pathogenic organisms, lowering serum cholesterol levels, and modulating the immune system have been documented (Sanders and Marco 2010).

Microbial colonization of the gut begins at birth through the microbiota in the mother's vagina, but also occurs through microorganisms on the mother's skin and through breastfeeding (Salazar et al. 2014, Mueller et al. 2015, Tanaka and Nakayama 2017). Breast milk contains several macro- and micronutrients that promote infant health, growth, and development. In addition, breast milk is a source of beneficial bacteria such as bifidobacteria and lactobacilli, which are very important for the development of the infant's immune system and defence against pathogens (Tanaka and Nakayama 2017). It has been shown that a composition of the gut microbiome with insufficient amounts of lactobacilli in the first months of life impairs immune responses and promotes the development of infant colic and atopic diseases (Savino et al. 2005). In addition, alteration of the gut microbiota in infants from 3 months of age may have several negative effects on the immune system and the development of allergies and autoimmune diseases such as type I diabetes (Francino 2014, Salazar et al. 2014, Vatanen et al. 2018). As a result, several species/strains of lactobacilli have been developed and used as probiotics that promote human health (Linares et al. 2017, Rajoka et al. 2017).

Received: January 17, 2023. Revised: May 24, 2023. Accepted: June 9, 2023

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Lactobacilli is not only one of the most important bacterial group attributed with probiotic properties (Butler et al. 2020), but is also used in the fermentation of various foods, including dairy products. Fermented foods such as yogurt and cheese have also been shown to be a suitable medium for dietary intake of probiotic bacteria (Coelho et al. 2022). However, for these bacteria to retain their probiotic capabilities and provide health benefits, they must be able to withstand fermentation and storage of processed foods. Their stability is influenced by various factors such as pH, temperature, and other ingredients in foods (Del Piano et al. 2006). Although numerous probiotic lactobacilli have been isolated from human gastrointestinal tract and dairy products, there are few reports on isolation from human milk (Damaceno et al. 2017, Gunyakti and Asan-Ozusaglam 2019, Kang et al. 2020, Liu et al. 2020). The few studies that have isolated lactobacilli from human milk have focused mainly on their ability to survive under simulated gastrointestinal conditions and their sensitivity to antibiotics, but there have been no studies that have comprehensively investigated their technological, antimicrobial and health-promoting properties. There is also limited information on how these lactobacilli in breast milk can have a positive effect on the health of the breastfed baby. A comprehensive knowledge of the health benefits of these lactobacilli, including the technological possibilities for their use in food fermentation, will contribute to a deeper understanding of their effect on the health of the infant and to the development of probiotic milk products that have the advantage of containing lactobacilli isolated from breast milk. Therefore, the aim of this study is to characterize and evaluate the probiotic potential of lactobacilli isolated from breast milk. This includes the technological possibilities for application in food fermentation and the *in vitro* health-promoting potential of the lactobacilli.

Materials and methods

Subjects and sample collection

Participants were healthy, breastfeeding women who had delivered their babies either vaginally or by caesarean section. Mothers were asked to clean the breast with water and express 5–10 mL of milk into a sterile container provided. Milk samples were stored at 4°C and used for bacterial isolation within 24 h of donation. Participation was voluntary, and informed consent was obtained before the start of the study. This study was approved by the Ethics Committee of the University of the Azores (PARECER 17/2021).

LAB isolation

Breast milk samples were plated directly (1 mL) on de Man, Rogosa, and Sharpe agar (MRS, Biokar, Beauvais, France) supplemented with 0.05% cysteine-HCl (Sigma-Aldrich, St. Louis, MO, USA), which were incubated under anaerobic conditions using an AnaeroGen kit (Oxoid, Milan, Italy) at 37°C for 2–5 days. Colonies were purified by successive streaking on MRS agar. Individual colonies were subjected to Gram staining and cell morphology was observed under the microscope. All isolates that matched to the basic characteristics of the LAB group, Gram-positive, catalase, and oxidase-negative were used for identification. Isolates were stored at –80°C in MRS broth supplemented with 30% (v/v) glycerol and acti-

vated by two consecutive transfers in MRS broth containing 0.05% cysteine before assay.

Identification of LAB

Isolates were identified by sequencing of the 16S rRNA gene as described by Ribeiro et al. (2014). Total genomic DNA was extracted and purified using the Ultra-Clean Microbial DNA Isolation Kit according to the manufacturer's protocol (MoBio, Carlsbad CA, USA). The 16S ribosomal RNA (rRNA) gene was amplified using universal bacterial primers 46F and 1409R, and Taq DNA polymerase (Fermentas, Life Sciences, Waltham, MA, USA). The amplified PCR fragment of the 16S rRNA gene of all isolates were sequenced (STAB Vida, Portugal) and analysed using the program BLAST on the NCBI website (<http://www.ncbi.nlm.nih.gov>). Based on the 16S rRNA gene sequences, isolates BM1, BM2, BM3, BM4, BM5, BM6, and BM7 were identified as lactobacilli and deposited in GenBank under accession numbers OP880191–OP880197. Finally, the 16S rRNA gene sequence of isolates were compared with the sequences from the LAB-type strains contained in GenBank through a MEGA analysis.

Physiological and biochemical tests

Microbial growth and acidifying ability were evaluated in sweet whey according to Ribeiro et al. (2021). Briefly, isolates were inoculated in MRS broth (50 mL) and incubated for 18 h under anaerobic conditions at 37°C. Then, the growth culture was centrifuged at 4000 × g for 20 min at 20°C (Centrifuge 5804R, Eppendorf, Hamburg, Germany). After washing with phosphate buffered saline (PBS, pH 7.2), the bacterial pellet was then resuspended in whey and the optical density (OD) was adjusted to 0.1 at 600 nm. Aliquots of 200 µL of the resulting suspension were placed in 96-well microplates in quadruplicate and incubated at 37°C in a plate reader (Fluorstar Omega, BMG Labtech, Ortenberg, Germany) to measure OD at 600 nm for 72 h. Growth curves were estimated, according to the model of Baranyi and Roberts (1994) using MicroFit Software Version 3.5. (<https://browser.combase.cc/DMFit.aspx>). For the acidification curves, 200 µL of the bacterial suspension was added to a whey solution containing bromocresol blue. Absorbance (Abs) was measured at 430 nm in the plate reader at 37°C for 24 h. Abs values were converted to pH values by prior calibration, using buffer solutions with pH values between 4 and 7. The calibration curve used was as follows: $Abs = -0.1019 \text{ pH} + 0.73$ ($R^2 = 0.9918$).

Isolates were also evaluated for the production of active enzymes (APIZYM kit, Bio-Mérieux, Marcy-l'Étoile France), their ability to ferment various sugars (API 50 CHL, Bio-Mérieux), and production of exopolysaccharides (EPS), according to Domingos-Lopes et al. (2017).

For EPS screening, isolates were plated on different MRS agar plates with glucose, fructose, sucrose, and lactose as the only carbon source. Plates were incubated at 37°C for 48 h and colonies were screened for mucoid or ropy properties. Colonies were classified as ropy if threads of 5 mm or more were detected when the colony was touched with an inoculation loop (Zivkovic et al. 2015, Manini et al. 2016).

Safety evaluation of isolates

Safety was assessed by screening isolates for haemolytic, DNase, and gelatinase activities according to the methodology of Yadav et al. (2016). In addition, isolates were tested

for the presence of virulence genes: *gelE* (gelatinase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), *ace* (adhesion of collagen); antibiotic resistance genes: *vanA* and *vanB* (both related to vancomycin resistance); and amino acid decarboxylase genes: *hdc* (histidine decarboxylase). Primers and fragment sizes were used as described in Ribeiro et al. (2014). Genomic DNA from *E. faecalis* L2B21K3 and L3A21M8 (Ribeiro et al. 2014), *E. faecium* (Jaouani et al. 2015), *E. faecium* C1434 and C1231 (López et al. 2009) were used as positive controls.

Antibiotic resistance was also determined by phenotypic testing using the agar diffusion method (Bauer 1966). Aliquots (100 µL) of each bacterial suspension were inoculated into 5 mL of MRS broth and incubated at 37°C for 24–48 h. At the end of this time, OD was adjusted to 0.5 on the McFarland scale and 400 µL was withdrawn into a new tube containing 5 mL MRS broth. Then, the contents of each tube were spread on two plates containing Mueller-Hinton agar (AES BioMérieux, Marcy l’Etoile, France). The plates were thoroughly dried in a laminar flow cabinet for 1 h. The antibiotic discs (Oxoid, UK) were applied to the plate with sterile forceps. Fifteen antibiotics were tested: Penicillins—penicillin G (10 IU per disc), and ampicillin (10 µg per disc); Glycopeptides—vancomycin (30 µg per disc); Aminoglycosides—kanamycin (30 µg per disc), gentamicin (10 µg per disc), and streptomycin (10 µg per disc); Quinolones—ciprofloxacin (5 µg per disc), levofloxacin (5 µg per disc), norfloxacin (5 µg per disc); Streptogramins—quinupristin/dalfopristin (15 µg per disc); Lincosamides—clindamycin (2 µg per disc); Amphenicols—chloramphenicol (30 µg per disc); Macrolides—erythromycin (15 µg per disc); Rifamycins—rifampicin (5 µg per disc); Tetracyclines—tetracycline (30 µg per disc). After 24 h of incubation at 37°C, the clear zones around each antibiotic were measured with a digital caliper (Absolute Digimatic Caliper, Mitutoyo, Neuss, Germany). Reference values were used to interpret the results (CLSI 2016). Bacteria were classified into two categories based on the diameter of the zone of inhibition: resistant (R) and sensitive (S).

Evaluation of probiotic properties

Resistance to pH 2.5 (to assess acidic conditions in the stomach) and to bile acids and pancreatin (intestinal conditions) was evaluated to assess survival of lactobacilli under simulated gastrointestinal conditions. Isolates were inoculated into MRS broth (50 mL) and incubated overnight (18 h) at 37°C. Then, the culture media were centrifuged at 4000 × *g* for 20 min at 20°C (Centrifuge 5804R). The pellet was washed with PBS buffer (pH 7.2) and centrifuged under the same conditions. The pellet containing the bacteria was then diluted in 1 mL of PBS to obtain the bacterial suspension. An aliquot (100 µL) of this suspension was placed in two tubes: one of the tubes contained 9.9 mL of a PBS solution with a pH of 2.5 and the other tube contained 9.9 mL of a PBS solution (pH 7.2) with 0.3% (w/v) bile salts and 0.1% pancreatin. The tubes were incubated at 37°C for 1, 2, and 3 h. After the indicated times (including time = 0), viable bacteria were counted by decimal dilution in peptone water and plating out on MRS agar medium.

Cell surface hydrophobicity of the isolates was also assessed using xylene, following the method reported by Rink-

inen et al. (2003). Briefly, isolates were inoculated into MRS broth (50 mL) and incubated at 37°C for 24 h. Cultures were centrifuged at 4000 × *g* for 20 min at 20°C and washed with PBS. The initial absorbance (Abs initial) was set between 0.5 and 0.6. Then 3 mL was added to a sealed test tube containing 1 mL of xylene, shaken for 2 min allowed to settle for 10 min. The organic surface layer was removed and the absorbance (Abs final) of the aqueous phase was measured at 600 nm. This test was performed in duplicate for each isolate. Hydrophobicity (%) was calculated using the following formula:

$$\% \text{Hydrophobicity} = 1 - \frac{\text{Abs final}}{\text{Abs initial}} \times 100.$$

Antimicrobial activity

The antibacterial activity of the isolates was determined using the agar diffusion method according to Ribeiro et al. (2014). The following reference bacteria were used as sensitive/indicator strains in antibacterial assay: *Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090, *Escherichia coli* ATCC 15922, *E. coli* ATCC 8739, *E. coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *S. enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Bacillus cereus* ATCC 11778, *Bacillus spizizenii* ATCC 6333, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* subsp. *aureus* ATCC 29523.

Isolates were inoculated into MRS broth (50 mL) and incubated at 37°C for 48 h. After this time, the cultures were centrifuged at 4000 × *g* (20 min, at 4°C). The supernatant was filtered using sterile 0.2 µm filters. To obtain neutralized supernatants, the pH was adjusted to 6.5–7.0 with NaOH (1 M) and filtered. Reference bacteria (target cultures) were inoculated into Nutrient Broth (Himedia, Dindori, India) and incubated at 37°C for 18 h. These cultures were then diluted in Nutrient Broth to reach a value of 0.5 on the McFarland scale. Then, 100 µL of the diluted culture was pipetted into 200 mL Plate Count Agar (Biokar, Beauvais, France). Wells with a diameter of 6 mm were made and 60 µL of sample (supernatant) was added to each well. For each isolate, both the cell-free supernatant (CFS) and the neutralized CFS were tested. Plates were incubated at 37°C for 12 h. A digital calliper (Absolute Digimatic Caliper) was used to measure the diameter of the inhibition zones.

The antifungal activity of the isolates was evaluated using the agar diffusion method described by Manini et al. (2016) with some modifications. The fungal species used to determine antifungal activity were *Aspergillus flavus* MUM16.106, *Penicillium nordicum* MUM16.93, *Aspergillus chevalieri* MUM 00.07, *Penicillium commune* MUM 16.56 and *Penicillium brevicompactum* MUM 9906, and were provided by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal). A spore suspension was prepared by growing the moulds on Potato Dextrose Agar (PDA, Oxoid, Basingstoke, England) at 37°C for 3–4 days. The lactobacilli isolates were previously inoculated with a sterile swab in two 5 cm rows in MRS agar plates. The inoculated plates were incubated for 48 h at 37°C under aerobic conditions. The plates were then overlaid with 10 mL of yeast extract peptone dextrose agar (YEPA, Hi-Media, Mumbai, India) inoculated with fungal spore suspension (5 × 10⁴ spores mL⁻¹). The plates were incubated under aerobic conditions at 37°C for 48–72 h

and examined for zones of inhibition around the bacterial swabs. Based on the observed zones of inhibition, the following classifications were made:—no inhibition; +/- weak inhibition (zone of inhibition < 0.5 cm); + low inhibition (zone of inhibition: 0.5–1 cm); ++ medium inhibition (zone of inhibition > 1 cm, fungal growth > 50% of plate); +++ high inhibition (zone of inhibition > 1 cm, fungal growth < 50% of plate).

Cholesterol lowering and bile salt hydrolase activities

The isolates were tested for their ability to reduce cholesterol in broth MRS-CHO according to (Domingos-Lopes et al. 2020). Briefly, isolates were grown overnight in MRS broth (50 mL) at 37°C, harvested by centrifugation (5000 × g, 10 min, 4°C), washed with PBS and incubated for 30 h in MRS-CHO broth (2%, OD = 0.5) at 37°C. MRS-CHO was prepared by adding 0.05% cysteine HCl (Sigma–Aldrich) and 0.5 mg mL⁻¹ cholesterol (Sigma–Aldrich) to MRS broth. The initial inoculum was 10⁷ CFU mL⁻¹. A control was prepared without the bacterial cells. Cholesterol concentration in the culture supernatant (after incubation) was determined by an enzymatic colorimetric method (CHOD-PAP cholesterol kit; NS Biotec, Cairo, Egypt). The percentage of cholesterol removal was calculated using the following formula:

$$\text{Cholesterol removal (\%)} = \left(1 - \frac{\text{Cholesterol level after incubation}}{\text{Cholesterol level in control sample}} \right) \times 100.$$

Cultures were also tested for bile salt hydrolase activity (BSH) according to Domingos-Lopes et al. (2020). Briefly, 10 µL of an overnight culture suspension was spotted on plates containing BSH screening medium and incubated at 37°C for 48 h. Plates were examined for the presence of precipitated bile salts around the colonies (opaque halo) and the diameters of the precipitated zones were measured.

Angiotensin converting enzyme (ACE) inhibitory activity

The angiotensin-I converting enzyme (ACE) inhibitory activity of isolates in fermented milk samples was measured in duplicate using the method of Cushman and Cheung (1971) and Ramchandran and Shah (2010) with some modifications. These methods are based on the release of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL; Sigma–Aldrich). Fermented milk was prepared by inoculating isolates (10%) in reconstituted skim milk (VWR-BDH chemicals, Leuven, Belgium) and incubated at 37°C for 24 h. The fermented milk was then centrifuged at 4000 × g for 30 min at 4°C. The supernatants were collected for subsequent determination of ACE activity. The assay mixture contained 0.2 mL of 3.8 mM HHL (in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) as substrate, 0.04 mL of ACE enzyme solution (from rabbit, 0.1 U mL⁻¹, Sigma–Aldrich), and 0.04 mL of sample. The mixture was then incubated at 37°C for 60 min. After stopping the reaction, the hippuric acid was extracted with 1.7 mL of ethyl acetate. The absorbance was measured at 228 nm using a UV/Vis spectrophotometer (Fluostar Omega). A negative control (no inhibition) was performed with non-fermented skim milk containing the enzyme and substrate. The percent-

age of inhibition was calculated using the following formula:

$$\text{ACE inhibition (\%)} = 100 \times \frac{(B - A)}{(B - C)},$$

where A is the absorbance of the sample (fermented milk) with ACE and HHL, B is the absorbance of non-fermented skim milk with ACE and HHL (negative control), and C is the Abs of substrate (HHL) and without ACE.

β-galactosidase activity

The β-galactosidase activity of the isolates was determined at least in triplicate as described by Li et al. (2012) with minor modifications. An overnight culture of each isolate (50 mL) was centrifuged at 4000 × g for 15 min at 4°C, washed twice, and resuspended in PBS to adjust Abs₆₀₀ between 0.5 and 0.6. An aliquot (20 µL) of the cell suspension was mixed with 80 µL of permeabilization solution pre-warmed to 37°C. Then, 600 µL of substrate solution (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 1 mg mL⁻¹ o-nitrophenyl-β-D-galactoside (ONPG), and 2.7 µL mL⁻¹ β-mercaptoethanol) were added to start the reaction. The reaction was carried out at 37°C and stopped by adding 700 µL 1 M Na₂CO₃ when a faint yellow colour was observed. The reaction time was recorded and both Abs were measured at 420 nm (Abs₄₂₀) and 550 nm (Abs₅₅₀). If Abs₅₅₀ values exceeded 0.050, the sample was centrifuged again and remeasured. The β-galactosidase activity was calculated using the following equation and expressed in Miller units (MU):

$$\text{MU} = 1000 \times \frac{\text{Abs}_{420}}{\text{Abs}_{600} \times \text{volume (0.02)} \times \text{reaction time}}.$$

CLA production

LAB isolates were screened for CLA production according to Ribeiro et al. (2018). Briefly, LAB was grown for 24 h at 37°C in MRS broth and then inoculated (1%) in MRS broth supplemented with linoleic acid (0.5 mg mL⁻¹, Sigma–Aldrich). After incubation at 37°C for 48 h, 1 mL of the culture medium was centrifuged at 4500 × g for 20 min and the supernatant was vortexed with 2 mL of isopropanol. Fatty acids were extracted by adding 1.5 mL of hexane, vortexing and allowing to stand for a further 3 min. For detection of CLA, 230 µL of each sample was pipetted into a 96-well plate (Costar, Corning, NY, USA) and absorbance was measured at 233 nm using a plate reader (Fluorstar Omega). Four replicates were made for each sample. A standard curve was prepared using the *cis*-9, *trans*-11 CLA isomer (Sigma–Aldrich, Saint Louis, USA).

Antioxidant activities

Antioxidant activities were estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, Steinheim, Germany) radical scavenging assay and the hydroxyl radical scavenging assay as described by Aarti and Khusro (2019) with minor modifications. The DPPH solution (1 mL, 0.2 mM) was added to 1 mL of cell suspension (Abs₆₀₀ = 0.6). The reaction mixture was incubated in the dark at room temperature for 30 min. The DPPH solution was used as a control while the cells with methanol were used as a blank. Absorbance was measured at 517 nm using a plate reader (Fluorstar Omega). The percentage of DPPH scavenging capacity was calculated using the fol-

following formula:

$$\text{DPPH scavenging capacity (\%)} = \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}}\right) \times 100.$$

For the evaluation of hydroxyl radical scavenging activity, 1.0 mL of freshly prepared cells ($Abs_{600} = 0.6$) was inoculated into 4.5 mL reaction mixture (1 mL 0.5 mM Brilliant Green, 2.0 mL 0.5 mM $FeSO_4$, and 1.5 mL H_2O_2). The mixture was incubated at room temperature for 15 min and absorbance was measured at 624 nm using a plate reader (Fluorstar Omega). The hydroxyl radical scavenging activity was calculated using the following formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{(A_1 - A_0)}{(A - A_0)} \times 100.$$

where A_1 is the absorbance of sample, A_0 is the absorbance of the control, and A is the absorbance without the sample and the Fenton reaction system.

Statistical analysis

At least three independent replicates were performed in all experiments and results were expressed as mean values \pm SEM. Comparisons between isolates were analysed by one-way analysis of variance (ANOVA). Data of probiotic evaluation (resistance to low pH and to bile acids and pancreatin) were subjected to factorial ANOVA, including isolates and time as factors. When statistically significant differences were found ($P < 0.05$), post-hoc Tukey's test was used to discriminate between isolates. Statistical tests were performed using IBM SPSS Statistics, version 25 (IBM Corporation, Armonk, NY, USA).

Results

Phylogenetic analysis

Based on physiological and biochemical characteristics and 16S rRNA gene sequence analysis results, six isolates were identified as *Lactocaseibacillus paracasei* (isolates BM1–BM6), and one isolate was identified as *Lactobacillus gasseri* (BM7). A phylogenetic tree utilizing the neighbouring (Maximum Likelihood) method was constructed based on their 16S rDNA sequences plus three of 16S partial nucleotide sequences from Genbank (*L. gasseri* ATCC 33323, *L. casei* ATCC 393 and *L. paracasei* ATCC 25302). As shown in Fig. 1, isolates of *Lactocaseibacillus* clustered together with *L. paracasei* ATCC, while the isolate identified as *L. gasseri* associated with ATCC strain in the same taxa.

Characterization of isolates

The growth parameters—lag time (λ), maximum specific growth rate (μ_{max}) and maximum OD value (X_{max}), as well as the corresponding values for acidification, were calculated from the values density (OD), and are presented in Table 1. Overall, the isolates showed high growth rates and the *L. gasseri* isolate had no lag phase. Three *L. paracasei* (isolates BM4, BM5, and BM6) also presented high acidifying ability, reducing pH by over two units after 6 h, while *L. gasseri* BM7 showed moderate acidifying capacity, reducing pH by one unit after 6 h and two units after 12 h.

The isolates were also evaluated for enzymatic patterns determined with the APIZym system, their ability to ferment sugars, and produce EPS (Fig. 2). Principal component analysis (PCA) was applied to the APIZym results, resulting in a clear separation of the two species (Fig. 2, A and C). All isolates were negative for trypsin, α -chymotrypsin, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase, and consequently they were not included in PCA. *L. gasseri* (BM7) distinguished from *L. paracasei* isolates by the absence of several enzymes such as alkaline phosphatase, acid phosphatase, lipase (C14), β -glucuronidase, α -glucosidase, and low activity of esterase (C4), esterase lipase (C8), and valine arylamidase.

Regarding the ability to ferment various sugars (Fig. 2C and D), all isolates showed a similar pattern, with exception of *L. paracasei* BM4 and *L. gasseri* BM7. These isolates presented a low ability to ferment raffinose, in contrast to the other *L. paracasei* (BM1, BM2, BM3, BM5, and BM6). Glucose and mannitol were fermented by all isolates, unlike xylose, rhamnose, starch and glycerol which were not fermented by any of the isolates. Lactose, ribose, sorbitol, dextrin, and inulin were fermented by all isolates except for *L. gasseri* BM7. Furthermore, none of the isolates produced EPS from the sugars tested, with the exception of *L. paracasei* BM3 and BM5, which produced EPS from lactose.

Safety evaluation

Phenotypical tests for virulence factors revealed that all isolates were negative for haemolytic, DNase, and gelatinase activities (results not shown). Isolates also tested negative for all the virulence genes (Table S1). In addition, all isolates were susceptible to most of antibiotics tested except for vancomycin and kanamycin (Table S1).

Probiotic properties

In vitro digestion simulators were used to screen the probiotic potential of lactobacilli. Fig. 3 (A and B) shows the number of viable lactobacilli under simulated gastrointestinal conditions for 3 h (simulating digestion time). Overall, the isolates showed greater resistance to bile acids and pancreatin than to acidic pH. No significant differences ($P > 0.05$) were observed between *L. paracasei* isolates in terms of resistance to the acidic conditions of stomach. All isolates survived at pH 2.5 for up to 1 h (Fig. 3A). However, no viable colonies were detected after 2 h. In contrast, all isolates showed viable colonies after 3 h bile acid and pancreatin solution. Some isolates showed a slight decrease in number after 1 h but remained stable until 3 h (Fig. 3B).

The ability of bacteria to adhere to intestinal cells was evaluated by determining cell surface hydrophobicity. All isolates showed a good adhesion capacity, as they presented hydrophobicity values ranging 60%–90% (Fig. 3C).

Antimicrobial activity

The inhibitory activity of CFS produced by isolates against various human and foodborne pathogens is presented in Table 2. All isolates showed a distinct zone of inhibition (with a diameter of 10 mm or more) against *Listeria* strains, with isolate BM2 showing the largest inhibition diameter. Remarkably, CFS of all *L. paracasei* isolates showed high inhibition against all pathogens tested. In contrast, *L. gasseri* BM7 showed no antagonistic effects against other pathogens apart from *Listeria*. In addition, the neutralized CFS showed no in-

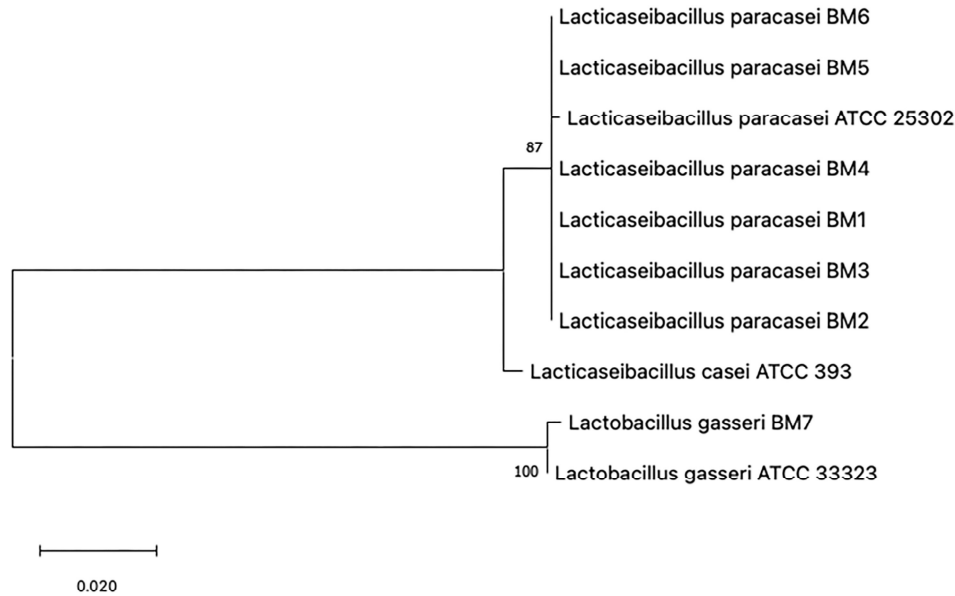


Figure 1. Position of the selected seven isolates in the neighbor joining phylogenetic tree. The phylogenetic tree was constructed on the basis of 16S rDNA sequences. The scale bar 0.01 indicates the nucleotide substitution rate at each site. Bootstrap probabilities were determined using 1000 replicates and presented as the percentage values.

Table 1. Growth parameters and Δ pH in milk whey of lactobacilli isolated from breast milk.

Isolate	Growth parameters			Δ pH		
	Lag (λ)	Rate (μ_{\max} h $^{-1}$)	X $_{\max}$ (OD)	3h	6h	12h
<i>L. paracasei</i> BM1	4.446 \pm 3.933	0.0131 \pm 0.00 087	1.057 \pm 0.0492	0.032	0.119	0.323
<i>L. paracasei</i> BM2	3.542 \pm 3.031	0.0138 \pm 0.00 080	0.930 \pm 0.0154	0.236	0.239	1.044
<i>L. paracasei</i> BM3	1.111 \pm 2.858	0.0117 \pm 0.00 049	1.117 \pm 0.0909	0.292	0.392	0.635
<i>L. paracasei</i> BM4	8.112 \pm 2.522	0.0192 \pm 0.00 113	1.134 \pm 0.0184	1.660	2.033	2.804
<i>L. paracasei</i> BM5	8.492 \pm 2.337	0.0177 \pm 0.00 083	1.275 \pm 0.0280	1.535	2.097	2.885
<i>L. paracasei</i> BM6	8.864 \pm 1.469	0.0263 \pm 0.00 099	1.454 \pm 0.0131	1.393	2.238	2.792
<i>L. gasseri</i> BM7	0.0 \pm 0.0	0.0141 \pm 0.00 053	1.076 \pm 0.0302	0.444	1.113	2.311

Table 2. Spectrum of antibacterial activity of lactobacilli isolates against indicator strains by spot bioassays. Inhibition zone diameters (mm) obtained from CFS of isolates correspond to the mean of two independent replicates \pm SEM.

Indicator strains*	<i>L. paracasei</i> BM1	<i>L. paracasei</i> BM2	<i>L. paracasei</i> BM3	<i>L. paracasei</i> BM4	<i>L. paracasei</i> BM5	<i>L. paracasei</i> BM6	<i>L. gasseri</i> BM7
<i>L. innocua</i> ATCC 33090	12.2 \pm 0.2 ^a	14.0 \pm 0.6 ^a	13.9 \pm 0.3 ^a	13.7 \pm 0.0 ^a	13.8 \pm 0.4 ^a	14.3 \pm 0.1 ^a	13.0 \pm 1.2 ^a
<i>L. monocytogenes</i> ATCC 7644	17.5 \pm 0.8 ^{ab}	19.4 \pm 0.1 ^a	16.6 \pm 1.1 ^{ab}	18.1 \pm 1.7 ^{ab}	10.1 \pm 3.6 ^b	13.5 \pm 1.0 ^{ab}	9.8 \pm 0.3 ^b
<i>E. coli</i> ATCC 15922	10.0 \pm 0.1 ^c	10.8 \pm 0.2 ^{bc}	10.1 \pm 0.0 ^c	11.6 \pm 0.2 ^{ab}	12.2 \pm 0.4 ^a	12.5 \pm 0.1 ^a	0.0 \pm 0.0 ^d
<i>E. coli</i> ATCC 8739	10.8 \pm 0.1 ^b	12.2 \pm 0.2 ^a	12.1 \pm 0.4 ^{ab}	10.7 \pm 0.2 ^b	11.4 \pm 0.1 ^{ab}	12.5 \pm 0.5 ^a	0.0 \pm 0.0 ^c
<i>E. coli</i> ATCC 25 922	11.3 \pm 0.1 ^a	11.2 \pm 0.1 ^a	11.6 \pm 0.6 ^a	10.3 \pm 0.1 ^a	11.7 \pm 0.7 ^a	13.2 \pm 1.3 ^a	0.0 \pm 0.0 ^b
<i>S. Typhimurium</i> ATCC 14028	10.3 \pm 1.3 ^a	11.7 \pm 0.2 ^a	10.2 \pm 0.7 ^a	9.3 \pm 1.0 ^a	12.6 \pm 0.1 ^a	12.1 \pm 0.6 ^a	0.0 \pm 0.0 ^b
<i>S. Enteritidis</i> ATCC 13076	8.6 \pm 0.4 ^b	9.2 \pm 0.0 ^{ab}	8.8 \pm 0.0 ^b	9.2 \pm 0.4 ^{ab}	9.0 \pm 0.1 ^b	10.5 \pm 0.4 ^a	0.0 \pm 0.0 ^c
<i>B. cereus</i> ATCC 11778	9.0 \pm 1.2 ^b	9.5 \pm 0.5 ^{ab}	12.5 \pm 0.0 ^a	11.0 \pm 0.5 ^{ab}	11.2 \pm 0.4 ^{ab}	9.0 \pm 0.0 ^b	0.0 \pm 0.0 ^c
<i>B. spizizenii</i> ATCC 6333	10.7 \pm 0.2 ^a	11.2 \pm 0.4 ^a	10.2 \pm 0.6 ^a	9.8 \pm 0.3 ^a	9.9 \pm 0.5 ^a	9.7 \pm 0.0 ^a	0.0 \pm 0.0 ^b
<i>P. aeruginosa</i> ATCC 27853	9.0 \pm 0.9 ^a	9.2 \pm 1.0 ^a	4.4 \pm 4.4 ^a	4.6 \pm 4.6 ^a	9.5 \pm 0.0 ^a	7.6 \pm 0.0 ^a	0.0 \pm 0.0 ^a
<i>S. aureus</i> ATCC 29523	8.1 \pm 0.1 ^a	9.5 \pm 0.2 ^a	8.6 \pm 0.5 ^a	8.9 \pm 0.8 ^a	9.4 \pm 0.2 ^a	9.6 \pm 0.7 ^a	0.0 \pm 0.0 ^b

*Different letters within each line indicate significant ($p < 0.05$) differences between isolates.

hibitory effect against any of the indicator strains (results not shown).

Concerning antifungal activity, the culture overlay test performed with the lactobacilli showed different inhibition values against the *Aspergillus* and *Penicillium* strains (Table 3). The

isolates of *L. paracasei* showed a broad spectrum of antifungal activity, with isolates BM1 and BM2 presenting the highest antagonistic activity. In contrast, *L. gasseri* BM7 showed no activity against any of the mould strains tested with the exception of *P. brevicompactum*.

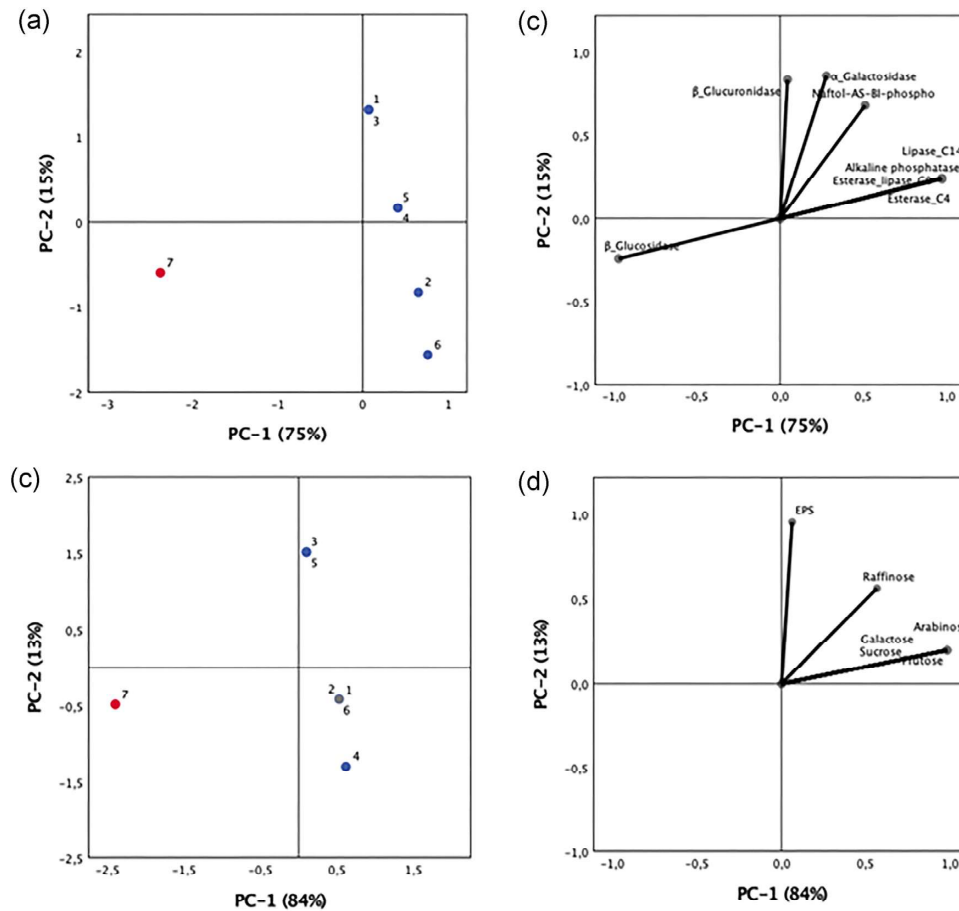


Figure 2. (a) Principal component analysis scores plot and (b) PCA loadings plot showing the isolates according to the enzymatic patterns determined with the APIZym system. PC1 and PC2 explained 75% and 15% of the total variance, respectively. (c) PCA scores plot and (d) PCA loadings plot showing the isolates according to their ability to ferment different sugars and produce EPS from lactose. PC1 and PC2 explained 84% and 13% of the total variance, respectively. The blue dots represent *L. paracasei* (isolates BM1–BM6), and the red dots represent *L. gasseri* (BM7).

Table 3. Antifungal activity of lactobacilli isolates against indicator strains of *Aspergillus* spp. and *Penicillium* spp.

Indicator strains	<i>L. paracasei</i> BM1	<i>L. paracasei</i> BM2	<i>L. paracasei</i> BM3	<i>L. paracasei</i> BM4	<i>L. paracasei</i> BM5	<i>L. paracasei</i> BM6	<i>L. gasseri</i> BM7
<i>Aspergillus flavus</i> MUM16.106	+++	+++	+++	+++	+++	-	-
<i>Aspergillus chevalieri</i> MUM 00.07	+++	+++	+++	+++	+++	+++	-
<i>Penicillium nordicum</i> MUM16.93	+++	+++	+++	+++	+++	+++	-
<i>Penicillium commune</i> MUM 16.56	+++	+++	++	+	+/-	++	-
<i>Penicillium brevicompactum</i> MUM 9906	+++	+++	++	+++	+++	+++	++

Potential health promotion characteristics

In addition to the high nutritional value of breast milk, recent research emphasizes the importance of the milk microbiome in the short- and long-term health benefits that breastfeeding brings to infants. Therefore, several potential health-promoting properties of lactobacilli were investigated in the present study (Table 4).

Overall, the lactobacilli showed high cholesterol-lowering efficacy, ranging from 50% to 60% of cholesterol reduction after 24h, although no differences ($P > 0.05$) were observed between isolates (Table 4). In addition, all *L. paracasei* isolates showed high BSH activity in contrast to *L. gasseri* (BM7), which showed no BSH activity (Table 4).

Isolates also presented high β -galactosidase activities, with isolate *L. paracasei* BM2 having the highest enzymatic activity ($P < 0.05$, 1427 MUs mL⁻¹).

Lactobacilli also produced hydrolysates from milk with high ACE inhibitory activity, ranging from 40% to 97% (Table 4). Isolates BM1, BM2, BM5, and BM7 showed the highest levels of ACE inhibitory activity (over 90%). Concerning CLA production, the isolates of *L. paracasei* produced low levels of CLA, ranging from 1.2 to 2.6 $\mu\text{g mL}^{-1}$. In addition, the *L. gasseri* isolate was unable to produce CLA (Table 4).

The antioxidative activities of whole cells of the tested lactobacilli isolates is also shown in Table 4. All isolates presented high antioxidant activity as measured by DPPH rad-

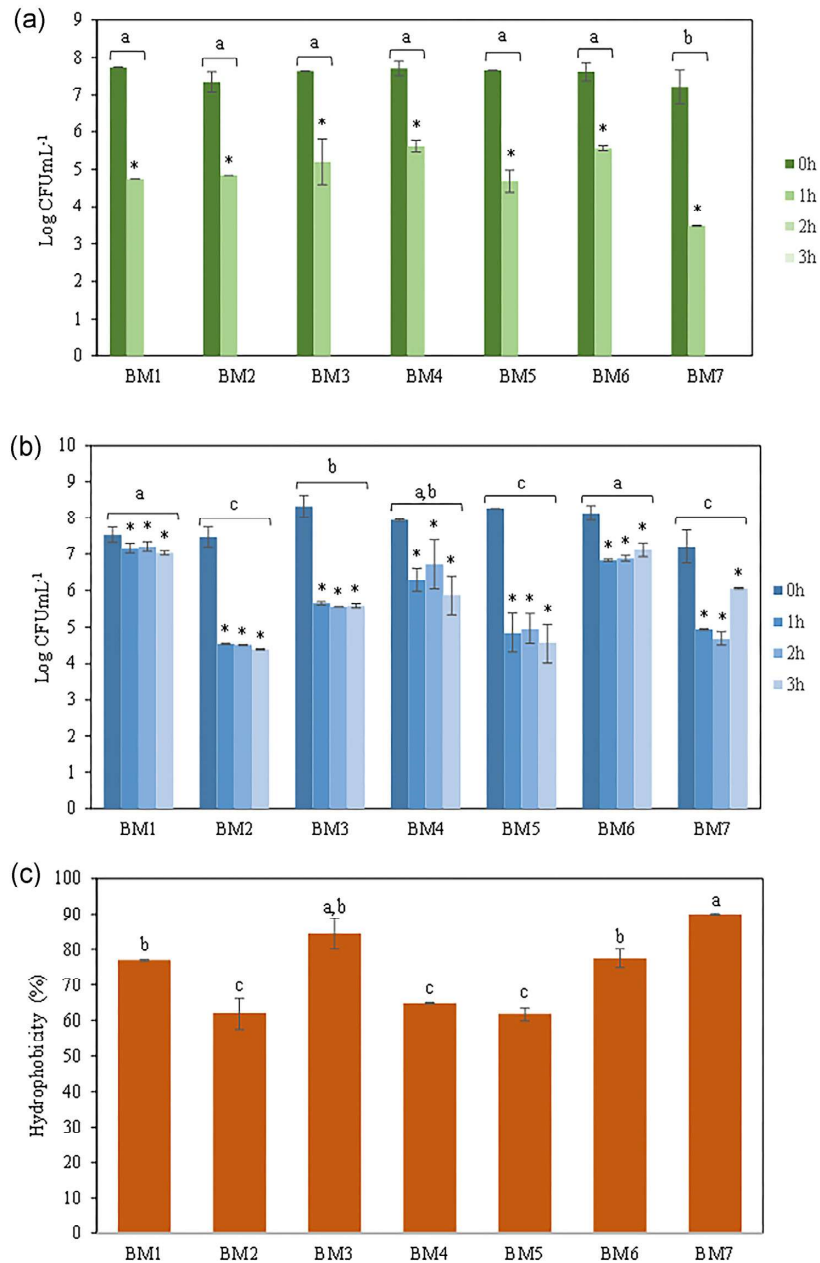


Figure 3. Survival of lactobacilli under simulated gastrointestinal conditions: (a) pH 2.5; (b) bile acids and pancreatin; (c) cell surface hydrophobicity of lactobacilli. Error bars indicate SEM of three independent experiments. *Indicate significant differences ($P < 0.05$) between the initial time point ($t = 0$) and different exposure times. Different lower case letters indicate significant differences ($P < 0.05$) between strains.

ical scavenging, with no significant differences ($P > 0.05$) between them. In contrast, the antioxidant activity of the isolates assessed by hydroxyl radical scavenging activity differed between them ($P < 0.05$). *L. paracasei* BM2 and BM3 showed the highest hydroxyl radical scavenging activity, while *L. paracasei* BM6 showed the lowest.

Discussion

In the present study, six *L. paracasei* and one *L. gasseri* were isolated from human milk and characterized with respect to their technological, probiotic, and health-promoting properties. As expected, the lactobacilli showed high growth rates

and acidifying ability in milk whey. Previous studies have also shown that LAB such as lactobacilli have equivalent growth rates in both sweet whey and milk (Ribeiro et al. 2021). Sweet whey contains most of the nutrients from milk (only casein was removed by rennet coagulation), maintains a similar pH of milk and supports growth and acid production by lactobacilli. The high growth of *L. gasseri* in whey contradicts numerous reports that this species cannot grow well in milk without any supplementation (Arakawa et al. 2015). In fact, *L. gasseri* showed a distinct pattern in the ability to ferment various sugars, such as the inability to ferment lactose. These results are consistent with other reports indicating that most *L. gasseri* strains are unable to utilize lactose (Francl et al.

Table 4. Ability to reduce cholesterol (%), β -galactosidase activity (MU mL⁻¹), ACE inhibitory activity (%), CLA production (μ g mL⁻¹) and antioxidant activities estimated from DPPH scavenging capacity (%) and hydroxyl radical scavenging activity (%) of lactobacilli isolates. Results are the average \pm SEM of three independent experiments.

	Cholesterol reduction (%) [*]	BSH (mm) [*]	β -galactosidase activity (MU mL ⁻¹) [*]	ACE inhibition (%) [*]	CLA (μ g mL ⁻¹) [*]	DPPH scavenging capacity (%) [*]	Hydroxyl radical scavenging activity (%) [*]
<i>L. paracasei</i> BM1	57.6 \pm 3.7 ^a	11.9 \pm 0.1 ^a	1192 \pm 56 ^{ab}	96.77 \pm 0.76 ^a	1.2 \pm 0.5 ^a	43.4 \pm 1.0 ^a	16.0 \pm 0.5 ^{ab}
<i>L. paracasei</i> BM2	60.2 \pm 5.0 ^a	11.4 \pm 0.4 ^a	1427 \pm 15 ^c	92.33 \pm 1.84 ^a	2.2 \pm 0.4 ^a	43.7 \pm 1.1 ^a	18.6 \pm 0.9 ^a
<i>L. paracasei</i> BM3	59.6 \pm 5.1 ^a	10.9 \pm 0.0 ^a	1221 \pm 28 ^{ab}	76.32 \pm 0.63 ^b	2.3 \pm 0.6 ^a	45.9 \pm 0.4 ^a	18.3 \pm 1.2 ^a
<i>L. paracasei</i> BM4	57.3 \pm 4.7 ^a	11.5 \pm 0.6 ^a	1154 \pm 23 ^{ab}	69.42 \pm 4.13 ^b	2.6 \pm 0.5 ^a	44.3 \pm 0.2 ^a	15.1 \pm 1.0 ^{abc}
<i>L. paracasei</i> BM5	57.6 \pm 3.2 ^a	11.6 \pm 0.1 ^a	1138 \pm 21 ^{ab}	93.72 \pm 0.76 ^a	1.8 \pm 0.4 ^a	45.1 \pm 1.8 ^a	15.1 \pm 0.5 ^{abc}
<i>L. paracasei</i> BM6	49.5 \pm 2.5 ^a	11.5 \pm 0.6 ^a	1252 \pm 61 ^{bc}	40.31 \pm 0.06 ^c	2.3 \pm 0.7 ^a	45.3 \pm 2.0 ^a	12.1 \pm 0.3 ^c
<i>L. gasseri</i> BM7	51.4 \pm 9.3 ^a	0.0 \pm 0.0 ^b	1071 \pm 25 ^a	95.56 \pm 0.06 ^a	0.0 \pm 0.0 ^b	43.7 \pm 0.6 ^a	14.3 \pm 0.1 ^{bc}

^{*}Different letters within column indicate significant ($P < 0.05$) differences between isolates.

2012, Pan et al. 2020). Arakawa et al. (2015) reported that *L. gasseri* requires peptides for growth, which could explain the high growth observed in the present study, as peptides are produced by the action of rennet in the production of sweet whey.

Interestingly, the absence of enzymes such as trypsin, α -chymotrypsin, N-acetyl- β -glucosaminidase, α -glucosidase, and β -glucuronidase in *L. gasseri* and some other lactobacilli could be considered an advantageous feature (Bujňáková and Kmeč 2012). For example, the activity of β -glucuronidase in the colon may increase the likelihood of tumour induction (Gill and Rowland 2002). Also, α -glucosidase catalyses the final step in the digestion of carbohydrates, and thus its absence can delay the uptake of dietary carbohydrates and suppress postprandial hyper-glycemia (Monteagudo-Mera et al. 2011). In contrast, the presence of β -glucosidase activity—expressed by all isolates, although in higher expression by *L. gasseri* (BM7), may have an important role in the cleavage of glycosidic bonds of certain disaccharides and oligosaccharides and exert a beneficial effect to the host (Son et al. 2018). In addition, the presence of β -galactosidase can be of interest to mitigate lactose intolerance and produce galacto-oligosaccharides with a prebiotic effect (Monteagudo-Mera et al. 2011).

Isolates *L. paracasei* BM3 and BM5 stand out for their ability to produce EPS from lactose. EPS production is an important probiotic attribute as these polysaccharides produced in the intestine from lactose may have health-promoting effects such as prebiotic, immunomodulatory and anti-inflammatory (Jurášková et al. 2022). Furthermore, the ability to produce EPS plays an important role in the persistence of the producing bacteria in the intestinal environment (Castro-Bravo et al. 2018).

Although lactobacilli have been assigned the qualified presumption of safety (QPS) by the European Food Safety Authority (EFSA) (Panel et al. 2022), safety concerns have been raised about some virulence genes that can be transferred among bacteria of human commensal microbiota and to pathogenic bacteria (Campedelli et al. 2019, Moradi et al. 2022). From these, the main concern is related to the absence of acquired genes conferring resistance to clinically relevant antimicrobials (Panel et al. 2022). Overall, the isolates did not present virulence factors neither resistance to widely used antibiotics in human clinical medicine, apart from vancomycin and kanamycin, which resistance in lactobacilli is intrinsic and

non-transferable (Campedelli et al. 2019). In addition, all isolates were sensitive tetracycline and erythromycin that are two of the most widely used antimicrobials in human and animal therapy and their genetic determinants are usually associated with mobile genetic elements (Chopra and Roberts 2001, WHO 2019, Moradi et al. 2022).

To be classified as probiotics administered through the digestive system, it is important that the bacteria have the ability to survive at low pH to tolerate the initial stress in the stomach (low pH) and intestinal environment (presence of bile acids and pancreatin). The tested isolates presented poor resistance to low pH, but it should be noted that the lactobacilli cells were washed before subjected to the gastrointestinal conditions and were not tested as incorporated into the food. Being ingested in milk, it is possible that they could survive the acidic conditions of the stomach for longer, as the food matrix protects microorganisms against the conditions of the digestive tract (Silva et al. 2015). Thus, although isolates (washed cells) do not show a long resistance to low pH, when incorporated into foods, resistance to acidic pH can increase considerably. Thus, it can be concluded that these isolates have a good ability to survive gastrointestinal conditions.

The hydrophobicity is also a very important parameter to assess the probiotic potential, as it measures the ability of bacteria to adhere to gut cells (Miljkovic et al. 2015, Rajoka et al. 2017). According to Rajoka et al. (2017), probiotics require a minimum hydrophobicity percentage value of 40%. Thus, all isolates have a good adhesion capacity, as they presented hydrophobicity values $>40\%$.

Inhibition of pathogenic bacteria is an important prerequisite for probiotic bacteria. After adhesion to the intestine, probiotics can produce extracellular antimicrobial compounds (e.g. bacteriocins) with activity against various pathogens such as *E. coli*, *Staph. aureus*, *L. monocytogenes* and *Salm. enterica*. Our results suggest that the inhibitory activity obtained with the non-neutralized CFS is not due to the production of bacteriocins (sensitive to the presence of protease) but probably due to the production of organic acids such as lactic, acetic, and propionic acids by the LAB. The observed difference in the antimicrobial activity of CFS between the two species could be due to the difference in metabolism, as *L. gasseri* is homofermentative and produces only lactic acid, while *L. paracasei* is facultative heterofermentative and can convert a variety of acids into lactic and acetic acids (Mikelsaar et al. 2016). Therefore, it is possible that the greater antibacterial activity

of *L. paracasei* isolates is due to the production of acids other than lactic acid, such as acetic and propionic acids. The high acidifying ability presented by some isolates is also a beneficial characteristic because the production of organic acids may result in a broad-spectrum inhibitory activity against Gram-positive and Gram-negative pathogenic bacteria. At an intestinal pH of 5.8, the undissociated form of these acids can penetrate the microbial cell membrane and elicit a synergistic antimicrobial effect (Naidu et al. 1999).

Isolates were also tested for activity against filamentous molds, as these raise health concerns due to possible production of mycotoxins or allergenic spores and mycelia. Overall, *L. paracasei* showed a broad spectrum of antifungal activity, in contrast to *L. gasseri* BM7. The antifungal activity of lactobacilli has been described in several studies and attributed to the production of organic acids such as lactic, acetic and phenyllactic acids (Guimarães et al. 2018, Marie et al. 2018). However, it has been hypothesized that a synergistic mechanism between organic acids and other metabolites may significantly enhance the overall antimicrobial activity of some strains (Salas et al. 2019).

Cumulative evidence suggests a link between the milk microbiota and a healthy gut microbiome that protects infants from diarrhoea (Moubareck 2021). As lactobacilli are among the most important bacteria in breast milk, they may be involved in reducing gastrointestinal infections as they can successfully compete with potentially harmful strains in the infant's gut. In fact, several studies have shown that giving lactobacilli to infants can lead to a reduction in the risk of gastrointestinal infections and other types of infections (Wan et al. 2020). In the present study, the isolates of *L. paracasei* showed high antagonistic activity against foodborne pathogens such as *L. monocytogenes*, *E. coli*, *Salm. enterica* and *B. cereus*. In addition, *L. paracasei* showed antagonistic activity against *Staph. aureus*, suggesting that it could be used to prevent or treat infectious mastitis during lactation. Recently, García-Ricobaraza et al. (2021) reported that oral ingestion of different strains of lactobacilli isolated from human milk reduced the number of pathogenic bacteria and improved mastitis symptoms caused by *Staph. aureus*.

In terms of health-related properties, the isolated lactobacilli were found to lower cholesterol levels and have comparable efficacy to the lactobacilli described in the study by (Domingos-Lopes et al. 2020). The lowering of cholesterol levels by the probiotic bacteria in the gastrointestinal tract (GIT) may help to reduce dietary cholesterol intake, which has a positive effect on hypercholesterolaemia. In addition, the BSH activity of the probiotic bacteria may have a cholesterol-lowering effect by reducing cholesterol absorption and cholesterol catabolism (Jones et al. 2013). Therefore, the ability of LAB to hydrolyse bile salts has been included among the criteria for selecting probiotic strains (Peres et al. 2014). Both the ability to bind cholesterol and to hydrolyse bile salts could explain the association between the consumption of fermented dairy products (with lactobacilli) and the lowering of serum cholesterol levels (Andrade and Borges 2009, Ataie-Jafari et al. 2009, Sadrzadeh-Yeganeh et al. 2010, Ziaei et al. 2021).

One of the most established probiotic traits corresponds to the use of selected LAB strains in relieving the symptoms of lactose intolerance (Oak and Jha 2019). The values of β -galactosidase activity presented by lactobacilli isolates were comparable to those of other authors for lactobacilli, although the results of the Miller test may depend strongly on the ex-

perimental conditions, such as the carbon source (Giacomini et al. 1992, Son et al. 2017) and pretreatment with lysozyme (Uzelac et al. 2015). These lactobacilli are thus good candidates for use as probiotics in individuals with lactose intolerance. It is noteworthy that the *L. gasseri* BM7 showed intracellular β -galactosidase activity although it was unable to ferment lactose. This could be due to the lack of lactose transport found in several strains (Francl et al. 2012).

The use of lactobacilli in milk fermentation has also been shown to be beneficial in controlling cardiovascular disease caused by hypertension, through the hydrolysis of milk proteins into peptides with ACE inhibitory activity (Şanlıer et al. 2019). ACE inhibitors are widely prescribed for the treatment of hypertension as they exert their antihypertensive effect with minor hemodynamic alterations (Rangoonwala and Rosenthal 1998, Pihlanto et al. 2010, Chaves-López et al. 2014). However, the ACE inhibitory effects of peptides produced by lactobacilli from milk proteins can vary widely (Rai et al. 2017). In the present study, all isolates produced peptides from milk with high ACE inhibitory activity. These values are higher than those reported by several authors with bacteria isolated from dairy products (Regazzo et al. 2010, Chaves-López et al. 2014, Solieri et al. 2015), while other studies have also observed strong ACE inhibitory activity in fermented yoghurt by selected strains of *Bifidobacterium* and lactobacilli (Gonzalez-Gonzalez et al. 2011). Similarly, Kumar et al. (2022) reported >80% of ACE inhibitory activity of *L. casei* and *L. delbrueckii* strains isolated from human milk, showing that some strains are able to produce peptides with high bioactivity.

In vitro studies have shown that some strains of lactobacilli can produce conjugated linoleic acid (CLA) isomers from linoleic acid (Kuhl and De Dea Lindner 2016). Since CLA has been shown to have health benefits (Koba and Yanagita 2014), its biosynthesis by LAB could provide a probiotic trait and an alternative to increase the content of these isomers in fermented foods. In this study the isolates were not able to produce CLA or produced reduced levels of CLA compared to those reported by other authors (Alonso et al. 2003, Yang et al. 2014, Ribeiro et al. 2018). Although these isolates were not the most efficient for the conversion of linoleic acid to CLA, the administration of these bacteria could contribute to the *in situ* production of CLA and its intestinal bioavailability.

The antioxidant activity of the LAB strains that colonize the intestinal tract may play an important role in protecting against free radicals. Indeed, several *in vitro* studies suggest that some strains of lactobacilli exert antioxidant activity that benefits host health after they have colonized and multiplied in the human gastrointestinal tract (Ren et al. 2014, Mu et al. 2018). In addition, the antioxidant activity of lactobacilli is also a useful property that can be utilized in food fermentation, as it scavenges free radicals and helps to reduce oxidative damage to food.

In conclusion, all lactobacilli isolated from human milk exhibited excellent probiotic and technological properties *in vitro* based on their tolerance to simulated gastrointestinal conditions, cell surface hydrophobicity, ability to ferment milk, high antimicrobial activity and lack of undesirable properties such as unsuitable antibiotic resistance and enzymatic activity. Among them, two isolates, namely *L. paracasei* BM3 and *L. paracasei* BM5, stand out for their ability to produce EPS from lactose. All *L. paracasei* displayed very similar characteristics, but *L. gasseri* (BM7) differed from the other isolates by the absence of several enzymes and the in-

ability to ferment lactose, although they possessed intracellular β -galactosidase activity. In addition, *L. gasseri* BM7 showed a reduced spectrum of antimicrobial activity compared to the *L. paracasei* isolates. Furthermore, all isolates showed health-promoting potential *in vitro*, as evidenced by high cholesterol-lowering efficacy, high ACE inhibitory activity and pronounced antioxidant activity. All tested isolates can therefore be considered suitable probiotic candidates. However, further experiments are needed to investigate the probiotic potential of these strains *in vivo*. Human trials are also important to assess how these strains may affect children's health in the short and long term.

Acknowledgements

We thank the Micoteca da Universidade do Minho (MUM) for providing the filamentous fungi.

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest

None declared.

Funding

This work was funded by the Portuguese Foundation for Science and Technology (FCT)—project UID/CVT/00153/2019. S. C. Ribeiro gratefully acknowledges financial support from the Regional Fund for Science and Technology (FRCT)—grant M3.1.a/F/017/2018. S. P. M. Silva received support from grant SFRH/BD/139525/2018 and D. Jurášková received support through grant UI/BD/151108/2021, both from the Portuguese Foundation for Science and Technology (FCT).

Author contributions

Vanessa Pires (Data curation, Formal analysis, Investigation, Writing – original draft), Susana C. Ribeiro (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft), Sofia P. M. Silva (Formal analysis, Investigation), Dominika Juraskova (Formal analysis, Investigation) and Celia C. G. Silva (Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing).

Data availability

Data available on request from the authors.

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