

Isolation, identification and characterisation of three novel probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) from the faeces of exclusively breast-fed infants

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Abstract

The aim of the present study was to isolate, identify and characterise novel strains of lactic acid bacteria and bifidobacteria with probiotic properties from the faeces of exclusively breast-fed infants. Of the 4680 isolated colonies, 758 exhibited resistance to low pH and tolerance to high concentrations of bile salts; of these, only forty-two exhibited a strong ability to adhere to enterocytes *in vitro*. The identities of the isolates were confirmed by 16S ribosomal RNA (rRNA) sequencing, which permitted the grouping of the forty-two bacteria into three different strains that showed more than 99% sequence identity with *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Bifidobacterium breve*, respectively. The strain identification was confirmed by sequencing the 16S–23S rRNA intergenic spacer regions. Strains were assayed for enzymatic activity and carbohydrate utilisation, and they were deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) of the Institute Pasteur and named *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036. The strains were susceptible to antibiotics and did not produce undesirable metabolites, and their safety was assessed by acute ingestion in immunocompetent and immunosuppressed BALB/c mouse models. The three novel strains inhibited *in vitro* the meningitis aetiological agent *Listeria monocytogenes* and human rotavirus infections. *B. breve* CNCM I-4035 led to a higher IgA concentration in faeces and plasma of mice. Overall, these results suggest that *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 should be considered as probiotic strains, and their human health benefits should be further evaluated.

Key words: Breast-fed infants: *Bifidobacterium*: *Lactobacillus*: Faeces: Probiotics

The FAO of the UN and the WHO (FAO/WHO) define ‘probiotics’ as ‘live micro-organisms which when administered in adequate amounts confer a health benefit to the host’⁽¹⁾. Lactic acid bacteria (LAB) and bifidobacteria constitute a significant proportion of probiotics, and many of the former are normal and non-pathogenic inhabitants of the human intestine. Among probiotics, *Lactobacillus* and *Bifidobacterium* are the most studied genera^(2,3). Members of these genera are active against gastrointestinal pathogens,

such as *Helicobacter pylori*, rotavirus and urogenital pathogens^(4,5), in both *in vitro* and *in vivo* assays. In addition, some probiotic strains have been proven to be useful in the prevention of various disorders, including diarrhoea, allergy and inflammatory diseases⁽⁶⁾. Moreover, probiotics, as well as their metabolites, have been suggested to play an important role in the formation and establishment of a well-balanced intestinal microbiota in human newborns and adults^(7,8). Researchers have focused their attention on the isolation and

Abbreviations: ATCC, American Type Culture Collection; BHI, Brain Heart Infusion; BSH, bile salt hydrolase; CNCM, Collection Nationale de Cultures de Microorganismes; DMEM, Dulbecco’s modified Eagle’s medium; LAB, lactic acid bacteria; MRS, Man, Rogosa and Sharpe; rRNA, ribosomal RNA.

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characterisation of novel potential probiotic strains from different sources, primarily the gastrointestinal tracts of animals and human subjects, human milk and, less frequently, fruits and fermented foods^(9–13).

To be considered a probiotic, a strain should be able to colonise the gastrointestinal tract and promote host health through its metabolic activities. Specifically, probiotics should survive the acidic conditions of the stomach, resist relatively high levels of bile salts and adhere to the gut epithelium. Attempts to obtain a consensus and guidelines for probiotic safety evaluation have been made by the FAO/WHO⁽¹⁾ and the European Union (EU)-funded Product Safety Enforcement Forum of Europe (EU-PROSAFE) project⁽¹⁴⁾. These groups have recommended that the genus and species of the micro-organism must first be definitively determined by phenotypic and genotypic techniques and those strains should be deposited in an internationally recognised culture collection. For safety, at a minimum, the probiotic strains should also be characterised with respect to their antibiotic resistance patterns, certain metabolic activities (e.g. D-lactate production, bile salt deconjugation and biogenic amines), safety through the use of acute ingestion studies in murine models and the estimation of potential side effects during human studies. Similarly, because probiotics must confer a health benefit to the host⁽¹⁾, it is necessary to ascribe a functional role to the potential probiotic strain, such as evaluating its potential activity against the growth of a human pathogen^(15,16).

The aim of the present study was to isolate, identify and characterise novel LAB strains with potential probiotic properties from the faeces of healthy, exclusively breast-fed infants. The isolated strains were selected primarily for their acid resistance, bile tolerance and ability to adhere to intestinal cells. Sequencing the 16S ribosomal RNA (rRNA) genes and 16S–23S rRNA intergenic spacer regions and assessing enzymatic properties then identified the isolated strains. Following the FAO/WHO guidelines, the isolated strains were deposited in an international type culture collection (Institut Pasteur). Furthermore, to ensure that the selected strains are not harmful to human health and confer a potential benefit to the host, a detailed toxicological characterisation, including metabolic activities, antibiotic resistance and acute ingestion assays, in murine models were performed. Also evaluations of their activity against human pathogens were conducted in *Listeria monocytogenes* and rotavirus. *L. monocytogenes* is an aetiological agent of meningitis and it is characterised by a high case-fatality rate and well-defined risk groups, including pregnant women, newborn infants and neonates⁽¹⁷⁾. Rotavirus is the leading cause of severe acute gastroenteritis among children worldwide⁽¹⁸⁾. Finally, a preliminary immunological study followed by a study on mice on the role of the isolated potential probiotics in enhancement of immunity was conducted.

Materials and methods

Subjects

A total of twelve healthy, exclusively breast-fed infants, aged 1 month, were selected for the study at the Clinic Hospital of

the University of Granada. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethical Committee of the University of Granada. Written informed consent was obtained from the parents after a careful explanation of the nature of the study.

Faecal samples and bacterial growth conditions

The fresh faecal samples were treated according to Thompson-Chagoyan *et al.*⁽¹⁹⁾. All samples were diluted and plated on Beerens medium⁽²⁰⁾ and Bifidobacterium Medium (BFM) medium⁽²¹⁾, which are both selective for *Bifidobacterium* spp., and on Rogosa medium (Oxoid), which is selective for *Lactobacillus* spp. The plates were incubated for 48–72 h at 37°C in an anaerobic or enriched CO₂ atmosphere. For each faecal culture sample, 100 colonies (fifty lactobacilli and fifty bifidobacteria) were randomly selected and incubated in Man, Rogosa and Sharpe (MRS) broth medium (Oxoid) for *Lactobacillus* spp. and in MRS broth medium (Oxoid) supplemented with 0.05% (w/v) cysteine (Sigma-Aldrich) (MRS plus cysteine (MRS-C) medium) for *Bifidobacterium* spp. After centrifugation, the cells were stored at –80°C in glycerol until further analysis.

Characterisation of isolated lactic acid bacteria as potential probiotics

Resistance to low pH and bile salts. The resistance of the isolated bacteria to low pH and different concentrations of bile salts (Oxgall, Sigma-Aldrich) was evaluated by monitoring bacterial growth. Briefly, 900 µl of PBS, which was adjusted to pH 2.0, 2.5, 3.0 or 7.0 (control) or supplemented with 0.3, 0.5 or 0.7 (w/v) Oxgall (Sigma-Aldrich), was inoculated with 100 µl of a 48 h culture, previously washed three times with PBS. After of 3 h culture at 37°C under anaerobic conditions, 50 µl of the diluted cultures was spread on plates of selective culture media and incubated for 48–72 h at 37°C, followed by colony counting. The percentage of viable bacteria was calculated. The assays were performed in three independent experiments.

Only those strains that survived the resistance tests were assayed for adherence to intestinal cells; the commercial strains *Lactobacillus rhamnosus* GG American Type Culture Collection (ATCC) 53103 and *Bifidobacterium longum* BB536 (Morinaga & Company Limited) were grown and used as controls for these assays.

Adhesion to HT-29 cells. The assay for bacterial adhesion to HT-29 cells was performed on the surviving strains described earlier. The cells were grown in twenty-four-well tissue culture plates (NUNC) in Dulbecco's modified Eagle's medium (DMEM); (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), 2 mM-L-glutamine, 1% (w/v) non-essential amino acid preparation (Sigma-Aldrich), 66 µg penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). The cells were cultured at 37°C in an atmosphere of 5% (v/v) CO₂ and 95% air until a confluent monolayer was reached.



Before the adhesion assay, the HT-29 cell monolayers were washed twice with antibiotic-free DMEM. Each well was inoculated with 250 μ l of 48 h LAB culture, washed three times with PBS, diluted in DMEM to an optical density at 600 nm of 0.8 units (equivalent to 10^{10} bacteria) and incubated for 90 min at 37°C in a 5% (v/v) CO₂ atmosphere. After the incubation, each well was washed with PBS and 100 μ l of trypsin-EDTA (Sigma-Aldrich) was added. An aliquot of 50 μ l of the homogenate was spread on plates containing *Lactobacillus* spp.- or *Bifidobacterium* spp.-specific medium. The percentage of bacteria that adhered to the wells was calculated. The assays were performed in three independent experiments.

Identification and biochemical characterisation of isolated strains

The isolated strains that survived low pH, exhibited bile salt resistance and adhered well to intestinal cells were identified by sequencing the 16S rRNA gene and 16S–23S rRNA intergenic spacer region and characterised by enzymatic activities and carbohydrate utilisation. A DNA extraction procedure was performed using the phenol–chloroform method⁽²²⁾. The DNA was checked for purity using standard methods. The 16S rRNA from the selected strains was amplified by PCR with the universal primers 27f 1492r and 39f, as reported previously^(23,24). The 16S–23S intergenic spacer region was amplified with the primers LactoF (5'-ACACCGCCCGTCACAC-CATG-3') and LactoR (5'-CCHSTTCGCTCGCCGCTACT-3') for *Lactobacillus*⁽²³⁾ and the primers F_allbif_IS and R_allbif_IS for *Bifidobacterium*⁽²⁴⁾. The amplification mixture (50 μ l) contained 1 μ l (1 nmol/ μ l) of each primer; 1 μ l (1 U/ μ l) *Taq* DNA polymerase (Biotools B&M Labs); 5 μ l 10 × reaction buffer (Biotools); 200 μ M of each deoxynucleoside triphosphate (Biotools) and 1 μ l DNA template. The DNA template was amplified by an initial incubation at 80°C for 5 min, denaturation at 94°C for 2 min, thirty-five cycles of denaturation at 94°C for 30 s, annealing at 55°C (27f-1492r), 65°C (39f-1391r) or 60°C (LactoR-LactoF; F_allbif_IS-R_allbif_IS) for 30 s and extension at 72°C for 1.5 min (27f-1492r; 39f-1391r) or 1 min (LactoR-LactoF; F_allbif_IS-R_allbif_IS), followed by a final extension at 72°C for 10 min. Controls devoid of DNA were included in the amplification process. The integrity of the PCR products was assayed by detection of single bands following electrophoresis. The PCR products were purified with the Illustra™ GFX™ PCR DNA and Band Purification Kit (General Electric Healthcare) and sequenced by the Genomic Service of the Institute of Parasitology and Biomedicine 'Lopez-Neyra' (Consejo Superior de Investigaciones Científicas).

The resulting sequences were automatically aligned, inspected by eye and compared with the online tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The strains were identified based on the highest hit scores. The 16S rRNA sequences of *L. paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 were deposited in the National Center for Biotechnology Information (NCBI) nucleotide sequence database under the accession no. JQ621984, JQ621983 and JQ621982, respectively.

Carbohydrate fermentation and the enzymatic activities of each LAB strain were analysed with the API 50 CHL System Kit and API ZYM Kit, according to the manufacturer's protocol (BioMérieux).

Sensitivity to antibiotics

The sensitivity of each strain to twenty antibiotics was determined according to the European Food Safety Authority's (EFSA) recommendations⁽²⁵⁾. The minimum inhibitory concentration values were determined in LAB susceptibility test medium (LSM) broth formulation⁽²⁶⁾ using the broth dilution antimicrobial susceptibility test established by the Clinical and Laboratory Standards Institute. The antibiotics were tested over a concentration range of 0.125–512 mg/l. The assays were performed in three independent experiments.

Toxicological study

Non-desired metabolite production

Lactic acid isomers production. Lactic acid production was determined in supernatants from a 17 h culture of each bacterial strain grown in their optimum conditions with a commercial kit (D-lactic acid/L-lactic acid; Megazyme). The assays were performed in three independent experiments.

Deconjugation of bile salts. Bile salt hydrolase (BSH) activity of intact and sonicated cells, as well as cell-free supernatants, was evaluated with the substrates glycocholate and taurocholate, according to the technique of Kumar *et al.*⁽²⁷⁾. The assays were performed in three independent experiments.

Formation of biogenic amines. The formation of biogenic amines cadaverine, histamine, putrescine and tyramine in the cell-free supernatants of 17 h cultures was detected following the chromatographic method described by Eerola *et al.*⁽²⁸⁾. The assays were performed in three independent experiments.

Acute ingestion study in immunocompetent and immunosuppressed mice. All procedures involving animals were conducted in accordance with the regulations established by the European Community Council on the protection of animals used in experimental and scientific applications (Regulation 86/609/EEC), and the experimental protocol was approved by the Biopolis Ethics Committee. Acute ingestion study was performed according to Chenoll *et al.*⁽²⁹⁾. In brief, the assays were performed in 7 week-old pathogen-free male BALB/c mice. Immunosuppressed group were achieved by intraperitoneal administration of cyclophosphamide (40 mg/kg per d), 5 d prior to the first bacterial administration and daily throughout the study, and kept inside containment units under positive pressure. After 2 weeks of acclimatisation, the mice were fed each day for 6 d with the three isolated strains (5.5×10^8 CFU) or placebo (lyophilised skin milk with sucrose (5%, w/v)) in a total volume of 200 μ l by oral gavage.

Mortality and morbidity were noted twice a day and individual body weights were recorded at the beginning and end of the trial. At day 7 of the study, blood from each mouse was collected by submandibular venipuncture and immediately

the mice were killed by cervical dislocation. For the bacterial translocation assessment, liver, spleen and mesenteric lymph nodes were removed under aseptic conditions to avoid any cross-contamination and bacterial counts at 37°C on MRS agar for *Lactobacillus* strains and MRS-C for *Bifidobacterium* strains were obtained. For the histological examination, the duodenum–jejunum, the proximal colon, the distal ileum and the distal colon were immediately excised, fixed (10% (v/v) buffered formalin) and embedded in paraffin. Pieces of 5 µm were stained with haematoxylin and eosin and examined by direct microscopy.

Inhibition of pathogens by lactobacilli and bifidobacteria supernatants

Obtaining the supernatants. To obtain supernatants, the bacterial strains were grown anaerobically for 17 h and 24 h at 37°C in MRS medium (lactobacilli) or MRS-C (bifidobacteria). After centrifugation at 12 000 g for 10 min, supernatants were neutralised to pH 6.5 with NaOH (1 M) and concentrated to 10 × by freeze-drying. Concentrated supernatants were sterilised by filtration through a 0.22 µm pore-size filter and stored at –20°C until use.

***Listeria monocytogenes* strains and growth conditions.** *L. monocytogenes* strains CECT 935, CECT 4031 and CECT 911 were obtained from the Spanish Type Culture Collection (CECT). *L. monocytogenes* was grown in Brain Heart Infusion (BHI) broth and incubated aerobically for 24 h at 37°C.

Cells lines and viruses. The human colon carcinoma cell line HT-29 was grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). The human rotavirus Wa strain (TC adapted; ATCC VR-2018) was obtained from the ATCC. The human rotaviruses Ito and Va70 were kindly provided by Dr Javier Buesa (Hospital Clínico Universitario). The viral stocks of the human rotavirus strains were propagated by infecting HT-29 cells in the presence of 1 µg/ml trypsin (type IX; Sigma). Aliquots of the viruses were stored at –80°C until use.

Activity of lactobacilli and bifidobacteria supernatants against *Listeria monocytogenes*. Assays were performed in polystyrene ninety-well (volume, 200 µl/well) multiwell plates (Maxisorp; Nunc). BHI broth was inoculated with a 5% (v/v) concentrated *L. monocytogenes* cell solution, and the neutralised supernatant was added to a final concentration of 0.4, 2 or 4% (v/v). The ability of the strains to inhibit *L. monocytogenes* was evaluated by monitoring bacterial growth at 37°C in BHI medium in ninety-six-well plates. Bacterial growth was analysed at 655 nm in a Multiskan microplate reader (Thermo Fisher Scientific). The percentage resistance was calculated in each case by comparing the final optical density with that of the corresponding controls, which were grown in BHI.

Rotavirus propagation and *in vitro* inhibition assays

Inhibition assays. Infection assays were carried out in HT-29 cell line according to Moreno Muñoz *et al.*⁽⁴⁾. Briefly, supernatants (1X) were added to HT-29 cell monolayers. After 1 h at 37°C, the supernatant was removed and the viral inoculum was added. After 1 h at 37°C DMEM medium was

added and the plate was incubated for 15–18 h at 37°C with 5% CO₂. Prior to the immunoperoxidase assay, the infected HT-29 cell monolayers were fixed with methanol–acetone (1:1 (v/v)) for 15 min.

Immunoperoxidase assays. Following Moreno Muñoz *et al.*⁽⁴⁾, to detect viral antigens anti-VP6 monoclonal antibody 2F3E7 was added in fixed cells. The mixture was incubated at 37°C for 1 h and peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) (100 µl/well, diluted 1/2000 in PBS-bovine serum albumin) was added and incubated at 37°C for 1 h. After washing with PBS, 100 µl/well of diamine-benzidine substrate was added. The infectious, peroxidase-stained foci were counted through an inverted microscope using five defined fields per well. The arithmetic mean was calculated to determine the number of foci per microscopic field, and these values were compared with the number of infectious foci in an untreated virus control.

Immunological studies

Experimental animal group. As described previously, all procedures involving animals were conducted in accordance with Regulation 86/609/EEC. All assay conditions (bacterial cell culture, housing of the mice, assigning the groups and killing the animal) were conducted as explained in the acute ingestion study. After 2 weeks of acclimatisation, the mice were fed with 200 µl of the three isolated strains or placebo by oral gavage every 48 h during 4 weeks. Mortality and morbidity were noted twice a day throughout the study. Individual body weights were recorded at the beginning and end of the trial.

Determination of total IgA and cytokine production. On the last day of the assay, faeces were collected. Determination of total IgA was performed as in Grewal *et al.*⁽³⁰⁾. After killing, the spleen was removed under laminar flow and homogenised in PBS–bovine serum albumin buffer. The resulting homogenates were filtered through a cell strainer 70 µm (BD Falcon). Erythrocytes were lysed and splenocytes were inoculated in six-well plates at a concentration of 10⁶ cells/well. Plates were incubated during 24 h at 37°C with 5% CO₂. Then, cultures were stimulated with ionomycin and PMA for 48 h. After this time, cell supernatants were recovered and cytokines interleukin (IL)2, IL-4, IL-10, IL-12 and interferon-γ were quantified by flow cytometry by using the FlowCytomix kit (Bender MedSystems).

Statistical analysis

The results are expressed as the mean and standard deviation. Differences between the mean values for different treatments with the isolated strains or their supernatants were analysed by one-way ANOVA. The least significant difference test was used for a posteriori *r*-paired comparison of the means. The statistical analysis was performed with Statgraphics plus (version 5.1) software (Manugistics).

For the murine assays, the organ weights were compared among the groups using Tukey's multiple comparison tests.



Results

Isolation and identification

A total of 4680 colonies were isolated from twelve faeces samples from breast-fed infants. Of these, 758 were resistant to low pH and were also tolerant to bile salts; these bacteria were tested for adhesion to HT-29 cells. In total, forty-two out of the 758 selected colonies strongly adhered to the cells, and consequently, their 16S rRNA genes were sequenced, resulting in the identification of three different strains that had greater than 99% sequence identity to *B. breve*, *L. paracasei* and *L. rhamnosus* species. The three isolated strains were deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) of Institute Pasteur. These strains were considered unique and were named as follows: *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036

Carbohydrate utilisation and enzymatic activities

An analysis of carbohydrate fermentation by the isolated LAB strains was done using the API 50 CHL System kit (see Materials and methods section for details). Both *Lactobacillus* strains utilised ribose, *N*-acetyl-glucosamine, arbutin, cellobiose, esculin, D-fructose, D-galactose, D-glucose, lactose, mannitol, D-mannose, melezitose, rhamnose, salicin, sorbitol, L-sorbose, trehalose, D-tagatose and D-turanose. Dulcitol, inulin and sucrose were utilised by the *L. paracasei* CNCM I-4034 strain but not by the *L. rhamnosus* CNCM I-4036 strain. Gentibiose, gluconate, maltose and α -methyl-D-glucoside were variably utilised by both strains, and adonitol, amygdalin and D-xylose were utilised only by *L. paracasei* CNCM I-4034 strain. The *B. breve* CNCM I-4035 strain utilised *N*-acetyl-glucosamine, amygdalin, L-arabinose, D-arabitol, L-arabitol, cellobiose, esculin, L-fucose, D-fructose, D-galactose, gentiobiose, gluconate, D-glucose, lactose, D-mannose, maltose, mannitol, melezitose, α -methyl-D-glucoside, D-raffinose, ribose, salicin, trehalose and D-turanose.

The enzymatic activities of the isolated LAB strains were also evaluated using the API ZYM kit (see Materials and methods section for details). *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036 strains exhibited

acid phosphatase, alkaline phosphatase, arylamidase, cystine naphthol-AS-BI-phosphohydrolase, esterase (C4), esterase lipase (C8), β -galactosidase, α -glucosidase, leucine arylamidase and valine arylamidase activities. Only *L. rhamnosus* CNCM I-4036 strain exhibited α -chymotrypsin, α -fucosidase, α -galactosidase, β -glucuronidase and β -glucosidase activities. The strongest α -glucosidase, leucine arylamidase and valine arylamidase activities were exhibited by *L. paracasei* CNCM I-4034 strain. The strongest acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), α -fucosidase, β -glucosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities were exhibited by *L. rhamnosus* CNCM I-4036 strain. The *B. breve* CNCM I-4035 strain exhibited acid phosphatase, esterase (C4), esterase lipase (C8), α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, leucine arylamidase, lipase (C14) and naphthol-AS-BI-phosphohydrolase activities. In particular, α -galactosidase, β -galactosidase and α -glucosidase activities were very strong in this strain.

Resistance to low pH, tolerance to bile salts and adhesion studies

The *in vitro* resistance of the isolated LAB strains to low pH and bile salts (using Oxgall, Sigma-Aldrich) were compared with that of two commercial strains (*L. rhamnosus* GG and *B. longum* from Morinaga & Company Limited (see results in Table 1). The five strains were able to grow in the presence of bile salts. The *B. breve* CNCM I-4035 and the *L. paracasei* CNCM I-4034 strain, as well as the two commercial strains, survived at pH 3.0. From all the tested strains, *L. rhamnosus* CNCM I-4036 was the most resistant to low pH, with 76.2% survival at pH 2.0. Also the results of the adhesion of the isolated LAB strains to HT-29 cells are shown in Table 1. Results indicate that isolated LAB strains adhered to HT-29 cells more efficiently than the two commercial analysed strains.

Sensitivity to antibiotics

The isolated strains did not show resistance to antibiotics (results not shown), except for 256 μ g/ml of metronidazole, nalidixic acid and sulphonamide, and both the isolated *Lactobacillus* strains were resistant to 256 μ g/ml of vancomycin.

Table 1. Resistance to pH and bile salts and adhesion to HT-29 cells of the *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 strains, and the control strains *L. rhamnosus* GG and *Bifidobacterium longum*

Strain	Survival (%)						Adhesion to HT-29 cells
	pH			Bile salt levels (Oxgall, Sigma-Aldrich)			
	3.0	2.5	2.0	0.3%	0.5%	0.7%	
<i>L. paracasei</i> CNCM I-4034	112.4	0	0	114.7	106.0	154.3	7.5
<i>B. breve</i> CNCM I-4035	139.6	0	0	86.9	98.4	123.0	16.7
<i>L. rhamnosus</i> CNCM I-4036	101.8	81.4	76.2	101.5	105.1	105.0	11.6
<i>L. rhamnosus</i> GG	106.2	0	0	57.8	231.8	76.1	4.8
<i>B. longum</i>	104.8	0	0	100.4	95.9	91.9	9.1

CNCM, Collection Nationale de Cultures de Microorganismes.

Table 2. Detection of undesirable metabolites in both cells and supernatants of the *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 strains, and the control strains *L. rhamnosus* GG and *Bifidobacterium longum* (Mean values and standard deviations, *n* 3)

Strains	BSH activity (nmol/μg)																	
	Lactic acid (g/l)				Cell-free supernatant				Sonicated cells				Biogenic amines (mg/ml supernatant)					
	D-Lactic acid		L-Lactic acid		BSH (Taurine)		BSH (Gly)		BSH (Taurine)		BSH (Gly)		Cadaverine		Histamine		Tyramine	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<i>L. paracasei</i> CNCM I-4034	0.60	0.01	13.40	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.01	3.15	0.50	5.00	0.20
<i>L. rhamnosus</i> CNCM I-4036	0.70	0.03	15.23	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.70	0.00	3.50	0.40	4.85	0.20
<i>B. breve</i> CNCM I-4035	0.07	0.00	0.74	0.12	0.00	0.00	0.14	0.01	0.00	0.00	0.14	0.01	0.60	0.01	2.90	0.60	6.70	2.30
<i>B. longum</i> Morinaga	0.03	0.01	5.10	0.63	10.67	0.00	8.67	0.00	0.28	0.00	0.15	0.00	0.53	0.05	0.51	0.06	1.80	0.20
LGG	0.68	0.06	12.10	2.05	0.00	0.00	0.15	0.02	0.00	0.00	0.00	0.00	0.93	0.12	0.56	0.05	0.29	0.24

CNCM, Collection Nationale de Cultures de Microorganismes; BSH, bile salt hydrolase.

Toxicological evaluation

Ex vivo assays. The results of lactic acid production assays are shown in Table 2. The *B. breve* CNCM I-4035 strain produced lower levels of L-lactic acid than the commercial strain *B. longum* Morinaga. D-Lactic acid levels were very low in both bifidobacteria. *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036 produced slightly more L-lactic acid than *Lactobacillus* GG strain, whereas the levels of D-lactic acid produced by both strains were similar to that obtained from the commercial strain. There was no detectable BSH activity with either taurocholate or glycocholate in the supernatants, intact cells or sonicated cells from 17 h cultures of *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036. BSH activity was observed in the supernatant from 17 h cultures and sonicated cells of *B. breve* CNCM I-4035 in the presence of glycocholate. Of the commercial strains, only the supernatant of *Lactobacillus* GG exhibited BSH activity in the presence of glycocholate, and the BSH activity of the *B. longum* Morinaga strain was higher than that of the *B. breve* CNCM I-4035 strain (Table 2).

The results of the biogenic amine quantification in cell-free supernatants are summarised in Table 2. Putrescine was not detected from any of the isolated strains. For other amines, the values ranged from 0.70 to 4.85 for *L. rhamnosus* CNCM I-4036, 0.60 to 5.00 for *L. paracasei* CNCM I-4034 and 0.60 to 6.70 for *B. breve* CNCM I-4035. The values of these three amines for the *B. longum* strain were lower than those obtained for *B. breve* CNCM I-4035. For *Lactobacillus* GG strain, the values of cadaverine and histamine were lower than those obtained for *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036. Of the considered amines, tyramine levels were the highest for the three isolated strains.

In vivo assays. Neither mortality nor adverse clinical signs were observed during the study. There were no statistically significant differences in body weight gain between the placebo and the treated groups (Table 3). In the immunosuppressed groups, there was a statistically significant loss in body weight throughout the study. None of the bacterial counts of the three isolated strains were found in the blood, spleen and liver. There were slight, but not significant, difference in the duodenum/jejunum and the ileum villus height and depth of the colonic crypts of the proximal and distal colon in the immunocompetent and immunosuppressed mice (data not shown). The Lieberkühn crypts had a uniform and constant aspect. However, the Brunner and Lieberkühn glands exhibited hyperplasia in the duodenum of the immunosuppressed mice, an effect that was not observed in immunocompetent mice. In the immunosuppressed mice treated with placebo, hyperplasia appeared in all cases (Table 3).

The three isolated strains yielded slight but not significantly different numbers of goblet cells in the small intestine. In the immunocompetent mice inoculated with the three isolated strains, the lymphoid tissue associated with the intestinal mucosa exhibited a higher number of lymphatic follicles and lower number of germinal centres than in the immunosuppressed mice. In both the immunosuppressed and immunocompetent mice, there were no statistically significant



Table 3. Results of acute ingestion assays of the *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 strains, and the control strains *L. rhamnosus* GG and *Bifidobacterium longum* in immunocompetent and immunosuppressed mice† (Mean values and standard deviations)

	Weight gain (g)†				No. of lymphatic follicles‡								No. of goblet cells in colon							
	IC***		IS***		IC***		IS***		IC***		IS***		IC		IS					
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD				
<i>L. paracasei</i> CNCM I-4034	-0.5	0.2	-3.6	0.2	10.7	2.5	6.7	2.3	6	16	0/6	3/6	4.5	0.8	11.8	1.9	6.0	0.6	9.5	0.8
<i>B. breve</i> CNCM I-4035	-0.7	0.2	-3.3	0.3	9.2	3.5	6.3	2.3	4	12	0/6	2/6*	7.5	2.3	13.8	1.6	5.0	0.6	10.3	0.5
<i>L. rhamnosus</i> CNCM I-4036	-0.7	0.3	-3.4	0.1	7.8	3.1	5.3	1.0	5	12	0/6	1/6*	5.6	2.7	11.3	2.9	5.6	0.8	10.2	1.3
Placebo	-0.8	0.0	-3.8	0.1	12.3	7.4	6.2	2.3	12	10	0/6	6/6*	4.7	0.5	10.5	2.5	5.8	0.8	12.6	0.8

CNCM, Collection Nationale de Cultures de Microorganismes; IC, immunocompetent mice; IS, immunosuppressed mice.

Values were significantly different: * $P < 0.05$; *** $P < 0.001$.

† Body weight gain is expressed as the final weight minus the initial weight.

‡ Data are expressed as the means and standard deviations of six doses.

§ Total germinal centres.

differences among groups treated with different isolated strains. The *L. paracasei* CNCM I-4034 and placebo treatment groups tended to have a higher number of lymphatic follicles. The *L. rhamnosus* CNCM I-4036-treated groups exhibited the lowest number of lymphatic follicles in both the immunosuppressed and immunocompetent groups. The immunosuppressed mice had more germinal centres than the immunocompetent mice. There were no statistically significant differences among the different treatments in the immunocompetent and immunosuppressed mice (Table 3).

Pathogen inhibition assays

Assay of activity against *L. monocytogenes*. The activity of the analysed supernatants against the three different strains of *L. monocytogenes* was variable and specific to the strain (Table 4). The 17 h supernatant of *L. paracasei* CNCM I-4034 primarily inhibited two *L. monocytogenes* strains (CECT 935 and CECT 4031). The 24 h supernatant of *L. rhamnosus* CNCM I-4036 at 2 and 4% inhibited all of the *L. monocytogenes* strains. The *L. monocytogenes* CECT 911 strain was inhibited by both *Lactobacillus* strains at all supernatant percentages. The supernatant of *B. breve* inhibited *L. monocytogenes* CECT 4031 regardless of the supernatant percentage, and the less-concentrated supernatant (0.4% (v/v)) inhibited *L. monocytogenes* CECT 911.

Rotavirus inhibition assays. HT-29 cells infected with the human rotavirus strains Ito, Va70 and Wa were used in inhibition assay using 1 × supernatants from 17 and 24 h cultures (Table 5). All three viruses were inhibited by 24 h supernatants of *L. rhamnosus* CNCM I-4036. *L. paracasei* CNCM I-4034 inhibited only the Wa and Va70 virus strains, whereas the *B. breve* supernatant did not inhibit any of the viruses.

Immunological effects

Also, in this case, there were no statistically significant differences in body weight gain between the placebo and treated groups (data not shown). The measurement of IgA concentration in faeces was higher in the case of the group fed with the *B. breve* CNCM I-4035 strain (results not shown). No statistical differences were detected between the four experimental groups for the measurement of most of the different assayed cytokines (IL-2, IL-4, IL-12 and interferon- γ). Only in the case of IL-10, there was a higher production by the *B. breve* CNCM I-4035 strain (results not shown).

Discussion

The beneficial properties of probiotics and the increased human consumption of these products have augmented efforts to identify potential probiotic strains. Selection and identification criteria for probiotic strains are now considered essential. In selecting potential probiotic strains, species identification by 16S rRNA gene and 16S–23S intergenic spacer region sequence analyses and the evaluation of their physico-chemical, safety and functional properties, such as

Table 4. *In vitro* activity of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 strain supernatants from 17 and 24 h cultures (0.4, 2 and 4% (v/v)) against *Listeria monocytogenes* strains (Mean values and standard deviations, n 3)

Bacteria	0.4% (v/v)				2% (v/v)				4% (v/v)					
	17 h		24 h		17 h		24 h		17 h		24 h			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
<i>L. monocytogenes</i> CECT 935														
<i>L. paracasei</i> CNCM I-4034	0.00	0.00	0.00	0.00	33.09***	0.32	6.64	0.56	0.00	0.00	1.89	0.63		
<i>B. breve</i> CNCM I-4035	0.69	0.36	0.00	0.00	1.24	0.01	0.00	0.00	0.03	0.02	0.00	0.00		
<i>L. rhamnosus</i> CNCM I-4036	1.51	0.08	3.21*	0.70	8.43**	0.94	13.86**	0.03	2.45*	0.83	30.31***	0.06		
<i>L. monocytogenes</i> CECT 4031														
<i>L. paracasei</i> CNCM I-4034	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.48**	0.20	0.00	0.00		
<i>B. breve</i> CNCM I-4035	3.32*	0.26	16.63**	0.26	1.06	0.95	9.88**	0.04	4.54**	0.34	28.05***	0.31		
<i>L. rhamnosus</i> CNCM I-4036	0.00	0.00	0.00	0.00	1.24	0.49	10.67**	1.56	8.21**	0.66	15.29**	0.82		
<i>L. monocytogenes</i> CECT 911														
<i>L. paracasei</i> CNCM I-4034	2.60*	0.33	5.73**	0.25	7.31**	1.84	9.92***	0.38	25.64**	0.38	16.81***	0.59		
<i>B. breve</i> CNCM I-4035	4.22**	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
<i>L. rhamnosus</i> CNCM I-4036	8.46**	0.56	5.18**	0.13	11.99**	0.88	18.36**	3.50	15.53**	0.33	28.13***	0.21		

CNCM, Collection Nationale de Cultures de Microorganismes.
P*<0.05; *P*<0.01; ****P*<0.001.

resistance to gastric acid and tolerance to bile salts, are highly important⁽¹⁾.

There are many molecular tools for the identification of micro-organism species. Among them, 16s rRNA gene sequencing is the most frequently used, because it is extremely useful for determining phylogenetic relationships among organisms from the level of domains to the level of moderately closely related species^(31,32). By comparing the 16S ribosomal DNA sequences of the isolated strains with the sequences available in NCBI/BLAST (100% homology), the isolated strains were identified as *L. paracasei*, *L. rhamnosus* and *B. breve* species, respectively. These bacteria are known to be present in the faeces of breast-fed infants, in which the genus *Bifidobacterium* accounts for 40–60% of the total microbiota and *B. breve* species are present in a high percentage⁽³³⁾. Additionally, a relatively high percentage of *Lactobacillus*, mainly

L. casei, *L. paracasei* and *Lactobacillus acidophilus*, has been described^(34,35).

In the present study, the API 50CH fermentation system was used to test the carbohydrate fermentation ability of the isolated LAB strains. This system permits the metabolic characterisation of strains on a wide range of individual substrates, an important component of lactobacilli characterisation. The API ZYM system allows strains to be characterised with respect to enzymatic type and activity level. In the analysis of carbohydrate utilisation, the three probiotic strains exhibited high β-galactosidase activity, which is extremely relevant to the utilisation of lactose and could potentially serve to alleviate lactose intolerance in human subjects. Strains *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 exhibited high α-galactosidase activity, which is important for the hydrolysis of α-D-galactosyl-oligosaccharides, which are found in relatively

Table 5. *In vitro* activity of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 strain supernatants from 17 and 24 h cultures against the human rotaviruses Ito, Va70 and Wa in HT-29 cells (Mean values and standard deviations, n 3)

Bacteria	17 h				24 h			
	Control		Supernatant		Control		Supernatant	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Rotavirus Ito								
<i>L. paracasei</i> CNCM I-4034	8.35 × 10 ⁴	1.95 × 10 ⁴	6.00 × 10 ⁴	2.24 × 10 ⁴	7.35 × 10 ⁴	6.66 × 10 ³	7.43 × 10 ⁴	7.51 × 10 ³
<i>B. breve</i> CNCM I-4035	1.05 × 10 ⁵	3.87 × 10 ⁴	8.58 × 10 ⁴	1.93 × 10 ⁴	5.95 × 10 ⁴	6.36 × 10 ³	3.77 × 10 ⁴	1.00 × 10 ⁴
<i>L. rhamnosus</i> CNCM I-4036	1.72 × 10 ⁵	2.20 × 10 ⁴	1.68 × 10 ⁵	4.81 × 10 ⁴	1.70 × 10 ⁵	3.94 × 10 ⁴	7.05 × 10 ⁴ *	7.07 × 10 ²
Rotavirus Wa								
<i>L. paracasei</i> CNCM I-4034	7.50 × 10 ²	7.07 × 10 ¹	7.67 × 10 ²	1.02 × 10 ²	4.10 × 10 ³	6.56 × 10 ²	1.01 × 10 ³ *	1.14 × 10 ²
<i>B. breve</i> CNCM I-4035	1.12 × 10 ⁴	3.04 × 10 ³	7.70 × 10 ³	1.43 × 10 ³	3.57 × 10 ³	5.12 × 10 ²	3.23 × 10 ³	7.51 × 10 ²
<i>L. rhamnosus</i> CNCM I-4036	9.00 × 10 ²	1.00 × 10 ²	7.00 × 10 ²	2.82 × 10 ¹	2.53 × 10 ³	9.29 × 10 ²	1.47 × 10 ³ *	5.69 × 10 ²
Rotavirus Va70								
<i>L. paracasei</i> CNCM I-4034	1.23 × 10 ⁴	3.54 × 10 ²	6.80 × 10 ³ *	1.70 × 10 ³	1.30 × 10 ⁴	1.27 × 10 ³	9.40 × 10 ³ *	1.67 × 10 ³
<i>B. breve</i> CNCM I-4035	8.67 × 10 ³	1.99 × 10 ³	7.53 × 10 ³	1.84 × 10 ³	9.63 × 10 ³	2.35 × 10 ³	6.00 × 10 ³	8.62 × 10 ²
<i>L. rhamnosus</i> CNCM I-4036	1.45 × 10 ⁴	2.25 × 10 ³	4.83 × 10 ³ *	1.08 × 10 ³	1.24 × 10 ⁴	3.11 × 10 ³	4.50 × 10 ³ *	1.42 × 10 ³

CNCM, Collection Nationale de Cultures de Microorganismes.
**P*<0.05.

high amounts in human milk (1 g/l)⁽³⁶⁾ and permit the selective growth of *Bifidobacterium* in the intestine⁽³⁷⁾. Of particular interest is β -glucuronidase activity, a known carcinogenic enzyme. It was not detected in *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 or *L. rhamnosus* CNCM I-4036. In general, we can state that the three isolated probiotic strains exhibited low enzymatic activities on mannose, fucose and glucuronides, instead preferring carbon sources like lactose and glucose. However, *L. rhamnosus* CNCM I-4036 showed high activity for α -fucosidase, and *B. breve* metabolised L-fucose. Several neutral oligosaccharides in human milk contain fucose. In fact, the most abundant human milk oligosaccharide is 2-fucosyl-lactose^(36,38), and these strains could contribute to the metabolism of human milk oligosaccharides in infants.

The carbohydrate fermentation assays demonstrated that the isolated *Lactobacillus* strains have similar profiles as the commercial *L. rhamnosus* GG control and other *Lactobacillus* strains⁽³⁹⁾. These strains fermented L-rhamnose, an activity found in *L. rhamnosus* and *L. paracasei* strains. Interestingly, the *L. paracasei* CNCM I-4034 strain utilised inulin, an activity that was absent in the commercial *L. rhamnosus* GG control. This polysaccharide is classified as a prebiotic and is used in functional foods because it has been reported to increase the prevalence of *Bifidobacterium* in the colon^(40–43). Although some *Lactobacillus* species have been reported to grow in the presence of this prebiotic^(43,44), this trait is quite uncommon in this genus.

Probiotics must be able to survive the passage through the upper digestive tract to reach the large intestine^(45,46); thus, the isolated strains were exposed for 3 h to pH 3.0, 2.5 or 2.0 to select strains that were most resistant to a low pH environment. The *in vitro* survival test revealed that the three strains were resistant to pH 3.0 even after 3 h of exposure, although 2 h may be sufficient for passage through the stomach. However, only the *L. rhamnosus* CNCM I-4036 strain exhibited resistance to pH 2.5 and 2.0. The resistance of probiotics to low pH varies greatly depending on the species and strain. In general, *Lactobacillus* strains exhibit better resistance to low pH than *Bifidobacterium* strains^(47,48). Resistance to low pH is an important characteristic for the food industry, especially in the design of functional foods, because probiotics will be able to withstand acidic environments for long periods, such as in fermented foods in which post-acidity could affect strain viability⁽⁴⁹⁾.

Bile salts play a fundamental role in the defence mechanism of the gut. The relevant physiological concentrations of human bile range from 0.3 to 0.5%^(50,51). For *Bifidobacterium* and *Lactobacillus*, the resistance to bile salts varies between species and strains⁽⁵²⁾. In the present study, the three isolated strains and commercial controls exhibited a high tolerance to bile salts, up to 0.7% (w/v). The adaptation to bile salts is related to changes in carbohydrate fermentation, glycosidase activity⁽⁵³⁾, exopolysaccharide production^(54,55), the composition of membrane proteins and fatty acids⁽⁵⁶⁾, increased adhesion to human mucus and inhibition of pathogen adhesion^(57,58).

An important characteristic of probiotics within the intestinal microbiota is their capacity for adhesion to the intestinal epithelium; this avoids their elimination by peristaltic movement. Additionally, adhesion is a prerequisite for colonisation⁽⁵⁹⁾ and is a factor in the competitive exclusion of enteropathogens⁽⁶⁰⁾, stimulation of the immune system⁽⁶¹⁾ and antagonistic activity against enteropathogens⁽⁶²⁾. The reported adhesion ability of *Bifidobacterium* and *Lactobacillus* strains varies depending on the *in vitro* method utilised⁽⁶³⁾. In the present study, HT-29 cells were used to study the adhesion ability of the isolated strains. The commercial control, the *Lactobacillus* GG strain, exhibited half (4%) of the adhesion level of the commercial control *B. longum* (8%), and the three isolated strains exhibited almost twice the level of *in vitro* adhesion of the commercial controls. Although the results of the *in vitro* studies cannot be directly applied to the *in vivo* situation, there is evidence to support an association between them⁽⁶⁴⁾.

Once the three potential probiotic strains were isolated, identified and characterised, their safety was assessed. Although these species belong to the list of taxonomic units proposed for qualified presumption of safety status⁽⁶⁵⁾ and thus considered innocuous, a detailed toxicological study was performed following the FAO/WHO recommendations⁽¹⁾. The resistance to antibiotics was similar to that previously reported⁽⁶⁶⁾. The strains for which the minimum inhibitory concentration was above the breakpoints recommended by the European Food Safety Authority⁽²⁵⁾ require further investigation. Whole-genome sequencing of the three strains would be useful to ensure the absence of antibiotic resistance genes in plasmids or between mobile genetic elements. The European Food Safety Authority has not published upper levels for D-lactic acid isomer and BSH activity, and comparisons with commercial probiotic strains could be a good approach to evaluating the safety of novel strains with respect to undesirable metabolite production. The level of production of both D- and L-lactic acid isomers was similar in the commercial *L. rhamnosus* GG strain and the isolated lactobacilli. Moreover, the level of D-lactic acid production in the *B. breve* strain was similar to that in the *B. longum* Morinaga strain, but L-lactic acid production was lower. The *B. breve* CNCM I-4035 strain displayed BSH activity with glycocholate in the supernatant and the sonicated cells, but the activity was lower than that of the commercial *B. longum* strain and that previously reported for other bifidobacteria⁽⁶⁷⁾. The lactobacilli strains did not display BSH activity, which is consistent with previous studies in which only certain strains of lactobacilli exhibited this activity⁽⁶⁸⁾.

Biogenic amine levels were determined from supernatants; putrescine was not detected in any strain, and cadaverine, histamine and tyramine levels were negligible compared with the maximums recommended by the FAO/WHO. The *in vivo* acute ingestion study demonstrated a lack of mortality and morbidity after the inoculation of mice with the isolated strains, even in immunosuppressed mice. Moreover, the administration of the three strains did not lead to bacterial loads in organs or changes in histomorphology.

To study their capacity to inhibit the growth of pathogens, we tested the isolated strains against bacteria and viruses. *L. monocytogenes* is a Gram-positive pathogen that serves as an important model for understanding host immune resistance to intracellular bacteria. This intracellular bacterium infects human subjects through the ingestion of contaminated food and, in risk groups, including neonates, can cause meningitis with a high case-fatality rate⁽¹⁷⁾. The isolated strains inhibited *L. monocytogenes* in a strain-specific manner. *L. rhamnosus* CNCM I-4036 inhibited the three *L. monocytogenes* used in the present study, whereas *L. paracasei* CNCM I-4034 and *B. breve* CNCM I-4035 inhibited at least one strain of *L. monocytogenes*. Other authors have reported the capacity of some LAB to protect against experimental listeriosis^(69–71).

Rotavirus infects mature enterocytes of the intestinal villus, and consequently, crypt cells are spared⁽⁷²⁾. Rotaviral diarrhoea has been attributed to different mechanisms and accounts for an estimated 2 million hospitalisations per year⁽¹⁸⁾. The probiotics isolated in the present study decreased the propagation of three rotavirus strains in an *in vitro* assay. These results agree with those of other authors^(4,72), who reported that probiotics can produce bioactive compounds that can modulate the protection of epithelial cells from rotavirus infection. Probiotics have been used to prevent some intestinal pathogenic infections, such as *Salmonella*, *Shigella*, *Escherichia coli*, *Listeria* and *H. pylori*^(73–77). The possible mechanisms of action for this protection include the production of acid^(78,79) and other by-products of bacterial metabolism^(76,80,81).

Finally, a preliminary study on the role of the isolated potential probiotics in enhancement of immunity was conducted. Results indicated that *B. breve* CNCM I-4035 strain has some properties of interest that needs to be confirmed in future experiments.

In conclusion, the results presented here demonstrate the capacity of the strains *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036, which were isolated from the faeces of exclusively breast-fed infants, to resist low pH and high bile salt concentrations and adhere to the colon. In addition, these novel strains inhibit some strains of *L. monocytogenes* and the infection of human cells with rotavirus *in vitro*. Also, the *B. breve* CNCM I-4035 strain has some promising immunological properties. Furthermore, toxicological studies demonstrated that these strains fulfil the main criteria required for safe human consumption. These strains could be useful in the prevention and treatment of diarrhoea due to rotavirus in infancy and in the prevention of meningitis mediated by *L. monocytogenes*. Further studies are needed to evaluate the *in vitro* and *in vivo* activities of the isolated strains against different enteropathogens, and human clinical trials must be performed before these strains can be commercialised.

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were involved in the isolation and characterisation of bacterial strains. J. M. selected the infants and was responsible for obtaining the faecal sample and E. M., M. J. B. and F. R. participated in the process of strain characterisation. E. C., S. G. and D. R. were involved in the safety and *L. monocytogenes* and human rotavirus infection. M. B.-B. and C. G.-L. helped in the bacterial characterisation and statistical analysis. D. R. and A. G. were the leader scientists of the present work and contribute equally to the design and supervision of the experiments and results; they also were involved in writing the manuscript. The present work was supported by the HERO Group through its company HERO Spain S. A (contract no. 3143 signed with the Fundación General Universidad de Granada Empresa contract and a private contract signed with the Spanish Biotechnology Company Biópolis S. L.). Hero Spain S. A in turn was funded by the CDTI, Spanish Ministry of Health. C. G.-L. is a recipient of a postdoctoral fellowship from Plan Propio of the University of Granada. E. M., M. J. B. and F. R., are members of the Hero Global Technology Centre for Infant Nutrition. This centre forms part of the food company HERO with headquarters in Switzerland. E. C., S. G. and D. R. are members of Biópolis S. L. (Spain), a spin-off of the High Scientific Research Council (Consejo Superior de Investigaciones Científicas), Ministry of Education, Spain. No other authors declare conflict of interest.

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