



Safety, functional properties and technological performance in whey-based media of probiotic candidates from human breast milk

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Received: 11 June 2018 / Revised: 1 November 2018 / Accepted: 7 November 2018 / Published online: 19 November 2018
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Abstract

We aimed at isolating and characterising microorganisms present in human breast milk with probiotic potential. In an 8-week postpartum sampling period, two strains of bifidobacteria (*Bifidobacterium longum* LM7a and *Bifidobacterium dentium* LM8a') and four strains of lactobacilli were isolated, all during the first 4-week postpartum. *B. longum* LM7a and *B. dentium* LM8a', together with four strains previously isolated from breast milk (*Bifidobacterium lactis* INL1, INL2, INL4 and INL5), were considered for further studies. Susceptibility of the strains to tetracycline, erythromycin, clindamycin, streptomycin, vancomycin and chloramphenicol was evaluated and the isolates exhibited, in general, the same properties as previously reported for bifidobacteria. All isolates showed low hydrophobicity and *B. lactis* and *B. longum* strains had satisfactory resistance to gastric digestion and bile shock, but not to pancreatin. *B. lactis* INL1, *B. longum* LM7a and *B. dentium* LM8a' were selected for some comparative technological studies. In particular, *B. lactis* INL1 displayed technological potential, with satisfactory growth in cheese whey-based media in biofermentor and resistance to freeze-drying, accelerated storage conditions and simulated gastric digestion.

Keywords Bifidobacteria · Cheese whey · Freeze-drying · Gastrointestinal digestion · Human breast milk · Probiotics

Introduction

Functional foods are described as those claimed to have positive effects on health beyond basic nutrition. Such products continue to gain widespread popularity and acceptance throughout the developed world and their global market has been in fast growth since the early 2000's, particularly in the USA, Europe and Japan (Saad et al. 2013). Breast milk is a complex and complete food, and it may be considered as the best example of a natural functional food (Gotteland et al.

2011). In addition to nutrients, hormones, growth factors, immunoglobulins, cytokines and enzymes, which contribute to immune maturation and child welfare (Le Huërou-Luron et al. 2010), breast milk contains significant amounts of microorganisms (Boix-Amorós et al. 2016). So far, about 700 bacterial species have been detected in breast milk, although the number of cultivable species present in an individual is much lower, in a range of 2–18 different species (Cabrera-Rubio et al. 2012; Jeurink et al. 2013). These bacteria, including bifidobacteria and lactobacilli, are responsible in part for the gastrointestinal colonisation of the newborn and for the adequate maturation of the gut mucosal immune system. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al. 2014). Specific strains of lactobacilli and bifidobacteria are the most studied microorganisms worldwide and the most used in food and pharma as probiotics. In 2008, a Spanish company, Puleva Food S.L. (www.puleva.es), launched the first formula containing a probiotic lactobacilli isolated from breast milk, *Lactobacillus fermentum* CECT 5716. To the best of our knowledge, except in this case, so far commercial infant formulas containing probiotic microorganisms have been made with strains isolated from

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10123-018-00046-0>) contains supplementary material, which is available to authorized users.

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fermented food or faecal microbiota of children (Chassard et al. 2014). In this context, the use of strains isolated from human breast milk is attractive and the administration of bacteria from this source to infants could provide opportunities to help to establish a healthy intestinal microbiota more similar to that of breast milk fed ones, reducing the risk of intestinal disorders (Maldonado et al. 2012), when breastfeeding is impaired or limited. For a microorganism to be regarded as probiotic, safety and the capacity to induce a beneficial effect are required, but resistance to the technological processes found from production to consumption is also of importance (Heller 2001). Finally, in addition to providing added value to food, probiotics need to be cost-effectively produced, which implies maximising substrate-to-biomass yield and stability during processing and shelf life (Jankovic et al. 2010). The use of cheese whey, an abundant by-product of the dairy industry, for probiotic biomass production and drying has been evaluated previously as a lower cost alternative to synthetic media or protectants (Doleyres and Lacroix 2005; Lavari et al. 2014).

The aim of this work was to isolate and characterise microorganisms present in human breast milk with probiotic and technological potential, with emphasis on bifidobacteria, for their use in functional foods or supplements.

Materials and methods

Isolation of bacteria from breast milk

Human breast milk samples were voluntarily donated by mothers attending different health institutions in Santa Fe city (Argentina), with full knowledge and written consent about their use. This study was performed in conformity with the Declaration of Helsinki and was approved by the Advisory Committee on Research Ethics and Safety of the Faculty of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Santa Fe, Argentina. Mothers were surveyed about some characteristics of the delivery (vaginal or by C-section, full term or premature) and probiotics consumption habits. Mothers that received antibiotics or consumed probiotic products during pregnancy or postpartum were excluded. Ten mothers provided periodically samples of breast milk, from delivery to the eighth week postpartum (PP), in order to include in the sampling period the three stages of lactation: colostrum (days 1–4 PP), transitional milk (day 5—week 2 PP), and mature milk (week 2 PP onwards). Sample collection and plating were performed according to Zacarías et al. (2011). Colonies presenting typical lactobacilli or bifidobacteria morphology (examined by phase-contrast microscopy, $\times 1000$) were isolated and purified and assessed for Gram-staining, mobility and catalase activity. Presumptive lactobacilli or bifidobacteria isolates were stored frozen in

MRS broth (Biokar, Beauvais, France) added with 20% (*v/v*) glycerol at $-70\text{ }^{\circ}\text{C}$ for subsequent studies.

Identification of isolates

Total DNA of isolates was obtained from overnight cultures by using the GenElute-Bacterial Genomic DNA kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Purified DNA samples were stored at $-20\text{ }^{\circ}\text{C}$ until use. The identity of isolates was analysed by amplifying, sequencing and comparing their 16S rRNA gene (Edwards et al. 1989). A 1500 bp fragment of 16S rRNA gene was amplified using primers pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pH 5'-AAGGAGGTGATCCAGCCGCA-3'. All PCR reactions were performed using 2 μL of diluted (1:50) DNA as template, 2.5 U Taq DNA polymerase (GE Healthcare, Little Chalfont, UK), 200 nMdNTPs (GE Healthcare) and 400 nM each primer (Sigma-Genosys, The Woodlands, TX, USA) in a final volume of 50 μL . Amplifications were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at $94\text{ }^{\circ}\text{C}$, 36 cycles of 1 min at $94\text{ }^{\circ}\text{C}$, 2 min at $51\text{ }^{\circ}\text{C}$ and 2 min at $72\text{ }^{\circ}\text{C}$, and a final step of 7 min at $72\text{ }^{\circ}\text{C}$. The PCR products were separated on 1% (*w/v*) agarose gels in TBE buffer, stained with GelRed (Biotium, Hayward, CA, USA) and visualised under UV light (Sambrook and Russell 2001). Amplicons were purified with QIAquick Gel Extraction (QIAGEN, Hilden, Germany) and their nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity of isolates was checked by nucleotide-nucleotide BLAST of the NCBI database (www.ncbi.nlm.nih.gov/blast) and 16S rRNA partial sequences from new isolates were deposited in GenBank (Table S1).

Random amplification of polymorphic DNA (RAPD) analysis

Genotypic diversity among isolates was analysed by RAPD-PCR, using four single arbitrary primers, in independent reactions: (i) primer B08 with sequence 5'-GTCCACACGG-3'; (ii) primer B10 with sequence 5'-CTGCTGGGAC-3'; (iii) primer M13 with sequence 5'-GAGGGTGGCCGGTTCT-3', and (iv) primer 1254 with sequence 5'-CCGCAGCCAA-3'. Amplifications for the primers B08 and B10 were carried out according to Binetti et al. (2007) and for M13 and 1254, according to Giraffa et al. (2004). PCR reactions were performed in a total volume of 25 μL with 1- μL template-diluted DNA, 2.5 U Taq Polymerase (GE Healthcare) and 200 nM of each dNTP (GE Healthcare). The primer final concentrations were 500 nM for B08 and B10, 2000 nM for M13 and 800 nM for 1254. In all cases, a tube without template was included as negative control. Amplification products were analysed by

electrophoresis on 1% (w/v) agarose gels on TBE buffer, following standard protocols.

Safety and functional characterisation of breast milk bifidobacteria

Strains

Two bifidobacteria strains isolated and identified during this work were used. Additionally, four breast milk derived-strains of *Bifidobacterium animalis* subsp. *lactis*, isolated previously (strains INL1, INL2, INL4 and INL5; Zacarías et al. 2011), were also studied in this work. Before use, the strains were sub-cultured two times in MRS broth supplemented with 0.1% (w/v) L-cysteine hydrochloride (Biopack, Buenos Aires, Argentina; MRSc) for 18 h under anaerobiosis (Anaeropack-Anaero, Mitsubishi Gas Chemical Co., Inc., Japan) at 37 °C.

Antibiotic resistance

Susceptibility to clindamycin, chloramphenicol, erythromycin, streptomycin, tetracycline and vancomycin was evaluated by using the E-test (AB Biodisk, Oxoid Inc., Ontario, Canada) method as previously described (Mättö et al. 2006). The inoculum was prepared by suspending colonies from cultures grown on LSM + cys agar for 2 days in LSM + cys broth to a cell density corresponding to 1 McFarland standard. The suspension was spread evenly on the pre-reduced agar plates by using a sterile cotton swab. The E-test strips were placed on the air-dried agar surface. The plates were incubated under anaerobic conditions at 37 °C for 48 h. The minimum inhibitory concentration (MIC) for each antibiotic was read as the lowest antibiotic concentration in which the growth was inhibited. Resistance to tetracycline was corroborated using a broth microdilution method, according to Cardamone et al. (2011). Briefly, the antibiotic stock solution was prepared in distilled water and then filter-sterilised (0.22-µm pore diameter) (Millipore, Sao Paulo, Brazil). The strains were grown overnight at 37 °C in MRSc and then diluted in LSM broth in order to reach a final inoculum of 10⁵ CFU/ml in the microplates. After incubation, the MIC was determined as the lowest antibiotic concentration at which no growth was observed. Interpretation of results was based on the cut off values adopted by the updated technical guidance of FEEDAP (European Food Safety Authority 2012).

Hydrophobicity

The ability of the strains to adhere to hydrocarbons as a measure of their hydrophobicity was determined according to Vinderola and Reinheimer (2003). The partition of bacterial cells between organic (n-hexadecane) (Merck, Darmstadt,

Germany) and aqueous phases was determined by measurement of optical density at 560 nm (OD₅₆₀ nm). Assays were performed in triplicate and hydrophobicity (H%) was calculated as follows: $H \% = [(OD_0 - OD) / OD_0] \times 100$, where OD₀ and OD are the optical density before and after extraction with n-hexadecane, respectively.

Resistance to simulated gastrointestinal digestion (SGID)

Gastric digestion (step 1) was performed as described by Burns et al. (2014). Briefly, overnight (18 h) cultures (40 ml) of the strains under study in MRSc broth were centrifuged (2750 g, 10 min, 5 °C), washed twice with PBS (pH 7.2) and resuspended in 20 ml of 10% (w/v) sterile skim milk, and 20 ml of a simulated gastric juice containing 0.6% (w/v) porcine pepsin (Merck) and 0.5% (w/v) sodium chloride were added. The cell suspensions were incubated in a thermal bath at 37 °C and pH was gradually decreased by the addition of HCl (1 and 0.1 N), until pH 2.0 in a time period of 90 min. Cultures were then centrifuged and resuspended in a 1% (w/v) bovine bile (Sigma) PBS (0.1 M, pH 8.0) solution and incubated at 37 °C for 10 min (step 2: bile shock). The cultures were finally centrifuged and resuspended in a 0.3% (w/v) bovine bile (Sigma) + 0.1% (w/v) pancreatin from porcine pancreas (Sigma) PBS (0.1 M, pH 8.0) solution and kept 90 min at 37 °C (step 3: intestinal digestion). At the beginning of the experiment and at the end of each step, colony counts on MRSc agar were performed.

Technological characterisation

Growth in in-house and whey-based media

B. lactis INL1, *B. longum* LM7a and *B. dentium* LM8a' were used in this assay. MRS broth was prepared in-house using ingredients manufactured by local providers (Microquin S.A. and Cicarelli, both from Santa Fe, Argentina). Also, two whey-based culture media were prepared by supplementing 5% (w/v) reconstituted cheese whey powder (Verónica, Lehmann, Argentina) with either 1% (YE1) or 5% (YE5) (w/v) yeast extract (Microquín). The aim of assessing formulated growth media using ingredients from local providers (less expensive than imported commercial media) or a dairy by-product as cheese whey, was to determine their capacity to promote the growth of the strains under study in lower cost culture media, compared to commercial MRS. In all cases, growth media were supplemented with 0.1% (w/v) L-cysteine hydrochloride. Overnight (18 h) cultures of the strains (MRSc, 37 °C, 18 h, anaerobiosis) were centrifuged (2750 g, 10 min, 5 °C), washed twice and resuspended in PBS solution (pH 7.2). Cell suspensions were inoculated (2% v/v) in in-house formulated MRS and in YE1 and YE5 and incubated overnight (37 °C, 18 h, anaerobiosis). Commercial

MRS (Biokar) was used as reference medium for assessing bifidobacteria growth capacity. Cell counts were performed on MRSc agar (37 °C, 72 h, anaerobiosis). The usefulness of successive previous transfers was assessed to improve growth in formulated culture media, when necessary.

Cheese whey as lyoprotectant

Overnight cultures (18 h, 37 °C, anaerobiosis) of *B. lactis* INL1, *B. longum* LM7a and *B. dentium* LM8a' were centrifuged, washed twice in PBS and resuspended in 10% (w/v) cheese whey (Verónica) or 10% (w/v) lactose (Merck) solution. Cell suspensions were aliquoted in glass vials (1 ml/vial), frozen at a freezing rate of approx. 1 °C/min and stored at –70 °C until freeze-drying. Samples were freeze-dried over 24 h in a single-chamber freeze drier (beta 2–16, Christ, Osterode, Germany). The drying vacuum applied was 37 Pa assuring product temperatures of –15 °C during primary drying and 25 °C in the secondary drying phase. At the end of the process, vials were sealed under vacuum. Moisture content was assessed gravimetrically in triplicate (101 °C, 20 h). Resistance to simulated gastric digestion (SGD) was assessed prior and immediately after freeze-drying and after 3 weeks of frozen (–20 °C) or an accelerated (25 °C) storage stability test. Freeze-dried cells were reconstituted with distilled water (1 ml/vial) and were allowed to stand for 15 min at room temperature for complete rehydration. Cell suspensions were brought to pH 2.0 with 1 N and 0.1 N HCl in the presence of 0.5% (w/v) NaCl and 0.5% (w/v) porcine pepsin (Merck) and incubated at 37 °C in a water bath for 90 min. Cell counts were performed before and after SGD.

Growth in biofermentor in cheese whey-based medium and resistance to SGD

B. lactis INL1 was used in this part of the study. In a preliminary series of fermentations, the strain was grown in a 1.5-l biofermentor (Biostat B, Sartorius Stedim Systems, Melsungen, Germany) in 5% (w/v) cheese whey supplemented with some of the ingredients found in MRS broth (yeast extract, glucose, and salts, in the concentrations they are found on MRS) and different pH values (6.5, 5.8 or 5.0) and modes (free or constant pH) were evaluated. Furthermore, the usefulness of cheese whey protein hydrolysis was also assessed. For enzymatic hydrolysis, 5% (w/v) cheese whey was prepared, transferred to the biofermentor, and 0.3% (w/w) pancreatic trypsin (Novo 6.0S, Type Salfree, Novozymes, Bagsvaerd, Denmark) was added. Cheese whey was incubated at 37 °C and pH 7.5 (adjusted with 8N NaOH) for 90 min to allow hydrolysis, prior to sterilisation. Finally, a formulation based on the composition of MRS broth, called W-MRS, was selected: 5% (w/v) cheese whey, hydrolysed with pancreatic trypsin, was supplemented with all MRS ingredients except for meat

extract and polypeptone (protein sources), and glucose content was decreased by half (from 2% (w/v) in MRS to 1% (w/v) in W-MRS). *B. lactis* INL1 was grown in W-MRS under two conditions: (a) pH 6.5 (15 h) followed by a moderate acid stress induced with lactic acid (Merck) (pH 5.0, 4 h) and (b) pH free (initial pH 5.8) (15 h) followed by addition of 8N NaOH (Merck) to keep pH at 5.0 for 4 h. The biofermentor was inoculated (2% v/v) with an overnight culture of the strain grown in MRSc, washed twice and resuspended in PBS. The initial concentration in the biofermentor was similar for both conditions (7.00 and 7.04 log CFU/ml, for condition a) and b), respectively. Biomass production was conducted at 37 °C with a CO₂ influx of 0.2 l/min and a stirring rate of 150 rpm. Samples of the fermentation medium (500 ml) were taken at 15 and 19 h of culture, centrifuged (2750 g, 10 min., 5 °C), washed twice with PBS and suspended in 10% (w/v) lactose solution. Cell suspensions were frozen at –70 °C until further processing. Samples were freeze-dried and the resistance to SGD was assessed as described in the previous item.

Statistical analysis

One-way ANOVA and Tukey's post hoc comparison were used to evaluate the resistance to SGID and data from growth capacity and resistance to freeze-drying, storage and SGD in cheese whey. Student's *t* test was used to analyse the growth in biofermentor. Data were analysed using GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Isolation and identification of bacteria from breast milk

Ten mothers (identified as LM1 to LM10) provided breast milk samples between day 1 and week 8PP. Information about donors and isolates is summarised in Table 1. RAPD analysis allowed us to evaluate the genetic diversity among isolates, prior to the molecular identification. In particular, primer B10 (Fig. 1) showed at least 2 RAPD types for LM1, LM4 and LM7, and at least 4 RAPD types for LM5 and LM8. Two bifidobacterial strains, *B. longum* LM7a (isolated on day 9 PP) and *B. dentium* LM8a' (isolated on day 20 PP), and four lactobacilli strains (*L. gasseri* LM8b, *L. salivarius* LM8a, *L. salivarius* LM8h and *L. vaginalis* LM8f, isolated between week 3 and 4 PP) were identified based on the nucleotidic sequence of their 16S rRNA gene. The differences between the two *L. salivarius* strains from mother LM8 could be corroborated through their RAPD profiles. The rest of the isolates belonged to the genera *Propionibacterium* and *Actinomyces* (Table 1).

Table 1 Breast milk sampling trial summary. Mothers were coded as LM followed by a number. Samples were taken periodically from day 1 to week 8th postpartum (PP). Mode and term of delivery: vaginal (V), C-section (C), in term (T), pre-term (P)

Donnor code	Mode of delivery	Term of delivery	Number of samples	Number of presumptive bifidobacteria or lactobacilli isolated	Identity of isolates
LM1	C	T	6	3	<i>Propionibacterium</i> sp. <i>Actinomyces odontolyticus</i>
LM2	V	T	1	N.D.	
LM3	C	P	4	N.D.	
LM4	C	P	3	4	<i>Propionibacterium</i> sp.
LM5	C	P	3	9	<i>Propionibacterium</i> sp.
LM6	V	T	6	N.D.	
LM7	V	T	5	2	<i>B. longum</i> LM7a (9 PP) <i>Actinomyces odontolyticus</i>
LM8	V	T	3	5	<i>B. dentium</i> LM8a' (20 PP) <i>L. gasseri</i> LM8b (27 PP) <i>L. salivarius</i> LM8a (27 PP) <i>L. salivarius</i> LM8h (20 PP) <i>L. vaginalis</i> LM8f (27 PP)
LM9	V	T	3	N.D.	
LM10	C	T	8	N.D.	

All *Bifidobacterium* and *Lactobacillus* isolates were found only once, in the postpartum day mentioned between brackets on the last column
N.D., presumptive bifidobacteria or lactobacilli non-detected

Safety and functional characterization of breast milk bifidobacteria

In this work, the E-test® method was used to analyse the susceptibility of the bifidobacteria strains to clindamycin, chloramphenicol, erythromycin, streptomycin, tetracycline and vancomycin. MIC values are shown in Table 2. TC resistance was also analysed by the microdilutions (MD) broth method (Table 2). The TC resistance of *B. lactis* INL1 was corroborated and was detected also for *B. lactis* INL2 (not detected before by the E-test®). When hydrophobicity was studied, all isolates showed low values ($20.1 \pm 2.0\%$ for *B. lactis* strains and 18.4 ± 0.4 and $3.7 \pm 0.2\%$ for *B. longum* LM7a and *B. dentium* LM8a', respectively). As shown in Table 3, all strains of *B. lactis* were able to resist the simulated gastric digestion and bile shock steps. However, the exposure to pancreatin combined with bile salts caused a reduction of 3 to 5 log orders in the cell counts of the strains of *B. lactis*, while the *B. longum* strain showed more resistance (0.4 log orders).

Technological characterization of the isolates

Growth in in-house and whey-based media

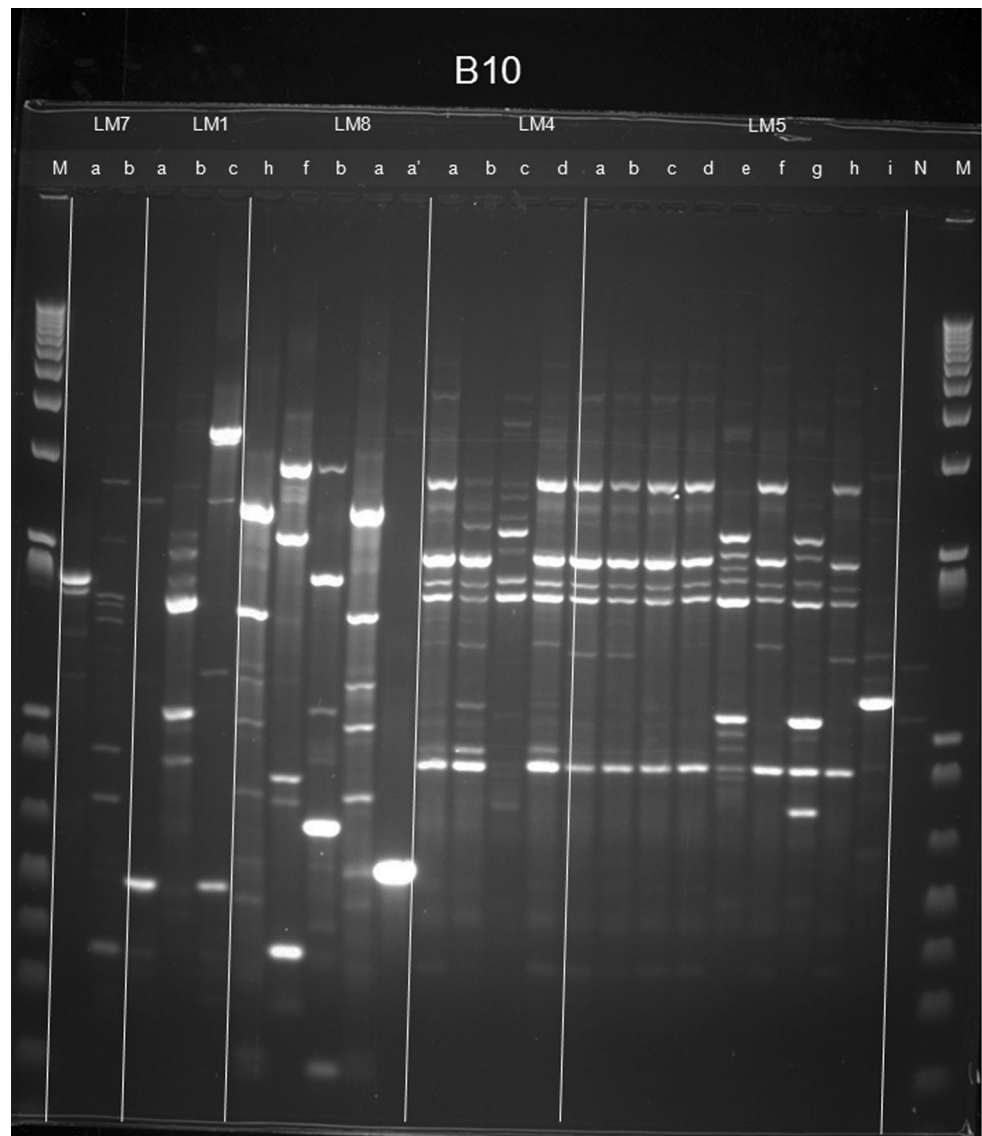
Figure 2 shows the levels of viable cells achieved by the strains in in-house MRS and in whey-based YE1 and YE5

formulated media, compared to commercial MRS (Biokar; reference medium). Overall, *B. lactis* INL1 was the strain with most satisfactory growth in all tested media.

Cheese whey as lyoprotectant

The usefulness of cheese whey as lyoprotectant, as compared to lactose, was assessed. Freeze-dried cultures were subjected to an accelerated storage test (storage at 25 °C, 3 weeks) and to SGD. *B. lactis* INL1 reached ca. 10 log orders after freeze-drying (FD), and these levels of viable cells were maintained after storage and SGD, regardless the lyoprotectant used (Fig. 3a). *B. longum* LM7a did not show any significant decay after FD, but a significant 2 log-order reduction was observed during the SGD of FD cultures (Fig. 3b), for both lyoprotectants. When stored at 25 °C, a significant higher loss in viability was observed for lactose (2.7 log orders) compared to cheese whey (1.2 log order). However, when these cultures were subjected to SGD, the reduction in viability was higher for cheese whey so, similar counts were obtained for both protectants after SGD (inactivation of 3.5 log orders). Finally, *B. dentium* LM8a' showed a higher sensitivity to freeze drying with viability losses of 2.8 and 2.1 log orders for lactose and cheese whey, respectively) and accelerated storage test (Fig. 3c).

Fig. 1 RAPD profile obtained with primer B10 for 23 breast milk isolates (named with letters) from five donating mothers (LM1, LM4, LM5, LM7 and LM8). N, negative control; M, TrackIt 1Kb plus DNA Ladder (Invitrogen). Isolates were subsequently identified by 16S rRNA sequencing as members of the genera *Bifidobacterium* (LM7a and LM8a') and *Lactobacillus* (LM8a, LM8b, LM8f and LM8h) or as *Propionibacterium* or *Actinomyces*



Growth in biofermentor in cheese whey-based medium and resistance to SGD

B. lactis INL1 was grown in a 1.5-l biofermentor in the in-house-prepared W-MRS broth under two different conditions. After 19 h of fermentation, similar cell counts (ca. 10^9 CFU/ml) were obtained under both fermentation conditions (Fig. 4). However, in the first 15 h of fermentation at pH 6.5, cell counts were significantly lower ($p = 0.043$) compared to growth in non-controlled pH conditions and, when pH was switched from 6.5 to 5.0 (and maintained for 4 h), *B. lactis* INL1 reached 9.0 log orders. For fermentation without pH control, 2 log orders of growth, from the inoculation level, were obtained in the first 15 h of culture and, when pH was brought to pH 5.0, the level of viable cells remained constant.

When freeze-dried cultures were subjected to SGD, cells grown without pH control + moderate acid stress (pH 5.0, 4 h), were the most resistant ones to SGD (Table 4). Cells from this fermentation but harvested after 15 h (prior to exposure to pH 5.0) were more sensitive to SGD, although losses of cell viability were of ca. 1 log order. In contrast, cells grown at pH 6.5 (15 h) were the most sensitive ones to SGD with a cell decay of ca. 4 log orders, and this sensitivity to acidity could not be overcome when cells underwent a moderate acid stress during growth.

Discussion

In the last years, the studies on breast milk microbiota have increased, focusing particularly on its composition and effects

Table 2 Antibiotic susceptibility (E-test® and microdilutions method: MD) of bifidobacteria isolated from breast milk. Clindamycin (CM), chloramphenicol (CL), erythromycin (ER), streptomycin (SM), vancomycin (VA) and tetracycline (TC)

Strain	MIC (µg/ml)						
	E-test						MD
	CM	CL	EM	SM	VA	TC	TC
<i>B. lactis</i> INL1	0.047	3	0.190	256	0.38	16	> 32.0
<i>B. lactis</i> INL2	0.047	0.25	0.064	768	0.75	0.25	32.0
<i>B. lactis</i> INL4	0.047	0.5	0.047	1024	0.38	0.38	< 0.5
<i>B. lactis</i> INL5	0.032	0.38	0.094	512	0.5	0.19	< 0.5
<i>B. longum</i> LM7a	0.25	1	> 256	96	1.5	1.50	4.0
<i>B. dentium</i> LM8a'	0.064	0.5	0.19	192	0.25	0.38	ND

Cut off values (µg/ml) for bifidobacteria according to EFSA (2012): CM: 1; CL: 4; EM: 1; SM: 128; VA: 2; TC: 8. The MICs of the three repeats for most antibiotics were within one log₂ dilution, except for SM MICs for *B. lactis* INL1 and INL2 (± 2 log₂ dilution)

on the infant health (Fernández et al. 2013; Pannaraj et al. 2017; Williams et al. 2017). In a lesser extent, the isolation and functional characterisation of potential new probiotics from this source has been performed, being the main features studied the antibiotic resistance, the adhesion capacity to epithelial cell lines, survival to simulated gastrointestinal passage and antagonistic activity against enteric pathogens (Arboleya et al. 2011; Kozak et al. 2015; Martín et al. 2005; Reis et al. 2016). Less attention has been paid to the characterisation of breast milk isolates from a technological point of view. In this study, bifidobacteria and lactobacilli strains with probiotic potential were isolated and diversity was evaluated by RAPD analysis. Whereas, primer B10 was useful to differentiate some of the isolates, primers M13 and 1254 were not as effective. As for primer B08, none of the presumptive bifidobacterial isolates in this trial showed a profile for this primer, whereas in our previous sampling trial (Zacarias et al. 2011) 4 out of 6 isolates, identified later as *B. lactis* (strains INL1, INL2, INL4 and INL5), were differentiated with it and from the commercial strain *B. lactis* Bb12. Following RAPD analysis, all the potential candidates were identified by partial sequencing of the 16S rRNA gene. No *Bifidobacterium* or

Lactobacillus strains were found on the colostrum stage, whereas positive isolates were found on the transitional milk and early mature milk stages. All strains isolated in this work belonged to species already described as part of breast milk microbiota (Jeurink et al. 2013).

In this study, *B. longum* LM7a and *B. dentium* LM8a' were isolated from different mothers at days 9 and 20 PP, and they were present in breast milk in levels of 1.48 and 2.51 log CFU/ml, respectively. These values are in the range of those found in our previous sampling trial (Zacarias et al. 2011) and of qPCR estimates reported by Grönlund et al. (2007), Gueimonde et al. (2007) and Martín et al. (2009). Several factors influence the composition of breast milk microbiota including the mode of delivery. Differences in the abundances of specific bacterial genera and a higher diversity have been described in the colostrum (Toscano et al. 2017) and breast milk (Cabrera-Rubio et al. 2015) from mothers with vaginal deliveries compared to C-section ones. An interesting observation that emerged from both trials performed by our group is the fact that all bifidobacteria or lactobacilli isolates came from mothers who had pregnancies in term and vaginal delivery. To further study the factors influencing microbiota composition in breast milk, a

Table 3 Resistance to simulated gastrointestinal digestion (SGID) of bifidobacteria isolated from breast milk

Strain	Cell counts (log CFU/ml)			
	t ₀	90 min pH 2.0 + 0.3% (p/v) pepsin	10 min 1% (p/v) bile	90 min 0.3% (p/v) bile + 0.1% (p/v) pancreatin
<i>B. lactis</i> INL1	9.0 ± 0.1 ^a	9.3 ± 0.2 ^a	8.9 ± 0.4 ^a	4.7 ± 0.4 ^d
<i>B. lactis</i> INL2	9.0 ± 0.1 ^a	8.4 ± 0.2 ^a	8.6 ± 0.6 ^a	3.6 ± 0.4 ^d
<i>B. lactis</i> INL4	8.1 ± 0.1 ^a	8.8 ± 0.6 ^a	8.5 ± 0.6 ^a	5.0 ± 0.6 ^c
<i>B. lactis</i> INL5	9.2 ± 0.1 ^a	9.1 ± 0.2 ^a	8.9 ± 0.4 ^a	5.4 ± 0.2 ^d
<i>B. longum</i> LM7a	9.0 ± 0.1 ^a	8.7 ± 0.1 ^a	8.8 ± 0.1 ^a	8.6 ± 0.1 ^b
<i>B. dentium</i> LM8a'	8.9 ± 0.1 ^a	6.2 ± 0.6 ^c	5.7 ± 0.1 ^c	5.5 ± 0.1 ^c

Values are the mean ± SD (n = 3). Different superscript lowercase letters in the same row indicate significant differences for the same strain (p < 0.05; p < 0.01; p < 0.005 and p < 0.001; ANOVA + Tukey's multiple comparison test)

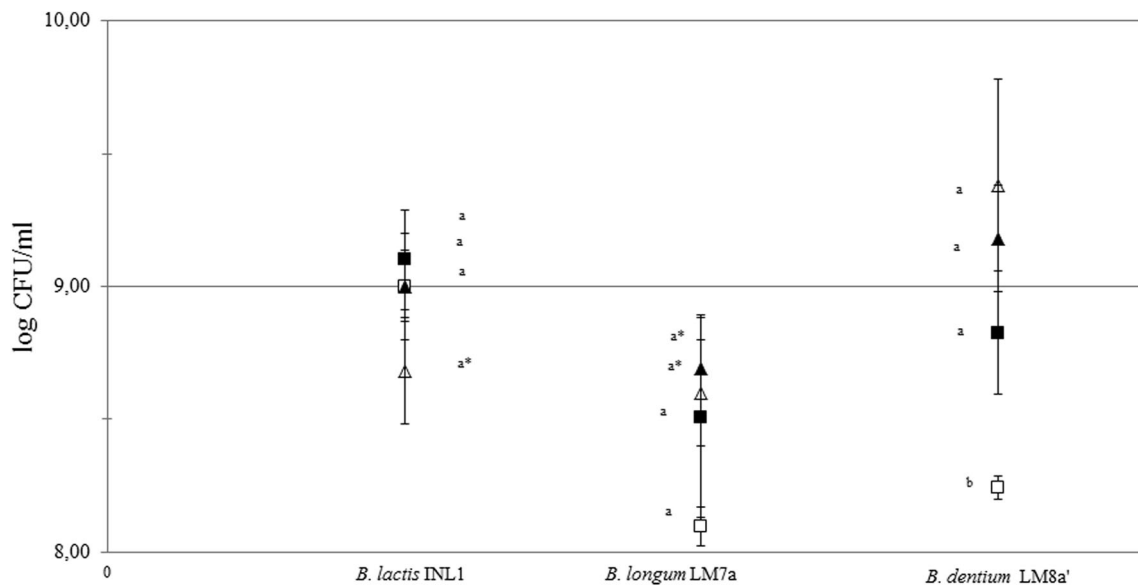


Fig. 2 Cell counts of *B. lactis* INL1, *B. longum* LM7a and *B. dentium* LM8a' in in-house (double dagger; white square) and commercial (black square) MRS broth (18 h; 37 °C, anaerobiosis), and in 5% (w/v) cheese whey + 1% (w/v) yeast extract (white triangle) or 5% (w/v) yeast extract (black triangle) (YE1 e YE5, respectively). Values are the mean \pm SD.

Strains

Superscript lowercase letters indicate values significantly different (ANOVA + Tukey's multiple comparative test; $p < 0.05$) for the same strain. A single asterisk indicates at least two successive subcultures needed. A double dagger indicates same composition of commercial MRS broth but prepared with ingredients from local companies

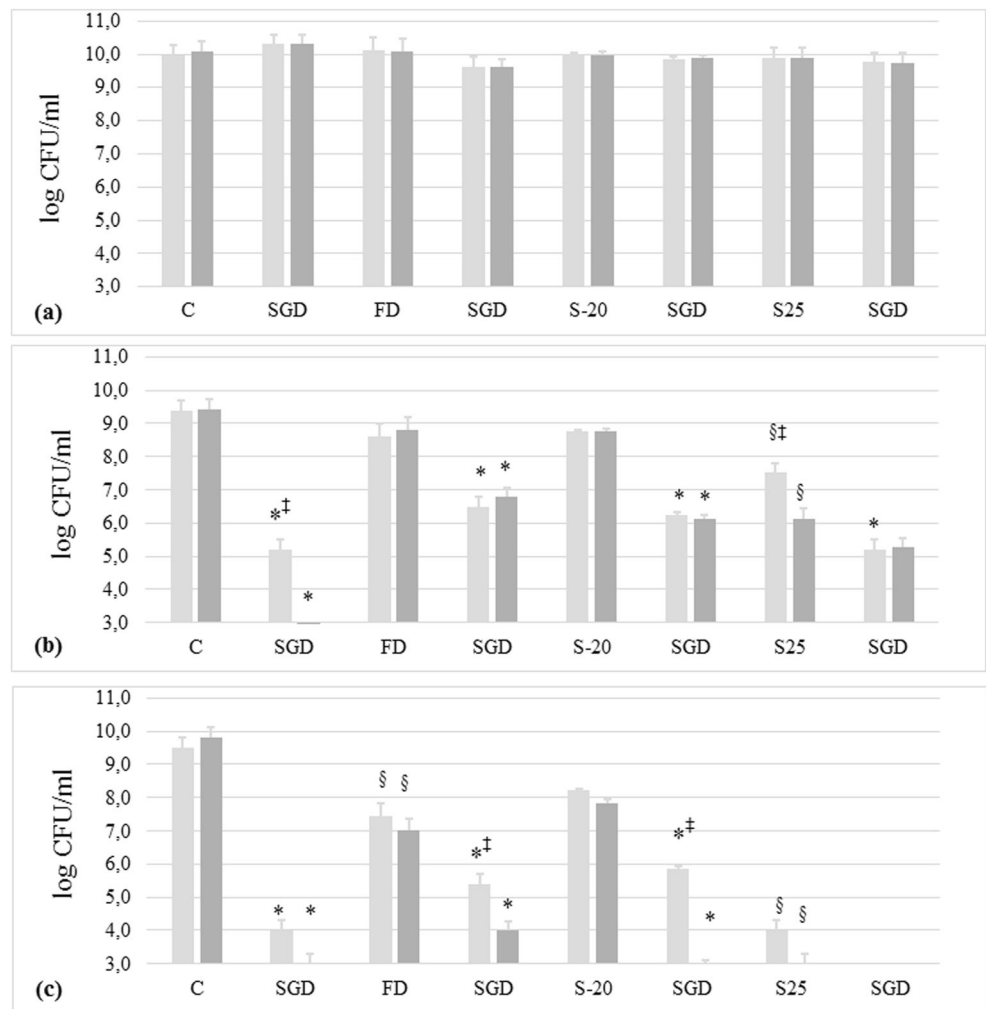
larger sample size and more complex tools as qPCR or metagenomics should be used. However, this kind of study goes beyond the scope of the present work.

Currently, the determination of antibiotic resistance is a safety criterion that must be studied for potential probiotic strains (EFSA 2008; FAO / WHO 2002). In 2012, the FEEDAP Panel (EFSA) issued a guide recommending the determination of MIC for some antimicrobial substances. As previously reported for *Bifidobacterium* (Ammor et al. 2008), in this study, the strains showed in general a high resistance to streptomycin and a marked sensitivity to clindamycin, chloramphenicol, erythromycin and vancomycin. MIC values detected were similar to those reported by Mättö et al. (2007) for strains of *B. lactis*. In particular, for *B. lactis* INL1 a marked resistance to tetracycline was observed, both by *E* test and the MD method, indicating some diversity among the isolates. Tetracycline (TC) resistance has been reported as the most common resistance within the genus *Bifidobacterium*, showing all strains of *B. lactis* studied so far an average degree of resistance to it (Gueimonde et al. 2010). As for *B. lactis* INL2, its resistance to TC was only detected by MD method. Although in general a good correlation between both methods has been observed for different antibiotics (Huys et al. 2010; Kushiro et al. 2009), MD is the method recommended by the EFSA guidance. Particularly, lower MICs were reported by the *E* test compared to the MD method for TC (Mayrhofer et al. 2008; Tosi et al. 2007). The most abundant genetic determinants responsible

for resistance to this antibiotic in bifidobacteria are the *tet* genes encoding ribosomal protection proteins. In order to ensure the safety of these strains for potential inclusion in food formulations, the presence of resistance genetic determinants and the nature of resistance, whether intrinsic or acquired, should be determined (EFSA 2012). The industrial implications of *tet* resistance genes in *B. lactis* strains has been approached, in particular considering the safety of commercial strains as *B. lactis* Bb12 and HNO19 (FDA GRAS Notices No. GRN 000049 (2005), and GRN 000445 (2013), respectively), but so far, the presence of these resistance genes does not change the GRAS status of *B. lactis* by FDA or the QPS by EFSA.

Adhesion of probiotics to the gastrointestinal surface is associated with the competitive exclusion of pathogens, persistence in the gut and modulation of the immune response (Lee and Puong 2002), and the measure of hydrophobicity has been used as a first indicator of adhesion capacity (Vinderola et al. 2004). The low values observed for all isolates in this study are in coincidence with previous reports for strains of *B. lactis* and *B. longum* (Mättö et al. 2004; Souza et al. 2013). The presence of reduced values of cell surface hydrophobicity in some probiotic strains as *B. lactis* Bb12 has been correlated with a defined balance of cell components, which apparently helps to ensure the resistance to adverse conditions, improves viability, and hence the overall probiotic properties of bacteria (Shakirova et al. 2013). Considering this, the low values found for the bifidobacteria isolates should not discourage their further study as probiotic candidates.

Fig. 3 Cell counts of a concentrated culture (C) of *B. lactis* INL1 (a), *B. longum* LM7a (b) and *B. dentium* LM8a (c), after freeze-drying (FD) in 10% (w/v) cheese whey (gray square) or 10% (w/v) lactose (dark-gray square), after simulated gastric digestion (SGD) and storage for 3 weeks at -20°C (S-20) or 25°C (S25). Each column named SGD indicates cell counts after simulated digestion of the culture on the left. Values are the mean \pm SD ($n = 3$). A single asterisk indicates significant differences for a condition and its respective DGS. A double dagger significant differences within the same condition between different lyoprotectants. A single section sign indicates significant differences between successive technological steps (concentration/freeze-drying; freeze-drying/storage) (ANOVA + Tukey’s multiple comparative test; $p < 0.05$)



The resistance to gastrointestinal transit is another in vitro selection parameter for potential probiotics. Currently, the available tests usually involve measuring the survival in a

medium acidified with HCl (pH 2–3) containing NaCl and pepsin, the subsequent use of bile salts (Mainville et al. 2005) and less frequently, the resistance to pancreatic

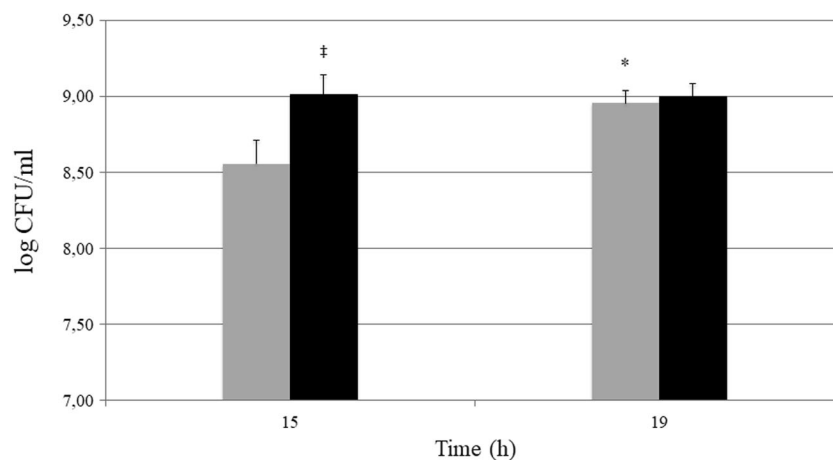


Fig. 4 Cell counts of *B. lactis* INL1 grown in a cheese whey-based culture medium (W-MRS) at pH 6.5 constant (15 h) + pH 5.0 (4 h) (gray square) and at pH free (initial pH 5.8, 15 h) + pH 5.0 (4 h) (black square). Values expressed as mean \pm SD ($n = 2$). A double dagger indicates

significant differences for the same time, between different fermentation conditions. A single asterisk indicates significant differences within the same fermentation condition with respect to harvesting time ($p < 0.05$; Student’s t test)

Table 4 Survival of freeze-dried cultures of *B. lactis* INL1 to simulated gastric digestion (SGD) when grown at pH 6.5 + acid stress (pH 5.0) and at pH free (initial pH 5.8 + pH 5.0)

Growth conditions	Total cultivation time (h)	Cell counts (log CFU/ml ± SD) at time zero and after SGD	
		0 min	90 min
pH 6.5	15	9.24 ± 0.31 ^a	5.13 ± 0.54 ^c
pH 6.5 followed by acid stress (pH 5.0, 4 h)	19	8.42 ± 0.24 ^a	4.60 ± 0.26 ^c
pH free (from pH 5.8)	15	9.16 ± 0.28 ^a	7.87 ± 0.42 ^c
pH free followed by pH 5.0	19	9.03 ± 0.44 ^a	8.26 ± 0.22 ^b

Different superscript lowercase letters in the same row indicate significant differences for the same culture condition ($p < 0.05$; $p < 0.01$ and $p < 0.005$; Student's *t* test). Values are the mean ± SD ($n = 3$)

digestion. All strains of *B. lactis* were found resistant to simulated gastric digestion and to exposure to bile salts. Resistance to acidity has been reported in previous studies for *B. lactis*, this tolerance may be due to an increase in the activity of H⁺-ATPase pump (Matsumoto et al. 2004). In a comparative test, Mättö et al. (2006) also observed reductions ≤ 1 log order for fresh cultures of two strains of *B. lactis* after 2 h at pH 2.0 + pepsin, whereas in the absence of pepsin the cultures showed a great susceptibility, so that pepsin would fulfil a protective role as a protein not yet activated by low pH. However, for other species of the genus *Bifidobacterium*, including a strain of *B. longum*, the protective effect of pepsin was not observed and the strains were highly sensitive to acidic pH, although in our study *B. longum* LM7a was highly resistant. Since bile salts have considerable antimicrobial activity at physiological concentrations, resistance to bile is important for the colonisation and persistence of intestinal microorganisms and is therefore one of the criteria for the selection of new probiotic bacteria (Grimm et al. 2014). When exposed to pancreatin and bile salts, all *B. lactis* strains were affected, whereas *B. longum* LM7a showed more resistance. This susceptibility to pancreatin in *B. lactis* is consistent with the results obtained by Masco et al. (2007), where a comparative study between members of the genus *Bifidobacterium* showed that strains belonging to the species *B. lactis* showed less tolerance towards the pancreatic enzyme, while a strain-dependent resistance was observed in case of *B. longum*.

The usefulness of cheese whey for biomass production of specific strains of lactobacilli (Lavari et al. 2014; Pérez Guerra et al. 2007) and bifidobacteria (Balciunas et al. 2016; Corre et al. 1992) was reported. When the growth in in-house and whey-based media was evaluated, no significant differences in the final cell counts of *B. lactis* INL1 were observed in all formulated media, although to be able to grow in YE1, at least one subculture (2% (v/v)) in this medium was needed. *B. longum* LM7a also showed similar growth in formulated and reference media but, while *B. lactis* INL1 showed satisfactory growth (ca. 2 log orders), *B. longum* LM7a only increased cell counts in ca. 1.5 log orders, irrespective of the culture medium. Also, *B. longum* LM7a had difficulties to grow satisfactorily in whey-based media when inoculated as

washed cells obtained in MRS, and the strain required 48 h of incubation plus at least one subculture in YE1 and YE5 to reach the same levels than in commercial MRS, suggesting perhaps the need of adaptation to this medium. Finally, for *B. dentium* LM8a', significant differences between in-house and commercial MRS were observed, and the strain showed difficulties to grow in the formulated medium. However, when grown in whey-based media, a satisfactory growth was observed although in YE1 two successive subcultures in this medium were needed in order to reach similar levels of viable cells compared to MRS and YE5. From the three bifidobacteria strains studied, *B. lactis* INL1 showed the most satisfactory growth in all formulated media, and only one previous subculture was needed for YE1 medium.

Cheese whey seemed to be a slightly better protectant than lactose, although statistical differences were only detected during the SGD of the freeze-dried cultures. The fact that cheese whey showed better results than lactose for *B. longum* LM7a in the accelerated storage test and during some SGD stages for both *B. longum* LM7a and *B. dentium* LM8a', could be explained by its composition. The protectant material composed of constituents of the same nature (carbohydrates or proteins), may not be as effective as when a mixture of carbohydrates and proteins is used (Young et al. 1993; Choi et al. 2010). The presence of molecules of different size, as occur in cheese whey, may contribute to a better protection compared to ingredients constituted by a single type of molecule (Carvalho et al. 2004). It can be hypothesised that all breast milk strains have functional relevance in the gut (and then they could all be potential probiotics) as they are naturally transferred from the mother to the child for early colonisation (Vael and Desager 2009) and maturation of the gut mucosal immune system (Romano-Keeler and Weitkamp 2015). However, from our results, it can be observed that not all breast milk isolates may display also technological potential to be manipulated in the laboratory or in the food industry. In this preliminary technological characterisation, the most satisfactory results were observed for *B. lactis* INL1. Some strains of *B. longum* have industrial importance not only for their function as probiotics (*B. longum* BB536 Morinaga, *B. longum* ES1) but also for their potential for the biosynthesis

of products with added value for the food industry such as β -galactosidase (Hsu et al. 2007; Han et al. 2014). However, the results obtained for *B. longum* LM7a in this study were not satisfactory and a first challenge would be to optimise the culture and dehydration conditions and then further evaluate its potential as probiotic. *B. dentium* was described as an opportunistic pathogen, since it is part of the ecology of the oral cavity (Ventura et al. 2009), and it was included in this study more for academic reasons rather than for a potential application, as safety issues were raised for this species.

The probiotic potential of *B. lactis* INL1 has been previously evaluated, showing immunomodulatory and protective properties in salmonellosis and acute and chronic colitis models (Burns et al. 2017; Zacarías et al. 2011, 2014, 2017). Based on this fact together with the results obtained in this study, the strain was selected for further technological characterisation. Results from biomass production in biofermentor showed and effect of the culture conditions particularly on the survival of the freeze-dried cultures to SGD. Cells grown at pH close to neutrality were more sensitive to SGD and this effect could not be overcome even when a mild acid stress was applied. The influence of growth conditions and other processing parameters on cell functionality has been previously described for probiotic bacteria. Vinderola et al. (2012) showed higher sensitivity to gastric acidity in *B. lactis* INL1 grown in MRS at pH 6.5 compared to cells grown at pH 5.0, for the same harvesting time. Deepika et al. (2009, 2012) found that other functionality parameters such as hydrophobicity and adhesion to Caco-2 cells were not only dependent on the pH at which the biomass of *L. rhamnosus* GG was produced, with a significant increase in this parameters when fermentations were carried out at pH 5.0, but also on the growth phase and temperature of fermentation. Moreover, Grześkowiak et al. (2011) demonstrated that the same probiotic strain, isolated from different food or pharma matrices, displayed different inhibitory capacity towards food pathogens, indicating that the manufacturing processes might have permanently altered the functional properties of the strain. In this regard, even if the use of different types of sub-lethal stress is a widely extended strategy to enhance the resistance to environmental stresses occurring during production, storage or digestion (Santos et al. 2016), it is necessary to evaluate the stability also in terms of functional properties to ensure that probiotics confer the expected health benefit (Gueimonde and Sánchez 2012).

In this study, the presence of bifidobacteria and lactobacilli in breast milk was observed only until week 4 postpartum. The isolates corresponding to the genus *Bifidobacterium* were studied and some safety, functional and technological characteristics were determined in order to detect potential probiotic candidates. Resistance to antibiotics was consistent with those reported by other authors, and in particular, resistance to tetracycline for strains *B. lactis* INL1 and INL2 should be further

studied. Low hydrophobicity and satisfactory resistance to gastrointestinal digestion were observed in general for all strains of *B. lactis* (INL1, INL2, INL4 and INL5) and *B. longum* LM7a. The use of cheese whey as ingredient for the biomass production of bifidobacteria is promising, although the composition of the media should be optimised on a strain basis. Furthermore, cheese whey was effective in protecting some strains during freeze-drying and simulated gastrointestinal digestion. Whereas *B. lactis* INL1 showed technological potential, with satisfactory growth in different culture media and resistance to freeze-drying, accelerated storage and acidity, *B. longum* LM7a and *B. dentium* LM8a' were more sensitive to these treatments. These results are encouraging to further study the potential of *B. lactis* INL1 and support the idea that not all isolates from breast milk may be potential probiotics, as many technological difficulties might arise for managing some of these strains outside their natural ecological niche.

Acknowledgements Authors are very grateful to the mothers and medical institutions that participated during the breast milk sampling trial.

Funding information María Florencia Zacarías received a research grant from DAAD (Deutscher Akademischer Austausch Dienst) and a CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas) doctoral scholarship. The present work was supported by the following projects: “Desarrollo de cultivos probióticos nacionales a partir de cepas autóctonas de lactobacilos y bifidobacterias,” Proyecto CAI + D Convocatoria 2011, Código 501 201101 00136 LI; “Cultivos microbianos autóctonos para la producción de alimentos funcionales para humanos y animales utilizando secado spray,” Proyecto PICT-2013-0260, ANPCyT Resolución N° 214/14.

Compliance with ethical standards

This study was performed in conformity with the Declaration of Helsinki and was approved by the Advisory Committee on Research Ethics and Safety of the Faculty of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Santa Fe, Argentina.

Conflict of interest The authors declare that they have no conflicts of interest.

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