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ORIGINAL ARTICLE

Characterization and in vitro properties of potential probiotic *Bifidobacterium* strains isolated from breast-fed infant feces

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Abstract Probiotics are live microorganisms that when administered in adequate amounts, confer health benefits on the host. In this study, 13 strains of *Bifidobacterium* were isolated from three samples of breast-fed infant feces. The isolates were identified based on conservative gene sequencing and phylogenetic analysis. In vitro tests included survival under simulated gastrointestinal tract conditions, aggregation, hydrophobicity, intestinal epithelial cell adhesion, antimicrobial activity, and antibiotic resistance according to international guidelines for probiotics. The results suggest that *B. bifidum*, *B. adolescentis*, and *B. breve* had high adhesive ability compared with *B. longum* and *B. catenulatum/B. pseudocatenulatum* group strains. In particular, *B. bifidum* IF3-211 has a highest adhesion index (8273 ± 247 and 18,009 ± 1476 adhering bacteria per 100 HT-29 and Caco-2 cells, respectively), far higher than the two reference strains, *B. lactis* Bb12 and *B. longum* BBMN68. *B. adolescentis* IF1-11 showed highest autoaggregation (82.52 ± 0.24 %) and coaggregation (45.59 ± 4.16 %) with *L. monocytogenes* among isolates. In conclusion, *B. bifidum* IF3-211 and *B. adolescentis* IF1-11 showed promising characteristics as probiotic candidates that have good potential for application in food industry.

Keywords *Bifidobacterium* · Probiotics · Infant feces · Adhesion · Aggregation

Introduction

Bifidobacteria represent one of the dominant groups of microorganisms colonizing the human intestine, constituting >1 % of the intestinal population in adults and representing up to 90 % of the fecal anaerobic bacteria in breast-fed infants (Mueller et al. 2006; Penders et al. 2006). Formation of the human gut microbiota starts at birth and is established after the first year of life; however, the composition and temporal patterns of the microbial communities vary largely among babies (Palmer et al. 2007). Thus, infant feces is a good source for the collection of wild bifidobacteria. Several selective techniques have been developed for the enumeration and isolation of bifidobacteria (Muñoa and Pares 1988; Beerens 1990; Hartemink et al. 1996; Silvi et al. 1996; Ferraris et al. 2010; Miranda et al. 2014). The United Nations Food and Agriculture Organization and the World Health Organization (FAO/WHO) define probiotics as "live micro-organisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2006). Certain members of the genus *Bifidobacterium* are the most frequently used human probiotics because of their health-promoting properties (Russell et al. 2011), such as improvement of the intestinal microbial balance of the host, lowering the risk of gastrointestinal diseases, assimilation of cholesterol, and immunomodulatory effects (Saavedra et al. 1994; Pereira and Gibson 2002; Fukuda et al. 2011; Fanning et al. 2012).

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To have functional effects in the intestine, probiotics have to survive the transit through the gastrointestinal tract (GIT). Thus, it is critical for bacteria to withstand the different challenges found along the GIT, mainly acidic pH and gastric enzymes in the stomach, and bile, pancreatin, and other intestinal enzymes in the small intestine (Sánchez et al. 2013). In addition, probiotics should adhere to the intestinal mucosa and significantly inhibit the adhesion of a variety of enteropathogenic bacteria (Del Re et al. 2000; Ouwehand et al. 2002). Adhesion, hydrophobicity and autoaggregation of bifidobacterial strains and *Lactobacillus* have been found to be strongly related (Del Re et al. 2000; Pan et al. 2006; Rahman et al. 2008), but with the exception in *Lactobacillus* (Tuo et al. 2013; García-Cayuela et al. 2014). Hydrophobicity and autoaggregation are based mainly on the proteins, glycoproteins, teichoic, and lipoteichoic acids on the cell-wall surface of bacteria, and secreted factors (Goh and Klaenhammer 2010).

As one of the most widely used probiotic bacteria, bifidobacteria are included in many functional foods and dietary supplements (Candela et al. 2008). However, few bifidobacterial strains have been commercialized due to their high sensitivity to environmental stresses (Scheller and O'Sullivan 2011). The commercial bifidobacteria strains in fermented milk products are mainly *Bifidobacterium animalis* (Raeisi et al. 2013), which is reported to be one of the most tolerant to environmental stresses (Sánchez et al. 2008), such as the widely used probiotic *B. animalis* subsp. *lactis* Bb12 (Garrigues et al. 2010). In contrast, the wild-type strains are generally sensitive to acid, bile salts, and oxygen (Simpson et al. 2005; Andriantsoanirina et al. 2013). In addition, probiotic safety issues such as virulence and transfer of antibiotic resistance need to be addressed (Saarela et al. 2000). The beneficial properties of probiotics and the increased human consumption of these products have augmented efforts to identify potential probiotic strains (Muñoz-Quezada et al. 2013). Selection of probiotics for food products should be based on their safety and technological and functional properties (Prasanna et al. 2014).

The number of formula-fed infants is on the rise in China and other countries (Blanchard et al. 2013; Tang et al. 2014). Harmful microorganisms and maturation of the intestinal immune system have an important influence on the infant's intestinal microbiome, and the number and diversity of bifidobacteria in formula-fed infants are generally low compared to those in breast-fed infants (Harmsen et al. 2000; Roger et al. 2010). Thus, supplementation of formula with probiotics is an important field of research (Braegger et al. 2011). However, most of the current commercial probiotic bifidobacterial strains are from limited species with less functional properties. In this study, 13 strains of *Bifidobacterium* were isolated from breast-fed infant feces collected from remote rural areas in China and were screened for desirable probiotic traits such as tolerance to simulated gastrointestinal juice, intestinal epithelial cell adhesion, aggregation activity, and antimicrobial activity, as well as antibiotic-resistance profiles. The candidate probiotic strains have potential for use as novel probiotic strains in the dairy industry, for example, incorporated into infant formulas.

Material and methods

Strain isolation and cultivation

Three samples of fresh feces from breast-fed, healthy infants (vaginally delivered) were collected—two from a 4-month-old infant in the rural area of Xinjiang Uygur autonomous region, China, and one from a 4-month-old infant in the rural area of Shandong province, China. The samples were diluted and plated on de Man, Rogosa, and Sharpe (MRS) medium supplemented with 0.05 % (w/v) L-cysteine hydrochloride, 3 g/L lithium chloride, and 5 mL/L propionic acid (MRScPL) (Hartemink et al. 1996; Silvi et al. 1996). The plates were incubated for 48 h at 37 °C under anaerobic (10 % H_2, 10 % CO_2, 80 % N_2) conditions. The colonies were incubated in MRS supplemented with 0.05 % L-cysteine hydrochloride (MRSc). After centrifugation, the cells were suspended in sterile 10 % (w/v) reconstituted skim milk, frozen in liquid nitrogen and stored at −80 °C until further use. To characterize the properties of the bifidobacterial isolates, the probiotics *B. lactis* Bb12 and *B. longum* BBMN68, cultured under the same conditions, were used as reference strains.

Bifidobacterial strain identification

Isolates were suggested to be bifidobacteria on the basis of their anaerobic requirement, cellular morphology, and Gram staining. For molecular biological identification of the isolates, total DNA was extracted by a previously described method (Zuo et al. 2013). Genus-specific PCR was performed to confirm strain assignment to the genus *Bifidobacterium* using the primers rpoB-F: 5'-AACATCGGTCTGATCGGCTC-3' and rpoB-R: 5'-GCTGCATGTTGGTACCCATC-3' (to detect the rpoB gene) (Kim et al. 2010). Species identification was performed by PCR amplification of the partial 16S rRNA gene using primers Bif164-F: 5'-GGGTGGTAATGCCGGATG-3' and Bif662: 5'-CCACCGTTACACCGGGAA-3' (Langendijk et al. 1995), and the partial transaldolase gene using primers ForTal: 5'-CGTCGCCTTCTTCTTCGTCTC-3' and RevTal: 5'-CTTCTCCGGCATGGTGTTGAC-3' (Requena et al. 2002). The PCR product was partially sequenced and compared to an all-nucleotide database using Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using the neighbor-joining distance method of MEGA program version 4.0.

Survival under conditions simulating the human GIT

Assays of tolerance to low pH or bile salts

All methods were based on Arboleya et al. (2011). A 5-mL aliquot of bacterial cells from overnight (16 h) culture was harvested by centrifugation (6000 *g*, 10 min, 4 °C), washed twice with 0.85 % NaCl and resuspened in 500 μL of the same buffer. A 100-μL aliquot of the bacterial suspension was added to 900 μL simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, and 3 g/L pepsin [Sigma], adjusted to pH 2.5 with HCl) or bile juice (45 mM NaCl, 1 g/L pancreatin [Sigma] and 3 g/L Oxgall [Sigma], adjusted to pH 8.0 with NaOH). Suspensions were then incubated under anaerobic conditions for 1 h. Plate counts in MRSc were performed at time 0 and after incubation, and results are presented as percent survival.

Bile salt hydrolysis assay

Fresh bacterial cultures were dropped onto MRS agar containing 0.5 % (w/v) taurodeoxycholic acid (Sigma T0875), then anaerobically incubated at 37 °C for 48 h. Strains with bile salt hydrolase activity were surrounded by a halo of precipitated deconjugated bile salts (Jones et al. 2008).

Autoaggregation and coaggregation assays

Autoaggregation assays were performed according to Del Re et al. (2000) with some modifications. Bifidobacterial strains were grown for 16 h at 37 °C in MRSc broth, the cells were harvested by centrifugation at 6000 *g* for 10 min, and the pellets were washed twice and suspended in phosphate buffered saline (PBS, pH 7.4) to yield an optical density at 600 nm (OD_{600}) of 1.0. After incubation at 37 °C for 2 h, 0.1 mL of the upper suspension was transferred to another tube with 1.9 mL PBS and OD_{600} was measured. Percent autoaggregation was expressed as 1 - (OD_{600} of upper suspension/OD_{600} of total bacterial suspension) × 100.

Preparation of cell suspensions for coaggregation was the same as for the autoaggregation analysis. Equal volumes (1 mL) of the cell suspensions of a bifidobacterial strain and the pathogen strain *Listeria monocytogenes* were mixed in a cuvette, and the OD_{600} was immediately measured (designated A_0). After incubation of the mixture at 37 °C for 2 h, the OD_{600} was measured again (designated A_t). Percent coaggregation was calculated using the equation of Nagaoka et al. (2007): coaggregation % = $(A_0 - A_t)/A_0 \times 100$.

Hydrophobicity assay

Hydrophobicity of the bifidobacterial strains was determined by xylene extraction according to Pablo et al. (1998) and Pan et al. (2006). After growth in MRSc broth for 24 h, bacterial cells were harvested by centrifugation at 6000 *g* for 5 min, and washed twice with 50 mM K_2HPO_4 (pH 6.5) buffer. Absorbance at 600 nm (A_{600}) was adjusted to 0.5 ± 0.05, then 0.6 mL xylene was added to 3 mL of bacterial suspension and vortexed for 180 s. The aqueous phase was removed after 1 h of incubation at room temperature and its A_{600} was measured. Affinity to hydrocarbons was reported as adhesion percentage according to the formula $[(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance before and after extraction with organic solvents, respectively.

Adhesion to HT-29 and Caco-2 cells

The adhesive activity of the bifidobacterial strains was assessed using HT-29 and Caco-2 cells as an intestinal epithelial cell model according to Tuo et al. (2013) with slight modifications. HT-29 and Caco-2 cells were seeded in 24-well cell culture plates at a concentration of 5×10^5 cells per well. The plates were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 %. After about 24 h of incubation, a confluent monolayer was obtained.

For adhesion assay, HT-29 and Caco-2 cell monolayers on the 24-well plates were washed twice with PBS (pH 7.4). Overnight-grown bifidobacterial strains were harvested by centrifugation at 6000 *g* for 5 min at 4 °C and washed twice with PBS (pH 7.4) and then resuspended in Dulbecco's Modified Eagle Medium (antibiotic-free, fetal bovine serum-free). Bacterial suspension (1 mL of 1×10^8 CFU/mL) was added to the 24-well plates and incubated for 1 h at 37 °C in a 5 % CO_2 atmosphere. After incubation, each well was washed six times with PBS (pH 7.4) to remove free, unattached bacterial cells. The monolayers were fixed in methanol, Gram-stained and examined microscopically under an oil-immersion lens. Adhesion was evaluated in 20 random microscopic fields and the mean ± standard deviation of adhering bacteria per 100 epithelial cells was determined.

Sensitivity to antibiotics

Bifidobacterial susceptibility to antibiotics was analyzed by minimum inhibitory concentration (MIC) assay. The different bifidobacterial strains were cultured in MRSc broth supplemented with various concentrations (0.125 to 1024 μg/mL) of antibiotics (including ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline, erythromycin, rifampicin, vancomycin) and examined in triplicate for growth in a microplate reader (OD_{600}) following a 24-h incubation period at 37 °C.

Antimicrobial activity against pathogens

The capacity of the strains to inhibit intestinal pathogens was determined by the agar-well diffusion method (Touré et al.

2003). Fresh overnight bifidobacterial MRSc culture supernatants were collected by centrifugation (12,000 *g*, 15 min, 4 °C). The cell-free supernatant (CFS) was divided into two aliquots, one adjusted to pH 6.5, and the other left unadjusted.

An initial inoculum of approximately 10^6 CFU/mL of the pathogen strain was incorporated into soft agar (1 %, w/v) plates with the appropriate medium for the target strain (BHI agar medium for *Staphylococcus aureus* ATCC25923 [Collado et al. 2005]; nutrient agar medium for *Salmonella enterica* ATCC13076 [Cheikhyoussef et al. 2009]; LB medium for *Escherichia coli* ATCC 8099). CFS (100 μL) was transferred in an Oxford cup on the surface of the agar. The plates were incubated at 37 °C for 12 h, and the diameter of the inhibition zone was measured. Tetracycline (10 μg/mL) was used as a positive control.

Statistical analysis

Data are presented as means per group ± standard errors of the means (SEM). Differences were considered significant at $p < 0.05$.

Results

Strain isolation and identification

Nearly 200 colonies were isolated from three feces samples collected from breast-fed infants. Of these, 30 were suggested to be bifidobacteria based on cellular morphology, rpoB gene amplification, and Gram staining. Their 16S rRNA and transaldolase genes were partially sequenced, resulting in the identification of five different species that had greater than 99 % sequence identity to *B. adolescentis*, *B. longum*, *B. breve*, *B. catenulatum/B. pseudocatenulatum* group, and *B. bifidum*, respectively. Among them, strains IF1-03, IF1-04, IF1-11, and IF1-12 were regarded as *B. adolescentis*, IF3-31, IF3-53, and IF3-111 as *B. longum*, IF2-141, IF2-191, and IF3-131 as *B. catenulatum/B. pseudocatenulatum* group strains, IF2-173, IF2-174 as *B. breve*, and IF3-211 as *B. bifidum* (Table 1). The phylogenetic tree of the identified bacteria based on partial 16S rRNA gene and transaldolase gene sequences provided the relative positions of the isolates (Fig. 1).

The partial 16S rRNA and transaldolase gene sequences of bifidobacteria were deposited in GenBank with accession numbers KP256207 to KP256219 and KP256220 to KP256232, respectively.

Resistance to simulated conditions of human GIT

Under the simulated GIT conditions, strains isolated from the breast-fed infants showed varied resistance to acid and bile salts. Tolerance to low pH was highly variable among strains, but all strains showed lower survival in simulated gastric juice after 1 h exposure than the two reference strains, *B. lactis* Bb12 and *B. longum* BBMN68 (Table 2). *B. adolescentis* IF1-12 displayed the highest survival, while *B. catenulatum/B. pseudocatenulatum* group and *B. breve* strains were more sensitive to low pH (Table 2). However, the isolated bifidobacterial strains were extremely sensitive to simulated bile juice. The survival rates after exposure for 1 h to bile juice were lower than 0.0001 % for all isolates, whereas the reference strain *B. lactis* Bb12 showed 0.46 % survival after bile juice challenge.

Aggregation property

All of the isolated bifidobacterial strains exhibited autoaggregation after 2 h incubation at 37 °C (Table 3). The strain *B. adolescentis* IF1-11 showed the highest autoaggregation activity, far higher than the two reference strains (*B. lactis* Bb12 and *B. longum* BBMN68; Table 3). After 16 h static cultivation in MRSc at 37 °C, *B. adolescentis* IF1-11 and *B. bifidum* IF3-211 cells aggregated and sank to the bottom of the Hungate tube (Supplementary Fig. 2).

The coaggregation ratios between bifidobacterial strains and *L. monocytogenes* are shown in Table 3. *B. adolescentis* strain IF1-11 showed the highest coaggregation ability with *L. monocytogenes*. All four *B. adolescentis* strains, three *B. longum* strains and the *B. bifidum* isolate showed significantly higher coaggregation ability than those of the two reference strains (Table 3).

Hydrophobicity distribution

Cell-surface hydrophobicity showed big differences, as measured by xylene extraction, among the bifidobacterial strains (Table 3). All of the isolated strains showed lower hydrophobicity than *B. lactis* Bb12 (Table 3). However, most of the strains showed significantly higher hydrophobicity than *B. longum* BBMN68 (Table 3), especially *B. bifidum* IF3-211, which presented hydrophobicity approaching that of *B. lactis* Bb12.

Adhesion to intestinal epithelial cells

Adhesion of the bifidobacterial strains to HT-29 and Caco-2 cells was evaluated and the results are presented in Table 3. The strains did not exhibit similar adhesion abilities, despite being in the same genus. In general, *B. longum* and *B. catenulatum/B. pseudocatenulatum* group strains had low adhesive ability compared with *B. bifidum*, *B. adolescentis*, and *B. breve*. The most adhesive strain was *B. bifidum* IF3-211, showing significantly higher adhesion than the two reference strains (Table 3). This was followed by *B. breve* IF2-173 and *B. adolescentis* IF1-11 cells (Table 3).

Table 1 The strains used in this study

Strains	Description	Source
Bifidobacterium longum BBMN68	Isolated from a centenarian's intestinal tract	Hao et al. 2011
Bifidobacterium lactis Bb12	Commercial probiotic	Chr. Hansen Ltd. (Hørsholm, Denmark)
Bifidobacterium adolescentis IF1-03	Infant feces isolate	This study
Bifidobacterium adolescentis IF1-04	Infant feces isolate	This study
Bifidobacterium adolescentis IF1-11	Infant feces isolate	This study
Bifidobacterium adolescentis IF1-12	Infant feces isolate	This study
Bifidobacterium longum IF3-31	Infant feces isolate	This study
Bifidobacterium longum IF3-53	Infant feces isolate	This study
Bifidobacterium longum IF3-111	Infant feces isolate	This study
Bifidobacterium breve IF2-173	Infant feces isolate	This study
Bifidobacterium breve IF2-174	Infant feces isolate	This study
Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum IF2-141	Infant feces isolate	This study
Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum IF2-191	Infant feces isolate	This study
Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum IF3-131	Infant feces isolate	This study
Bifidobacterium bifidum IF3-211	Infant feces isolate	This study

Antibiotic-resistance profiles

Table 4 shows the MICs of the tested bifidobacterial strains against antibiotics of different groups: RNA-synthesis inhibitor (rifampicin), cell-wall inhibitors (ampicillin and vancomycin), and protein-synthesis inhibitors (kanamycin, streptomycin, tetracycline, erythromycin, and chloramphenicol). Strains were considered resistant when they showed MIC values higher than the MIC breakpoints established by the European Food

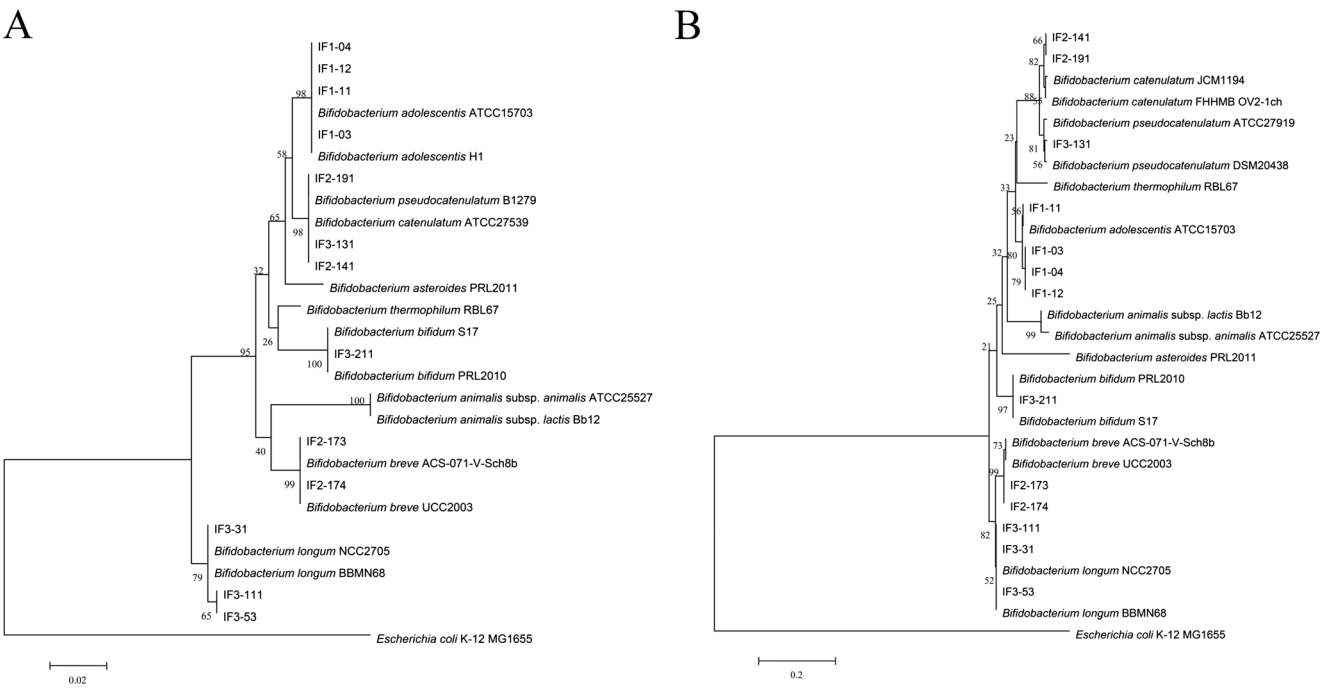

Fig. 1 Phylogenetic trees were constructed using the neighbour-joining distance and 1000 bootstrap samples of MEGA4 software based on partial 16S rRNA gene sequences (**a**) and partial transaldolase gene sequences (**b**). *Escherichia coli* K-12 MG1655 was used as an outgroup

Table 2 Resistance of different bifidobacterial strains to exposure to simulated gastric and bile juices

Strains	Tolerance to gastric juice (%, mean ± SD)	Tolerance to bile juice (%, mean ± SD)	Bile salt hydrolytic activity[a]
B. adolescentis IF1-03	11.78 ± 0.39	$8.0524 \times 10^{-5} \pm 1.5668 \times 10^{-5}$	+
B. adolescentis IF1-04	15.16 ± 0.66	$9.1403 \times 10^{-5} \pm 1.8147 \times 10^{-5}$	+
B. adolescentis IF1-11	11.67 ± 0.13	0	+
B. adolescentis IF1-12	46.97 ± 4.29	$9.7353 \times 10^{-5} \pm 9.7054 \times 10^{-7}$	+
B. longum IF3-31	28.91 ± 0.51	0	+
B. longum IF3-53	26.59 ± 0.83	0	+
B. longum IF3-111	7.82 ± 0.76	0	+
B. breve IF2-173	0.57 ± 0.00	0	+
B. breve IF2-174	1.57 ± 0.02	$6.0767 \times 10^{-6} \pm 4.4271 \times 10^{-7}$	+
B. catenulatum/B. pseudocatenulatum IF2-141	1.71 ± 0.40	0	+
B. catenulatum/B. pseudocatenulatum IF2-191	2.14 ± 0.84	0	+
B. catenulatum/B. pseudocatenulatum IF3-131	0.02 ± 0.00	0	+
B. bifidum IF3-211	<0.01	$7.2360 \times 10^{-5} \pm 6.4534 \times 10^{-6}$	+
B. lactis Bb12	70.13 ± 0.26	0.4588 ± 0.0060	+
B. longum BBMN68	60.50 ± 0.0011	$1.3266 \times 10^{-5} \pm 5.9050 \times 10^{-6}$	-

[a] + positive; - negative

Safety Authority (EFSA 2008). Accordingly, all of the isolated bifidobacterial strains could be characterized as susceptible to ampicillin and chloramphenicol, but resistant to kanamycin, streptomycin, and vancomycin (Table 4).

Antimicrobial activity

All of the bifidobacterial strains were tested for antimicrobial activity against selected pathogens in well-diffusion assays. As indicated in Table 5, CFS of all of the *Bifidobacterium*

Table 3 Autoaggregation, coaggregation with *Listeria monocytogenes*, hydrophobicity, and adhesion ability of bifidobacterial strains

Strains	Autoaggregation (%)	Coaggregation (%)	Hydrophobicity (%)	Adhesion index[a]	
				HT-29	Caco-2
B. adolescentis IF1-03	25.58 ± 4.24	32.67 ± 2.83	6.05 ± 0.75	3633 ± 497	1348 ± 219
B. adolescentis IF1-04	14.60 ± 6.72	26.36 ± 0.95	8.06 ± 2.97	3647 ± 166	578 ± 56
B. adolescentis IF1-11	82.52 ± 0.24	45.59 ± 4.16	18.62 ± 5.08	937 ± 144	1834 ± 244
B. adolescentis IF1-12	40.02 ± 2.72	27.30 ± 5.22	4.98 ± 2.35	1409 ± 280	696 ± 58
B. longum IF3-31	43.48 ± 2.51	28.23 ± 2.91	7.80 ± 2.93	256 ± 4	999 ± 132
B. longum IF3-53	42.22 ± 2.93	28.42 ± 1.71	46.07 ± 1.56	138 ± 18	852 ± 78
B. longum IF3-111	33.85 ± 1.33	24.93 ± 1.64	14.22 ± 0.44	79 ± 4	1242 ± 44
B. breve IF2-173	19.74 ± 1.86	16.10 ± 1.20	35.84 ± 1.52	3849 ± 376	822 ± 18
B. breve IF2-174	17.95 ± 0.00	7.69 ± 1.11	56.14 ± 5.82	604 ± 46	822 ± 52
B. catenulatum/B. pseudocatenulatum IF2-141	28.38 ± 1.91	13.39 ± 3.79	18.71 ± 1.30	304 ± 22	673 ± 19
B. catenulatum/B. pseudocatenulatum IF2-191	27.44 ± 4.41	14.17 ± 1.18	22.26 ± 2.92	416 ± 21	283 ± 23
B. catenulatum/B. pseudocatenulatum IF3-131	21.45 ± 1.61	6.82 ± 1.93	76.69 ± 0.85	670 ± 37	960 ± 161
B. bifidum IF3-211	34.62 ± 8.88	19.39 ± 2.04	96.82 ± 2.27	8273 ± 247	18009 ± 1476
B. lactis Bb12	22.97 ± 1.91	13.77 ± 2.10	98.84 ± 0.87	3467 ± 7	3038 ± 745
B. longum BBMN68	32.16 ± 2.73	17.30 ± 1.24	10.65 ± 3.84	234 ± 19	1102 ± 370

Values are means of three replicates ± SD

[a] The value presents mean numbers ± SD of adhering bacteria per 100 epithelial cells

Table 4 Bifidobacterial strain sensitivities to different antibiotics

Strains	MIC (μg/mL)							
	Ampicillin	Chloramphenicol	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Rifampicin	Vancomycin
B. adolescentis IF1-03	0.125	2	>1024	128	8	>128	16	128
B. adolescentis IF1-04	0.125	2	>1024	256	16	>128	16	>128
B. adolescentis IF1-11	0.125	1	1024	256	2	>128	16	128
B. adolescentis IF1-12	0.125	2	>1024	256	2	>128	32	128
B. longum IF3-31	1	1	>1024	128	16	32	8	128
B. longum IF3-53	1	1	>1024	128	32	8	8	128
B. longum IF3-111	1	1	>1024	256	16	64	8	128
B. breve IF2-173	1	2	1024	128	32	>128	>128	>128
B. breve IF2-174	1	1	512	128	32	>128	8	>128
B. catenulatum/B. pseudocatenulatum IF2-141	1	1	>1024	512	32	16	16	128
B. catenulatum/B. pseudocatenulatum IF2-191	0.5	1	>1024	512	4	32	64	128
B. catenulatum/B. pseudocatenulatum IF3-131	2	1	>1024	512	4	8	>256	256
B. bifidum IF3-211	0.25	1	1024	32	32	>256	16	256

strains inhibited *Salmonella enterica* ATCC13076, and CFS of *B. adolescentis* and *B. breve* weakly inhibited *Escherichia coli* and *Staphylococcus aureus* ATCC25923. However, no inhibition was observed for any of the CFS in which the pH had been neutralized (data not shown).

Discussion

The wide use of infant formula in China carries potential health-risk concerns (Tang et al. 2014); infant formula is less efficient at assisting in the development of the gut microbiota and the immune system than breast milk (Harmsen et al. 2000). Increasing bifidobacterial levels is considered a target in infant formula development through, for example, supplementation with live *Bifidobacteria* (Braegger et al. 2011). Thus, isolation of bifidobacterial strains from breast-fed infants is of great importance. In China, however, wild bifidobacterial strains have been only scarcely isolated and characterized from human feces (Pan et al. 2006; Liu et al. 2013). Here we confirmed the identity of 13 *Bifidobacterium* strains isolated from breast-fed infant feces by partial sequencing of the 16S rRNA and transaldolase genes; these included strains of

Table 5 In vitro inhibition of pathogens by bifidobacterial strains

Strains	Diameter (mm) of inhibition zones		
	Salmonella enterica ATCC13076	*Staphylococcus aureus* ATCC25923	*Escherichia coli*
B. adolescentis IF1-03	8.21	7.74	9.85
B. adolescentis IF1-04	7.39	7.74	12.36
B. adolescentis IF1-11	7.11	7.74	11
B. adolescentis IF1-12	8.91	7.74	7.74
B. longum IF3-31	15.83	0	10.95
B. longum IF3-53	12.31	0	7.74
B. longum IF3-111	7.74	0	7.74
B. breve IF2-173	7.74	7.74	7.74
B. breve IF2-174	7.74	0	9
B. catenulatum/B. pseudocatenulatum IF2-141	7.74	0	7.74
B. catenulatum/B. pseudocatenulatum IF2-191	7.74	0	8.24
B. catenulatum/B. pseudocatenulatum IF3-131	12.04	0	0
B. bifidum IF3-211	7.74	0	0

B. adolescentis, *B. catenulatum*/*B. pseudocatenulatum* group, *B. longum*, *B. breve*, and *B. bifidum*. Differentiation of *B. catenulatum* and *B. pseudocatenulatum* is difficult; strains IF2-141 and IF2-191 were regarded as *B. catenulatum*, IF3-131 was regarded as *B. pseudocatenulatum* according to phylogenetic tree based on partial transaldolase gene (Fig. 1b). In spite of this, a much better method to discriminate them, such as multi-gene-based analysis, is needed (Kim et al. 2010).

To test the potential of these wild bifidobacterial strains for use as probiotics, in vitro functional characterization and safety assessment were carried out according to the guidelines established by the FAO/WHO working group (FAO/WHO 2006). Bifidobacteria of human intestinal origin have been proposed to be more suitable for probiotic applications, but they are sensitive to environmental stresses such as low pH and bile salts as they enter the GIT (Scheller and O'Sullivan 2011). Survival under GIT conditions is crucial for probiotic strains. In this study, all of the isolated strains showed high sensitivity to bile salts, albeit generally lower sensitivity than reported in previous studies (Arboleya et al. 2011; Andriantsoanirina et al. 2013). This suggests that resistance to bile salts is highly strain- and species-dependent, although all isolates showed bile salt hydrolase activity. It should be noted that there are many methods to improve the viability of wild bifidobacteria for use in the food industry, such as stress adaptation and microencapsulation (Hansen et al. 2004; Noriega et al. 2004; Picot and Lacroix 2004; Sánchez et al. 2007).

In analyses of cell-surface properties, *B. adolescentis* IF1-11 exhibited the highest autoaggregation values and highest coaggregation with *L. monocytogenes*. *B. catenulatum*/*B. pseudocatenulatum*group strain IF3-131 and *B. bifidum* IF3-211 had the highest hydrophobicity values, with the latter value being close to that of the reference strain *B. lactis* Bb12. In most cases, hydrophobicity and autoaggregation abilities are strongly related to the adhesion properties of bifidobacteria and lactobacilli (Del Re et al. 2000; Tuo et al. 2013). Adhesion to the intestinal mucosa is considered one of the main criteria for the selection of potential probiotics, as it may increase their persistence in the intestine, giving the probiotic time to exert its effects (Kolida et al. 2006). *B. bifidum* IF3-211 showed the highest adhesion levels to HT-29 and Caco-2 cells, far exceeding those of *B. lactis* Bb12 and *B. longum* BBMN68; this corresponds to the high hydrophobicity of *B. bifidum* IF3-211, but not to its low autoaggregation. However, *B. bifidum* IF3-211 did exhibit high autoaggregation when cells were grown to the stationary phase (Fig. 2) or in low pH conditions (data not shown), suggesting that the autoaggregation characteristic of *B. bifidum* IF3-211 is dependent on environmental conditions, mainly pH, as described previously (Canzi et al. 2005; Guglielmetti et al. 2009). Most of the *B. adolescentis* strains and *B. breve* IF2-173 showed relatively higher adhesion ability than *B. longum* BBMN68. Except for *B. adolescentis* IF1-11, which had high autoaggregation values, the other three *B. adolescentis* strains had relatively low autoaggregation and hydrophobicity. But *B. adolescentis* IF1-11 showed lower adhesion ability to HT-29 cells and higher adhesion ability to Caco-2 cells than the other *B. adolescentis* strains. *B. breve* IF2-173 showed far higher adhesion ability to HT-29 cells than *B. breve* IF2-174, but its hydrophobicity was lower. We could, therefore, hypothesize that the aggregation and hydrophobicity phenotypes are not always correlated with adhesion abilities, and they are not the only mechanism involved in adhesion. For example, adhesion of *Lactobacillus rhamnosus* GG and *Lactococcus lactis* TIL448 was found to be mediated by pili, structures known to mediate the adhesion of many pathogens (Kankainen et al. 2009; Meyrand et al. 2013). Remarkably, the tight adherence (Tad) pili and sortase-dependent pili are responsible for the adhesion of *B. breve* UCC2003 and *B. bifidum* PRL2010 to the intestinal epithelium, respectively (Motherway et al. 2011; Turroni et al. 2013). In addition, the moonlighting protein transaldolase has been shown to play a role in the autoaggregation and adhesion of *B. bifidum* to mucin (Gonzalez-Rodriguez et al. 2012). However, *B. longum* and *B. catenulatum*/*B. pseudocatenulatum* group strains showed relatively lower adhesion ability. In fact, the adhesion properties of these two bifidobacteria are generally poorer than those of other species such as *B. breve* and *B. bifidum* (Del Re et al. 2000; He et al. 2001). Thus, to select new potential probiotic strains, a case-by-case assessment is required. Other mechanisms related to adhesion and pathogen-exclusion properties of these wild bifidobacterial strains need to be further investigated.

The production of antimicrobial compounds against pathogens by bifidobacterial strains was determined by agar diffusion assay. All of the selected bifidobacteria's supernatants could inhibit the foodborne pathogen *Salmonella enterica*, and inhibition of *E. coli* and *S. aureus* was found with some bifidobacterial secretions. However, no inhibition was observed for any of the CFS when the pH was neutralized, indicating that the inhibition effect is mainly due to organic acids, such as short-chain fatty acids, lactic acid, and acetic acid (Midtvedt and Midtvedt 1992; Makras and De Vuyst 2006). Bifidobacterial antimicrobial activity is due to a number of metabolites, organic acids, and most importantly, bacteriocins, although only limited classes of bacteriocins produced by bifidobacteria have been characterized in depth (Martinz et al. 2013). The capacity to produce antimicrobial compounds is one of the critical characteristics of bifidobacteria in terms of effectively and competitively excluding pathogens in the intestine, and exerting their probiotic effect on the host (Ouwehand and Salminen 1998).

It is important to determine the safety of wild bifidobacterial strains, particularly their antibiotic resistance profiles (Sanders et al. 2010). Antibiotic MIC assay suggested

that all of the isolated bifidobacterial strains are susceptible to ampicillin and chloramphenicol, but resistant to kanamycin, streptomycin, and vancomycin. *B. lactis* Bb12 is resistant to kanamycin, streptomycin, and vancomycin (Zhou et al. 2005), and some other studies suggest that bifidobacteria were resistant to kanamycin and streptomycin (Sharma et al. 2014). Kiwaki and Sato (2009) suggest that bifidobacteria resistance to streptomycin might be caused by mutations in the *rpsL* gene, but sequencing of *rpsL* genes from all the isolates plus the two reference strains didn't find any mutations at nucleotide position 128 (data not shown). Thus, the *rpsL* gene was not responsible for streptomycin resistance in these strains. Testing them for the presence of the enterococcal *vanA* and *vanB* genes by PCR using the primers provided by Klein et al. (2000) gave a negative result (data not shown), similar with the previously reports (Klein et al. 2000; Zhou et al. 2005). Some strains of *B. animalis* subsp. *lactis* and *B. bifidum* have shown acquisition of the tetracycline resistance gene *tet*(W) (Meile et al. 2008), and some commercial probiotic strains, including *B. longum*, *B. bifidum*, and *B. thermophilum*, are also resistant to the antibiotics erythromycin, streptomycin, and chloramphenicol (Sato and Iino 2009; Mayrhofer et al. 2011; Wei et al. 2012). Although none of the *Bifidobacterium* species with qualified presumption of safety (QPS) status, including *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* (EFSA 2012), have been associated with human clinical disease, their antibiotic resistance phenotypes should be thoroughly characterized to prevent the potential transfer of antibiotic resistance genes to other bacteria, especially pathogens, in the intestinal habitat (Ammor et al. 2008).

The main objective of this study was to find new probiotic candidates for use in functional fermented food, such as infant formula. In summary, we characterized 13 bifidobacterial strains isolated from breast-fed infant feces, phenotypically and genotypically, according to international guidelines for probiotics. In addition, in vitro tests were performed to assess the probiotic potential of these strains. Our results suggest that some of the strains isolated from breast-fed infant feces, notably *B. bifidum* IF3-211 and *B. adolescentis* IF1-11, may have valuable probiotic potential in functional food products, although further safety evaluation and human studies would be needed.

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Compliance with ethical standards

Conflict of interest None of the authors had a conflict of interest.

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