

# Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns

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**Abstract** Several studies support the use of probiotics for the treatment of minor gastrointestinal problems in infants. Positive effects on newborn colics have been evidenced after administration of *Lactobacillus* strains, whereas no studies have been reported regarding the use of bifidobacteria for this purpose. This work was therefore aimed at the characterization of *Bifidobacterium* strains capable of inhibiting the growth of pathogens typical of the infant gastrointestinal tract and of coliforms isolated from colic newborns. Among the 46 *Bifidobacterium* strains considered, 16 showed high antimicrobial activity against potential pathogens; these strains were

further characterized from a taxonomic point of view, for the presence and transferability of antibiotic resistances, for cytotoxic effects and adhesion to nontumorigenic gut epithelium cell lines. Moreover, their ability to stimulate gut health by increasing the metabolic activity and the immune response of epithelial cells was also studied. The examination of all these features allowed to identify three *Bifidobacterium breve* strains and a *Bifidobacterium longum* subsp. *longum* strain as potential probiotics for the treatments of enteric disorders in newborns such as infantile colics. A validation clinical trial involving the selected strains is being planned.

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## Introduction

Several studies support the use of probiotics as therapeutic agents against a number of diseases, in particular enteric disorders but also human diseases which are not apparently linked to the microbial gut composition, such as allergies and autoimmune diseases (Taylor et al. 2006; Roessler et al. 2008). The intestinal microbiota plays an important role in human health by providing nutrients that would not be otherwise available to the host, by stimulating the development of the immune system and by creating a barrier for the colonization of pathogens (Palmer et al. 2007; Roncaglia et al. 2011). A balanced composition of the gut microbiota has a positive influence on the health status of the host, both in adults and infants (Guarner 2006; Serikov et al. 2010).

Bifidobacteria are the major components of the microbiota of infants fed exclusively with breast milk and are commensal bacteria of the large intestine of humans and animals (Ventura et al. 2004). They are widely used as probiotics for therapeutic

purposes considering their capabilities of colonizing the gastrointestinal tract and their long history of safe use (Sanders et al. 2010). As regards to *Bifidobacterium* spp. administration to newborns, in early 2002 the *Food and Drug Administration* has accepted as “safe” the use of *Bifidobacterium lactis* in formula milks and has given to this microorganisms the “Generally Regarded As Safe (GRAS)” status (Hammerman et al. 2006). Positive effects on the administration of *B. lactis* Bb12 on the reestablishment of a balanced composition of the gut microbiota were found on preterm, full-term infants and toddlers (Mohan et al. 2006). Among the different species belonging to this genus, *Bifidobacterium breve* appears to be one of the most used in infants. Li et al. (2004) concluded that the very early administration (at the first days of life) of a *B. breve* strain to low birth weight infants was useful in promoting the colonization of the bifidobacteria and the formation of a normal intestinal flora. A later study (Wang et al. 2007) evidenced that the administration of a *B. breve* strain to low birth weight infants reduces the production of butyric acid, which may be helpful in protecting these infants from necrotizing enterocolitis (Lin 2004). The beneficial effects of *B. breve* strain Yakult have also been evidenced in immunocompromized pediatric patients on chemotherapy (Wada et al. 2010). These young patients suffered from infectious complications, which were preceded by bowel colonization of pathogenic bacteria followed by translocation through the gut mucosa and systemic dissemination. Following probiotic administration, the use of antibiotics was lower and the gut habitation of anaerobes was enhanced.

Recent results evidenced that probiotics may be also useful for the treatment of minor gastrointestinal problems of newborns such as colics (Indrio et al. 2008; Savino et al. 2010). Infantile colics are characterized by an excessive and inconsolable crying without an identifiable cause in healthy newborns in the first 3 months of life. It affects up to 30 % of infants and it causes considerable stress and concern for parents. The pathogenesis of the conditions remains largely unknown although evidences suggested multiple independent causes, including modification of the gut microbiota with an increased number of gas-forming coliforms in colicky infants with respect to healthy controls (Savino et al. 2009). This finding suggested a role of coliform colonic fermentation in excessive intra-intestinal air production and pain typical of colic infants. The daily administration of *Lactobacillus reuteri* DSM 17938 in early breastfed infants was found to improve symptoms of infantile colics (Savino et al. 2010) and a recent study evidenced that other *Lactobacillus* spp. strains possess the ability to in vitro inhibit gas-forming coliforms and therefore have the potential of being used as probiotics for colics treatment (Savino et al. 2011). No studies have been presented up to now on the possibility of using *Bifidobacterium* spp. strains for this purpose, although, differently from *Lactobacillus* spp., *Bifidobacterium* spp. systemic infections upon administration

in infants have never been reported (Hammerman et al. 2006).

In this work, a number of bifidobacteria strains were screened for desirable functional properties for their application as probiotics against enteric disorders and, in particular, colic disease in newborns. The antimicrobial activity against coliforms isolated from colicky newborns and against bacteria most frequently cause of diarrhea in infants was studied, as well as their antibiotic susceptibility, adhesion, and toxicity towards gut epithelial cells and their ability to stimulate the metabolic activity and immune response of epithelial cells. The most interesting strains were also checked for transmission of antibiotic resistance traits.

## Materials and methods

### Strains and culture conditions

Forty-six strains of *Bifidobacterium* spp. were included in this study; the majority of them derived from infant feces (Table 1). Forty-two of them were obtained from the Bologna University Scardovi Collection of Bifidobacteria (BUSCoB), available at the University of Bologna, whereas four were from the American Type Culture Collection (ATCC 15697, ATCC 15707, ATCC 15708, ATCC 27917). Thirty-six of the BUSCoB strains have been previously characterized with phenotypic analyses and by means of the electrophoretic pattern of transaldolase and 6-phosphogluconic dehydrogenase (Scardovi et al. 1979). The remaining six strains (B7710, B7740, B7840, B7947, B7958, B8452) were isolated from preterm newborn feces and characterized as members of the *Bifidobacterium* genus by means of phenotypic analyses and the fructose 6-phosphate phosphoketolase assay (unpublished results). *Bifidobacterium* strains were cultivated in tryptone, peptone, yeast extract medium (TPY prepared according to Biavati and Mattarelli 2006) and incubated at 37 °C under anaerobic conditions using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany). Four strains have been deposited to the DSMZ culture collection with the following collection numbers: DSM 24706 (*B. breve* B632), DSM 24707 (*B. breve* B2274), DSM 24708 (*B. breve* B7840), DSM 24709 (*Bifidobacterium longum* subsp. *longum* B1975).

The strains used as antagonistic microorganisms were: *Escherichia coli* ATCC 11105, *Salmonella enteritidis* M94 strain and *Clostridium difficile* M216 strain (both isolated from hospitalized patients and available at BuSCoB), *Campylobacter jejuni* CIP 70.2<sup>T</sup> (from the Collection de l’Institut Pasteur, Paris, France) and two gas-forming coliforms isolated from feces of colicky infants, *Klebsiella pneumoniae* GC23a and *Enterobacter cloacae* GC23a (Savino et al. 2011). The *E. coli*, *S. enteritidis*, *K. pneumoniae*, and *E. cloacae* strains were cultivated in nutrient broth (NB; Oxoid, Basingstoke, UK)

**Table 1** List of the 46 *Bifidobacterium* spp. strains used in this study and evaluation of their antimicrobial activity against four antagonistic strains (*E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. enteritidis*) expressed as *radius* (in centimeter) of the inhibition halos obtained on TPY plates in the agar spot test

Species	Strain	Origin	Antimicrobial activity*				Average inhibition radius (cm)
			<i>E. coli</i> ATCC 11105	<i>E. cloacae</i> GC 6a	<i>K. pneumoniae</i> GC 23a	<i>S. enteritidis</i> M 94	
<i>B. bifidum</i>	B1968	Infant feces	0.2	0.3	0.2	0.2	0.22 (gh)
<i>B. bifidum</i>	B2009	Infant feces	0.4	0.4	0.3	0.4	0.37 (eh)
<i>B. bifidum</i>	B2531	Infant feces	0	0	0	0	0 (h)
<i>B. bifidum</i>	B2091	Infant feces	0.6	0.6	0.6	0.7	0.62 (bg)
<i>B. breve</i>	B2274 <sup>a</sup>	Infant feces	0.8	1	1	1.3	1.02 (ab)
<i>B. breve</i>	B2021	Infant feces	0.6	0.9	0.9	1	0.85 (ae)
<i>B. breve</i>	B632 <sup>b</sup>	Infant feces	1.2	0.8	0.9	1.2	1.02 (ab)
<i>B. breve</i>	B1501	Infant feces	0.5	0.1	0.2	0.1	0.22 (gh)
<i>B. breve</i>	B2150	Infant feces	0.6	1	0.8	1	0.85 (ae)
<i>B. breve</i>	B2142	Infant feces	0.4	0.4	0.5	0.5	0.45 (dh)
<i>B. breve</i>	B2228	Infant feces	0.2	0.3	0.1	0.2	0.20 (gh)
<i>B. breve</i>	B626	Infant feces	0.1	0.3	0.1	0.3	0.20 (gh)
<i>B. breve</i>	B633	Infant feces	0.2	0.2	0.1	0	0.12 (gh)
<i>B. breve</i>	B2136	Infant feces	0.4	0.3	0.3	0.6	0.40 (dh)
<i>B. breve</i>	B2023	Infant feces	0.7	0.2	0.2	0.7	0.45 (dh)
<i>B. breve</i>	B2195	Infant feces	0.5	0.9	0.7	1.1	0.80 (af)
<i>B. breve</i>	B2210	Infant feces	0.2	0.3	0.1	0	0.15 (gh)
<i>B. longum</i> subsp. <i>infantis</i>	B1412 <sup>c</sup>	Infant feces	1.2	1.3	0.9	1	1.10(a)
<i>B. longum</i> subsp. <i>infantis</i>	B651	Infant feces	0.1	0.2	0.1	0.3	0.17 (gh)
<i>B. longum</i> subsp. <i>infantis</i>	B1915	Infant feces	0.1	0.2	0.1	0.3	0.17 (gh)
<i>B. longum</i> subsp. <i>infantis</i>	B1860	Infant feces	1.1	0.1	0.3	0	0.37 (eh)
<i>B. longum</i> subsp. <i>infantis</i>	Re 6	Infant feces	0.7	0	0	0	0.17 (gh)
<i>B. longum</i> subsp. <i>longum</i>	B1629	Infant feces	0.2	0.5	0.5	0.4	0.40 (dh)
<i>B. longum</i> subsp. <i>longum</i>	Re11	Infant feces	0	0	0	0	0 (h)
<i>B. longum</i> subsp. <i>longum</i>	Re12	Infant feces	0.9	0.8	0.8	1	0.87 (ad)
<i>B. longum</i> subsp. <i>longum</i>	B2101	Infant feces	0.9	0.9	1	1	0.95 (ac)
<i>B. longum</i> subsp. <i>longum</i>	B1975 <sup>d</sup>	Infant feces	0.9	0.7	0.6	1.2	0.85 (ae)
<i>B. longum</i> subsp. <i>longum</i>	B1482	Infant feces	0.5	0	0.4	0	0.22 (gh)
<i>B. longum</i> subsp. <i>longum</i>	B2327	Infant feces	0.3	0.3	0	0.6	0.30 (gh)
<i>B. longum</i> subsp. <i>longum</i>	B2212	Infant feces	0.5	0	0.1	0	0.15 (gh)
<i>B. longum</i> subsp. <i>longum</i>	B2192	Infant feces	0.9	1	0.7	1.5	1.02 (ab)
<i>B. longum</i> subsp. <i>longum</i>	B2055	Infant feces	0.7	0.5	0.5	0.5	0.55 (cg)
<i>B. longum</i> subsp. <i>longum</i>	B1993	Infant feces	0.1	0.3	0.2	0	0.15 (gh)
<i>B. longum</i> subsp. <i>longum</i>	B1996	Infant feces	0.5	0.6	0.4	0.2	0.42 (dh)
<i>B. adolescentis</i>	B7311	Infant feces	0.3	0.4	0.5	0.7	0.47 (dh)
<i>B. adolescentis</i>	B7162	Infant feces	0.3	0	0	0.3	0.15 (gh)
<i>B. pseudocatenulatum</i>	B1279	Infant feces	0.5	0.5	0.4	0.4	0.45 (dh)
<i>Bifidobacterium</i> spp	B1391	Infant feces	0.1	0.1	0.3	0.2	0.17 (gh)
<i>Bifidobacterium</i> spp	B2529	Infant feces	0	0.1	0.1	0.3	0.12 (gh)
<i>Bifidobacterium</i> spp	B3225	Infant feces	0.5	0.3	0.2	0.3	0.32 (fh)
<i>Bifidobacterium</i> spp	B7710	Preterm feces	0	0	0	0	0 (h)
<i>Bifidobacterium</i> spp	B7740	Preterm feces	0	0.5	1	0.5	0.50 (dh)
<i>Bifidobacterium</i> spp	B7840 <sup>e</sup>	Preterm feces	0.7	1	0.6	1	0.82 (ae)
<i>Bifidobacterium</i> spp	B7947 <sup>f</sup>	Preterm feces	0.7	0.4	0.3	0.5	0.47 (dh)
<i>Bifidobacterium</i> spp	B7958 <sup>g1</sup>	Preterm feces	0.7	0.6	0.8	1.1	0.80 (af)

**Table 1** (continued)

Species	Strain	Origin	Antimicrobial activity*				
			<i>E. coli</i> ATCC 11105	<i>E. cloacae</i> GC 6a	<i>K. pneumoniae</i> GC 23a	<i>S. enteritidis</i> M 94	Average inhibition radius (cm)
<i>Bifidobacterium</i> spp	B8452 <sup>h</sup>	Preterm feces	0.6	0.1	0.6	0.2	0.37 (eh)

The average of the values obtained for each *Bifidobacterium* strain is presented in the right column; mean values followed by different letters (in brackets) are statistically different at  $P < 0.001$

<sup>a</sup> Strain deposited as DSM 24707

<sup>b</sup> Strain deposited as DSM 24706

<sup>c</sup> Strain identified as *B. longum* subsp. *longum* within this work

<sup>d</sup> Strain deposited as DSM 24709

<sup>e</sup> Strain identified as *B. breve* within this work and deposited as DSM 24708

<sup>f</sup> Strain identified as *B. breve* within this work

<sup>g</sup> Strain identified as *B. longum* subsp. *longum* within this work

<sup>h</sup> Strain identified as *B. pseudocatenulatum* within this work

aerobically at 37 °C. *C. difficile* M216 strain was grown in Brain Heart Broth (Merck) and incubated under anaerobic condition at 37 °C; *C. jejuni* CIP 70.2<sup>T</sup> strain was cultured as described in Santini et al. (2010).

#### Genetic typing of the strains

##### *Enterobacterial repetitive intergenic consensus PCR*

Total DNA was extracted from 10 mL of overnight pure cultures and purified using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) patterns of *Bifidobacterium* strains were obtained following the procedure described by Ventura et al. (2003). Primers ERIC-1 (5'ATG-TAAGCTCCTGGGGATTAC-3') and ERIC-2 (5'AAG-TAAGTACTGGGGTGAGCG-3') were used. The 20 µL reaction mixture contained 10 µL of HotStart Taq Plus Master Mix Kit (Qiagen, West Sussex, UK), 1 µM of each primer, 1.5 mM MgCl<sub>2</sub> (Qiagen). PCR reactions were run in a Veriti Thermal Cycler (Applied Biosystem, Foster City, CA, USA). The reference strains used in this study were: *Bifidobacterium pseudocatenulatum* ATCC 27917<sup>T</sup>, *Bifidobacterium catenulatum* ATCC 27539<sup>T</sup>, *B. breve* ATCC 15700<sup>T</sup>, *Bifidobacterium bifidum* DSM 20456<sup>T</sup>, *B. longum* subsp. *longum* ATCC 15707<sup>T</sup>, and *B. longum* subsp. *infantis* ATCC 15697<sup>T</sup>.

##### *PCR with genus-specific and specie-specific primers*

*Bifidobacterium* genus-specific PCR was performed on total DNA using 16S rDNA-targeted primers Bif64-f and Bif662-r (Satokari et al. 2001). Species identification was carried out using species-specific PCR primers described by Matsuki et al. (1999).

#### In vitro inhibition of antagonistic strains

##### *Agar spot test using living cells*

To assess the antimicrobial activity of *Bifidobacterium* spp. strains against selected bacteria (*E. coli* ATCC 11105, *S. enteritidis* M94, *K. pneumoniae* GC23a strain, and *E. cloacae* GC 6a were used for all the 46 strains, whereas *C. jejuni* CIP 70.2<sup>T</sup> and *C. difficile* M216 only for 16 selected strains) the protocol described by Santini et al. (2010) was employed. Briefly, *Bifidobacterium* spp. strains were spotted on TPY plates and, after strain growth, the plates were overlaid with a soft agar medium inoculated with each pathogen strain. Inhibition was evaluated after 24 h measuring the radius of the inhibition halo around the *Bifidobacterium* spot. Each assay was performed in triplicate.

##### *Antimicrobial activity of Bifidobacterium spp. culture supernatants*

This assay was performed with the 16 strains showing the most interesting antimicrobial activity in the previously described assay and, as a negative control, with a *Bifidobacterium* strain not showing any antagonistic activity in the spot agar assay (B7710). Cell-free supernatants were obtained by centrifuging TPY bifidobacteria o.n. cultures (15,000×g, 20 min, 4 °C) followed by filtration through a 0.22 µm pore size cellulose acetate filter. An aliquot of the supernatant was adjusted to pH 7. The antagonist strains used in this assay were: *E. coli* ATCC 11105, *S. enteritidis* M94, *K. pneumoniae* GC23a, and *E. cloacae* GC23a. The antagonistic strains were grown in NB until absorbance at 600 nm ( $A_{600}$ ) of 0.9 and used to inoculate 96-well plates. Each well contained: 100 µL of double concentrated NB, 25 or 50 µL of *Bifidobacterium*

spp. cell-free supernatant (both neutralized and non-neutralized), corresponding to a v/v percentage of 12.5 and 25, respectively, and water to 200  $\mu$ L of total volume. 1 % v/v inoculum of the antagonistic strain was added. Positive controls were prepared by using 50  $\mu$ L of fresh NB without any supernatants. Plates were incubated aerobically at 37 °C for 22 h;  $A_{620}$  was periodically evaluated in a multiwell plate spectrophotometer (Multiskan, Thermo Electron, Oy, Vaanta, Finland).

#### Antibiotic resistance profiles

##### *Minimal inhibitory concentration*

Minimal inhibitory concentration (MIC) for 12 antibiotics was determined with the microdilution assay in 96 well plates as described in Santini et al. (2010) on the 16 *Bifidobacterium* strains. Twelve antibiotics were employed, eight of which were suggested in the most recent European Food Safety Authority (EFSA) guidelines (EFSA 2008), i.e., tetracycline, cefuroxime, kanamycin, chloramphenicol, vancomycin, ampicillin, streptomycin, and erythromycin whereas the other four were antibiotics widely used in infant therapy (cefuroxime, amoxicillin, ceftriaxone, and clarithromycin). Antibiotics were used in the concentration range 1–512  $\mu$ g/mL. Growth or inhibition of the strains was determined by measuring the  $A_{620}$  at regular time intervals for a total incubation of 24 h at 37 °C. Antibiotics were all from Sigma-Aldrich, Milan, Italy.

##### *Screening of resistance genes*

The presence of known antibiotic resistance genes was determined by PCR using specific primers: *aph* (3'')-I, *aph* (3'')-II, *aph* (3'')-III coding for kanamycin and neomicine resistance (Ouoba et al. 2008), *aadA*, *aadE*, *ermA*, streptomycin, and erythromycin-resistance genes (Ouoba et al. 2008), *tet*(M), *tet*(O), *tet*(W) coding for tetracycline resistance (Masco et al. 2006) and blaCTX-M-g1, blaCTX-M-g2,  $\beta$ -lactam resistance (Van Hoek et al. 2008). The amplification conditions are from Ouoba et al. (2008). The annealing temperature varied in the range 45–64 °C, depending on the primer. *Lactobacillus casei* L9 was used as positive control for *aph*(3'')-III, *aadA*, *aadE* genes whereas *Bifidobacterium adolescentis* DSM 20087 was the positive control for *tet* (W) gene. PCR products were separated by electrophoresis on 1.5 % agarose gel.

##### *Plasmid detection*

Pure yield plasmid Miniprep System kit (Promega) was used to extract and purify plasmid DNA from the selected 16 *Bifidobacterium* strains. *B. longum* B2399, which was known to possess two plasmids, was used as positive control

for plasmid DNA extraction. Plasmids were separated after electrophoresis on a 0.7 % agarose gel.

##### *Evaluation of the transferability of the antibiotic resistance traits*

Four *Bifidobacterium* strains (B632, B1975, B2274, B7840) were used as donor strains, whereas *Bifidobacterium animalis* ATCC 27536, *B. longum* subsp. *suis* PCD733B (Santini et al. 2010), three *Bifidobacterium* strains from this study (B1412, B7840, B632), *Lactobacillus plantarum* PCS22 (Nissen et al. 2009), and *Enterococcus faecium* PCD71 (Santini et al. 2010) were used as recipient strains. The transferability of the antibiotic resistance traits was assayed following the protocols of Lampkowska et al. (2008) and Ouoba et al. (2008). A scheme of the experiments is outlined in Table 2. Donor and recipient strains were cultivated to mid-exponential growth phase in liquid medium with appropriate antibiotics, and then mixed in 1:1 ratio in a final volume of 200  $\mu$ L. The mixture was inoculated into 10 mL of TPY broth, which was incubated anaerobically for 24 h at 37 °C. Cells were then harvested by centrifugation (10 min at 6,000 rpm), resuspended in 1 mL of phosphate-buffered saline (PBS) and plated on donor- and recipient-selective agar plates and on selection plates, in which only recipient strains having acquired the antibiotic resistance can grow (Table 2). The same plates were used to estimate the frequency of spontaneous mutations in the recipient strain. To counter select lactic acid bacteria, the plates were incubated in aerobic conditions.

##### *In vitro interaction between Bifidobacterium strains and human cells*

##### *Growth and maintenance of cell line*

The cell lines used were: small intestinal human epithelial cell line H4, derived from human fetal tissue and supplied by Massachusetts General Hospital (Prof. WA Walker), and human blood monocytes/macrophages, referred to as TLT cell line (Cencic and Langerholc 2010). Cells were routinely grown in Dulbecco modified essential medium (DMEM) as described in Nissen et al. (2009). To perform biological assays, the cells were seeded in 96-well plates at the concentration of  $1 \times 10^6$  cells/mL and incubated for 24 h at 37 °C in 5 % CO<sub>2</sub>. When cell monolayers were obtained, the 16 selected *Bifidobacterium* strains, grown in TPY and suspended in DMEM at the concentration of  $1 \times 10^8$  CFU/mL, were inoculated in each well at the concentration of  $10^7$  CFU/mL. In most of the assays described, the well-known probiotic strain *Lactobacillus rhamnosus* GG (LGG) was used to compare the results obtained. Reagents used were purchased from Sigma-Aldrich.

**Table 2** Evaluation of the transferability of the antibiotic resistance traits from *B. breve* B632, B2274, and B7840 and *B. longum* B1975 to selected recipient strains

Donor strain	Antibiotic resistance assayed <sup>a</sup>	Recipient strain(s)	Selection plates
B632	Ampicillin	ATCC	TPY+AMP+TET
	(blaCTX-M-g1)	27536	
	Ampicillin	PCS22	MRS+AMP+aerobiosis <sup>b</sup>
	Kanamycin	B1412	TPY+KAN+AMO
B1975	Streptomycin	B7840	TPY+STR+TET
	Ampicillin	ATCC	TPY+AMP+TET
	(blaCTX-M-g1)	27536	
	Ampicillin	PCS22	MRS+AMP+aerobiosis
	Kanamycin	B1412	TPY+KAN+STR
B2274	(aph (3'')III)		
	Amoxicillin	PCD71	MRS+AMP+aerobiosis
	(blaCTX-M-g1)		
	Ampicillin	PCS22	MRS+AMP+aerobiosis
	Tetracycline	PCD71	MRS+TET+aerobiosis
	(tetW)		
	Kanamycin	B1412	TPY+KAN+TRIM
B7840	Streptomycin	B7840	TPY+STR+KAN
	Amoxicillin	B632	TPY+AMO+TRIM
	Amoxicillin	PCD71	MRS+AMO+aerobiosis
	Ampicillin	PCD733B	TPY+AMP+STR
	(blaCTX-M-g1)		
	Ampicillin	PCS22	MRS+AMP+aerobiosis
	Tetracycline	B632	TPY+TET+STR
	(tetW)		
	Tetracycline	PCD71	MRS+TET+aerobiosis
	Kanamycin	B1412	TPY+KAN+STR
Amoxicillin	B632	TPY+AMO+STR	
(blaCTX-M-g1)			
Amoxicillin	PCD71	MRS+AMO+aerobiosis	

AMO amoxicillin, AMP ampicillin, CEFT ceftriaxone, CEFU cefuroxime, CHL chloramphenicol, CLA clarithromycin, ERY erythromycin, KAN kanamycin, GEN gentamycin, STR streptomycin, TET tetracyclin, TRIM trimethoprim, VAN vancomycin

<sup>a</sup> The resistance genes indicated in brackets has been identified by PCR

<sup>b</sup> Plates were incubated in aerobic conditions to allow the growth only of lactic acid bacteria

### Cytotoxicity assays

*Bifidobacterium* strains were inoculated on H4 and TLT cell monolayers as described above and plates were incubated in 5 % CO<sub>2</sub> at 37 °C for 90 min. Unbound bacteria were eliminated by washing the cell layers three times with PBS. One hundred microliters of DMEM supplemented with L-glutamine (2 mM) was added to each well, and plates were incubated for 24 h. Cell viability was measured with crystal violet staining, measuring absorbance at 595 nm (A<sub>595</sub>), and compared with the A<sub>595</sub> of non treated cells (i.e., cells not exposed to probiotics).

### Adhesion assay

H4 and TLT cell monolayers were washed with PBS and *Bifidobacterium* strains were applied at a concentration of 9.4 log (CFU/m<sup>2</sup>). Plates were incubated for 90 min at 37 °C. The monolayers were washed with PBS, then cells with adherent bacteria were harvested with trypsin and the number of bacteria was counted on TPY agar plates. Results of attached bacteria cells were expressed as percentage of adherent bacterial cells compared to initial inoculum.

### Mitochondrial activity assay

The metabolic activity of H4 and TLT cell lines after exposure to *Bifidobacterium* strains was measured by evaluating their mitochondrial function as described by Ivec et al. (2007). After bacterial exposure to cell monolayers and incubation, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM was added to each well and incubated for 75 min. Solubilization of MTT reduction product (i.e., formazan) was achieved by addition of 0.04 % HCl in isopropanol and formazan was quantified at 650 nm. Results are expressed as:  $(A_{650} \text{ of treated wells} - A_{650} \text{ of untreated wells}) / A_{650} \text{ of untreated wells} \times 100$ .

### Determination of reactive oxygen species; NO, H<sub>2</sub>O<sub>2</sub>

NO released by cell monolayers after stimulation of *Bifidobacterium* strains was determined by measuring the accumulation of nitrate using a modified Griess reagent (Sigma-Aldrich), according to the Griess reaction (Pipenbaher et al. 2009). The release of H<sub>2</sub>O<sub>2</sub> was determined as described by Nissen et al. (2009). Constitutive H<sub>2</sub>O<sub>2</sub> production by bifidobacteria was evaluated by incubating bifidobacteria in DMEM; the amount of H<sub>2</sub>O<sub>2</sub> produced by bifidobacteria was subtracted from the amount produced by the cells.

### Dot blot for interleukin 6

Interleukin-6 (IL-6) in supernatants of H4 and TLT cells after probiotic exposure was detected using the dot blot technique as described by Ivec et al. (2007). Briefly, supernatants were blotted onto nitrocellulose membrane (Pierce, Rockford, USA) with a Bio-Rad Dot Blot apparatus (Bio-Rad Laboratories, Hercules, USA). After primary and the secondary antibodies addition, proteins were visualized with the supersignal West Pico chemiluminescent substrate system (Pierce) and BiomaxMR-1 film (Sigma-Aldrich). Supernatants of monolayers not exposed to bacteria were used as negative control, whereas *L. casei* Shirota and LGG were used as positive controls.

### Experimental design, statistical analysis, and strain selection criteria

For the different trials, the adopted experimental scheme was a fully randomized design. All the tests were performed in triplicate. Data on spot agar tests, cytotoxicity assay, adhesion test, mitochondrial activity test, and reactive oxygen species (ROS; NO, H<sub>2</sub>O<sub>2</sub>) production were subjected to one-way analysis of variance (ANOVA) by using the GLM procedure of the SAS statistical package. Means were subjected to Fisher's test (SAS 1988). When treatments were

significant according to Fisher's test, corresponding means were differentiated by the SNK multiple range test at the 0.05 level of probability.

The correspondence analysis (CA) was applied to the fingerprinting pattern obtained from ERIC-PCR of *Bifidobacterium* reference strains and investigated strains. CA is a statistical method for visualizing the association between levels of a two-way contingency table (Benzecri 1992). Banding profiles were scored as presence/absence of individual fragments in each investigated strain. The contingency table was analyzed by CA module of Statistica Software (ver. 7.1, StatSoft, Tulsa, OK, USA). Plotting the first two dimensions of the coordinates of cases (ERIC-PCR bands) and variables (strains) gave a global view of the correspondence among reference and investigated strains, and band patterns. The first and second dimensions explained 34 and 28 % of the total variability, respectively.

A first strain selection was based on antimicrobial activity against *E. coli*, *S. enteritidis*, *K. pneumonia*, and *E. cloacae* allowing the choice of the 16 best performing strains out of the original 46 strains. Among the 16 strains, four bifidobacteria were selected on the basis of a synthetic index, calculated as follows: the outputs of different analyses (spot agar tests, antibiotic resistance or sensitivity assay, cytotoxicity test, adhesion assay, mitochondrial dehydrogenase activity, NO and H<sub>2</sub>O<sub>2</sub> production) were transformed into relative percentages by giving the 100 value to the strain showing the best performance in each test. A correction factor of 0.5 was given to the mitochondrial dehydrogenase activity, NO and H<sub>2</sub>O<sub>2</sub> production tests, in order to give more importance to the other parameters, which are defined in the EFSA guidelines (EFSA 2005). IL-6 production was not considered in this evaluation as it is not a quantitative test. These procedures allowed to select four strains which were checked for the transferability of the antibiotic resistance traits to other gut bacteria and were then deposited to the DSMZ culture collection.

## Results

### Antimicrobial activity with the spot agar test

The results obtained with the 46 *Bifidobacterium* strains against *E. coli*, *E. cloacae*, *K. pneumonia*, and *S. enteritidis* evidenced antimicrobial activity to varying degrees (Table 1): three strains (B2531, Re11, B7710) did not show any inhibition halo against all the indicator strains, 27 strains showed inhibition halo's radius not higher than 0.5 cm, whereas 14 strains (Re12, B632, B1412, B1975, B2021, B2055, B2091, B2101, B2150, B2192, B2195, B2274, B7840, B7958) showed an average inhibition halo's radius

higher than 0.5 cm (Table 1). The elaboration of the results with the ANOVA test allowed to indicate these 14 strains as the most performing; however, we decided to include two more strains (B7947 and B8452) for further studies considering their high antimicrobial activity against *E. coli*, which is the most abundant coliform in the infant gut, and their potential interest as preterm isolated strains.

These 16 strains were then assayed against *C. jejuni* and *C. difficile* as antagonistic microorganisms. The results obtained (Table 3) evidenced that all *Bifidobacterium* strains except for B2101 were capable of inhibiting both antagonistic microorganisms. Among them, eight strains (B632, B1412, B1975, B2055, B2192, B2274, B7840, B8452) showed a marked activity against the two pathogens.

#### Antimicrobial activity of *Bifidobacterium* culture supernatants against coliforms and *S. enteritidis*

The culture supernatants, both neutralized (referred to as neutralized culture supernatant; NCS) and non-neutralized (referred to as CS), of the 16 *Bifidobacterium* strains showing the highest antimicrobial activity (listed in Table 3), plus one strain (B7710) as negative control, were used for evaluating the inhibiting activity towards *E. coli* ATCC 11105, *S. enteritidis* M94, *K. pneumoniae* GC23a, and *E. cloacae* GC6a. The majority of *Bifidobacterium* supernatants were

**Table 3** Antagonistic activity of 16 selected *Bifidobacterium* strains against *C. jejuni* LMG8841 and *C. difficile* M216 expressed as average radius (in centimeter) of the inhibition halos obtained on TPY plates in the agar spot test; mean values followed by different letters (in brackets) are statistically different at  $P < 0.01$  for *C. jejuni* and  $P < 0.001$  for *C. difficile*

Strains	<i>C. jejuni</i> LMG8841	<i>C. difficile</i> M216
Re 12	1.1 (a)	0.4 (ab)
B 632	0.8 (ab)	0.7 (a)
B1412	1.1 (a)	0.8 (a)
B1975	0.8 (ab)	0.7 (a)
B2021	1 (ab)	0.4 (ab)
B2055	1 (ab)	0.6 (a)
B2091	0.8 (ab)	0.4 (ab)
B2101	0.8 (ab)	0 (b)
B2150	0.8 (ab)	0.4 (ab)
B2192	1 (ab)	0.6 (a)
B2195	1.2 (a)	0.5 (a)
B2274	1 (ab)	0.7 (a)
B7840	1.4 (a)	0.6 (a)
B7947	0.3 (b)	0.3 (ab)
B7958	1.1 (a)	0.4 (ab)
B8452	0.8 (ab)	0.7 (a)
P	0.05 (*)	0.01 (**)
LSD	0.4	0.3

capable of exerting their inhibiting activity only when non-neutralized (data not shown), whereas the inhibitory activity of four strains (B632, B1975, B2274, and B7840) was evidenced both with CSs and NCSs. Figure 1 shows details of the experiments performed with B632: the inhibitory activity towards *E. coli* and *S. enteritidis* was clearly evident in the early hours of incubation (Fig. 1a, b) with no differences in the use of NC and NCS; the inhibitory activity towards *E. cloacae* and *K. pneumoniae* was also present although less marked with respect to the other target strains and, moreover, it was more evident when the non-neutralized supernatants was used. The profiles obtained with B1975, B2274, and B7840 were quite similar to that of B632, showing a higher inhibitory activity of NCS towards *E. coli* and *S. enteritidis* (data not shown). No inhibitory activity against all the antagonistic strains was evidenced by the B7710 strain (data not shown).

#### Genotypic and phenotypic characterization of the strains

The selected 16 strains were identified and classified at the species level using the ERIC-PCR approach proposed by Ventura et al. (2003). An accurate clustering and identification of the strains was achieved comparing ERIC-PCR banding patterns of the strains used in this work with those retrieved from reference strains. The CA and the scatterplot projections of variables (strains) and cases (ERIC-PCR bands) on the first two dimension evidenced four main clustering groups corresponding to different type strains (Fig. 2). One group was formed by the *B. pseudocatenulatum* type strain (ATCC 27917<sup>T</sup>) and the B8452 strain: it was the most divergent cluster due to the exclusive presence of eight DNA fragments. A second main group clustered with the *B. longum* strains including the *B. longum* subsp. *longum* and the *B. longum* subsp. *infantis* type strains: six strains clustered close to *longum* subspecies and were therefore identified as *B. longum* subsp. *longum* (B1412, B1975, B2055, B2101, B2192, B7958, Re12). A third cluster grouped with the *B. breve* type strain (B632, B2021, B2150, B2274, B2195, B7840, B7847). Finally, the B2091 strain clustered close to the *B. bifidum* type strain.

To confirm the results obtained with ERIC-PCR, the strain identification was compared with species-specific standard PCR. 16S-targeted species specific primers allowed to confirm the *Bifidobacterium* identification at the species level, except for the *B. pseudocatenulatum* strain which was only inserted in the “*catenulatum* group” with this technique.

#### Antibiotic resistance profiles

##### MIC and screening of resistance genes

The resistance or sensitivity of the selected 16 strains to 12 antibiotics and the relative MIC values obtained are shown in



**Fig. 1** Effect of culture supernatants (CS) and neutralized culture supernatants (NCS) of *E. coli* ATCC 11105 (a), *S. enteritidis* M94 (b), *E. cloacae* GC6a (c), *K. pneumoniae* GC23a (d) on the growth of *B. breve* B632 (filled diamond control with 50 μl NB, filled square 25 μl CS, empty square 50 μl CS, multiplication symbol 25 μl NCS, empty circle 50 μl NCS)

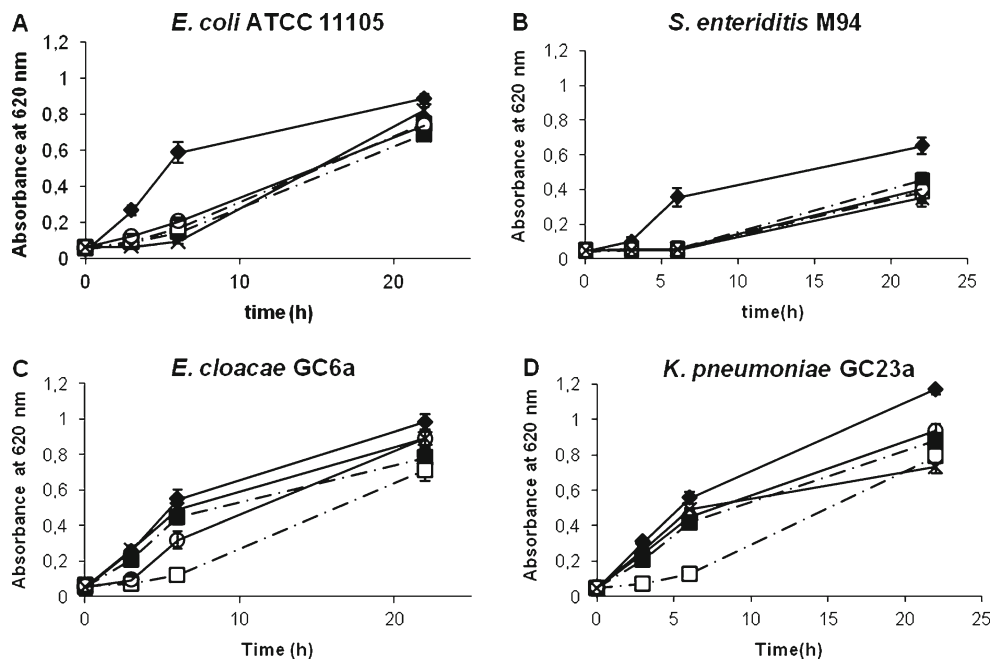
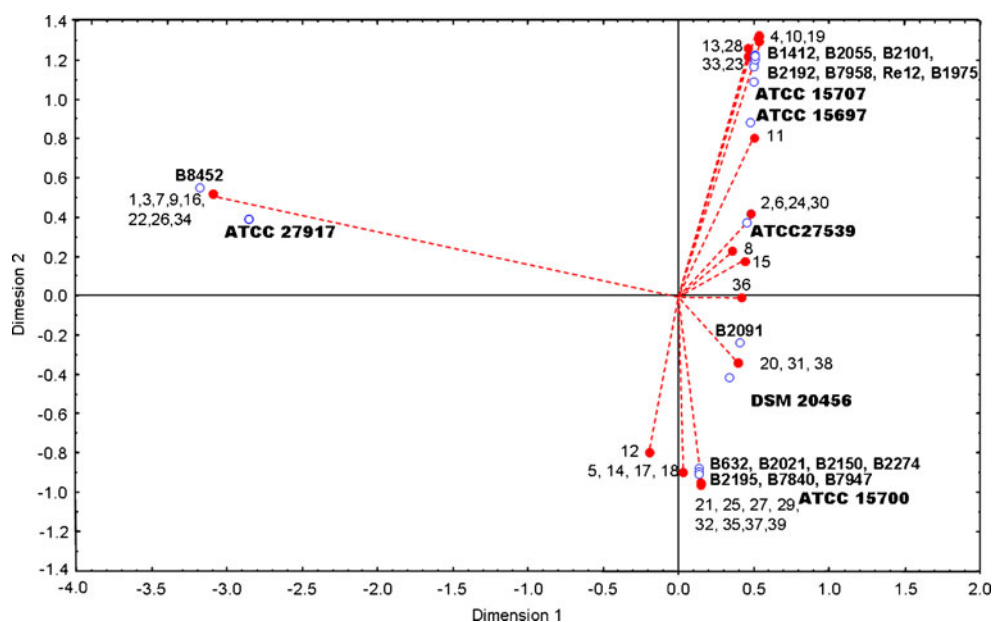


Table 4. All the strains were found to be sensitive to chloramphenicol, erythromycin, vancomycin (apart from B2091), and gentamycin according to most recent EFSA guidelines (EFSA 2008). Moreover, most of the strains were sensitive to tetracycline except a few strains (B2055, B2150, B2195, B2274, B7840, and B7958). All the strains were resistant to ampicillin and the majority of them to kanamycin (except B1412). Nine strains out of 16 were resistant to streptomycin. Regarding cefuroxime, ceftriaxone, and clarithromycin, whose break-points are not present in the mentioned EFSA guidelines, the majority of the strains presented low MIC values so they can be considered sensitive to them. All the strains but one (B632) presented a high MIC value for amoxicillin.

The screening of the resistance genes via PCR amplification of known genes in the 16 strains of bifidobacteria allowed to detect the *tet*(W) amplicon only in two (B2274 and B7840) of the six tetracyclin resistance strains, whereas none of them was positive to *tet*(M) and *tet*(O). Only three strains (B1975, B2192, and B7947) out of the 15 resistant to kanamycin were positive to *aph*(3'')-III amplification, whereas *aph*(3'')-I and *aph*(3'')-II were not amplified in any strain. With regard to the β-lactam-resistance determinants, almost all the tested strains carried *bla*CTX-M-g1 apart from B2021, B2101, B2150, B2274, and B7958. No strains were found to be positive to the amplification of the *aadA* and *aadE* streptomycin-resistance genes. The presence of plasmids was detected only

**Fig. 2** Relationships established among *Bifidobacterium* strains by means of CA based on ERIC-PCR band patterns. Numbers correspond to fingerprinting DNA fragments obtained after agarose gel electrophoresis following ERIC-PCR



**Table 4** MIC of various antibiotics of the selected strains

Strain	Minimum inhibitory concentration ( $\mu\text{g/mL}$ )													
	AMP (2)	CHL (4)	ERY (0.5)	TET (8)	VAN (2)	KAN (8)	STR (128)	GEN (64)	CEFU	AMO	CEFT	CLA		
Re12	$\geq 256$	R 2	S 0.5	S 4	S 2	S 64	R 32	S 8	S 8	ND $\geq 256$	ND 2	ND 2		
B632	$\geq 256$	R 4	S 0.1	S 1	S 0.5	S 64	R $\geq 256$	R 32	S 8	ND 2	ND 4	ND 2		
B1412	$\geq 256$	R 4	S 0.1	S 2	S 2	S 4	S $\geq 256$	R 32	S 2	ND $\geq 256$	ND 2	ND 2		
B1975	$\geq 256$	R 4	S 0.5	S 2	S 2	S 32	R 32	S 32	S 2	ND $\geq 256$	ND 2	ND 2		
B2021	$\geq 256$	R 4	S 0.25	S 2	S 2	S $\geq 256$	R $\geq 256$	R 32	S 8	ND $\geq 256$	ND 2	ND 2		
B2055	$\geq 256$	R 4	S 0.5	S 64	R 2	S 32	R 128	S 16	S 4	ND $\geq 256$	ND 4	ND 2		
B2091	$\geq 256$	R 4	S 0.5	S 8	S $\geq 4$	R $\geq 256$	R $\geq 256$	R 64	S 8	ND $\geq 256$	ND 2	ND 2		
B2101	$\geq 256$	R 2	S 0.5	S 8	S 2	S 128	R 64	S 64	S 2	ND $\geq 256$	ND 2	ND 2		
B2150	$\geq 256$	R 4	S 0.5	S 64	R 0.5	S $\geq 256$	R $\geq 256$	R 64	S 32	ND $\geq 256$	ND 8	ND 2		
B2192	$\geq 256$	R 4	S 0.5	S 2	S 2	S $\geq 256$	R 64	S 32	S 8	ND $\geq 256$	ND 4	ND 2		
B2195	$\geq 256$	R 4	S 0.5	S 32	R 2	S 128	R $\geq 256$	R 64	S 16	ND $\geq 256$	ND 8	ND 2		
B2274	$\geq 256$	R 4	S 0.5	S 32	R 2	S $\geq 256$	R $\geq 256$	R 32	S 32	ND $\geq 256$	ND 8	ND 2		
B7840	$\geq 256$	R 2	S 0.5	S 32	R 2	S $\geq 256$	R 16	S 32	S 32	ND $\geq 256$	ND 8	ND 2		
B7947	$\geq 256$	R 2	S 0.25	S 2	S 2	S $\geq 256$	R 256	R 32	S $\geq 256$	ND $\geq 256$	ND 2	ND 2		
B7958	$\geq 256$	R 4	S 0.5	S 32	R 2	S 128	R 128	S 32	S 4	ND $\geq 256$	ND 2	ND 2		
B8452	$\geq 256$	R 2	S 0.1	S 2	S 0.5	S $\geq 256$	R $\geq 256$	R 32	S 2	ND $\geq 256$	ND 2	ND 2		

Strains are characterized as sensitive (S) or resistant (R) according to the breakpoints defined by EFSA (2008)

AMP ampicillin, CHL chloramphenicol, ERY erythromycin, TET tetracycline, VAN vancomycin, KAN kanamycin, STR streptomycin, GEN gentamicin, CEFU cefuroxime, AMO amoxicillin, CEFT ceftriaxone, CLA clarithromycin

ND breakpoints not defined in EFSA (2008)

in *B. longum* subsp. *longum* B2192 strains, which possessed two plasmids (data not shown).

In vitro interaction between *Bifidobacterium* strains and human cells

#### Cytotoxicity and adhesion

Cytotoxicity assays showed that a number of strains (B1412, B2021, B2101, B2150, B2192, B7947, B7958, and Re12) at the bacterial concentration of  $10^7$  CFU/mL after 90 min incubation exerted a cytotoxic effect to the H4 monolayers higher than the control strain LGG ( $p < 0.05$ ). Referring to TLT monolayers, only B7958 strain was more cytotoxic than LGG ( $p < 0.05$ ; data not shown). A number of strains showed a low reduction of viability of TLT cells when compared to untreated cells, although not statistically significant; however, it has to be considered that a direct contact between the content of the intestinal lumen with macrophages is not an in vivo real condition. On the contrary, three *breve* strains (B632, B2274, and B7840), *B. longum* B2055, and *B. pseudocatenulatum* B8452 showed positive effects on both cell monolayers, in particular B632 and B2274 seem to increase the viability of cells after the exposure (data not shown).

All strains showed a good ability to adhere to polarized human epithelial H4 cells and TLT macrophages (data not shown). *B. breve* B632, *B. pseudocatenulatum* B8452, and *B. longum* B2192 showed a higher attachment to H4 cells with respect to the control LGG strain whereas the majority of *Bifidobacterium* strains presented an adhesion capability comparable to LGG or slightly higher.

#### Stimulation of cell activity: mitochondrial activity, production of ROS and of interleukin

The results of the mitochondrial activity enhancement with the MTT assay are shown in Fig. 3. The mitochondrial dehydrogenase activity of H4 cell lines increased after exposure to *B. breve* B632 and B2195 strains at the concentration of  $1 \times 10^7$  CFU/mL. B632 was the only strain able to strongly stimulate the activity of mitochondrial dehydrogenase of macrophages, whereas only a slight enhancement was obtained with B2021 and B2274, although not statistically significant. Therefore, the percentage of stimulation obtained for most of the *Bifidobacterium* strains, such as for the reference strain LGG was, generally, negligible apart from B632. However, the values obtained for all the strains were not as negative as those obtained with the potential pathogens *S. enteritidis* and *E. coli*.

Among the 16 *Bifidobacterium* strains applied at the concentration of  $10^7$  CFU/mL on H4 cell line, only B2274 induced an increase of NO production statistically higher

than LGG strain (Table 5). Except for B632, B2091, and B7840 strains, the remaining *Bifidobacterium* strains exhibited a lower stimulation effect on NO production than LGG strain. As concerns the stimulation of NO production on TLT cell line, the strongest induction was observed for B1412 strain (approximately five times higher with respect to LGG strain). A moderate increase of NO production, comparable with that observed for LGG strain, was reported for B2091, B2274, B7840, B7947, and B7958 strains. Twelve out of 16 *Bifidobacterium* strains stimulated  $H_2O_2$  production on H4 cell lines, while all *Bifidobacterium* induced an increase of hydrogen peroxide on TLT cell lines (Table 5). On H4 cell line, the B1412, B2021, B2055, B2150, and B2195 strains induced an increase of  $H_2O_2$  production statistically higher than LGG strain. In contrast, only one strain (B7947) was more efficacious in stimulating  $H_2O_2$  production of TLT cell line than LGG. *E. coli* and *S. enteritidis*, used as potential enteropathogens, induced the strongest stimulation of ROS production (i.e., nitric oxide and hydrogen peroxide) in both H4 and TLT cell lines (Table 5).

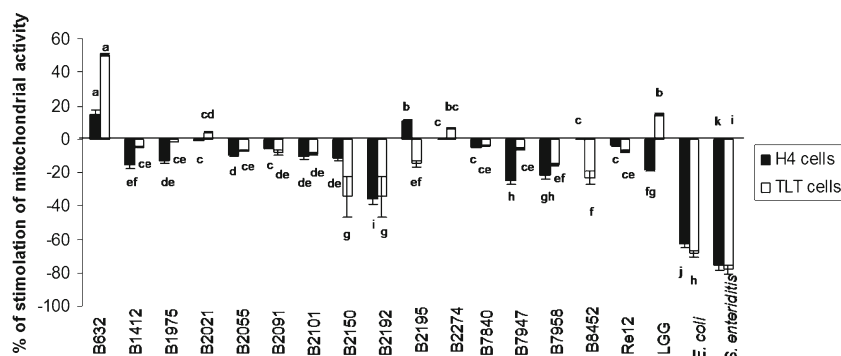
Dot blot was performed to determine the presence of pro-inflammatory cytokine IL-6 in cell-free culture supernatants after exposure of cells to the bacteria for 24 h. A notable production of IL-6 was achieved with H4 and TLT cells using all bacteria except for *B. longum* subsp. *longum* B1412. The highest IL-6 production was noted for B632 and B2055 (Fig. 4).

#### Selection of the best probiotic strains with the use of a synthetic index

The outputs of all the analyses described above were transformed into relative percentages as described in the “Materials and methods” section. The matrix thus completed allowed to calculate a synthetic index (see Supplementary material, Table S1). The strains with the highest synthetic index were selected, i.e., B632, B2274, and B7840. In addition, B1975 strain was also chosen for further studies because of its considerable synthetic index and its high antimicrobial activity against coliforms and potential pathogens.

#### Transferability of antibiotic resistance traits of selected strains

The capability of B632, B1975, B2274, and B7840 of transferring the antibiotic resistance traits to *Bifidobacterium* spp. strains and lactic acid bacteria (*L. plantarum* PCS22, *L. casei* L9, and *E. faecium* PCD71) was studied according to the scheme proposed in Table 2. No recipient strains could receive the antibiotic resistance trait from all the donors and, in addition, no spontaneous mutants of the four donor strains were detected (data not shown).



**Fig. 3** Effect of 16 *Bifidobacterium* spp. strains on the mitochondrial dehydrogenase activity of H4 and TLT cell monolayers. Results are expressed as the average of three independent experiments ( $\pm$ SD). LGG

strain is used as a reference strain for the evaluation of the probiotic effect on the cells, *E. coli* and *S. enteritidis* are used to evidence the effects of potential pathogen microorganisms on the cells

## Discussion

Probiotics are increasingly being used for the treatment of diseases and minor gastrointestinal problems in infants. A recent study has evidenced positive effects on infant colics after treatment of newborns with a *L. reuteri* strain (Savino et al. 2010), whereas no studies have been performed up to now regarding the use of bifidobacteria for this purpose. This work

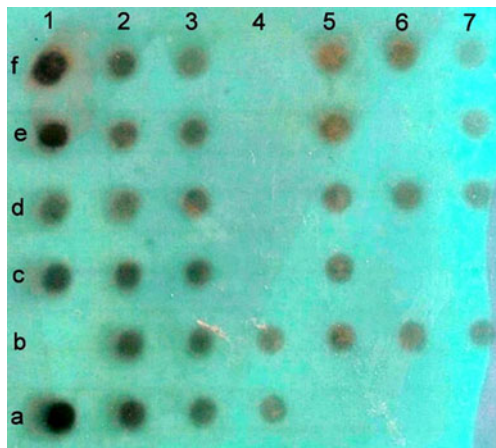
was therefore aimed at the characterization of *Bifidobacterium* spp. strains possessing in vitro capabilities of inhibiting the growth of pathogens typical of the infant gastrointestinal tract without exerting toxic activities on the gut epithelium and harmful effects to the host.

The majority of *Bifidobacterium* spp. strains used in this work derives from infant feces (Scardovi et al. 1979), i.e., from the source which constitutes the target population of the

**Table 5** ROS production (nitric oxide, hydrogen peroxide) by different intestinal cell lines (H4, TLT) as a function of the stimulation from different bacterial strains

Strains	Nitric oxide		Hydrogen peroxide	
	H4	TLT	H4	TLT
B632	7.40 (ce)	7.78 (dg)	6.53 (dh)	27.27 (fg)
B1412	-2.36 (e)	61.71 (c)	25.63 (c)	27.29 (fg)
B1975	-4.32 (e)	-3.35 (eh)	2.51 (fi)	20.51 (gi)
B2021	-5.62 (e)	-1.12 (dh)	9.55 (df)	14.10 (ij)
B2055	-3.01 (e)	-2.60 (dh)	17.59 (ce)	23.94 (fh)
B2091	2.19 (ce)	1.86 (dh)	4.23 (fi)	10.08 (jk)
B2101	-4.32 (e)	-5.58 (fh)	-4.86 (hj)	16.24 (ij)
B2150	-1.06 (de)	-2.60 (eh)	8.54 (df)	28.79 (ef)
B2192	-0.41 (de)	-7.06 (gh)	-14.57 (j)	6.48 (kl)
B2195	-6.92 (e)	-1.86 (dh)	18.39 (cd)	28.64 (ef)
B2274	18.14 (c)	2.60 (dh)	3.52 (fi)	34.62 (e)
B7840	15.21 (cd)	8.89 (df)	8.17 (dg)	28.64 (ef)
B7947	1.54 (de)	10.78 (de)	1.97 (fi)	54.55 (c)
B7958	-8.22 (e)	1.86 (dh)	-11.28 (j)	17.78 (hi)
B8452	-6.27 (e)	-8.55 (h)	5.93 (eh)	29.06 (ef)
Re12	-4.97 (e)	0.37 (dh)	-6.61 (ij)	2.10 (l)
LGG	3.49 (ce)	12.58 (d)	-4.02 (gj)	46.15 (d)
<i>E. coli</i>	223.87 (a)	199.68 (a)	138.33 (a)	146.87 (a)
<i>S. enteritidis</i>	160.51 (b)	143.67 (b)	111.15 (b)	123.67 (b)
<i>P</i>	0.001	0.001	0.001	0.001
LSD	16.56	14.89	12.29	7.12

The results are expressed as mean ratios (percentage) of ROS production with respect to controls (intestinal cell lines not exposed to bacterial strains). Mean values followed by different letters (in brackets) are statistically different at  $P < 0.001$



**Fig. 4** Dot blot of IL-6 detection. The experiment was performed with 16 *Bifidobacterium* spp. strains. LGG and *L. casei* Shirota were used as positive controls; negative controls do not have any applied *Bifidobacterium* strain (H4 or TLT untreated cells). 1a B632/H4, 1b B1412/H4, 1c B1975/H4, 1d B2021/H4, 1e B2055/H4, 1f 2101/H4. 2a B2150/H4, 2b B2192/H4, 2c B2195/H4, 2d B2274/H4, 2e B7840/H4, 2f 7958/H4, 3a B8452/H4, 3b Re12/H4, 3c B2091/H4, 3d B7947/H4, 3e LGG/H4, 3f LGG/H4, 4a *L.casei* Shirota/H4, 4b *L.casei* Shirota/H4, 4c neg control/H4, 4d neg control/H4, 4e neg. control/TLT, 4f neg control/TLT, 5<sup>a</sup> B1412/TLT, 5b B2091/TLT, 5c B1975/TLT, 5d B2021/TLT, 5e B2055/TLT, 5f 2101/TLT, 6a B2150/TLT, 6b B2192/TLT, 6c B2195/TLT, 6d B2274/TLT, 6e B7840/TLT, 6f B632/TLT, 7a B7947/TLT, 7b B7958/TLT, 7c B8452/TLT, 7d Re12/TLT, 7e LGG/TLT, 7f *L. casei* Shirota/TLT

potential probiotic (Arboleya et al. 2011). Preterm isolates were also included considering the high stressing environment of the preterm infant gut, which shows a higher prevalence of *C. difficile* compared with term infants (Penders et al. 2006). Sixteen strains out of the 46 assayed in this study were capable of effectively contrasting the growth of pathogens which are the main cause of infectious diarrhea of bacterial origin in infants, such as *E. coli*, *S. enteritidis*, *C. difficile*, and *C. jejuni* (Rowland 2008; Van Niel et al. 2002). Moreover, the same *Bifidobacterium* strains showed marked antimicrobial activity against gas producing coliforms isolated from stools of colicky infants. Considering that gas forming coliform concentration is higher in colicky infants with respect to healthy controls (Savino et al. 2009, 2011), the results obtained are interesting in the perspective of developing a probiotic based therapy for colic treatment in newborns. The number of *Bifidobacterium* strains showing antimicrobial activity was lower by using NCS. However, this experiment pointed out that at least in some strains, such as *B. breve* B632, the inhibitory activity may not result only from the production of acidic metabolites, but also from the action of other cell excreted metabolites such as bacteriocins. This result represents an interesting starting point for further studies aimed at the characterization of inhibitory molecules in this strain.

A clear taxonomic identification is necessary for the use of a probiotic strain in humans (Arboleya et al. 2011). The genotypic characterization approach used in this work

allowed to cluster the majority of the 16 strains into two species, i.e., *B. breve* and *B. longum* subsp. *longum*, whereas only two strains were clustered within the *B. pseudocatenulatum* and *B. bifidum* species. The results of this analysis confirm that *B. pseudocatenulatum* and *B. catenulatum*, which are indistinguishable by standard PCR, can be easily and quickly distinguished via the ERIC-PCR approach (Ventura et al. 2004). The strain B1412, which has been previously identified as *B. longum* subsp. *infantis*, has now been included in the *longum* subspecies.

According to the most recent EFSA guidelines (EFSA 2008), the spread of resistance to antimicrobials in bacteria requires the examination of the sensitivity/resistance to a number of antibiotics for potential probiotic strains as well as the risks of the resistance traits to be transferred to other bacteria. Except for a number of antibiotics for which the majority of the assayed *Bifidobacterium* strains are resistant, such as ampicillin, kanamycin, and amoxicillin or sensitive, such as chloramphenicol, erythromycin, and vancomycin, there is a great variability among strains also belonging to the same species, as already evidenced in the literature (Masco et al. 2006; Ammor et al. 2008). Intrinsic resistance to aminoglycosides such as streptomycin and kanamycin is commonly present in bifidobacteria (D'Aimmo et al. 2007); however, information on streptomycin-resistance genes is limited for *Bifidobacterium* strains (Kiwaki and Sato 2009). Aminoglycoside-resistance genes, including *aadE* which was evidenced in a *B. longum* strain (Ouoba et al. 2008), were not found in the genome of the assayed strains as well as the kanamycin-resistance genes *aph* (Ouoba et al. 2008). Conversely, all the strains were sensitive to the aminoglycoside gentamycin in agreement with the data present in the literature on bifidobacteria (Ammor et al. 2008). The MICs for tetracycline obtained for most of the tested strains suggested the presences of tetracycline-resistance genes. *Tet* genes, coding for ribosomal protection protein, are involved in resistance to tetracycline and *tet*(M) and *tet*(W) have been exclusively found in bifidobacteria (Aires et al. 2007). However, only two of the assayed strains, *B. breve* B2274 and B7840, presented the *tet*(W) amplicon. Bifidobacteria are usually susceptible to  $\beta$ -lactams, such as ampicillin and amoxicillin (Ammor et al. 2008; Matto et al. 2007), whereas the majority of the strains considered in this analysis are resistant. Consequently, resistance to some  $\beta$ -lactams can be considered an acquired resistance and therefore has the potential for lateral spread (EFSA 2008). There is very little information on the mechanisms responsible for horizontal gene transfer in anaerobic gut bacteria like bifidobacteria; however, the most widespread is the conjugation of plasmids carrying the antibiotic resistance genes. All the 16 *Bifidobacterium* spp. strains potentially considered interesting for the aims of this study did not carry any plasmids, although plasmids have been

identified in several bifidobacteria species and strains (Ventura et al. 2008). However, other genetic mechanisms can influence the likelihood of genetic transfer (Burrus and Waldor 2003), such as transposons, which can carry resistance genes and can move from chromosome to plasmids and vice versa, thereby increasing the mobility of these genes. Therefore, the transferability of the antibiotic resistance traits to *Bifidobacterium* spp. strains and lactic acid bacteria was assayed in the four strains which were selected as the most interesting ones for the aim of this study (*B. breve* B632, B2274, B7840 and *B. longum* subsp. *longum* B1975) and the results allowed to conclude that there was no transfer of the antibiotic resistances neither to the bifidobacteria nor to the lactic acid bacteria assayed.

Finally, adhesion and cytotoxic effects to human cells of the 16 putative probiotic strains was evaluated using non-tumorigenic cell lines, which have already been used as a reliable in vitro method for the selection of lactic acid bacteria with potential probiotic properties (Maragkoudakis et al. 2010; Nissen et al. 2009), but have never been tested with *Bifidobacterium* spp. strains. It is well assessed that the phenotype of tumorigenic cell lines traditionally used for this purpose distinguishes them profoundly from the normal gut epithelium (Tremblay and Slutsky 2007). The ability to adhere to the intestinal epithelium is one of the most important features as it allows to persist in the colon preventing the elimination by peristalsis and the adhesion of pathogenic bacteria. All the tested bacteria showed a good adhesion to both cell types, epithelial cells, and macrophages. Anyway adhesion cannot singly determine the biological activity of these putative probiotic strains. It is a combination of different factors which determines epithelial integrity, viability, and immunoresponse. Treatments with *B. breve* B632, B2274, and B7840, *B. longum* B2055, *B. pseudocatenulatum* B8452 manifested no cytotoxicity over H4 and TLT cell lines at the concentration of  $10^7$  CFU/mL. In addition, *B. breve* B632 and B2274 at the same concentration were able to increase the metabolic activity of cell mitochondria. These results indicate that these strains are not harmful when exposed to a healthy intestine. Most of the tested strains increased the production of ROS in small intestinal epithelial cells and in macrophages. The ability of probiotic bacteria to induce NO secretion from intestinal epithelium may offer a significant contribution to prevent the enteric pathogens from infecting the host. The ability to stimulate NO production in eukaryotic cells is not a common ability of the genera *Lactobacillus* and *Bifidobacterium*, but rather of individual strains (Pipenbaher et al. 2009). Furthermore, most of the bacterial strains tested induced  $H_2O_2$  release in both types of cells. Moderate production of  $H_2O_2$  and NO induced by probiotics could have a beneficial effect in maintaining a balance and increasing resistance to infections. However, it should be noted that high concentration

of  $H_2O_2$  and NO, as displayed by potential enteropathogens such as *E. coli* and *S. enteritidis* (Table 5), can cause tissue injury, disseminated intravascular coagulation, and shock (Park et al. 1999). Last but not least, there is extensive evidence that cytokines play pivotal roles in host defence, inflammatory response, and autoimmune disease (Park et al. 1999). Therefore, IL-6 production is likely to be a good indicator of a degree of macrophages and endothelial cells activation. In the present work, exposure of H4 and TLT cells to *Bifidobacterium* and *Lactobacillus* strains resulted in marked increase of IL-6 production.

In conclusion, the large array of aspects examined in this study has allowed the identification of four *Bifidobacterium* strains, *B. breve* B632 (DSM 24706), B2274 (DSM 24707), B7840 (DSM 24708), and *B. longum* subsp. *longum* B1975 (DSM 24709), as potential probiotics for the treatment of enteric disorders in newborns such as infantile colics or as preventive agents for infantile diarrhea of bacterial origin. They all possess strong antimicrobial activity against coliforms and other pathogenic bacteria, do not possess transmissible antibiotic resistance traits and are not cytotoxic for the gut epithelium. Studies are currently being performed in order to develop suitable ways of administering the selected probiotic strains to newborns with the aim of planning a validation clinical trial.

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