Ann Microbiol (2015) 65:1079–1086
DOI 10.1007/s13213-014-0953-5

ORIGINAL ARTICLE

Probiotic characteristics and in vitro compatibility of a combination of *Bifidobacterium breve* M-16 V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536

Marco Toscano · Elena De Vecchi · Arianna Gabrieli · Gian Vincenzo Zuccotti · Lorenzo Drago

Received: 23 May 2014 / Accepted: 28 July 2014 / Published online: 22 August 2014

Abstract The consumption of probiotic-based products has risen greatly in recent decades. Due to their probiotic characteristics, microorganisms such as lactobacilli and bifidobacteria are in daily use in the production of food supplements. In the present study, three bifidobacterial strains (*Bifidobacterium breve* M-16 V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536) were tested for growth compatibility, resistance to antimicrobial agents, antibacterial activity against pathogens, resistance to gastric acidity, bile salt hydrolysis and adhesion to the human intestinal epithelial cell line HT29. All of these strains were resistant to gentamycin, but none showed in vitro growth incompatibility or the presence of known resistance determinants. *B. breve* M-16 V had the best probiotic characteristics and, indeed, was the only strain possessing antibacterial activity against *Escherichia coli* and *Klebsiella pneumoniae*. All strains were resistant to simulated gastric juice, while only *B. longum* subsp. *longum* BB536 and *B. breve* M-16 V showed a bile salt hydrolytic activity. Interestingly, a strong adhesion to HT29 cells was observed in all *Bifidobacterium* strains. In conclusion, *B. breve* M-16 V, *B. longum* subsp. *longum* BB536 and *B. longum* subsp. *infantis* M-63 showed several promising characteristics as probiotic strains.

Keywords Probiotic · Bifidobacteria · Food supplement · Safety

M. Toscano · L. Drago (✉)
Department of Biomedical Sciences for Health, University of Milan, Via Mangiagalli 31, Milan, Italy
e-mail: microbio@unimi.it

E. De Vecchi · L. Drago
IRCCS Galeazzi Orthopaedic Institute, Via R. Galeazzi 4, Milan, Italy

A. Gabrieli
San Donato Foundation Group, IRCSS Galeazzi Orthopaedic Institute, Via R. Galeazzi 4, Milan, Italy

G. V. Zuccotti
Department of Biomedical and Clinical Sciences, Luigi Sacco Hospital, Via G.B. Sacco 74, Milan, Italy

Introduction

In recent decades the number of food supplements containing probiotic microorganisms has increased significantly, giving consumers the possibility of choosing among a large variety of products containing specific strains belonging to the genera *Lactobacillus* and *Bifidobacterium* (Schillinger et al. 2004). Interestingly, some probiotic bacteria, such as lactobacilli, are used in the production of chewing-gums, in order to exert beneficial effects on oral malodour assessed by organoleptic scores (Keller et al. 2012). Several studies have highlighted the beneficial effects of probiotics on human health, underlying how probiotic strains are involved in intestinal permeability (Mach 2006), urogenital infections (Reid 2008) and allergies (Vanderhoof 2008). In particular, bifidobacteria seem to be able to regulate intestinal microbial homeostasis, the production of vitamins, and also local systemic immune responses (Mayo and van Sinderen 2010) and the hydrolysis of bile salts (Tanaka et al. 1999).

Bifidobacteria are Gram-positive anaerobic bacteria that are ubiquitous and endosymbiotic inhabitants of the gastrointestinal tract, mouth and vagina (Mayo and van Sinderen 2010). Due to their beneficial effect on human health, bifidobacteria and lactic acid bacteria (LAB) are often employed in the production of probiotic products (Toscano et al. 2011). One of the most important characteristics of

probiotic strains used in food supplements is their ability to resist the stressful conditions encountered during passage through the gastrointestinal tract (Ouwehand et al. 2002). Indeed, the acidic environment of the stomach and the bile salts secreted in the duodenum are the major impediments to the survival of ingested living bacteria, which consequently lose their optimal functionality (Collado and Sanz 2007). Generally, bifidobacteria have low viability at pH values close to that of gastric juices, with survival rate varying with different species and strains (Matsumoto et al. 2004). In recent years, there has been increased use of coating materials and encapsulation to optimize viability of bifidobacteria subjected to different biological stresses (Collado and Sanz 2007). As a consequence, resistance to gastric acid is fundamental in the selection of probiotic strains for food use. Moreover, adherence to intestinal epithelial cells and bile salt hydrolytic (BSH) activity are also considered of great importance for probiotic microorganisms to exert their beneficial role in the gastrointestinal tract. Adherence of probiotic bacteria to the intestinal mucosa is the first step in gut colonisation, with adherence being mediated by a close interaction between surface adhesive proteins of the bacteria and the host cell surface (Westerlund and Korhonen 1993); furthermore, probiotic bacteria having a tight bond with host epithelial cells may be able to compete with pathogens for the same receptors, displacing pathogenic bacteria from host cells (Styriak et al. 2003).

The ability of probiotic strains to compete with pathogens is thought to be linked also to their ability to produce antimicrobial substances, such as organic acids (Tejero-Sariñena et al. 2012), and also to stimulate the host immune system (Gibson and Fuller 2000; Rowland et al. 2009). Several studies have evaluated the antibacterial action of probiotic bacteria against different pathogenic bacteria, demonstrating the ability of probiotics to inhibit the growth of pathogens such as *Escherichia coli* and *Clostridium difficile* (Tejero-Sariñena et al. 2012; Schoster et al. 2013). Obviously, bacteria used in probiotic products should not inhibit the growth of other probiotic strains. However, one of the most dangerous traits of bifidobacteria and LAB is the potential transfer of antibiotic resistance genes to pathogenic bacteria within the gastrointestinal flora (Teuber et al. 1999). A European Food Safety Authority (EFSA) document recommends that commercial strains used in the production of food supplements should not harbour transferable antibiotic resistance; for this reason, EFSA guidelines strongly recommended that minimum inhibitory concentrations (MICs) of the most important antimicrobial agents used in human care be evaluated (EFSA 2008). Many routes of antibiotic resistance transfer exist: integrons, transposons, insertional elements, bacteriophages and conjugative plasmids are all implicated in such mechanisms (EFSA 2008). The intense use of antimicrobial agents for medical use and also in animal husbandry has led to the selection of antibiotic-resistant strains within the gut microbiota of humans and livestock; consequently, in the last decades, there has been a massive spread of antibiotic-resistant bacteria worldwide (Devirgiliis et al. 2011). *Bifidobacterium breve* M-16 V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536 have already been demonstrated to maintain intestinal health by improving gut microbiota and the intestinal environment (Ishizeki et al. 2013). *B. breve* M-16 V, in particular, was able to prevent necrotising entrocolitis and infection in low-birth-weight infants (Satoh et al. 2007).

In this study, *B. breve* M-16 V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 were examined for growth compatibility, resistance to antimicrobial agents, resistance to gastric acidity, bile salt hydrolysis and adhesion to the human epithelial cell line HT29. Moreover, the antibacterial activity of these strains against various pathogens was evaluated. The present study can be regarded as an in vitro assessment of the use of *B. breve* M-16 V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 in combination as food supplements.

Materials and methods

Bacterial strains and culture conditions

Bifidobacterium breve M-16 V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536 were used in the study. All strains were obtained from Morinaga Milk Industry Co. (Tokyo, Japan). Each lyophilised strain was sent in a single sachet containing 1×10^9 viable cells. Strains were revitalised prior to each experiment in Wilkins-Chalgren (WC) broth. All strains were grown at 37 °C in anaerobiosis for 48 h.

Growth compatibility test

Bifidobacterium strains, alone and in combination (ratio 1:1:1), were inoculated in 10 mL WC broth in order to have an initial concentration of about 10^5 CFU/mL. Then, bacteria were incubated at 37 °C in anaerobiosis. After 24, 48 and 72 h of incubation, an aliquot was taken from each sample and 10-fold dilutions were seeded into Bifidus Selective Agar (BSM) (Sigma-Aldrich, Milan, Italy) and incubated in anaerobiosis at 37 °C for 48 h for bacterial counts. Finally, each bifidobacteria colony in the mixture compatibility assay was identified by mean of Pyrosequencing, as previously described by Jonasson et al. (2002). Briefly, DNA was isolated from colonies (50 colonies per plate) by heat, and variable regions V1 and V3 were sequenced by mean of Pyrosequencing. Then, the short sequences obtained, 20–40 nucleotides long, were compared with GenBank sequences using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Subspecies of the isolated strains

that belonged to *B. longum* were identified by PCR using primers Bifido For (5′-TTCCAGTTGATCGCATGGTC-3′) and Longum Rev (5′- GGGAAGCCGTATCTCTACGA-3′) for *B. longum* subsp. *longum* and Bifido For (5′-TTCCAG TTGATCGCATGGTC-3′) and Infantis Rev (5′- GGAAAC CCCATCTCTGGGAT-3′) for *B. longum* subsp. *infantis* (Satokari et al. 2003). Amplification products were analysed by electrophoresis on 1.5 % agarose gels and staining with ethidium bromide.

Antimicrobial susceptibility testing

Four antibiotics belonging to different classes were tested: erythromycin (macrolides), gentamicin (aminoglycosides), tetracycline and penicillin.

Minimum inhibitory concentrations (MIC) for erythromycin, penicillin, gentamycin and tetracycline were determined using E test (Oxoid, Hants, UK). A 0.5 McFarland bacterial inoculum was prepared and the surface of Brucella agar (BRU) plates was flooded with bifidobacterium suspension. Subsequently, the surface of the agar was allowed to dry before the strips were applied and plates incubated in anaerobiosis for 48 h at 37 °C. The MIC was considered as the lowest concentration at which the border of the elliptical inhibition zone intersected the scale on the strip. *Bacteroides fragilis* E-022248 (=DSM 2151=ATCC 25285) was used as a control for susceptibility testing on BRU (tested in three repeats for all antibiotics). Antibiotic susceptibility was evaluated by comparing MIC values to breakpoints suggested by EFSA (EFSA 2008). Breakpoints for penicillin were taken from NCCLS guidelines (National Committee for Clinical Laboratory Standards 2003).

Characterisation of antibiotic resistance

Those strains that were resistant to one or more antibiotics were investigated further for the presence of known resistance genes. The following genes were investigated: aac6-aph2, *aph3-III*, *ant6-I* (resistance to aminoglycosides); *tetL*, *tetM*, *tetS*, *tetW* (resistance to tetracycline); *ermA*, *ermB*, *ermC*, *mefA* (resistance to erythromycin). The presence of resistance determinants was detected by means of PCR, using primers and conditions previously reported (Kobayashi et al. 2001; Ouoba et al. 2008; Comunian et al. 2010). The PCR products were analysed by electrophoresis on 1.5 % agarose gel stained with ethidium bromide.

Agar well diffusion assay

The ability of bifidobacteria to inhibit *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 18833, and *Bacteroides fragilis* ATCC 25285 growth was assessed by an agar well diffusion assay estimating the inhibitory effect of cell-free supernatant obtained from probiotic culture. Bifidobacteria, alone and in combination (mixture), were grown overnight in WC broth. Then, 1 mL from each sample was centrifuged at 6,000 *g* for 20 min to recover the probiotic supernatant. *Escherichia coli* and *Klebsiella pneumoniae* were grown in brain-heart infusion broth (BHI) (Sigma-Aldrich) at 37 °C in aerobiosis for 24 h, while *Bacteroides fragilis* was grown in WC broth in anaerobiosis for 48 h. Overnight cultures of pathogens were diluted 10-fold to achieve semi-confluent growth. A 2-mL aliquot of the diluted suspension was then applied to Mueller-Hinton plates and the surface covered by rotating the plate. Consequently, 9-mm wells were made in each dish by removal of agar plugs, and 100 μL probiotic supernatant was added to each well. Cell-free broth was used as a negative control. The presence of an inhibition zone was assessed visually following 24–48 h of incubation at 37 °C in aerobiosis (*E. coli* and *K. pneumoniae*) and in anaerobiosis (*Bacteroides fragilis*). Each test was performed in triplicate.

Resistance to gastric acidity

A simulated gastric juice was prepared by suspending pepsin (3 g/L) (Sigma-Aldrich) in sterile saline and adjusting the pH to 3.0 with 4 M HCl. Overnight cultures (6 mL) of each *Bifidobacterium* strain were centrifuged at 6,000 *g* for 20 min and washed twice with phosphate buffered saline (PBS). The pellet was then resuspended in simulated gastric juice and vortexed. Samples were incubated at 37 °C in anaerobiosis. In the controls, simulated gastric juice was replaced by PBS and these samples were used to determine the initial cell counts. Aliquots were removed after 30, 60 and 180 min and viable counts were determined by plating serial 10-fold dilutions on BSM agar and counting colony numbers after having incubated the plates at 37 °C for 48 h under anaerobic conditions. Experiments were performed in duplicate and were repeated three times. Results are expressed as the mean and standard deviation of three determinations.

Bile salt hydrolysis assay

Bifidobacterium breve M-16 V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 were screened for their ability to hydrolyse bile salts. Strains were tested alone and in combination; 10 μL aliquots of overnight cultures were spotted on deMan Rogosa Sharpe agar (MRS) supplemented with 0.5 % (w/v) taurodeoxycholic acid sodium salts (TCDA) (Sigma-Aldrich) and 0.37 g/L $CaCl_2$ (Sigma-Aldrich). Plates were then incubated anaerobically for 48 h at 37 °C and strains forming precipitation zones were considered to have BSH activity.

HT29 cell culture

HT29 cells obtained from the Istituto Zooprofilattico Sperimentale (Brescia, Italy) were grown routinely in complete RPMI medium, supplemented with 2.0 g/L $NaHCO_3$, 10 % heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/mL streptomycin, and 100 μg/mL penicillin (Sigma-Aldrich). The medium was replaced every 2 days until a confluent monolayer was achieved. For adhesion assay, HT29 monolayers were trypsinised, transferred (10^5 cells per well) in 24-well tissue plates, and incubated under 5 % CO_2 in complete RPMI medium for 24 h. Then, the medium was removed, adhered HT29 cells were washed twice with PBS and 1 mL RPMI complete without antibiotics was added.

Adhesion of *Bifidobacterium* strains to HT29 cells

Overnight cultures of each strain grown in WC broth were diluted to 10^7 CFU/mL in RPMI complete medium, added to the wells to obtain an initial multiplicity of infection of 100:1 and incubated for 30 min at 37 °C under 5 % CO_2. Cells were then washed twice with PBS buffer to eliminate non-adherent bacteria, trypsinised and centrifuged at 10,000 *g* for 10 min. The pellet was suspended in 1 mL RPMI medium, serial dilutions were prepared, and each dilution was plated on BSM agar and incubated at 37 °C for 48 h. The percentage of adhesion was defined as the log of the number of adherent bacteria (log CFU) divided by the log of the total number of bacteria inoculated, multiplied by 100. Each determination was carried out in triplicate.

Results

Figure 1 shows the growth of *Bifidobacterium* strains alone (Fig. 1a) and in combination in the same broth (Fig. 1b). The compatibility assay showed that a single strain did not inhibit the growth of the other strains in the broth. Indeed, there was no significant difference in the growth curves when strains were grown alone (Fig. 1a) or together (Fig. 1b).

All strains were resistant to gentamicin, while none were resistant to erythromycin, tetracycline or penicillin. No known genes conferring antibiotic resistance to aminoglycosides were detected in the studied strains (Table 1).

Bifidobacterium longum subsp. *longum* BB536 and *B. longum* subsp. *infantis* M-63 did not show a zone of inhibition against pathogens tested in the study. Conversely, *B. breve* M-16 V showed a halo of inhibition against *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 1883. The zones of inhibition produced and observed in this test were between 1 mm and 2 mm in diameter.

The ability of bifidobacteria to survive the simulated gastric juice transit is summarised in Fig. 2. The survival of strains was slightly affected during exposure to gastric conditions, above all for *B. longum* subsp. *longum* BB536. This strain, indeed, showed a reduction in bacterial count of approximately 2.5 logs after 30 min of incubation, while after 60 min of incubation the bacterial load was halved. Conversely, *B. breve* M-16 V and *B. longum* subsp. *infantis* M-63 were more resistant to gastric acidity: a reduction of approximately 1.0 log was observed only after 60 min of incubation in simulated gastric juice (Fig. 2). All the tested strains showed a reduction in bacterial count from 3.5 to 4.0 logs after 90 min of incubation.

Screening of the *Bifidobacterium* strains for BSH activity showed that *B. breve* M-16 V and *B. longum* subsp. *longum* BB536 strains produced precipitation zones in the plate supplemented with TCDA and, more specifically, the zone of precipitation produced by *B. breve* M-16 V was bigger than that produced by *B. longum* subsp. *longum* BB536 (Fig. 3). Conversely, *B. longum* subsp. *infantis* M-63 had no BSH activity (Fig. 3). Regarding the bifidobacteria mixture, a clear zone of precipitation was observed but it was smaller than the halo produced by *B. breve* M-16 V.

All strains were able to adhere to HT29 cells (Fig. 4). The strongest in vitro adherence was observed for *B. breve* M-16 V (63 %), but also *B. longum* subsp. *longum* BB536 and *B. longum* subsp. *infantis* M-63 strains had high adherence to HT29 cells (57 % and 48 %, respectively).

Discussion

Recent decades have seen a significant increase in the use of living bacteria as food supplements; indeed, LAB and bifidobacteria are often used in the production of dietary supplements, fermented foods and beverages (Caplice and Fitzgerald 1999; Leroy and De Vuyst 2004). Bacterial strains used as dairy food supplements should have some functional characteristics that allow probiotic strains to survive in the gastrointestinal tract, colonise the gut and exert their beneficial role. In this study, no *Bifidobacterium* strain inhibited the growth of other strains when mixed together in the same broth; as a consequence, *B. breve* M-16 V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 could be used in the same probiotic mixture without affecting each other's functionality in the human gut.

One of the most important selection criteria for bacterial strains used as food supplements is the absence of transferable antibiotic resistance determinants. Selection of antimicrobial resistant bacteria resulting from the intensive use of antimicrobial agents appears to be one of the factors behind the

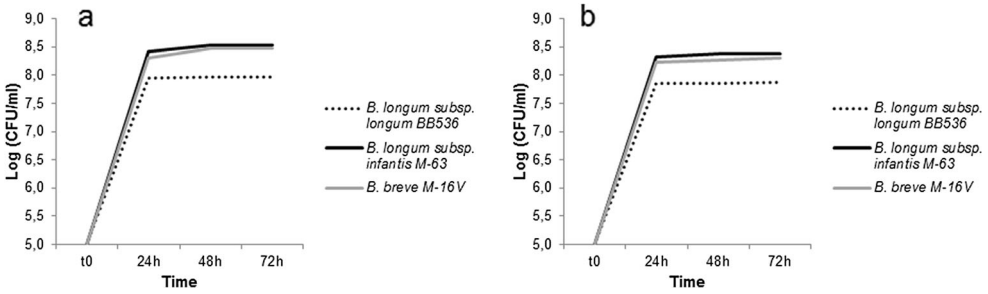

Fig. 1a,b Growth compatibility assay. Growth curves of bifidobacteria grown **a** alone or **b** together in the same broth

increase in antimicrobial resistance among bacteria (EFSA 2008); as a consequence, since 2006 the use of antimicrobial growth promoters has been banned in Europe. The most dangerous problem is that microorganisms used in food products can often act as reservoirs of resistance and transfer antibiotic resistance determinants to pathogenic bacteria located in the human gastrointestinal tract. A recent study demonstrated that ready-to-eat salads can also act as source of bacteria carrying antibiotic resistance determinants (Campos et al. 2013). Not only food supplements containing probiotic bacteria but also fresh vegetables are involved in the dissemination of microorganisms within the kitchen environment, becoming one of the most important vehicles for antibiotic resistant bacteria/genes of clinical interest (Campos et al. 2013). In our study, *B. breve* M-16 V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 showed resistance only to gentamicin, confirming the results of a previous study in which a high rate of resistance to gentamicin among probiotic bacteria was observed (Drago et al. 2013). Ouoba et al. (2008) observed that, among strains isolated from European products, but not in those isolated from African products, there were high levels of phenotypic resistance to aminoglycosides. It was hypothesized that the aforementioned resistance could be the result of selection due to the presence of a selective pressure for aminoglycoside resistance that is not present in the environment from which the African isolates had been recovered (Ouoba et al. 2008).

We screened all three *Bifidobacterium* strains by PCR for some known resistance genes but found no evidence of the presence of resistance determinants. All strains were susceptible to erythromycin and penicillin, confirming the results of Moubareck et al. (2005), who observed the absence of erythromycin and penicillin resistance in several *Bifidobacterium* strains isolated from humans, animals and probiotic products. Moreover, none of the strains were resistant to tetracycline. This data was quite different from those obtained in other studies, in which resistance to tetracycline was observed (Temmerman et al. 2003). Moreover, the present study showed that only *B. breve* M-16 V supernatant was able to inhibit growth of *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 1883 in an agar well diffusion assay. Strangely, the pH of broth after *B. breve* M-16 V growth was 7.0, so the inhibition observed during the experiment was pH-independent. Interestingly, several *Bifidobacterium* strains have been found to produce antibacterial substances in addition to organic acids (Makras and De Vuyst 2006); these compounds, such as low-molecular-mass, lipophilic molecules, could have a strong killing activity against several pathogens. The antimicrobial effect of *B. breve* M-16 V was already observed by Makras and De Vuyst (2006), who underlined the ability of *B. breve* M-16 V strain supernatant to inhibit growth of pathogenic bacteria, such as *E. coli*. Interestingly, the antimicrobial effect of bifidobacteria seems to be strain-specific and not species-specific (Ibrahim et al. 2005).

Regarding resistance to simulated gastric juice, *B. longum* subsp. *longum* BB536 strain was more susceptible to acidity than *B. breve* M-16 V and *B. longum* subsp. *infantis* M-63. Exposure of *B. longum* subsp. *longum* BB536 to the pepsin simulated gastric juice resulted in a significant decrease in viability within 30 min, whereas the other strains underwent only a slight decrease in viability. The low acidity resistance of *B. longum* subsp. *longum* BB536 had already been observed by Maus and Ingham (2003), who considered this particular

Table 1 Susceptibility to antibiotics (*R* resistant, *S* sensitive) and genes involved in antibiotic resistance (in brackets)

Strain	Antimicrobial agent			
	Erythromycin	Gentamicin	Tetracycline	Penicillin
Bifidobacterium breve M-16 V	S	R (unknown)	S	S
Bifidobacterium longum subsp. *infantis* M-63	S	R (unknown)	S	S
Bifidobacterium longum subsp. *longum* BB536	S	R (unknown)	S	S

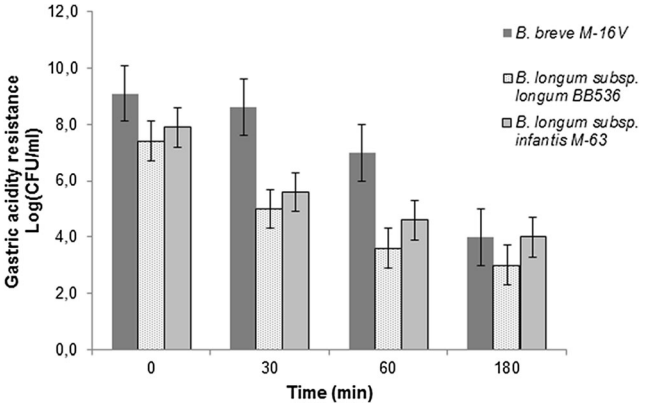

Fig. 2 Tolerance of *Bifidobacterium breve* M-16 V, *Bifidobacterium longum* subsp. *infantis* M-63 and *Bifidobacterium longum* subsp. *longum* BB536 to a simulated gastric juice (pH 3.0). Results are from three independent experiments. *Bars* Mean ± standard deviation (SD) of three determinations

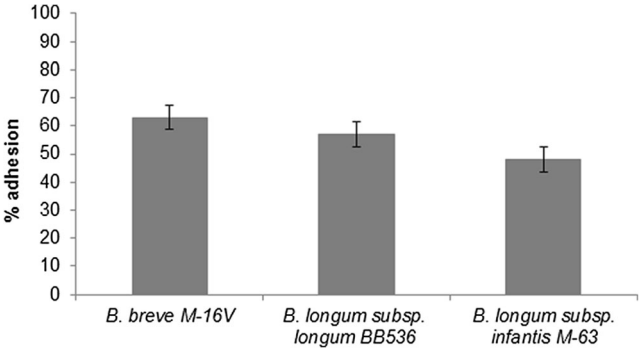

Fig. 4 Adhesion of *Bifidobacterium* strains to HT29 cells. Results are from three independent experiments. *Bars* Mean ± SD of three determinations

Bifidobacterium species to be a highly acid-sensitive species among bifidobacteria. However, the acid tolerance of bifidobacteria, included *B. longum* subsp *longum*, *B. breve* and *B. longum* subsp. *infantis* species, was also seen to be generally weak and strain-dependent in previous studies (Charteris et al. 1998; Matsumoto et al. 2004).

The resistance to bile salts is another important evaluation criterion to verify the ability of probiotic strains to survive the conditions in the small intestine. BSH activity allows bacterial strains to be more resistant to the toxicity of conjugated bile salts in the duodenum and, as a consequence, is a fundamental factor in colonisation (Schillinger et al. 2004). Moreover, deconjugation of bile salts could lead to a reduction in serum cholesterol either by increasing the demand for cholesterol for de novo synthesis of bile acids to replace those lost in faeces, or by reducing cholesterol solubility and thereby absorption of cholesterol through the intestinal lumen (Begley et al. 2006). Examination of BSH activity of the strains tested in the present study demonstrated that only *B. breve* M-16 V and *B. longum* subsp. *longum* BB536 strains were able to hydrolyse bile salts, with *B. breve* M-16 V showing a stronger BSH activity than *B. longum* subsp. *longum* BB536. Strangely, the ability of probiotic mixture to hydrolyse bile salts was lower than the BSH activity of *B. breve* M-16 V alone. The meaning of this result is still unclear. Furthermore, *B. longum* subsp. *infantis* M-63 did not show any BSH activity, contrary to the results of a previous study which found bile salt hydrolysing activity to be common in *Bifidobacterium* species (Tanaka et al. 1999). A strong correlation was previously observed between the habitat of a genus or a species and the presence of BSH activity. In general, bile salt hydrolase activity is common in species and strains located in the gut and isolated from the faeces of mammals (Tanaka et al. 1999).

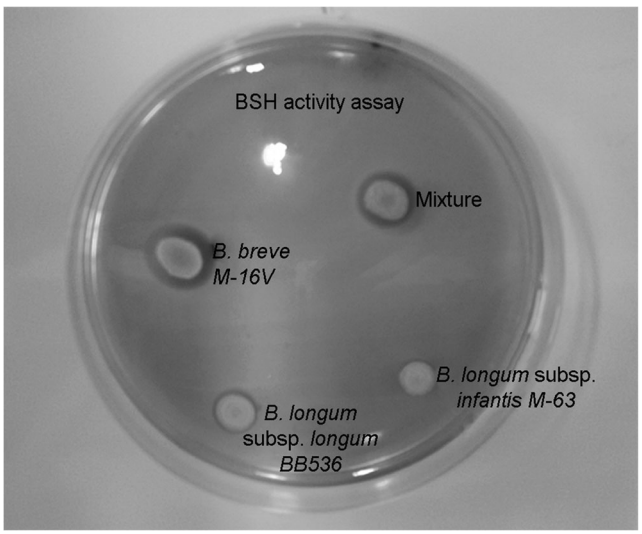

Fig. 3 Zone of precipitation produced by *Bifidobacterium* strains. *B. breve* M-16 V and *B. longum* subsp. *longum* BB536 produced precipitation zones in the plate supplemented with taurodeoxycholic acid sodium salts (TCDA), showing bile salt hydrolytic (BSH) activity. *B longum* subsp. *infantis* M-63 did not produce a precipitation zone

The last probiotic property evaluated was the ability of the tested strains to adhere to HT29 cells. All three strains were able to adhere to this cell line. The binding rates of *Bifidobacterium* strains to HT29 cells were quite high (48–63 %), especially if compared to the adhesion assay performed by other authors who used a different cell line (Del Re et al. 2000; Collado et al. 2005). The mechanism of adhesion of bifidobacteria seems to be different from that observed in lactobacilli, and the adhesin-like proteins of bifidobacteria involved in the adhesion to human cells are probably species-specific (Bernet et al. 1993).

In conclusion, the *Bifidobacterium* strains tested in our study showed some characteristics considered fundamental for probiotic bacteria used as food supplements. Further analyses will aim to investigate the molecular mechanisms behind the ability of bifidobacteria to adhere to human cells and, moreover, to evaluate the resistance of these strains to pancreatic juice, in order to simulate the passage through the intestinal environment.

Nevertheless, our findings highlight several pivotal features of *B. breve* M-16 V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 that could be used for a preliminary screening to identify probiotic mixtures potentially suitable for commercial purposes.

References

Begley M, Hill C, Gahan CG (2006) Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72:1729–1738

Bernet MF, Brassart D, Neeser JR, Servin AL (1993) Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Appl Environ Microbiol 59:4121–4128

Campos J, Mourão J, Pestana N, Peixe L, Novais C, Anyunes P (2013) Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. Int J Food Microbiol 166:464–470

Caplice E, Fitzgerald GF (1999) Food fermentations: role of microorganisms in food production and preservation. Int J Food Microbiol 50: 131–149

Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol 84:759–768

Collado MC, Sanz Y (2007) Induction of acid resistance in *Bifidobacterium*: a mechanism for improving desirable traits of potentially probiotic strains. J Appl Microbiol 103:1147–1157

Collado MC, Gueimonde M, Hernández M, Sanz Y, Salminen S (2005) Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. J Food Prot 68:2672–2678

Comunian R, Daga E, Dupré I, Paba A, Devirgiliis C, Piccioni V, Perozzi G, Zonenschain D, Rebecchi A, Morelli L, De Lorentiis A, Giraffa G (2010) Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. Int J Food Microbiol 138:151–156

Del Re B, Sgorbati B, Miglioli M, Palenzona D (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Lett Appl Microbiol 31:438–442

Devirgiliis C, Barile S, Perozzi G (2011) Antibiotic resistance determinants in the interplay between food and gut microbiota. Genes Nutr 6:275–284

Drago L, Mattina R, De Vecchi E, Toscano M (2013) Phenotypic and genotypic antibiotic resistance in some probiotics proposed for medical use. Int J Antimicrob Agents 41:396–367

EFSA (2008) The maintenance of the list of QPS microorganisms intentionally added to food or feed. Scientific opinion of the panel on biological hazards. EFSA J 923:1–48

Gibson GR, Fuller R (2000) Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. J Nutr 130:391–365

Ibrahim SA, Dharmavaram SR, Seo CW, Shahbazi G (2005) Antimicrobial activity of *Bifidobacterium longum* (NCFB 2259) as influenced by spices. Int J Food Safety 2:6–8

Ishizeki S, Sugita M, Takata M, Yaeshima T (2013) Effect of administration of bifidobacteria on intestinal microbiota in low-birth-weight infants and transition of administered bifidobacteria: a comparison between one-species and three-species administration. Anaerobe 23: 38–44

Jonasson J, Olofsson M, Monstein HJ (2002) Classification, identification and subtyping of bacteria based on pyrosequencing and signatura matching of 16S rDNA fragments. APMIS 110:263–272

Keller MK, Bardow A, Jensdottir T, Lykkeaa J, Twetman S (2012) Effect of chewing gums containing the probiotic bacterium *Lactobacillus reuteri* on oral malodour. Acta Odontol Scand 70:246–250

Kobayashi N, Alam M, Nishimoto Y, Urasawa S, Uehara N, Watanabe N (2001) Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*. Epidemiol Infect 126:197–204

Leroy F, De Vuyst L (2004) Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Technol 15:67–78

Mach T (2006) Clinical usefulness of probiotics against chronic inflammatory bowel diseases. J Physiol Pharmacol 57:23–33

Makras L, De Vuyst L (2006) The in vitro inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. Int J Dairy Sci 16:1049–1057

Matsumoto M, Ohishi H, Benno Y (2004) H + −ATPase activity in *Bifidobacterium* with special reference to acid tolerance. Int J Food Microbiol 93:109–113

Maus JE, Ingham SC (2003) Employment of stressful conditions during culture production to enhance subsequent cold- and acid-tolerance of bifidobacteria. J Appl Microbiol 95:146–154

Mayo B, van Sinderen D (2010) Bifidobacteria: genomics and molecular aspects. Caister Academic, Norwich, UK

Moubareck C, Gavini F, Vaugien L, Butel MJ, Doucet-Populaire F (2005) Antimicrobial susceptibility of bifidobacteria. J Antimicrob Chemother 55:38–44

National Committee for Clinical Laboratory Standards (2003) Performance standards for antimicrobial susceptibility testing; 13th informational supplement M 100-S 13. NCCLS, Villanova

Ouoba L, Lei V, Jensen L (2008) Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. Int J Food Microbiol 121:217–24

Ouwehand AC, Salminen S, Isolauri E (2002) Probiotics: an overview of beneficial effects. Antonie Van Leeuewenhoek 82:279–289

Reid G (2008) Probiotic lactobacilli for urogenital health in women. J Clin Gastroenterol 42:234–236

Rowland I, Capurso L, Collins K, Cummings J, Delzenne N, Goulet O, Guarner F, Marteau P, Meier R (2009) Current level of consensus on probiotic science-report of an expert meeting-London, 23 November 2009. Gut Microbes 1:436–439

Satoh Y, Shinohara K, Umezaki H (2007) Bifidobacteria prevents necrotizing enterocolitis and infection in preterm infants. Int J Probiot Prebiot 2:149–154

Satokari RM, Vaughan EE, Smidt H, Saarela M, Mättö J, de Vos WM (2003) Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. System Appl Microbiol 26:572–584

Schillinger U, Guigas C, Holzapfel WH (2004) In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. Int Dairy J 15:1289–1297

Schoster A, Kokotovic B, Permin A, Pedersen PD, Dal Bello F, Guardabassi L (2013) In vitro inhibition of *Clostridium difficile* and *Clostridium perfringens* by commercial probiotic strains. Anaerobe 20:36–41

Styriak I, Nemcová R, Chang YH, Ljungh A (2003) Binding of extracellular matrix molecules by probiotic bacteria. Lett Appl Microbiol 37:329–333

Tanaka H, Doesburg K, Iwasaki T, Mierau I (1999) Screening of lactic acid bacteria for bile salt hydrolase activity. J Dairy Sci 82:2530–2535

Tejero-Sariñena S, Barlowb J, Costabile A, Gibson GR, Rowland I (2012) In vitro evaluation of the antimicrobial activity of a range

of probiotics against pathogens: evidence for the effects of organic acids. Anaerobe 18:530–538

Temmerman R, Pot B, Huys G, Swings J (2003) Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int J Food Microbiol 81:1–10

Teuber M, Meile L, Schwarz F (1999) Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek 76:115–137

Toscano M, de Vecchi E, Rodighiero V, Drago L (2011) Microbiological and genetic identification of some probiotics proposed for medical use in 2011. J Chemother 25:156–161

Vanderhoof JA (2008) Probiotics in allergy management. J Pediatr Gastroenterol Nutr 47:38–40

Westerlund B, Korhonen TK (1993) Bacterial proteins binding to the mammalian extracellular matrix. Mol Microbiol 9:687–694