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Publication information
Beneficial Microbes: ISSN 1876-2883 (paper edition); ISSN 1876-2891 (online edition)

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Beneficial Microbes, June 2013; 4(2): 179-186

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In vitro evaluation of *Bifidobacterium* strains of human origin for potential use in probiotic functional foods

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Received: 2 October 2012 / Accepted: 23 December 2012
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Abstract

The present study investigated some *in vitro* properties for probiotic use of four strains of bifidobacteria isolated from faeces of healthy children (*Bifidobacterium longum* 5^{1A}, *Bifidobacterium breve* 110^{1A}, *Bifidobacterium pseudolongum* 119^{1A} and *Bifidobacterium bifidum* 162^{2A}). *In vitro* tests were carried out to compare growth rate, aerotolerance, antagonistic activity against pathogens, antimicrobial susceptibility profile and cell wall hydrophobicity. Mean doubling time of *B. longum* 5^{1A} was shorter compared to the other strains. All strains were aerotolerant up to 72 h of exposure to oxygen. *In vitro* antagonism showed that *B. longum* 5^{1A} and *B. pseudolongum* 119^{1A} were able to produce inhibitory diffusible compounds against all pathogenic bacteria tested, but not against *Candida albicans*. *B. longum* 5^{1A} was sensitive to all the antimicrobials tested, except neomycin. The hydrophobic property of the cell wall was highest for *B. bifidum* 162^{2A}. Based on these parameters, *B. longum* 5^{1A} showed the best potential for probiotic use among the tested strains, presenting the greatest sensitivity to antimicrobials, the best growth rate and the highest capacity to produce antagonistic substances against various pathogenic microorganisms.

Keywords: growth rate, aerotolerance, antimicrobial susceptibility, hydrophobicity, antagonism

1. Introduction

According to the definition recommended by the Food and Agriculture Organization and the World Health Organization, probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2002). Strains of the genera *Lactobacillus* and *Bifidobacterium* are the most widely used as probiotics in food, with the probiotic capacity being strain dependent within a given species (López *et al.,* 2010). The determination of the genome sequence of *Bifidobacterium longum* NCC2705 revealed several physiologic traits that could explain the successful adaptation of these bacteria to the human colon in terms of metabolic activity and immunomodulatory and adhesion capacities (Schell *et al.,* 2002).

Experiments on the benefits of the preventive administration of bifidobacteria suggest a range of potentially health benefits such as: (1) improvement of immune function and prevention of infections (Fukuda *et al.,* 2011; Souza *et al.,* 2012); (2) prevention of colon cancer (Coakley *et al.,* 2009); (3) lowering of blood cholesterol (Hasan Al-Sheraji *et al.,* 2012); (4) prevention of *Helicobacter pylori* infection (Gotteland *et al.,* 2006), antibiotic-associated diarrhoea (Corrêa *et al.,* 2005) and traveller's diarrhoea (Leahy *et al.,* 2005); and (5) treatment of inflammatory bowel diseases (Guglielmetti *et al.,* 2011) and constipation (Guerra *et al.,* 2011).

The first step, before the selection of a candidate for probiotic use, is the determination of its taxonomic classification, which may give indication of the origin, habitat

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and physiology of the strain. All these latter characteristics have important consequences on the selection of the novel strains (Morelli, 2007). Although numerous criteria have been recognised and suggested for the selection of probiotics, a general agreement exists concerning the following aspects: (1) safety (origin, pathogenicity and infectivity, virulence factors, antimicrobial susceptibility); (2) technological (production characteristics, organoleptic properties, resistance to processing and storage, phage resistance, genetic stability); (3) functional (resistance to gastrointestinal tract conditions, adherence to mucus and epithelial cells); and (4) beneficial effects on the host (antagonistic activity against pathogenic microorganisms, immunomodulation, hypocholesterolaemic effect, anti-mutagenic and anti-carcinogenic properties) (FAO/WHO, 2002; Vasiljevic and Shah, 2008). For the first aspect, *Lactobacillus* and *Bifidobacterium* are generally considered as safe due to their origin, and only determination of antimicrobial susceptibility of these potential probiotics is performed. Technological properties are rarely determined in the first steps of a probiotic selection, functional and beneficial aspects being more frequently used as initial criteria. Functional aspects must be evaluated essentially when the probiotic candidate has not been isolated from the gastrointestinal tract (e.g. food or environmental origin). Finally, the beneficial effects to be tested depends on the aim of the probiotic administration.

Various *in vitro* assays can be used for an initial selection based on the safety, technological viability, functionality and potential beneficial properties of probiotic candidates using simple and rapid methodologies. However, it is well-known, that results obtained in *in vitro* experiments are not always further confirmed *in vivo.* For this reason, these probiotic candidates must be posteriorly submitted to experiments in animal models and then assessed in well-controlled and conducted human clinical trials. Despite this, *in vitro* assays are among the tests recommended by the WHO and the FAO in their guidelines for the evaluation of probiotics (FAO/WHO, 2002).

In the present study, some *in vitro* assays were used to assess and compare technological viability (aerotolerance, growth rate), functionality (cell wall hydrophobicity to test adherence to mucus and epithelial cells), potential beneficial properties (antagonistic capacity) and safety (antimicrobial susceptibility) of four strains of *Bifidobacterium* isolated from healthy infant faeces.

2. Materials and methods

Microorganisms

B. longum 5^{1A}, *Bifidobacterium breve* 110^{1A}, *Bifidobacterium pseudolongum* 119^{1A} and *Bifidobacterium bifidum* 162^{2A} were isolated from faeces of different healthy children and identified by multiplex PCR. The strains were maintained at the Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil in De Man, Rogosa and Sharp (MRS; Difco, Sparks, NV, USA) broth supplemented with 20% (v/v) glycerol at -70 °C. Before use, the strains were cultured at least twice in MRS broth during 24 to 48 h in an anaerobic chamber (Forma Scientific Company, Marietta, GA, USA) containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2, at 37 °C.

For the experiments of *in vitro* antagonism, the following indicator strains were used: *Bacillus cereus* ATCC 11778, *Bacteroides fragilis* ATCC 25285, *Bacteroides vulgatus* ATCC 8482, *Candida albicans* ATCC 18804, *Clostridium difficile* ATCC 9689, *Clostridium perfringens* type A ATCC 13124, *Enterobacter aerogenes* ATCC 13048, *Enterococcus faecalis* ATCC 19433, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica* serovar Typhi ATCC 19430, *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Shigella sonnei* ATCC 11060. All the microorganisms were obtained from the Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil. In addition, *S.* Typhimurium, *Shigella flexneri* and *Vibrio cholerae* strains of human origin and obtained from the Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, were also used. The microorganisms were grown in brain heart infusion (BHI; Difco) or Sabouraud dextrose (Difco) broth for 18 or 48 h at 37 °C. For obligate anaerobic strains, BHI was supplemented with 0.5% yeast extract (Difco), 0.05 mg/ml hemin (Inlab, Diadema, Brazil) and 0.01mg/ml menadione (Inlab) (BHI-s) and incubation was performed in an anaerobic chamber for 48 h at 37 °C. All microorganisms were maintained at -70 °C in BHI or BHI-s broth containing 20% glycerol.

Growth rate determination

Four bottles containing 300 ml of MRS broth were inoculated with an overnight culture of each bifidobacteria strain (initial OD_{600nm} ca. 0.1). Cultures were kept at 37 °C in an anaerobic chamber and growth was periodically monitored over 72 h by determining OD (600 nm) and pH. Specific growth rate (μ) was calculated using the following equation $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, in which X_2 and X_1 are the cell density at time t_2 and t_1, respectively. Mean doubling time (T_d) was calculated as: $T_d = \ln 2/\mu$, expressed in hours (Shin *et al.,* 2000).

Oxygen tolerance assay

Oxygen tolerance assay was performed according to Farias *et al.* (2001) with modifications. Briefly, 3.0 μl sample of an overnight culture was spot-inoculated on the surface of experimental plates containing MRS agar and grown for 48 h at 37 °C under anaerobic conditions. Simultaneously, a second plate inoculated with the same strain was maintained

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in the anaerobic chamber as a control. After growth, the experimental plates were exposed to atmospheric oxygen up to 72 h under refrigeration at 4 °C. At appropriate time intervals (8, 24, 48 and 72 h) each spot was used to inoculate a MRS broth which was incubated in an anaerobic chamber for 48 h at 37 °C. Oxygen tolerance was evaluated by the observation of growth under anaerobiosis from each spot. Purity of the cultures was checked at the end of each assay by Gram staining and microscopic examination (100×).

Antagonism assay

The strains were tested for antagonistic activity using the double agar layer diffusion assay (Nardi *et al.,* 1999). A 5.0 µl sample of an overnight culture of each bifidobacteria strain was spot-inoculated onto the surface of MRS agar and grown for 48 h at 37 °C under anaerobiosis. The cells were inactivated by exposure to chloroform for 30 min and the residual chloroform was allowed to evaporate. For the determination of the antagonistic activity the plate was overlaid with 3.5 ml of BHI, BHI-s or Sabouraud dextrose soft agar (0.75% w/v) that had been inoculated with 10^7 cfu/ml of the indicator strain and incubated for 24 or 48 h at 37 °C. The antagonistic activity was determined as the presence of an inhibition zone around the spot. The diameter of the inhibitory zone was determined with a digital pachymeter (Digimatic Calipar; Mitutoyo, Suzano, Brazil).

Cell wall hydrophobicity assay

The ability of the organisms to adhere to hydrocarbons as a measure of their cell wall hydrophobicity was determined according to Vinderola and Reinheimer (2003) with modifications. Briefly, cultures of the strains were harvested in the stationary phase by centrifugation at 1,500×*g* for 10 min and the cells were washed twice in sterile phosphate buffered saline (PBS) solution and suspended in the same buffer. The cell suspension was adjusted to an OD_{560nm} value of approximately 1.0 with the buffer and 3.0 ml of the bacterial suspensions were put in contact with 0.6 ml of n-hexadecane (Sigma, St Louis, MO, USA) and vortexed for 120 s. Samples were allowed to stand at 37 °C for one hour. After phase separation, the lower aqueous phase was carefully removed with a syringe. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\% = [(OD_0\text{-}OD)/OD_0] \times 100$, in which OD_0 and OD are the optical density before and after contact with n-hexadecane, respectively.

Antimicrobial susceptibility assay

The susceptibility of bifidobacteria to antimicrobial agents was performed by the minimum inhibitory concentration (MIC) assay using the reference agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012). Antimicrobial agents included inhibitors of cell wall synthesis (penicillin G, cefoxitin, piperacillin, vancomycin), of protein synthesis (chloramphenicol, erythromycin, neomycin and tetracycline), and of nucleic acid synthesis (metronidazole). All the antimicrobial agents were from Sigma, except for metronidazole (All Chemistry, São Paulo, Brazil) and penicillin G (Fluka, St. Gallen, Switzerland). The results were expressed as MIC, i.e. the minimum concentration that completely inhibited bacterial growth. Increasing concentrations of antimicrobials were added to *Brucella* agar (Difco) supplemented with hemin (Inlab) and menadione (Inlab). The inoculum was standardised by suspending cells to achieve a turbidity equivalent to 0.5 McFarland standard and was delivered by a Steers replicator onto the agar plates (ca. 10^5 cfu/ml). *B. fragilis* ATCC 25285 was included as a control. Plates were incubated in the anaerobic chamber at 37 °C for 48 h. At the beginning of each series of tests, a plate without antimicrobial agents was inoculated to determine the viability of the organisms and to serve as a control for growth comparison. As endpoint readings may be sometimes hard to determine with anaerobes, MIC was determined as the lowest concentration of antimicrobial agent that resulted in either no growth or a few colonies or a significant drop-off in the amount of growth, as recommended by CLSI. The MIC results were interpreted according to the guidelines of CLSI and the breakpoints proposed by CLSI (2012) or Ouoba *et al.* (2008).

Statistical analysis

All the assays were performed at least in two independent experiments, each of them in duplicate. Statistical analyses were done using the program R version 2.13.1 (R Development Core Team, 2011) at a probability level of 0.05. The data were statistically analysed using the ANOVA test for hydrophobicity (Tukey post-test) and Cochran Q test for analysis of *in vitro* antagonism.

3. Results

Growth rate determination

Figure 1 shows the growth curves of the four *Bifidobacterium* strains under anaerobic conditions. Initial pH was 6.21±0.05 and decreased along the curve reaching a final value that ranged from 4.09±0.06 to 4.50±0.13 depending on the strain (data not shown). *B. longum* 5^{1A}, acidified the medium more rapidly than the other strains. *B. longum* 5^{1A} showed a higher growth rate (average doubling time 2.2±0.2 h) than the other strains (*B. breve* 110^{1A}, 6.8±1.5 h; *B. pseudolongum*, 119^{1A} 4.7±0.6 h; and *B. bifidum*, 162^{2A}: 7.2±1.6 h). After 24 h, *B. longum* 5^{1A} reached maximum growth; during the interval of 12 to 24 h an abrupt drop in pH medium was noted, which was not observed for the other bifidobacteria

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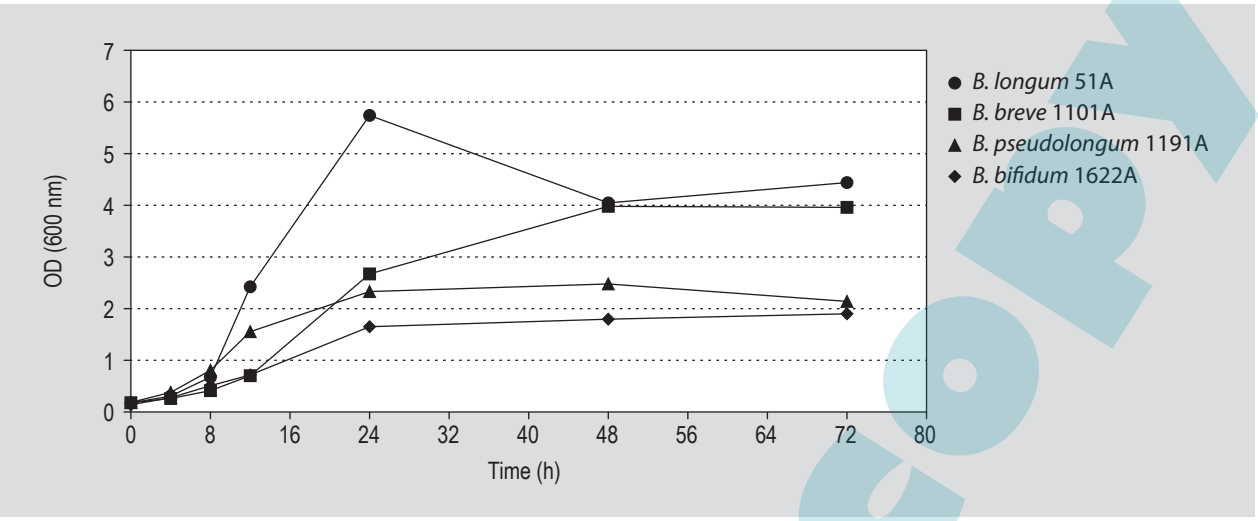

Figure 1. Growth kinetics of *Bifidobacterium longum* 5^{1A}, *Bifidobacterium breve* 110^{1A}, *Bifidobacterium pseudolongum* 119^{1A} and *Bifidobacterium bifidum* 162^{2A} in MRS broth at 37 °C under anaerobic conditions.

(data not shown). A drop in OD was also noted only for *B. longum* 5^{1A} after 24 h of incubation. Maximum growth was observed after 24 h for *B. pseudolongum* 119^{1A} and *B. bifidum* 162^{2A}, but not for *B. breve* 110^{1A}, which reached its stationary phase only after 48 h.

Oxygen tolerance assay

The ability of the strains to tolerate exposure to oxygen up to 72 h was assessed. All strains behaved similarly presenting high aerotolerance, independently of the time of exposure to oxygen up to 72 h (data not shown).

In vitro antagonism assay

According to Table 1, inhibition zones were detected against most pathogens, suggesting the production of inhibitory diffusible substances by all the bifidobacteria. However, not all indicator strains used were sensitive to antagonist activity of bifidobacteria. *E. faecalis* ATCC 19433, *B. vulgatus* ATCC 8482, *B. fragilis* ATCC 25285 and *S.* Typhimurium were not sensitive to the antagonistic substances produced by *B. breve* 110^{1A} and *B. bifidum* 162^{2A}. In addition, growth of *E. aerogenes* ATCC 13048 was not inhibited by *B. bifidum* 162^{2A}, and none of bifidobacteria strains were able to inhibit *C. albicans* ATCC 18804. *B. longum* 5^{1A} and *B. pseudolongum* 119^{1A} showed the highest inhibitory capacity against almost all pathogens used, while *B. breve* 110^{1A} and *B. bifidum* 162^{2A} inhibited 66.7 and 60%, respectively, of the patogens ($P<0.05$).

Cell wall hydrophobicity assay

Hydrophobicity of the cell surface of the four strains was characterised by using the partitioning coefficient in the phase partition assay. *Bifidobacterium* strains showed significant differences in cell surface hydrophobicity, with *B. bifidum* 162^{2A} presenting the highest probability of adhesion to the intestinal epithelium due to a high cell wall hydrophobicity (67.5±7.0%) ($P<0.05$) when compared to *B. breve* 110^{1A} (14.5±2.5%), *B. pseudolongum* 119^{1A} (22.2±6.2%) and *B. longum* 5^{1A} (24.4±3.1%).

Antimicrobial susceptibility assay

MIC results are presented in Table 2. All bifidobacteria showed sensitivity towards chloramphenicol, penicillin G, piperacillin and vancomycin. *B. longum* 5^{1A} was sensitive to all antimicrobials, except neomycin like the other bifidobacteria (MIC 32 µg/ml). *B. breve* 110^{1A} was resistant to cefoxitin, erythromycin and metronidazole (MIC 64, 128 and 512 µg/ml, respectively), *B. pseudolongum* 119^{1A} to erythromycin and tetracycline (MIC 128 and 64 µg/ml, respectively) and *B. bifidum* 162^{2A} to metronidazole (MIC >512 µg/ml).

4. Discussion

An important characteristic for a microorganism to be used as a probiotic is to have good technological properties allowing feasible biomass production, which is characterised, among other factors, by a high growth rate. This parameter can be used as a criterion in the search for probiotic candidates for industrial use (Ouwehand *et al.*, 2002). Obligate anaerobes generally exhibit a slower growth rate than facultative anaerobes, most often requiring an incubation period up to 48 h for maximum growth. Decrease in pH accompanying the growth of bifidobacteria is the result of fermentative metabolism promoting release of acetic and lactic acids in a theoretical ratio of 3:2. In addition to the slower growth rate, another technological problem for anaerobic bacteria is their tolerance to some

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Table 1. *in vitro* antagonistic activity of *Bifidobacterium* strains against pathogenic indicators.

Pathogenic indicator strains	Inhibition halo (mean diameter ± standard deviation in mm)			
	***Bifidobacterium longum* 5[1A]**	***Bifidobacterium breve* 110[1A]**	***Bifidobacterium pseudolongum* 119[1A]**	***Bifidobacterium bifidum* 162[2A]**
Bacillus cereus	22.1±2.0	16.2±3.2	33.3±3.7	36.2±1.6
Bacteroides fragilis	10.1±0.6	–[1]	15.5±1.6	–
Bacteroides vulgatus	13.1±2.4	–	16.5±2.3	–
Candida albicans	–	–	–	–
Clostridium difficile	26.4±3.6	12.9±2.1	36.3±1.8	24.2±2.1
Clostridium perfringens	36.8±2.1	23.2±1.5	34.5±3.2	29.0±2.5
Enterobacter aerogenes	46.6±4.1	16.0±1.1	25.5±2.0	–
Enterococcus faecalis	16.6±1.3	–	13.6±1.4	–
Listeria monocytogenes	29.3±1.3	29.4±2.0	27.0±3.4	19.6±2.7
Shigella flexneri[2]	31.7±1.2	17.9±1.6	41.3±4.5	42.1±4.0
Shigella sonnei	28.8±1.7	19.8±1.8	37.4±4.9	41.7±3.8
Salmonella Typhi	20.8±5.0	11.8±1.7	22.1±4.4	18.2±2.0
Salmonella Typhimurium	28.5±2.0	14.5±3.4	34.6±3.8	36.9±0.5
Salmonella Typhimurium[2]	36.3±4.0	–	23.7±2.7	–
Vibrio cholerae[2]	18.2±2.0	19.2±1.8	36.2±2.6	31.4±4.1

[1] – = no inhibition zone observed.
[2] Bacteria of human origin.

Table 2. Antimicrobial susceptibility as MIC (µg/ml) of four *Bifidobacterium* strains using the agar dilution method. *Bacteroides fragilis* was used as a control.

Antimicrobials	***Bifidobacterium longum* 5[1A]**	***Bifidobacterium breve* 110[1A]**	***Bifidobacterium pseudolongum* 119[1A]**	***Bifidobacterium bifidum* 162[2A]**	***Bacteroides fragilis* ATCC 25285**	Break-points
Cefoxitin	8.0	64.0	0.5	1.0	4.0	≥64[a]
Chloramphenicol	2.0	2.0	2.0	2.0	4.0	≥32[a]
Erythromycin	0.25	128.0	128.0	0.125	16.0	8[b]
Metronidazole	0.5	512.0	2.0	>512	0.25	≥32[a]
Neomycin	32.0	32.0	32.0	32.0	>64.0	16[b]
Penicillin G	0.125	0.25	0.06	0.015	>4.0	≥2[a]
Piperacillin	2.0	2.0	0.125	0.125	4.0	≥128[a]
Tetracycline	2.0	2.0	64.0	2.0	0.125	≥16[a]
Vancomycin	0.5	0.5	0.5	0.5	32.0	4[b]

[a] Breakpoints recommended by CLSI (2012).
[b] Breakpoints recommended by Ouoba *et al.* (2008).

oxygen levels during the production process. Bifidobacteria do not show cellular mechanisms efficient in removing oxygen, so exposure to oxygen causes accumulation of toxic metabolites (superoxide, hydroxyl anion, hydrogen peroxide) leading to cell death by oxidative damage. However, some variability has been observed in oxygen tolerance among species and even strains of the same species, indicating that oxygen tolerance is species/strain specific (Silva *et al.,* 2003). The oxygen sensitivity might influence levels of viable cells in dairy products by the time of consumption (Simpson *et al.,* 2005). In the present study, *B. longum* 5[1A] showed satisfactory growth and oxygen tolerance for use on industrial scale.

It is believed that the antagonism shown frequently by bifidobacteria results primarily from the action of lactic

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and acetic acids produced by fermentative metabolism. The antimicrobial activity of these acids is partly due to the fact that in the non-dissociated form they may cross the microbial cell membrane and dissociate inside the cell, leading to an intracellular pH reduction, which interferes with important cellular metabolic functions (Makras and Vuyst, 2006). However, many other studies have shown that antimicrobial compounds other than acids may also contribute to the antagonistic activity of bifidobacteria, such as bacteriocins (Yildirim *et al.*, 1999), hydrogen peroxide (Lahtinen *et al.*, 2007) and bacteriocin-like compounds (Cheikhyoussef *et al.,* 2008). The present study demonstrated that *B. longum* 5^{1A} and *B. pseudolongum* 119^{1A} produced antagonistic substance(s) against a very wide spectrum of bacterial pathogens. Antagonism of bacteria against yeasts is rarely described in the literature, and was not observed here. The results obtained may represent the antagonist activity of one or more substances, acting separately or together. Other studies using the same methodology showed that bifidobacteria are able to produce diffusible antagonist substances against various pathogens (Martins *et al.*, 2009). The addition of probiotics with a broad spectrum of antagonistic activity in dairy-based foods confers various advantages by increasing shelf life of the product, replacing artificial preservatives, and acting beneficially after ingestion in the digestive tract of the consumer (O'Riordan and Fitzgerald, 1998).

Another expected feature for probiotics is the ability to adhere to intestinal mucosa, allowing them to stay longer and act in the digestive ecosystem. Many mechanisms are involved in the adhesion of microorganisms to the intestinal epithelial cells, and one of them is the hydrophobic nature of the cell surface. Thus, the determination of cell wall hydrophobicity is a simple qualitative methodology that can be used to select probiotic candidates based on a higher probability to adhere to intestinal epithelial cells (Vinderola and Reinheimer, 2003). In our study, *B. bifidum* 162^{2A} showed the highest value of hydrophobicity. Similar results have been obtained for four strains of *B. bifidum*, which showed hydrophobicity between 46.7±0.9% and 64.7±2.1%, while three strains of *B. longum* showed lower values ranging from 22.5±0.9% to 28.9±2.3% (Vinderola and Reinheimer, 2003). Adhesion is probably an important factor for longer persistence of a probiotic in the digestive tract, but this property can bring also other benefits to the host by competitive exclusion or reduction of pathogen adhesion, as well as by increasing the modulatory contact of the probiotic with immune cells (Martins *et al.,* 2009).

Bacterial resistance to antimicrobials, especially among pathogenic microorganisms, is one of the major problems faced by modern medicine. In the search for new methods to combat gastrointestinal infections, an alternative is the use of probiotics that must be sensitive to antibiotics or their intrinsic resistance must not be transmissible. Therefore, the determination of antimicrobial susceptibility of a bacterial strain is an important prerequisite for its approval as a probiotic candidate (FAO/WHO, 2002). However, some authors claim that in case of co-administration with antimicrobials to prevent and treat antibiotic associated diarrhoea, probiotics should be resistant to certain antimicrobials to survive in the gastrointestinal tract (Danielsen and Wind, 2003), however, this opinion is controversial. Risks related to potential transfer of antibiotic resistance from probiotic strains to intestinal pathogens are a concern, especially after the report of a probiotic, *Enterococcus faecium* (Gaio), which was found to be a possible recipient of the resistance *vanA* gene (Lund and Edlund, 2001). In general, a large antibacterial sensitivity is suggested as a selection criterion for probiotics to prevent possible transmission of genetic elements of resistance in the digestive ecosystem (Ouoba *et al.,* 2008). The antimicrobial susceptibility of bifidobacteria has not been seriously questioned until now, and the few data available in the literature are heterogeneous and contradictory. Therefore, we evaluated the antimicrobial susceptibility profile of the bifidobacteria evaluated in the present study. Based on the MIC values, it was found that the susceptibility varies according to the species and antimicrobial tested. *Bifidobacterium* spp. are considered as resistant to aminoglycosides (neomycin, gentamicin, kanamycin and streptomycin), and this intrinsic resistance can be explained by the lack of transport of this drug (Flórez *et al.,* 2008). In relation to the susceptibility observed for chloramphenicol, penicillin, piperacillin and vancomycin, similar results were found by other authors (Delgado *et al.,* 2005; Ouoba *et al.,* 2008; Zhou *et al.,* 2005). Differences in susceptibility to cefoxitin, erythromycin, metronidazole and tetracycline have been demonstrated in other studies (Ammor *et al.,* 2008; Delgado *et al.,* 2005). Intrinsic and acquired resistance mutations have a low risk of horizontal transmitting, while the risk of transfer is maximal if the acquired resistance is mediated by exogenous genes. Exogenous DNA can be acquired by conjugation, transduction and transformation, and can be transferred by plasmids and transposons to other bacterial species or genera (Ammor *et al.,* 2008; Vankerckhoven *et al.,* 2008). Knowledge of the resistance patterns is necessary when administered probiotics are associated with antimicrobial therapy. In this case, the association of an antimicrobial with an intrinsically resistant probiotic strain can offer significantly better results.

The importance of certain technological and physiological characteristics for probiotic strains was recognised long time ago, and the evaluation of these properties is used as an initial step to select candidate microorganisms for probiotic use. The determination of these criteria is performed in *in vitro* assays, which must be completed and confirmed by further *in vivo* experiments using animal models. However, the evidence of beneficial effects is only obtained with well-conduced clinical trials (double-blind, randomised,

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placebo or formula-controlled trials). In two recent published studies, *B. longum* 5^{1A} has been demonstrated to be effective to treat children for functional constipation in such clinical trials (Guerra *et al.,* 2011) and to protect mice against *S.* Typhimurium infection in addition to inducing IgA+ cell proliferation in the gut (Souza *et al.,* 2012). In the present study, some *in vitro* selection criteria based on safety and technological, functional and beneficial aspects were evaluated in four *Bifidobacterium* strains. *B. longum* 5^{1A} was confirmed to be the best candidate for probiotic use, presenting the highest sensitivity to antimicrobials, the best growth rate and an ability to produce antagonistic substances against various pathogens, justifying its selection for animal and human studies to be conducted.

Acknowledgments

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento do Pessoal de Ensino Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). The authors are grateful to Clélia Nunes da Silva for valuable technical help. T.C. Souza and A.M. Silva were recipients of doctor fellowships from CNPq and/or CAPES/CONICET.

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