In Vitro Assessment of the Gastrointestinal Transit Tolerance of Taxonomic Reference Strains from Human Origin and Probiotic Product Isolates of *Bifidobacterium*

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ABSTRACT

Next to health promoting effects, the functional aspect of probiotic strains also involves their capacity to reach the colon as viable metabolically active cells. The present study aimed to assess the potential of 24 probiotic product isolates and 42 human reference strains of *Bifidobacterium* to survive gastrointestinal transit under in vitro conditions. The survival capacity of exponential and stationary phase cultures upon exposure to gastric and small intestinal juices was determined using a recently developed microplate-based assay in combination with the LIVE/DEAD BacLight Bacterial Viability kit. All 66 strains tested displayed a considerable loss in viability during exposure to an acidic pepsin containing solution (pH 2.0). Among the 10 taxa tested, cultures of *B. animalis* ssp. *lactis* appeared to be most capable to survive gastric transit. Although to a lesser extent, the presence of bile salts also affected the viability of most of the strains tested. Except for 3 strains, all 66 strains showed bile salt hydrolase activity using an agar-based assay. In contrast, the bifidobacterial strains used in this study appeared to possess a natural ability to survive the presence of pancreatin (pH 8.0). Although the effect was not significant, a slightly enhanced tolerance to gastrointestinal transit was observed when cells were in the stationary phase, especially when exposed to acid, compared with cells being in the exponential phase. Survival in the gastrointestinal tract appeared to be largely strain-dependent and hence implies that different strains will likely display a different behavior in functionality. The assay used in this study allows an initial assessment of strains for use as probiotic cultures prior to selecting potential candidate strains for further investigation in vivo.

Key words: fluorescent dye, *Bifidobacterium*, probiotic, gastrointestinal survival

INTRODUCTION

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (http://www.who.int/food_safety/publications/fs_management/en/probiotics.pdf; accessed Apr. 30, 2007). A wide range of dairy-based and dried probiotic products for human consumption is currently available on the market (Stanton et al., 2001; Agrawal, 2005). Although cellular or culture components of dead probiotic bacteria are also thought to mediate beneficial effects in the host, it has been argued that most health benefits associated with probiotics are exerted by viable metabolically active microorganisms (Ouwehand and Salminen, 1998). Prior to inducing any effect, however, most living probiotic cultures are orally administered upon which they need to survive gastrointestinal (GI) transit in sufficient numbers.

During GI passage, cultures are required to tolerate the presence of pepsin and the low pH of the stomach, the protease-rich conditions of the duodenum, and the antimicrobial activity of bile salts. Although the pH of the stomach may increase up to 6.0 or higher after food intake (Johnson, 1977), it generally ranges from 2.5 to 3.5 (Holzapfel et al., 1998). Fasting pH in the stomach may even be as low as 1.5 (Waterman and Small, 1998), which implies that survival in extreme acidic conditions is one of the first major physiological challenges faced by probiotic cultures upon oral administration. Following stomach passage, the small intestine is a second major barrier in the GI tract. Although the pH of the small intestine (i.e., 7.0 to 8.5; Thomson et al., 2003) is more favorable toward bacterial survival, the presence of pancreatin and bile salts may have adverse effects.

Traditionally, the ability of a probiotic candidate to survive GI transit is assessed using conventional plating techniques that provide information on the number of viable and reproductive cells during incubation in simulated GI juices (Charteris et al., 1998; Huang and Adams, 2004; Mättö et al., 2004). In the present study, a recently developed technique based on the use of 2

Received August 22, 2006.

Accepted April 16, 2007.

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fluorescent staining agents (Alakomi et al., 2005) was evaluated to assess the GI survival of probiotic cultures and human reference strains of *Bifidobacterium* under in vitro conditions. Together with several *Lactobacillus* species, bifidobacteria are among the most commonly used bacterial organisms in commercial probiotic products. Using a microplate-based assay, the relative degree of tolerance toward gastric and pancreatic juices and the ability to survive in the presence of bile salts was assessed by differentiation between viable and dead cells. In addition, an agar-based culture method was applied to determine the potential of *Bifidobacterium* strains to deconjugate bile salts.

MATERIALS AND METHODS

Bacterial Strains

A total of 66 *Bifidobacterium* strains were investigated in this study including 42 type and reference strains obtained from the BCCM/LMG Bacteria Collection, Ghent University, Belgium (<http://bccm.belspo.be/index.php>; accessed Dec. 4, 2006) and 24 isolates obtained from 23 commercial probiotic products (Masco et al., 2005). The species identity of all strains was previously checked by BOX-PCR fingerprinting (Masco et al., 2005). The selection encompassed the following *Bifidobacterium* (sub)species: *B. adolescentis* (n = 6), *B. angulatum* (n = 2), *B. animalis* ssp. *lactis* (n = 19), *B. bifidum* (n = 8), *B. breve* (n = 7), *B. catenulatum* (n = 2), *B. gallicum* (n = 1), *B. longum* biotype *infantis* (n = 7), *B. longum* biotype *longum* (n = 9), and *B. pseudocatenulatum* (n = 5). All strains were subcultured in de Man, Rogosa, and Sharpe (MRS) broth supplemented with 0.5 g/L L-cysteine-HCl (MRS-cysteine broth) at 37°C under anaerobic conditions (84% N₂, 8% H₂, 8% CO₂). Subsequently, overnight subcultures were grown in MRS-cysteine broth until they reached the early exponential or the stationary growth phase, respectively.

Microplate Assays

Survival rates in GI juices (i.e., gastric, pancreatic, and bile salt solutions) were assessed using a microplate-based fluorochrome assay in combination with the LIVE/DEAD BacLight Bacterial Viability (L/D) kit (L7012, Molecular Probes Inc., Eugene, OR) as previously described (Alakomi et al., 2005). The L/D kit combines the nucleic acid dyes propidium iodide (PI), a red-colored agent that is excluded from intact cells and SYTO9, a green-colored agent that is membrane-permeant and stains viable and nonviable cells. Because PI has a higher affinity for DNA than SYTO9, it is able to displace SYTO9 from the DNA. Hence, intact

viable cells will stain fluorescent green, whereas dead cells will color red.

Exponential and stationary phase cells were harvested by centrifugation, washed with 0.85% (wt/vol) NaCl and incubated with (challenged culture) or without (control culture) each of the GI juices. Gastric juice [0.3% (wt/vol) pepsine, 0.5% (wt/vol) NaCl, pH 2, adjusted with HCl] or pancreatic juice [0.1% (wt/vol) pancreatin, 0.5% (wt/vol) NaCl, pH 8, adjusted with NaOH] was added to the harvested cells and aliquots were taken after 1, 90, and 180 min of incubation under microaerobic (6% O₂) or anaerobic conditions, respectively. Cells were treated with bile salt solution [0.3% (wt/vol) bovine bile, 0.5% (wt/vol) NaCl, pH 8, adjusted with NaOH] during 60 min in anaerobic conditions. After incubation, cells were harvested by centrifugation, washed, and resuspended in 0.85% (wt/vol) NaCl solution. Of each bacterial cell suspension, 100 µL was pipetted in triplicate into separate wells of a white 96-well fluorescence microplate. Staining solutions of PI and SYTO9 were prepared according to the manufacturer's instructions. Aliquots of 100 µL of staining solution were added to each well and mixed. Subsequently, plates were incubated in the dark at room temperature for 15 min. Fluorescence measurements were performed using a fluorescence microplate reader (HTS 7000 Bio Assay Reader, Perkin-Elmer, Waltham, MA). Intensities of green (535 nm) and red (635 nm) emission were recorded after excitation at 485 nm. Following fluorescence background subtraction, the mean ratio of green to red fluorescence emission (**ratio_{G/R}**), which is proportional to the relative numbers of live bacteria and hence the survival rate, was calculated from 3 measurements. For every in vitro test, *B. animalis* ssp. *lactis* LMG 18314^T was included for reproducibility assessment.

Bile Salt Hydrolase Assay

Strains were tested for taurodeoxycholic acid (TDCA) hydrolase activity using MRS-cysteine agar plates to which 0.3% TDCA sodium salt was added (MRS-TDCA). Overnight grown MRS-cysteine broth cultures were inoculated by quadrant streaking on MRS-TDCA and incubated for 24 h at 37°C under anaerobic conditions. Strains were scored positive for bile salt hydrolase (BSH) activity when a white precipitate of deoxycholic acid beneath and around the colonies was observed. Growth performance was compared with cultures grown on MRS-cysteine agar.

Statistical Analysis

Statistical analysis was composed of significance testing of the difference between means of trendline slopes

Table 1. Effect of simulated gastric juice on the viability of 66 *Bifidobacterium* strains as calculated from the trendline slopes¹

Species (no. of strains)		Exponential growth phase	Stationary growth phase
		Mean slope $\pm$ SD	Mean slope $\pm$ SD
<i>B. adolescentis</i> (6)		-0.0033 ± 0.0015	-0.0054 ± 0.0025
<i>B. angulatum</i> (2)		-0.0028 ± 0.0022	-0.0027 ± 0.0022
<i>B. animalis</i> ssp. <i>lactis</i>	Reference strains (4)	-0.0016 ± 0.0012	-0.0007 ± 0.0006
	Probiotic isolates (15)	-0.0009 ± 0.0015	-0.0012 ± 0.0028
<i>B. bifidum</i>	Reference strains (5)	-0.0046 ± 0.0013	-0.0055 ± 0.0033
	Probiotic isolates (3)	-0.0026 ± 0.0031	-0.0019 ± 0.0014
<i>B. breve</i>	Reference strains (6)	-0.0054 ± 0.0014	-0.0047 ± 0.0015
	Probiotic isolate (1)	-0.0008 ± 0.0001	-0.0001 ± 0.0001
<i>B. catenulatum</i> (2)		-0.0035 ± 0.0023	-0.0013 ± 0.0026
<i>B. gallicum</i> (1)		-0.0063 ± 0.0000	-0.0051 ± 0.0002
<i>B. longum</i> biotype <i>infantis</i>	Reference strains (6)	-0.0044 ± 0.0019	-0.0044 ± 0.0021
	Probiotic isolate (1)	-0.0050 ± 0.0000	-0.0042 ± 0.0000
<i>B. longum</i> biotype <i>longum</i>	Reference strains (5)	-0.0039 ± 0.0016	-0.0016 ± 0.0019
	Probiotic isolates (4)	-0.0047 ± 0.0006	-0.0024 ± 0.0011
<i>B. pseudocatenulatum</i> (5)		-0.0036 ± 0.0010	-0.0040 ± 0.0021
	Total (66)	-0.00356 ± 0.00162	-0.00302 ± 0.00183
	Reference strains (42)	-0.00394 ± 0.00134	-0.00355 ± 0.00181
	Probiotic isolates (24)	-0.00281 ± 0.00201	-0.00198 ± 0.00150

¹Trendline slopes are expressed as the mean value of the measurements of the tested strains. Individual trendlines were calculated based on triplicate measurements of the log (% ratio_{G/R}) values at times 0, 1, 90, and 180 min.

calculated from the logarithmic (% ratio_{G/R}) values using the nonparametric Kruskal-Wallis H and Mann-Whitney U-test.

RESULTS

Despite some exceptions, there was a general tendency indicating that GI survival under in vitro conditions as determined from ratio_{G/R} values was strain-specific among the selected *Bifidobacterium* strain set. The overall mean coefficient of variation of triplicate measurements of the ratio_{G/R} was $\pm 0.94\%$ with a maximum variation of 3.63%. Although most strains performed slightly better in presence of gastric juice when being in the stationary phase (Mean slope_{STAT}: -0.00302 ± 0.00020) compared with exponential phase cells (Mean slope_{EXP}: -0.00356 ± 0.00016 ; $P > 0.05$), all strains tested showed significant loss in viability after incubation for 180 min ($P < 0.001$; Table 1). Of all strains tested, reference strains and probiotic isolates of *B. animalis* ssp. *lactis* displayed the highest survival rates during simulated gastric transit compared with the other taxa ($P < 0.05$). Only single strains of *B. adolescentis* (LMG 10502^T), *B. angulatum* (LMG 10503^T), *B. bifidum* (LM 588), *B. breve* (LM 646), *B. catenulatum* (LMG 11043^T), *B. longum* biotype *longum* (LMG 13196), and *B. pseudocatenulatum* (LMG 10505^T) exhibited survival rates comparable with those observed for the *B. animalis* ssp. *lactis* strains. Remarkably, probiotic product isolates of *B. bifidum* and *B. breve* LM 646

showed higher survival rates compared with the *B. bifidum* and *B. breve* reference strains ($P < 0.05$; Figure 1), respectively.

A subset of 30 strains, encompassing the 10 *Bifidobacterium* taxa that exhibited variable survival rates during simulated gastric transit, was selected for in vitro analysis of the small intestinal transit (Tables 2 and 3). For most strains tested, no loss in viability was witnessed after 180 min of incubation in presence of pancreatin when in the stationary phase compared with exponential phase cells that were more susceptible ($P < 0.05$). Exponential phase cells belonging to *B. angulatum*, *B. animalis* ssp. *lactis*, and *B. catenulatum* as well as *B. bifidum* probiotic product isolates and *B. breve* reference strains showed a significant decrease in viability ($P < 0.05$). As was the case for gastric transit, the survival capacity in the presence of pancreatin proved to be largely strain dependent. However, cultures of *B. animalis* ssp. *lactis* now grouped among the least pancreatin tolerant strains. Differences in survival rates were noted between probiotic product isolates and reference strains of the same species, the latter showing higher survival rates ($P < 0.05$).

During testing of conjugated bile salt tolerance, a general slight decrease in viability was witnessed after 60 min of incubation (Mean slope_{EXP}: -0.0112 ± 0.0065 ; Mean slope_{STAT}: -0.0110 ± 0.0071 ; $P < 0.05$; Table 3). Of the 30 strains tested, only *B. longum* biotype *infantis* strains LMG 18902 (Mean slope_{STAT}: 0.0007 ± 0.0004) and LM 418 (Mean slope_{STAT}: 0.0060 ± 0.0001) and *B.*

Figure 1. Survival behavior of probiotic product isolate *Bifidobacterium breve* LM 646 compared with *B. breve* reference strains (stationary phase cells) over a 180-min incubation period in simulated gastric juice. For each strain, trendlines were calculated based on the log of the mean ratio of green to red fluorescence emission (% ratio_{G/R}) values of 4 time points (0, 1, 90, and 180 min), which are expressed as the mean $\pm$ standard deviation (bars indicated when >0.1) of 3 measurements. Comparison and statistical analysis were based on the slopes of these trendlines (slope range for *B. breve* reference strains: -0.0028 to -0.0074 and *B. breve* LM 646: -0.0001).

Table 2. Effect of simulated pancreatic juice on the viability of 30 *Bifidobacterium* strains as calculated from the trendline slopes¹

Species (no. of strains)		Exponential growth phase	Stationary growth phase
		Mean slope $\pm$ SD	Mean slope $\pm$ SD
<i>B. adolescentis</i> (3)		-0.000078 ± 0.00102	0.00066 ± 0.00061
<i>B. angulatum</i> (2)		-0.000083 ± 0.00008	-0.00027 ± 0.00019
<i>B. animalis</i> ssp. <i>lactis</i>	Reference strains (2)	-0.00057 ± 0.00045	0.00098 ± 0.00019
	Probiotic isolates (3)	-0.00111 ± 0.00045	-0.00096 ± 0.00085
<i>B. bifidum</i>	Reference strain (1)	0.00170 ± 0.00035	0.00110 ± 0.00000
	Probiotic isolates (2)	-0.00085 ± 0.00035	0.00082 ± 0.00032
<i>B. breve</i>	Reference strains (2)	-0.00035 ± 0.00014	0.00070 ± 0.00143
	Probiotic isolate (1)	0.00147 ± 0.00023	0.00003 ± 0.00006
<i>B. catenulatum</i> (2)		-0.00050 ± 0.00026	0.00033 ± 0.00077
<i>B. gallicum</i> (1)		0.00060 ± 0.00000	0.00067 ± 0.00006
<i>B. longum</i> biotype <i>infantis</i>	Reference strains (3)	0.00083 ± 0.00080	0.00080 ± 0.00082
	Probiotic isolate (1)	-0.00067 ± 0.00029	0.00033 ± 0.00012
<i>B. longum</i> biotype <i>longum</i>	Reference strains (2)	0.00058 ± 0.00058	0.00008 ± 0.00079
	Probiotic isolates (2)	-0.00023 ± 0.00052	-0.00053 ± 0.00015
<i>B. pseudocatenulatum</i> (3)		-0.00014 ± 0.00026	0.00068 ± 0.00082
	Total (30)	0.00004 ± 0.00083	0.00036 ± 0.00059
	Reference strains (21)	0.00020 ± 0.00072	0.00057 ± 0.00041
	Probiotic isolates (9)	-0.00028 ± 0.00103	-0.00006 ± 0.00070

¹Trendline slopes are expressed as the mean value of the measurements of the tested strains. Individual trendlines were calculated based on triplicate measurements of the log (% ratio_{G/R}) values at times 0, 1, 90, and 180 min.

Table 3. Effect of conjugated bile salt on the viability of 30 *Bifidobacterium* strains as calculated from the trendline slopes¹

Species (no. of strains)		Exponential growth phase	Stationary growth phase
		Mean slope $\pm$ SD	Mean slope $\pm$ SD
<i>B. adolescentis</i> (3)		-0.0099 $\pm$ 0.0086	-0.0095 $\pm$ 0.0049
<i>B. angulatum</i> (2)		-0.0093 $\pm$ 0.0073	-0.0210 $\pm$ 0.0011
<i>B. animalis</i> ssp. <i>lactis</i>	Reference strains (2)	-0.0120 $\pm$ 0.0004	-0.0151 $\pm$ 0.0064
	Probiotic isolates (3)	-0.0206 $\pm$ 0.0011	-0.0128 $\pm$ 0.0039
<i>B. bifidum</i>	Reference strain (1)	-0.0111 $\pm$ 0.0002	-0.0068 $\pm$ 0.0001
	Probiotic isolates (2)	-0.0167 $\pm$ 0.0042	-0.0073 $\pm$ 0.0067
<i>B. breve</i>	Reference strains (2)	-0.0154 $\pm$ 0.0018	-0.0083 $\pm$ 0.0007
	Probiotic isolate (1)	-0.0018 $\pm$ 0.0001	-0.0057 $\pm$ 0.0001
<i>B. catenulatum</i> (2)		-0.0174 $\pm$ 0.0017	-0.0180 $\pm$ 0.0069
<i>B. gallicum</i> (1)		-0.0238 $\pm$ 0.0003	-0.0222 $\pm$ 0.0004
<i>B. longum</i> biotype <i>infantis</i>	Reference strains (3)	-0.0033 $\pm$ 0.0022	-0.0068 $\pm$ 0.0057
	Probiotic isolate (1)	-0.0118 $\pm$ 0.0001	0.0060 $\pm$ 0.0001
<i>B. longum</i> biotype <i>longum</i>	Reference strains (2)	-0.0054 $\pm$ 0.0035	-0.0081 $\pm$ 0.0057
	Probiotic isolates (2)	-0.0052 $\pm$ 0.0075	-0.0150 $\pm$ 0.0018
<i>B. pseudocatenulatum</i> (3)		-0.0049 $\pm$ 0.0075	-0.0150 $\pm$ 0.0053
	Total (30)	-0.0112 $\pm$ 0.0065	-0.0110 $\pm$ 0.0071
	Reference strains (21)	-0.0113 $\pm$ 0.0063	-0.0131 $\pm$ 0.0059
	Probiotic isolates (9)	-0.0112 $\pm$ 0.0078	-0.0070 $\pm$ 0.0082

¹Trendline slopes are expressed as the mean value of the measurements of the tested strains. Individual trendlines were calculated based on triplicate measurements of the log (% ratio_{G/R}) values at times 0 and 60 min.

longum biotype *longum* LM 257 (Mean slope_{EXP}: 0.0016 $\pm$ 0.0001) were able to retain their metabolically active state during this incubation period. Within the species *B. bifidum*, the human reference strain performed better than the probiotic product isolates, whereas the opposite was the case for the *B. breve* strains ($P < 0.05$). For the other species tested, no pronounced differences in ratio_{G/R} were witnessed between probiotic product isolates and reference strains at a level of $P = 0.05$. Likewise, no significant differences were noted between stationary phase cells and exponential phase cells.

Of the 66 strains tested, only 3 strains (*B. gallicum* LMG 11596^T and *B. longum* biotype *infantis* LMG 8811^T and LMG 11588) did not display TDCA hydrolase activity in the BSH assay. Of note, a slight reduction in growth performance was witnessed in the presence of TDCA compared with growth on the MRS control plates.

DISCUSSION

Together with safety and technological aspects, functionality screening plays a key role in the selection of potential probiotic strains for human use. Next to health promoting characteristics, the functional aspect of probiotic strains also involves their capacity to reach the colon in a metabolically active state. In the present study, a number of in vitro tests were used to screen a large number of *Bifidobacterium* strains of intestinal and food origin for their ability to survive in the pres-

ence of pepsin, pancreatin, and bile salts. Survival rates were assessed using a recently described microplate scale fluorochrome assay (Alakomi et al., 2005) following incubation in each of the GI juices. This approach proved to be highly reproducible and provides a rapid alternative to laborious plate count techniques. As a general tendency, survival rates of the bifidobacterial strains tested proved to be largely strain-dependent. As previously reported for bifidobacteria of human origin, most of the strains were susceptible to low pH and bile salts, and an apparently intrinsic ability to survive the presence of pancreatin was witnessed (Charteris et al., 1998).

Of all strains tested, representatives of *B. animalis* ssp. *lactis* appeared to be most capable to survive gastric transit, which is probably due to their enhanced acid tolerance compared with other *Bifidobacterium* species (Matsumoto et al., 2004; Mättö et al., 2004; Ventura et al., 2004; Sánchez et al., 2006). When exposed to acidic conditions, bacteria try to maintain a pH homeostasis by discharging H⁺ from the cell by H⁺-ATPase (Booth, 1985). It has previously been shown that upon incubation under acidic conditions, the H⁺-ATPase activity in *B. animalis* ssp. *lactis* increases, whereas that of other, nonacid-tolerant bifidobacteria, such as *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. longum* biotype *infantis* and *longum*, and *B. pseudocatenulatum*, diminishes and results in a general decrease or loss of viability (Matsumoto et al., 2004). These results suggest that many *B. animalis* ssp. *lactis* cultures incorporated in

probiotic food products are sufficiently acid tolerant to reach the intestinal tract in a metabolically active state after oral administration. For probiotic strains with limited acid tolerance, gastric passage can be enhanced by the presence of milk proteins due to a buffering or protective effect, suggesting that milk-based products constitute an important carrier of probiotic strains (Charteris et al., 1998). Other studies have demonstrated that probiotic cultures can be significantly protected by the addition of (cryo)protectants during spray- and freeze-drying or via encapsulation in milk proteins and complex (prebiotic) carbohydrates (Ross et al., 2005). Furthermore, the upregulation of genes involved in stress responses has been shown to enhance acid tolerance of probiotic bacteria (Kullen and Klaenhammer, 1999). Perhaps, this type of adaptation to acidic stress conditions might explain why the relative decrease in viability after 180 min of incubation was lower than after 1 min for all strains tested in our study. Finally, food-grade genetic manipulation has been used to improve probiotic performance (Desmond et al., 2004), which leaves the option to further exploit promising cultures that are sensitive to gastric transit.

Despite considerable loss of viability during simulated gastric transit, most of the bifidobacterial strains used in this study appeared to possess a natural ability to survive the presence of pancreatin. The survival of lactobacilli (Charteris et al., 1998) and dairy propionibacteria (Huang and Adams, 2004) also seems unaffected when incubated with a simulated pancreatin-containing solution. Consequently, the presence of pancreatin in the small intestine does not appear to confer an insuperable barrier for probiotic cultures. In contrast, our results indicate that the presence of bile salts slightly reduces the viability of most of the strains tested. After synthesis from cholesterol and conjugation to glycine or taurine in the liver, conjugated bile salts are secreted into the small intestine and undergo extensive chemical modifications upon arrival in the colon due to microbial activity. Bile salt hydrolase catalyzes the hydrolysis of conjugated bile salts into the bile salt and the amino acid residue. Although the functions of this enzyme and the physiological impact on its host are far from understood, conjugated bile salt hydrolysis is a commonly observed phenomenon among GI bacteria, including the genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Fusobacterium*, *Lactobacillus*, and *Peptostreptococcus* (Aries and Hill, 1970; Hy-lemon, 1985; Chateau et al., 1994). Deconjugation of bile salts is an important metabolic reaction in the bile salt metabolism of mammals and has been associated with a reduction of serum cholesterol (Anderson and Gilliland, 1999; Pereira and Gibson, 2002). On the other hand, excessive bile salt deconjugation can also exert

pathological effects. Deconjugated bile salts are thought to be involved in the formation of gallstones (Thomas et al., 2000) and the development of colorectal cancer (Singh et al., 1997). In line with this functional paradox, it has been suggested that the release of deconjugated bile salts seems to have a higher antimicrobial effect than the unmodified bile salts (Grill et al., 1995, 2000). Although most bifidobacterial strains tested in the present study exhibited BSH activity, we observed a slight overall sensitivity toward bile salts, which confirms earlier findings (Kociubinski et al., 1999). Because BSH activity is expressed constitutively within the genus *Bifidobacterium* (Grill et al., 1995, 2000), the absence of detectable BSH activity in 2 *B. longum* biotype infantis strains and in the type strain of *B. gallicum* might indicate absence, inactivation, mutation, or low expression of the BSH gene. Although interest has been shown to use strains that produce BSH to lower serum cholesterol levels (De Smet et al., 1994), FAO/WHO guidelines (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf; accessed Apr. 30, 2007) only recommend to test BSH activity of probiotic candidates but do not include a statement whether this is a criterion for probiotic selection or not. Given the wide distribution and high activity of BSH in bifidobacteria compared with other probiotic groups (Grill et al., 1995, 2000; Tanaka et al., 1999), the lack of BSH as a selection criterion seems controversial and might need revision.

Stress responses of bacterial cultures generally vary with the growth phase. Bacterial cells that enter the stationary phase tend to develop a general stress resistance and are thus more resistant to various types of stress factors (van de Guchte et al., 2002) including the ones encountered during GI transit. When exposed in vitro to GI juices, the *Bifidobacterium* strains tested in our study exhibited a slightly more tolerant profile during the stationary phase than during the exponential phase; however, these differences were not significant.

In conclusion, the results obtained during this study indicate that tolerance toward bile salts is potentially more important during probiotic selection compared with gastric and pancreatic tolerance. With the development of new delivery systems and the use of specific foods, acid-sensitive strains can be buffered through the stomach. In addition, bifidobacteria seem to possess a natural tolerance toward pancreatin. Consequently, the potential of a probiotic strain to survive passage through the GI tract after ingestion may largely depend on its ability to resist the antimicrobial action of bile salts. Furthermore, the strain-dependent tendency observed for transit survival implies that different strains will likely display a different behavior in functionality. For this reason, preliminary characterization of strains

for use as probiotic cultures through in vitro screening is of great value in selecting functional candidate strains for further in vivo studies.

ACKNOWLEDGMENTS

This research was financially supported by a PhD grant from the Flemish Institute for the Promotion of Innovation by Science and Technology (IWT - Vlaanderen, Brussels, Belgium). G. Huys is a postdoctoral fellow of the Fund for Scientific Research—Flanders (Belgium; F.W.O.-Vlaanderen).

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