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ORIGINAL ARTICLE

Probiotic properties of bifidobacteria and lactobacilli isolated from local dairy products

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Abstract Fourteen strains of lactobacilli and bifidobacteria were isolated from raw milk and fermented dairy products produced by local traditional industries. These strains were evaluated for potential use as probiotics based on their adhesion to intestinal epithelial cells, resistance towards acidic and bile conditions, and antimicrobial activities. All strains exhibited varying levels of adhesion properties on mucin that were strain-dependent. Although most strains tolerated acidic conditions of pH 2, 3, and 4, the viability of the *Lactobacillus* and *Bifidobacterium* strains was significantly ($P<0.05$) reduced at pH 2. Most strains also tolerated bile conditions similar to that of the gastrointestinal tract. A higher inhibition was observed in the presence of deconjugated bile, such as cholic acid, compared to deconjugated bile, such as taurocholic acid. In addition, most strains also showed antimicrobial activity towards intestinal pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Salmonella typhimurium*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. The findings from this study show that strains of lactobacilli and bifidobacteria isolated from local dairy products may be promising probiotics for use as dietary adjuncts or the development of new functional foods.

Keywords Probiotics · Mucin · Acid · Bile · Antimicrobial

Introduction

Probiotics are defined as "live microorganisms that when ingested in adequate amounts, confers beneficial effects to the host by improving its intestinal microbial balance" (Liong and Shah 2005). Probiotics are well documented to have health-promoting benefits that include modulation of the immune system, lowering of the serum cholesterol level, and alleviation or prevention of intestinal disorders, such as lactose intolerance and antibody-associated diarrhea (Guo et al. 2010). At the present time, lactobacilli and bifidobacteria are common probiotics associated with health-promoting "functional foods" as well as therapeutic, prophylactic and growth supplements for animals and humans (Kesarcodi-Watson et al. 2008). Many early reports have shown that selected strains of lactobacilli and bifidobacteria are increasingly being introduced into various food products because they are considered to be non-pathogenic and safe. The underlying rationale for this perception is that such strains are naturally present in the host intestinal tract and also their long history of safe use in food and fermented products (Vaughan et al. 1999).

The genus *Lactobacillus* comprises Gram-positive, rod-shaped, catalase-negative, non-motile, and non-sporulating microorganisms. Lactobacilli are strictly fermentative facultative anaerobes that are tolerant to very small amounts of oxygen up to strictly anaerobic conditions (Singh et al. 2009). Lactic acid is the main metabolic acid produced, enabling the members of this genus to better adapt to acidic conditions. The most studied probiotic strains include members of the *Lactobacillus acidophilus* group (*L. rhamnosus*, *L. casei*, *L.*

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paracasei, *L. gasserii*, *L. reuteri*, and *L. plantarum*). Bifidobacteria (genus *Bifobacterium*), on the other hand, are predominant bacteria in the human intestines and have been found to proliferate and colonize neonatal intestines after birth and then to decrease throughout life (Liu et al. 2007). Bifidobacteria are rod-shaped, Gram-positive, non-spore forming, non-motile catalase-negative, and pleomorphic anaerobic microorganisms (Cheikhyoussef et al. 2008). Common bifidobacteria used as probiotics include *Bifidobacterium bifidum*, *B. longum*, and *B. infantis*. Both lactobacilli and bifidobacteria are not naturally present in milk, but may be present in raw milk due to fecal and dairy farm environmental cross-contamination (Delcenserie et al. 2005; Kagkli et al. 2007).

Before the strains of lactobacilli and bifidobacteria are able to exert such proposed benefits in the intestines, they need to fulfill several criteria as well as to survive the harsh environments of the gastrointestinal tract, which include the acidic conditions of the stomach and bile salts. Probiotics need to persist in the intestines to confer health benefits to the host. The stomach and intestines contain gastric juices, digestive enzymes, and bile salts that pose as threats to such bacteria. Survival through the stomach and small intestine enables the lactobacilli and bifidobacteria to reach the large intestines in considerable amounts and subsequently to thrive and adhere to the mucosa of intestinal tissues. Enterocyte-like Caco-2 cells and HT29 cells are the most common cell lines used for studies on the adhesion properties of probiotics. In this regard, *L. plantarum* 423 has been shown to adhere to Caco-2 cells via adhesions such as EF-Tu and the glycolytic enzymes GAPDH and TPI. *Lactobacillus plantarum* 423 has also been found to be able to competitively displace *Clostridium sporogenes* LMG 13570 and *Enterococcus faecalis* LMG 13566 by 81 and 91%, respectively (Ramiah et al. 2008). Adhesion then leads to the colonization of these bacteria, which are resistant to pathogens through their production of antimicrobial substances, such as bacteriocins, organic acids, hydrogen peroxide, and bacteriocin-like substances (Oh et al. 2000). Important natural bacteriocins, including Lactacin B, Lactacin F, Brevicin 37, Buchnericin LB, Lacticin A, Helveticin J, Sakacin A, Plantaricin A, and Gassericin A, are also produced by probiotics to act as protection against pathogenic bacteria and as preservatives in food products (Singh et al. 2009; Giraffa et al. 2010).

The aim of the study reported here was to evaluate certain strains of lactobacilli and bifidobacteria that are more durable and resistant for their ability to adhere to intestinal cells and to colonize the intestines, their tolerance towards human gastric juice and bile, and their antagonistic activity towards intestinal pathogens.

Materials and methods

Dairy sources and isolation of cultures

Different types of fermented milk and yoghurt and raw milk samples were purchased from local traditional industrial outlets in Penang, Malaysia. Aliquots of the dairy samples were then cultured in sterile de Mann, Rogosa, Sharpe (MRS) medium (Himedia, Mumbai, India) for 48 h at 37 and 42°C. Cultures successfully grown in MRS medium were transferred to Bifidobacteria Selective Media (BSM) (Sigma-Aldrich, St. Louis, MO) for selective enumeration of bifidobacteria that produced purple–brown colonies, while lactobacilli were selectively enumerated using the *Lactobacillus* Selective Agar (LBS) (BD Diagnostics, Franklin Lakes, NJ). *Lactobacillus delbrueckii* subsp. *bulgaricus* cultures were obtained upon incubation at 42°C. Colonies were identified using API 50 CHL medium (bioMérieux, Durham, NC) (Pelinescu et al. 2009).

Maintenance of cultures

Isolated strains (*Lactobacillus gasserii* FTDC 8131, *L. fermentum* BT 8219, *L. acidophilus* FTDC 8933, *L. delbrueckii* subsp. *bulgaricus* FTDC 8913, *L. acidophilus* FTDC 8633, *L. delbrueckii* subsp. *bulgaricus* FTDC 8611, *L. acidophilus* FTDC 8033, *L. delbrueckii* subsp. *bulgaricus* FTDC 8011, *L. acidophilus* FTDC 2131, *L. acidophilus* FTCC 0291, *L. acidophilus* BT 1088, *L. casei* BT 1268, *Bifidobacterium longum* FTDC 8643, and *Bifidobacterium* spp. FTDC 8943) were propagated three times in sterile MRS broth (Himedia) using 1 % (v/v) inoculum and incubated for 24 h at 37°C prior to use. The sterile MRS broth was supplemented with 0.15% (w/v) filter-sterilized L-cysteine hydrochloride (Himedia) solution. Stock cultures were stored in 40% (v/v) sterile glycerol at −20°C.

Adhesion properties of *Lactobacillus* and *Bifidobacterium* strains

Mucin and plate preparations The adhesion properties of the cultures were evaluated using the method of Azcarate-Peril et al. (2009) as previously described. Briefly, 100 μL of a 10 mg/mL solution of partially purified type III porcine gastric mucin (Sigma-Aldrich) was immobilized in 96-well microtiter plates by incubation overnight at 4°C. Excess mucin was removed by pipetting, and the wells were washed twice with 200 μL of phosphate buffer solution (Gibco, Auckland, NZ).

Preparation of Lactobacillus and Bifidobacterium cultures for adhesion assay Activated cultures of lactobacilli and bifidobacteria (100 μL) were added to each well. The plates

were then incubated for 3 h at 37°C. Each well was washed five times with 200 μL of sterile phosphate buffered saline (PBS) to remove unbound bacteria and then treated with 200 μL of a 0.05% (v/v) Triton X-100 (Sigma-Aldrich) solution to desorb the bound bacteria. A 100-μL sample of the contents in each well was removed, diluted in peptone water (Merck, Darmstadt, Germany), and plated on MRS agar plates. The plates were then incubated at 37°C for 24 h. Cultures of *B. longum* FTDC 8643 and *Bifidobacterium* spp. FTDC 8943 were incubated under anaerobic conditions, using anaerobic jars (BD Diagnostics, Sparks, MD) with gas-generating kits (Merck).

Acid tolerance of *Lactobacillus* and *Bifidobacterium* strains

Acid tolerance of the cultures was evaluated using the method of Teh et al. (2009), as previously described. Activated cultures of lactobacilli and bifidobacteria (10% v/v inoculum) were incubated for 3 h at 37°C in pepsin-supplemented MRS broths that were adjusted to a pH of 2.0, 3.0, or 4.0. The pour plate method was used for monitoring growth. Cultures were sampled every 30 min, serially diluted, plated onto sterile MRS agar supplemented with 0.15 % (w/v) L-cysteine hydrochloride, and incubated at 37°C. Cultures of *B. longum* FTDC 8643 and *Bifidobacterium* spp. FTDC 8943 were incubated under anaerobic conditions.

Bile tolerance of *Lactobacillus* and *Bifidobacterium* strains

Bile tolerance of the cultures was evaluated using the method of Teh et al. (2009), as previously described. Four types of bile salts were used, namely, oxgall, cholic acid, taurocholic acid (Sigma-Aldrich), and glycocholic acid (Merck). Sterile MRS broth containing 0.30 % (w/v) oxgall, cholic acid, taurocholic acid, or glycocholic acid was incubated with each strain and incubated at 37°C. The control comprised MRS broth without bile salts. Bacterial growth was monitored once every hour for 7 h by measuring the absorbance with a spectrophotometer (Shimadzu, Kyoto, Japan) at 620 nm. The obtained absorbance values were plotted against incubation time, and the bile tolerance of each strain was determined as the time required for absorbance value to increase by 0.3 U. pH values were measured at time=0, and another measurement was taken after the absorbance had increased by 0.3 units for all samples.

Antibacterial activity of *Lactobacillus* and *Bifidobacterium* strains

Antibacterial activity of the cultures was evaluated by the disc-diffusion assay method (Blažeka et al. 1991). Cultures of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Salmonella typhimurium*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* were obtained from the Culture Collection Centre of School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia. The test pathogens were cultured in a tryptone soya broth (Himedia) for 24 h and diluted with sterile peptone water. Then, 20 μL of lactobacilli or bifidobacteria culture broth was applied to sterile filter discs (6 mm) which were placed on the surface of solidified tryptone soya agar (Himedia) seeded with 100 μL of test microorganisms that had been cultured for 12–14 h. The plates were incubated at 37°C for 14–16 h and the diameter of zones of inhibition then measured.

Statistical analysis

Data analysis was carried out with SPSS software (ver. 14.0; SPSS, Chicago, IL). A repeated measures analysis of variance (ANOVA) was used for the time-based analyses. One-way ANOVA was used to determine significant differences between means at a significance level of $\alpha=0.05$. Tukey's test was used to perform multiple comparisons between means. All data are presented as the mean ± standard error of means (SEM). All analyses were performed in triplicate ($n=3$).

Results and discussion

Adhesion properties of lactobacilli and bifidobacteria

The adhesion of lactobacilli and bifidobacteria onto mucin was used to evaluate the ability of strains to colonize the intestines. In our tests, porcine gastric mucin was used as it mimics the mucus-secreting cells of the human intestines. *Lactobacillus casei* BT 1268 (78.89%), *L. acidophilus* FTDC 0291 (78.24%), *L. acidophilus* FTDC 2131 (76.27%), *L. acidophilus* BT 1088 (75.35%), *L. acidophilus* FTDC 8933 (74.23%), and *L. delbrueckii* subsp. *bulgaricus* FTDC 8913 showed a higher adherence to mucin than the other strains studied (Table 1; $P<0.05$). Adherence properties can be attributed to various factors, such as entrapment of cells in the mucus (Gopal et al. 2001), high expression of mucus adhesion genes (e.g., *Mub* and *MapA*), and a higher excretion of surface-layer proteins (Jakava-Viljanen and Palva 2007; Ramiah et al. 2007). Lactobacilli have been shown to have the elongation factor Tu (EF-Tu) and the chaperonin protein complex GroEL, both of which are involved in the synthesis of surface proteins responsible for mucin and human epithelial cell adhesion (Ramiah et al. 2008).

The adhesion properties of lactobacilli and bifidobacteria are vital factors for the selection of probiotics, as probiotics need to adhere to the intestinal epithelial cells for retention

Table 1 Attachment properties of *Lactobacillus* and *Bifidobacterium* strains to mucin

Strain	Number of cells at time 0 (CFU/mL)	Number of cells after incubation for 3 h at 37°C (CFU/mL)	Cells attached to mucin[a] (%)
Lactobacillus acidophilus FTDC 8633	10.35±0.20	7.08±0.44	68.41±2.98 d,e,f
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8011	9.43±0.03	6.79±0.07	72.00±0.97 b,c,d,e,f
Lactobacillus acidophilus FTDC 2131	9.27±0.09	7.07±0.19	76.27±1.36 a,b,c
Lactobacillus acidophilus BT 1088	9.86±0.09	7.43±0.21	75.35±2.78 a,b,c,d
Lactobacillus acidophilus FTCC 0291	9.19±0.07	7.19±0.15	78.24±2.22 a,b
Lactobacillus casei BT 1268	9.40±0.04	7.51±0.11	78.89±0.84 a
Bifidobacterium longum FTDC 8643	10.05±0.08	6.96±0.21	69.25±1.65 c,d,e,f
Lactobacillus acidophilus FTDC 8933	10.09±0.07	7.49±0.10	74.23±0.47 a,b,c,d,e
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8611	9.53±0.47	5.34±0.11	56.03±1.44 g
Lactobacillus gasserii FTDC 8131	9.27±0.16	6.26±0.14	67.53±2.58 e,f
Lactobacillus fermentum BT 8219	9.88±0.04	6.75±0.13	68.32±1.07 d,e,f
Bifidobacterium spp. FTDC 8943	10.40±0.15	6.78±0.30	65.21±1.92 f
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8913	10.06±0.06	7.35±0.13	73.06±1.75 a,b,c,d,e
Lactobacillus acidophilus FTDC 8033	9.95±0.05	6.54±0.21	65.73±1.80 f

[a] Means within the column followed by different lowercase letters are significantly different ($P<0.05$)

in the gastrointestinal tract. Adhered probiotic cells have been reported to interact with the immunomodulatory cells of the mucosal immune system, such as the enhanced leucocyte, to exert phagocytic activity against pathogens (Vaughan et al. 1999; Azcarate-Peril et al. 2009). Indigenous lactobacilli and bifidobacteria have been found to have a competitive adherence advantage and a lengthier probiotic effect. The presence of mucin in the intestines of hosts often provides protection from the adherence of enteric pathogens via the steric hindrance specific binding domains for bacteria or viruses (Larson et al. 2003). Larson et al. (2003) and Mack et al. (1999) stated that co-incubation of a *Lactobacillus* spp. with human intestinal epithelial cells increased the expression of MUC3 and thus inhibited the adherence of intestinal pathogenic *E. coli.*

Acid tolerance of *Lactobacillus* and *Bifidobacterium* strains

In order to exert their beneficial effects in the host, probiotics must remain alive during both ingestion and their transit prior to reaching the large intestines. Thus, tolerance towards unfavorable conditions, such as low pH, is essential for the survival of the lactobacilli and bifidobacteria. The pH of simulated gastric fluid was maintained at pH 2, 3, or 4 for 3 h. The low pH mimics the conditions in the stomach during fasting as well as during digestion. It has been found that the fasting gastric pH could be as low as 1.87 in humans (Husebye et al. 1992; Husebye 2005).

We found that a low pH level of 2 had consistent effects on the viability of the probiotic cells, with higher viability log reduction compared to higher pH levels of 3 and 4 (Table 2). *Lactobacillus acidophilus* FTDC 8633, *L. delbrueckii* subsp. *bulgaricus* FTDC 8011, *L. acidophilus* FTDC 2131, *L. acidophilus* BT 1088, and *L. acidophilus* FTDC 0291 showed the greatest acid tolerance ($P<0.05$) in all pH level studies. *Lactobacillus delbrueckii* subsp. *bulgaricus* FTDC 8611 and *B. longum* FTDC 8643 were the most acid-sensitive strains, showing no growth after 30 and 60 min, respectively, at pH 2. Many strains of *Lactobacillus* have been reported to exhibit a tolerance to acidic conditions due to their having a cytoplasmic buffering capacity at pH 3.72–7.74 (Kalaisapathy and Chin 2000), which enables them to tolerate cytoplasmic pH changes and thus achieve stability in acidic environments. Although only two *Bifidobacterium* strains were evaluated, both strains did not show a strong tolerance under acidic conditions compared to the *Lactobacillus* strains studied. Bifidobacteria are fastidious and non-competitive, making them very susceptible to environmental constraints, such as low pH and exposure to oxygen. Due to their strict growth requirements, bifidobacteria normally have a low survival rate in dairy products and during their transit through the gastrointestinal tract (Doleyres and Lacroix 2005).

Bile tolerance of lactobacilli and bifidobacteria

Cholic acid is a deconjugated bile whereas taurocholic and glycocholic acids are conjugated biles. Oxgall is the combination of both conjugated and deconjugated bile. The largest pH decrease was observed upon fermentation in media supplemented with glycocholic and taurocholic

Table 2 Effect of pH 2, 3, and 4 on the viability of *Lactobacillus* and *Bifidobacterium* strains

Strain[a]	pH[b]	Viable count (log CFU/mL)[c] at different time (h)							Log reduction
		0	30	60	90	120	150	180	
Lactobacillus acidophilus FTDC 8633 A	2 c	10.09±0.01	10.08±0.08	9.77±0.07	9.68±0.02	9.18±0.26	9.13±0.06	9.11±0.42	0.98±0.41
	3 b	9.67±0.25	9.64±0.05	9.59±0.18	9.56±0.05	9.55±0.04	9.43±0.03	9.43±0.15	0.24±0.10
	4 a	9.49±0.18	9.35±0.29	9.35±0.08	9.29±0.20	9.27±0.48	9.19±0.17	9.15±0.15	0.34±0.04
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8011 A,B	2 c	10.07±0.09	9.42±0.02	9.42±0.01	9.42±0.01	9.38±0.03	9.37±0.00	9.34±0.07	0.73±0.15
	3 b	9.13±0.08	9.10±0.12	9.04±0.12	9.03±0.12	8.99±0.42	8.99±0.19	8.96±0.07	0.17±0.00
	4 a	9.49±0.17	9.29±0.15	9.16±0.36	9.06±0.49	9.00±0.07	8.90±0.15	8.84±0.15	0.65±0.02
Lactobacillus acidophilus FTDC 2131 A,B	2 c	9.98±0.10	9.45±0.02	9.45±0.04	9.42±0.01	9.41±0.01	9.35±0.06	9.27±0.00	0.71±0.08
	3 b	9.38±0.90	9.32±0.47	9.27±0.77	9.26±0.66	9.25±0.70	9.16±0.59	9.14±0.82	0.24±0.08
	4 a	9.40±0.13	9.38±0.49	9.32±0.08	9.30±0.57	9.30±0.28	9.19±0.09	9.13±0.01	0.27±0.14
Lactobacillus acidophilus BT 1088 A,B	2 c	10.59±0.23	9.97±0.26	9.76±0.38	9.04±0.08	8.85±0.62	8.85±0.62	8.76±0.08	1.83±0.16
	3 b	9.36±0.27	9.27±0.26	9.20±0.41	9.19±0.46	9.19±0.73	9.15±0.28	9.13±0.07	0.23±0.20
	4 a	9.56±0.19	9.54±0.02	9.54±0.06	9.50±0.04	9.47±0.14	9.45±0.22	9.34±0.24	0.22±0.05
Lactobacillus acidophilus FTCC 0291 A,B	2 c	10.03±0.56	9.60±0.05	9.59±0.38	9.57±0.45	9.50±0.02	9.36±0.12	9.24±0.71	0.79±0.15
	3 b	9.65±0.20	9.61±0.19	9.58±0.01	9.53±0.17	9.50±0.09	9.47±0.02	9.46±0.13	0.19±0.07
	4 a	9.82±0.24	9.79±0.21	9.73±0.09	9.64±0.04	9.58±0.04	9.57±0.16	9.51±0.04	0.31±0.20
Lactobacillus casei BT 1268 B,C	2 c	10.27±0.07	9.32±0.60	9.29±0.49	9.27±0.41	9.22±0.44	9.12±0.24	6.85±0.21	3.42±0.28
	3 b	9.30±0.25	9.27±0.04	9.24±0.30	9.14±0.26	9.10±0.32	9.09±0.07	9.06±0.14	0.24±0.12
	4 a	9.50±0.22	9.50±0.27	9.50±0.27	9.43±0.29	9.36±0.11	9.20±0.26	9.12±0.12	0.38±0.34
Bifidobacterium longum FTDC 8643 F	2 c	9.14±0.40	8.00±0.64	6.51±0.05	ND	ND	ND	ND	9.14±0.35
	3 b	7.64±0.08	7.63±0.29	7.38±0.03	7.31±0.07	7.27±0.01	7.26±0.01	7.24±0.01	0.40±0.09
	4 a	8.26±0.02	8.23±0.00	8.09±0.19	8.04±0.15	7.92±0.12	7.98±0.22	7.88±0.04	0.38±0.02
Lactobacillus acidophilus FTDC 8933 D	2 c	10.11±0.33	8.85±0.48	8.10±0.11	7.89±0.21	8.72±0.48	7.71±0.15	7.00±0.00	3.11±0.33
	3 b	9.18±0.15	8.87±0.37	8.77±0.90	8.58±0.34	8.52±0.32	8.52±0.63	8.47±0.25	0.71±0.40
	4 a	9.69±0.23	9.65±0.32	9.61±0.26	9.59±0.19	9.58±0.26	9.58±0.27	9.54±0.52	0.15±0.30
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8611 G	2 c	10.21±0.18	9.27±0.09	ND	ND	ND	ND	ND	10.21±0.28
	3 b	8.86±0.62	8.76±0.66	8.75±0.81	8.73±0.61	8.70±0.45	8.66±0.48	8.64±0.23	0.22±0.40
	4 a	9.43±0.21	9.42±0.15	9.28±0.09	9.26±0.55	9.25±0.41	9.22±0.08	9.19±0.33	0.24±0.11
Lactobacillus gasserii FTDC 8131 C,D	2 c	9.82±0.30	9.66±0.41	9.17±0.06	8.57±0.31	7.99±0.26	7.52±0.31	7.51±0.05	2.31±0.26
	3 b	9.35±0.38	9.31±0.10	9.31±0.10	9.27±0.11	9.26±0.18	9.25±0.02	9.16±0.13	0.19±0.25
	4 a	9.61±0.21	9.60±0.19	9.56±0.19	9.53±0.09	9.51±0.20	9.46±0.25	9.28±0.24	0.33±0.45
Lactobacillus fermentum BT 8219 C,D	2 c	10.10±0.07	8.72±0.01	8.74±0.19	8.62±0.44	8.43±0.11	8.38±0.18	8.23±0.04	1.87±0.11
	3 b	10.05±0.11	10.02±0.05	10.01±0.03	9.87±0.04	9.85±0.24	9.76±0.39	9.59±0.03	0.46±0.13
	4 a	9.67±0.23	9.65±0.32	9.62±0.27	9.55±0.07	9.50±0.15	9.50±0.18	9.22±0.17	0.45±0.06
Bifidobacterium spp. FTDC 8943 E	2 c	8.71±0.04	7.87±0.069	6.26±0.00	6.17±0.10	6.15±0.28	6.14±0.14	6.07±0.10	2.64±0.16
	3 b	8.20±0.19	7.64±0.03	7.41±0.04	7.37±0.56	7.35±0.02	7.29±0.01	7.27±0.10	0.93±0.29
	4 a	8.30±0.09	8.05±0.00	8.02±0.05	7.98±0.16	7.96±0.18	7.95±0.17	7.92±0.04	0.38±0.04
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8913 C,D	2 c	10.44±0.03	9.93±0.06	9.05±0.11	8.54±0.09	8.12±0.60	8.00±0.43	7.70±0.00	2.74±0.04
	3 b	9.68±0.02	9.43±0.30	9.30±0.34	9.24±0.58	9.09±0.28	9.03±0.40	9.02±0.25	0.66±0.23
	4 a	9.39±0.43	9.32±0.38	9.30±0.06	9.30±0.34	9.20±0.42	9.20±0.00	9.17±0.19	0.22±0.62
Lactobacillus acidophilus FTDC 8033 D	2 c	10.36±0.34	10.22±0.07	8.50±0.14	7.85±0.22	7.70±0.00	7.09±0.12	6.94±0.34	3.42±0.00
	3 b	9.14±0.11	9.19±0.32	9.16±0.48	9.08±0.31	9.03±0.22	9.01±0.14	8.96±0.20	0.18±0.12
	4 a	9.66±0.27	9.55±0.03	9.50±0.35	9.45±0.19	9.45±0.01	9.34±0.29	9.33±0.26	0.33±0.01

Statistical significance effect: Effects of time: $P<0.001$. Effects of strains: $P<0.001$. Effects of strains × time: $P<0.001$

[a] Means between different strains followed by different uppercase letters are significantly different at $P<0.05$

[b] Means between different pH within the same strain followed by different lowercase letters are significantly different at $P<0.05$

[c] Results are expressed as the mean ± standard deviation; Each data point is the average of two repeated measurements from 3 independent experiments ($n=3$)

ND, Not detected

Table 3 Effect of different biles on the viability of *Lactobacillus* and *Bifidobacterium* strains

Strain	Growth medium[a]														
	MRS broth			MRS broth+0.3% cholic acid			MRS broth+0.3% glycocholic acid			MRS broth+0.3% taurocholic acid			MRS broth+0.3% oxgall		
	pH[b]			pH[b]			pH[b]			pH[b]			pH[b]		
	h[c]	T1	T2	h[c]	T1	T2	h[c]	T1	T2	h[c]	T1	T2	h[c]	T1	T2
Lactobacillus acidophilus FTDC 8633	2.21±0.17 c,d,e,f,A	5.9	4.8	2.00±0.36 a,A	5.8	5.9	4.76±0.37 h,A	5.9	5.5	2.34±0.14 a,b,A	6.0	4.9	22.89±3.54 d,e,B	6.1	6.1
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8011	1.03±0.06 a,A	6.0	5.0	11.01±0.32 b,c,d,D	5.8	6.0	2.60±0.04 b,c,d,e,B	6.1	5.0	2.29±0.03 a,b,B	6.0	5.1	5.02±0.13 a,b,C	6.0	6.0
Lactobacillus acidophilus FTDC 2131	1.24±0.06 a,b,A	6.0	5.0	8.39±0.91 a,b,c,B	5.8	5.9	2.35±0.01 a,b,c,d,A	6.0	5.0	2.23±0.23 a,b,A	6.1	5.1	14.40± 0.52 b,c,d,e,C	6.0	6.0
Lactobacillus acidophilus BT 1088	1.86±0.09 b,c,d,e,A	6.0	5.1	19.05±1.05 d,e,f,C	5.9	5.9	3.18±0.11 f, g,A	6.0	5.0	2.88±0.18 a,b,A	6.1	6.0	6.43±0.81 a,b,c,B	6.0	5.7
Lactobacillus acidophilus FTCC 0291	1.74±0.15 a,b,c,d,A	6.0	4.9	27.61±1.59 g,B	5.8	6.0	1.86±0.13 a,A	6.0	5.0	2.35±0.21 a,b,A	6.1	5.1	3.25±0.09 a,A	6.0	6.0
Lactobacillus casei BT 1268	1.23±0.03 a,b,A	6.0	5.1	4.50±0.06 a,b,C	5.8	6.0	2.56±0.04 b,c,d,e,B	6.0	5.0	2.59±0.03 a,b,B	6.0	5.0	4.35±0.06 a,b,C	6.1	6.0
Bifidobacterium longum FTDC 8643	2.52±0.08 e,f,A	6.0	4.9	27.74±4.39 g,C	5.9	6.0	2.07±0.15 a,b,A	6.0	5.1	2.03±0.14 a,b,A	6.1	5.1	13.16±0.07 a,b,c,d,B	6.1	5.9
Lactobacillus acidophilus FTDC 8933	2.47±0.08 d,e,f,A	6.0	4.9	11.57±3.73b c,d,B	5.9	6.0	2.10±0.11 a,b,c,A	6.1	5.1	2.03±0.06 a,A	6.0	5.0	9.91±0.03 a,b,c,B	6.1	6.0
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8611	2.05±0.31 d,e,f,A	5.9	5.1	23.08±0.34 e,f,g,B	5.9	6.0	2.37±0.18 a,b,c,d,A	6.1	5.0	2.33±0.11 a,b,A	6.1	5.0	15.46±4.53 c,d,e,B	6.0	5.9
Lactobacillus gasserii FTDC 8131	1.18±0.07 ab,,A	6.0	5.0	24.91±1.29 f,g,C	5.9	6.0	2.56±0.13 b,c,d,e,A	6.0	5.1	2.92±0.64 b,A	6.1	5.0	8.50±1.10 a,b,c,B	6.0	6.0
Lactobacillus fermentum BT 8219	1.80±0.09 a,b,c,d,e,A	6.0	5.0	14.99±2.28 c,d,e,A,B	5.9	6.0	1.90±0.09 a,A	6.0	5.1	2.31±0.05 a,b,A	6.0	5.1	24.23±7.93 e,B	6,0	6.0
Bifidobacterium spp. FTDC 8943	2.91±0.59 f,A	6.0	4.9	16.06±1.20 c,d,e,C	5.9	6.0	3.01±0.08 e,f,g,A	6.1	5.0	2.92±0.21 b,A	6.1	5.0	5.23±0.02 a,b,c,B	6.1	5.5
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8913	1.88±0.13 b,c,d,e,A	6.0	5.1	11.48±0.23 b,c,d,C	5.9	5.9	2.80±0.04 d,e,f,A	6.1	5.1	2.92±0.33 b,A	6.0	5.0	8.29±1.21 a,b,c,B	5.9	5.7
Lactobacillus acidophilus FTDC 8033	2.10±0.11 c,d,e,A	5.9	4.9	16.06±1.56 c,d,e,B	5.9	6.0	2.63±0.10 c,d,e,f,A	6.0	5.0	2.50±0.05 a,b,A	6.1	5.0	3.76±0.06 a,A	6.1	5.4

Means in the same *column* followed by different lowercase letters are significantly different at $P<0.05$. Means in the same *row* followed by different uppercase letters are significantly different at $P<0.05$

[a] Results of Time (h) required to increase absorbance by 0.3 units at 620 nm are expressed as mean ± standard error of mean (SEM); each data point is the average of repeated measurements from three independently replicated experiments (n=3). Means in the same column followed by different lowercase letters are significantly different at $P<0.05$. Means in the same row followed by different uppercase letters are significantly different at $P<0.05$

[b] pH at time=0 (T1) and at time after absorbance was increased by 0.3 units (T2)

[c] Time (h) required to increase absorbance by 0.3 units at 620 nm

acids, whereas media supplemented with cholic acid showed an increase in pH (Table 3). These changes occurred in tandem with the growth of the strains, where all strains showed faster growth in MRS broth containing glycocholic and taurocholic acids, but slower growth in the presence of cholic acid. *Lactobacillus acidophilus* FTDC 8633, *L. casei* BT 1268 and *L. acidophilus* FTDC 2131 were the most bile-tolerant strains in the presence of cholic acid, whereas *B. longum* FTDC 8643, *L. acidophilus* FTDC 0291, *L. gasserii* FTDC 8131, and *L. delbrueckii* subsp. *bulgaricus* FTDC 8611 were the least tolerant ($P<0.05$). The two *Bifidobacterium* strains studied were also more bile sensitive than the *Lactobacillus* strains studied. Due to their fastidious nature, bifidobacteria were also found to have a lower tolerance towards bile salts, thus exhibiting inhibited growth. Inhibition of growth was also higher in deconjugated bile (cholic acid) compared to conjugated bile (glycocholic acid and taurocholic acid). Deconjugated bile salts have been reported to have a greater tendency to damage cell membranes due to their hydrophobicity (Yokota et al. 2000), where deconjugated and free bile acids disaggregate the ordered structure of biological membranes, leading to a higher toxic effect compared to conjugated bile acids.

Antibacterial activity of lactobacilli and bifidobacteria

Antibacterial activity is vital for the successful colonization of lactobacilli and bifidobacteria in the intestinal mucosa as they provide a barrier effect and defense against pathogens (Vaughan et al. 1999). Inhibition of pathogens as evidenced by the disc-diffusion test indicated that the inhibitory metabolites produced by lactobacilli and bifidobacteria were extracellular and diffusible, as the test was conducted

Table 4 Antibacterial activity of *Lactobacillus* and *Bifidobacterium* strains against test pathogenic microorganisms

Strain	Inhibition zone (mm)[a]					
	Escherichia coli	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Bacillus subtilis*
Lactobacillus acidophilus FTDC 8633	7.50±0.35 a,b,c	6.63±0.18 a,b,c	7.13±0.18 a	6.50±0.00 a	6.25±0.35 b,c	6.63±0.18 a
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8011	7.88±0.18 a,b	7.63±0.18 a,b,c	6.88±0.18 a	6.50±0.71 a	7.75±0.00 a	6.25±0.00 a
Lactobacillus acidophilus FTDC 2131	6.38±0.53 a,b,c	6.63±0.88 a,b,c	6.50±0.71 a	ND	6.88±0.18 a,b,c	6.63±0.53 a
Lactobacillus acidophilus BT 1088	7.25±0.35 a,b,c	6.88±0.18 a,b,c	6.50±0.35 a	6.25±0.35 a	6.63±0.88 a,b,c	6.50±0.35 a
Lactobacillus acidophilus FTCC 0291	6.75±0.71 a,b,c	6.38±0.53 b,c	6.75±0.71 a	6.38±0.53 a	6.25±0.35 b,c	6.38±0.18 a
Lactobacillus casei BT 1268	7.50±0.35 a,b,c	7.75±0.00 a,b	6.50±0.00 a	ND	7.25±0.35 a,b,c	6.38±0.53 a
Bifidobacterium longum FTDC 8643	6.25±0.35 b,c	6.38±0.53 b,c	6.38±0.18 a	ND	6.63±0.18 a,b,c	6.25±0.35 a
Lactobacillus acidophilus FTDC 8933	7.50±0.35 a,b,c	7.50±0.71 a,b,c	6.25±0.00 a	ND	6.38±0.53 b,c	6.63±0.18 a
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8611	6.38±0.53 a,b,c	6.38±0.53 b,c	6.88±0.88 a	6.38±0.18 a	6.38±0.18 b,c	6.13±0.18 a
Lactobacillus gasserii FTDC 8131	8.00±0.00 a	7.63±0.53 a,b,c	6.75±0.00 a	7.13±0.18 a	7.38±0.53 a,b	6.50±0.35 a
Lactobacillus fermentum BT 8219	7.63±0.18 a,b,c	6.13±0.18 c	7.13±0.18 a	6.13±0.18 a	6.75±0.71 a,b,c	6.50±0.00 a
Bifidobacterium spp. FTDC 8943	6.88±0.18 a,b,c	6.88±0.18 a,b,c	6.38±0.53 a	6.25±0.35 a	6.75±0.00 a,b,c	6.50±0.35 a
Lactobacillus delbrueckii subsp. *bulgaricus* FTDC 8913	7.13±0.18 a,b,c	8.13±0.18 a	7.38±0.53 a	6.38±0.53 a	6.38±0.53 b,c	6.25±0.20 a
Lactobacillus acidophilus FTDC 8033	6.13±0.18 b,c	7.00±0.00 a,b,c	6.13±0.18 a	6.38±0.18 a	6.25±0.00 b,c	6.88±0.18 a

[a] Results are expressed as mean ± standard error of mean; each data point is the average of repeated measurements from three independently replicated experiments (n=3). Means in the same column followed by different lowercase letters are significantly different at P<0.05

ND, Not detected

via diffusion through a layer of agar (Tadesse et al. 2005). The highest diameter of inhibition zones were observed for *L. delbrueckii* subsp. *bulgaricus* FTDC 8913 (8.13 mm) against *Staphylococcus aureus*, followed by *L. gasserii* FTDC 8131 (8.00 mm) against *E. coli* and *L. delbrueckii* subsp. *bulgaricus* FTDC 8011 (7.75 mm) against *Salmonella typhimurium* (Table 4). The most common extracellular metabolites of lactobacilli and bifidobacteria are organic acids, such as acetic and lactic acids, which contribute to a pH-lowering effect. The inhibitory action of undissociated organic acid molecules has been reported to be one of the main mechanisms for inhibiting the colonization of intestinal pathogens (Cui et al. 2000; Tambekar and Bhutada 2010).

Lacobacillus acidophilus FTDC 2131, *L. casei* BT 1268, *L. acidophilus* FTDC 8933, and *B. longum* FTDC 8643 showed no inhibition towards *P. aeruginosa.* In addition, all strains of lactobacilli and bifidobacteria studied also did not show any inhibition of *K. pneumoniae, P. aeruginosa*, and *B. subtilis*. Curtis and Sperandio (2011) demonstrated that *P. aeruginosa* utilizes an autoinducer regulatory system, based on the production of 2-alkyl-4(1*H*)-quinolones that acted as antibiotics and inhibited the growths of *S. aureus* and *Candida albicans*. Also, some microorganisms have been shown to produce a protection response system that provides protection against severe inhibition conditions over long periods (Tadesse et al. 2005). These may have provided greater tolerance towards the inhibitory effects of lactobacilli and bifidobacteria.

Conclusions

Based on the results of our study, six *Lactobacillus* and *Bifidobacterium* strains have potential value as probiotics. These include *L. casei* BT 1268, *L. acidophilus* FTDC 0291, *L. acidophilus* FTDC 2131, *L. acidophilus* BT 1088, *L. acidophilus* FTDC 8933, and *L. delbrueckii* subsp. *bulgaricus* FTDC 8913, all of which showed good colonization and adhesion to mucin, tolerance to acid and bile, as well as the production of potential antimicrobial substances towards certain enteric pathogens. These strains could be further assessed for possible benefits in vivo and used as cultures for the development of new dairy products.

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