The Journal of Microbiology (2012) Vol. 50, No. 4, pp. 603–612
DOI 10.1007/s12275-012-2045-1

Screening for Probiotic Properties of Strains Isolated from Feces of Various Human Groups

Sathyaseelan Sathyabama*, Rajendran Vijayabharathi, Palanisamy Bruntha devi, Manohar Ranjith kumar, and Venkatesan Brindha Priyadarisini

Clinical Biotechnology Laboratory, Department of Microbial Biotechnology, Bharathiar University, Coimbatore, India

(Received January 30, 2012 / Accepted April 23, 2012)

The present study searched for potential probiotic strains from various human fecal samples. A total of 67 aerobic and 38 anaerobic strains were isolated from 5 different categories of human feces. Systematic procedures were used to evaluate the probiotic properties of the isolated strains. These showed about 75–97% survivability in acidic and bile salt environments. Adhesion to intestinal cell line Caco-2 was also high. The isolates exhibited hydrophobic properties in hexadecane. The culture supernatants of these strains showed antagonistic effects against pathogens. The isolates were resistant to a simulated gastrointestinal environment *in vitro*. Of the 4 best isolates, MAbB4 (*Staphylococcus succinus*) and FIdM3 (*Enterococcus fecium*), were promising candidates for a potential probiotic. *S. succinus* was found to be a probiotic strain, which is the second such species reported to date in this particular genus. A substantial zone of inhibition was found against *Salmonella* spp., which adds further support to the suggestion that the probiotic strain could help prevent intestinal infection. This study suggested that the human flora itself is a potential source of probiotics.

Keywords: probiotics, bile tolerance, Caco-2, hydrophobicity, antimicrobial activity

Introduction

Probiotics are live microorganisms that alter the enteric microbiota and confer a beneficial effect on the health of all age groups when supplied in adequate amounts (Park *et al*., 2002; Liu *et al*., 2007). They are fastidious in nature and their survival at high numbers during the passage through the human gastrointestinal tract (GIT) is a major challenge for effective delivery of these beneficial bacteria (Annan *et al*., 2008). The beneficial bacteria associated with probiotic activity have frequently been lactic acid bacteria (LAB) or bifidobacteria.

During the first few weeks of life, a complex microbial community develops in the human GIT, which is dominated by bifidobacteria and later, when exposed to the environment, another microflora starts developing as the bifidobacteria decrease (Embleton and Yates, 2008). About 100 trillion bacterial cells from at least 400 different species are found in the human intestine, far exceeding the numbers of host cells (Backhed *et al*., 2005). Throughout adult life, the homeostatic state of the gut can be altered by environmental pollution and other stress conditions in the modern life style, which may lead to acute and chronic disorders. Extrinsic factors affecting the microbial pattern in infants were found to be: mode of delivery, type of infant feeding, gestational age, infant hospitalization and antibiotic use (Penders *et al*., 2006). Hence, to combat this problem, probiotics can be administered to re-establish the bacterial population in the GIT by restoring the balance of the microflora, thereby avoiding further infection or some intestinal disorders. For bacteria to act as a probiotic, a number of issues of safety, function and technology should be fulfilled. The beneficial effect of probiotic strains depends on their ability to survive during passage through the stomach, their ability to establish themselves in the GIT and to compete with pathogens (Liu *et al*., 2007).

Probiotic bacteria often belong to the genera *Lactobacillus* or *Bifidobacteria*, though some probiotic strains of *Streptococcus*, *Candida*, and *E. coli* have also been reported. Though it is generally accepted that they provide health effects, knowledge about the mechanism of action is limited. Suggested modes of action for their health effects include the production of antimicrobial substances that might be bacteriocins or organic acids, competition for nutrients by siderophore formation, competitive exclusion of pathogen binding and modulation of the immune system (Parvez *et al*., 2006; Bao *et al*., 2010). There is an increasing interest in the development of adjunct or alternative therapies based on bacterial replacement, using probiotics isolated from the natural intestinal flora (Forestier *et al*., 2001; Collado *et al*., 2007).

In an attempt to obtain probiotic strains, bacteria were isolated from fecal samples taken from male and female volunteers in various categories. These isolates were screened for probiotic properties by determining their tolerance to pH and bile salts, antimicrobial susceptibility, antibiotic sensitivity, *in vitro* adhesion ability to intestinal cells and some additional physiological properties. This study also examined the transit tolerance of strains and characterized them taxonomically.

*For correspondence. E-mail: sathyabama85@gmail.com; Tel.: +9198651 47072

Materials and Methods

Sample collection

Fecal samples were collected from various groups of human volunteers, viz male and female adults, male and female children and pregnant women. Five samples were collected from each category. The adults were aged between 25 to 50 years and children were aged between 3 months to 10 years.

Isolation of intestinal bacteria

For each sample, one gram of freshly voided feces was collected, homogenized in sterile saline solution (0.85% NaCl w/v) and stirred for 10 min. They were further serially diluted and plated on MRS agar (deMan Rogosa and Sharpe, Himedia) for anaerobes in an anaerobic jar at 37°C for 48 h and BHI agar (Brain Heart Infusion, Himedia, India) for aerobes incubated at 37°C for 24 h (Gu *et al.*, 2008). Morphologically different colonies were selected randomly. The selected colonies were purified by repeated streaking on the appropriate agar media and subcultured periodically.

Screening for acid and bile tolerance

Initial screening of the 105 isolated microorganisms for their probiotic features was performed by determining the tolerance of cultures against various concentrations of acid and bile salt. Tolerance for pH was studied by incubating the isolates in appropriate medium adjusted to pH 2.0 and 3.0. One milliliter of overnight bacterial suspension was adjusted to 0.6 OD at 620 nm using a UV-Visible spectrophotometer, then inoculated into 10 ml sterile medium and incubated at 37°C. Samples were withdrawn periodically (at 0, 30, 60, 90, and 120 min) to determine the cell concentration by measuring OD at 620 nm (Liu *et al.*, 2007). The 20 most pH tolerant isolates, based on survival rate, were further studied for tolerance to bile salt concentration (0.3, 0.5, and 0.8% of bile salt in BHI/MRS broth) by determining the cell concentration at the same time intervals as above (measuring OD at 620 nm) (Aswathy *et al.*, 2008).

Tolerance to other inhibitory substances

The best four isolates of the 20 tested, which were tolerant to both pH and bile, were selected, based on their overall ranking from the pH and bile testing (MS Excel, 2010) and were tested for tolerance to NaCl (3, 6, 9, and 12%) and phenol (0.2, 0.4, 0.6%) as before (Aswathy *et al.*, 2008). Overall ranking of the isolates was performed using the average of survival rate and stability to 120 min, with the help of the Microsoft Excel tool.

Cell culture

Caco-2 cells were used to determine the adhesion capacity of the isolates. Cells were purchased from NCCS, Pune, India and were grown in Dulbecco-modified Eagle's Minimal Essential Medium (25 mM-glucose) (DMEM) (Sigma, USA), supplemented with 20% (v/v) inactivated (30 min 56°C) fetal bovine serum (Sigma). Cells were seeded at a concentration of 4×10^4 cells/cm^2. Monolayers of Caco-2 cells were prepared on glass coverslips, which were placed in six-well tissue-culture plates. The culture medium was changed every 24 h. To determine the number of Caco-2 cells in a monolayer, cells were trypsinized for 10 min at 22°C and counted using a haemocytometer.

Adhesion assay

Caco-2 monolayers were washed twice with phosphate-buffered saline (PBS) (138 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). For each adhesion assay, 0.5 ml of bacterial suspension was mixed with DMEM medium (0.5 ml) and the final concentration of bacteria was 2×10^8 bacteria/ml. The bacterial suspension was added to each well of the tissue culture plate, which was then incubated at 37°C in 5 % CO_2. After incubation for 1h, cells were washed five times with sterile PBS, fixed with methanol, Gram stained and examined microscopically under oil immersion. Each assay was conducted in triplicates. For each glass coverslip monolayer, the number of adherent bacteria was counted in 20 random microscopic areas. Adhesion of bacteria was expressed as number of bacteria adhering to 100 Caco-2 cells (Chauviere *et al.*, 1992).

Treatment of bacteria with various agents

To characterize the bacterial binding determinants, the bacterial cultures were subjected to various treatments. Bacterial cells were incubated with trypsin, lipase and pepsin (2.5 mg/ml, Himedia) for 60 min at 37°C and with sodium metaperiodate (10 mg/ml, Himedia) for 60 min at room temperature. These bacteria were then used for the adhesion assay as described above (Chauviere *et al.*, 1992).

Scanning Electron Microscopy (SEM)

Cells for scanning electron microscopy were grown on glass coverslips. The specimen was then examined with a scanning electron microscope (Chauviere *et al.*, 1992) to confirm the adhesion of the isolates to the Caco-2 human intestinal cells.

Antimicrobial activity assay

The inhibitory potential of the isolated strains was investigated using a modified agar well assay method as described by Schillinger and Lucke (1987). Indicator organisms such as *Salmonella*, *Escherichia coli*, *Klebsiella* etc. were used. Overnight cultures of these pathogens were swabbed on nutrient agar plates in which wells were cut. Supernatants (50 µl) of 12 h isolated cultures grown in BHI/MRS broth were added to the wells. They were further incubated for 24 h at 37°C. Activity was quantified by measuring the diameter of any clear zone. Supernatant from medium broth without inoculum was used as control.

Antibiotic resistance study

Antibiotic resistance patterns of the strains were determined by a disk diffusion method using the Kirby-Bauer technique (Bauer *et al.*, 1966). Muller-Hinton agar plates were plated evenly with 50 µl of isolates using a sterile swab. Antibiotic discs were placed over the plates, which were then incubated

Table 1. Effect of pH on the growth of the selected isolates (OD at 620 nm)

Isolates	0 min		30 min		60 min		90 min		120 min	
	2	3	2	3	2	3	2	3	2	3
MAbB4	0.22±0.02	0.15±0.04	0.19±0.03	0.15±0.04	0.19±0.02	0.16±0.03	0.18±0.09	0.16±0.02	0.17±0.01	0.16±0.05
MAdB1	0.10±0.03	0.19±0.05	0.09±0.04	0.11±0.03	0.11±0.03	0.11±0.02	0.11±0.02	0.11±0.03	0.11±0.06	0.11±0.08
MAdB2	0.09±0.01	0.13±0.02	0.09±0.03	0.08±0.04	0.10±0.03	0.09±0.03	0.10±0.03	0.10±0.04	0.09±0.02	0.09±0.01
MIaB1	0.10±0.02	0.10±0.01	0.12±0.04	0.12±0.05	0.12±0.04	0.12±0.02	0.10±0.04	0.14±0.05	0.07±0.02	0.14±0.05
MIaB2	0.17±0.02	0.17±0.03	0.19±0.06	0.19±0.09	0.19±0.05	0.19±0.03	0.09±0.05	0.19±0.05	0.09±0.02	0.19±0.06
MIeB1	0.15±0.04	0.13±0.04	0.17±0.07	0.15±0.01	0.18±0.02	0.16±0.04	0.17±0.04	0.16±0.02	0.16±0.07	0.14±0.06
MIeB4	0.20±0.06	0.38±0.03	0.20±0.04	0.18±0.02	0.16±0.02	0.13±0.05	0.16±0.02	0.07±0.07	0.14±0.06	0.07±0.03
FIaB1	0.29±0.04	0.12±0.07	0.18±0.05	0.13±0.06	0.17±0.03	0.14±0.03	0.17±0.03	0.13±0.03	0.15±0.05	0.12±0.02
FIaB3	0.23±0.06	0.25±0.08	0.15±0.06	0.15±0.08	0.16±0.04	0.15±0.07	0.17±0.04	0.13±0.05	0.13±0.03	0.13±0.02
FIcB1	0.24±0.03	0.14±0.04	0.14±0.07	0.14±0.03	0.14±0.03	0.16±0.06	0.14±0.01	0.16±0.07	0.14±0.04	0.16±0.03
FIdB3	0.26±0.04	0.21±0.07	0.21±0.06	0.21±0.02	0.21±0.05	0.21±0.05	0.21±0.02	0.20±0.08	0.21±0.02	0.19±0.02
FAaB1	0.23±0.02	0.21±0.09	0.22±0.08	0.18±0.03	0.23±0.06	0.18±0.01	0.23±0.03	0.19±0.05	0.21±0.09	0.19±0.01
FAbB2	0.32±0.03	0.14±0.04	0.15±0.05	0.14±0.04	0.14±0.07	0.13±0.02	0.14±0.02	0.12±0.02	0.11±0.03	0.11±0.04
FAbB3	0.33±0.03	0.18±0.05	0.22±0.06	0.20±0.02	0.21±0.03	0.18±0.02	0.20±0.01	0.18±0.03	0.18±0.04	0.17±0.03
PWaB3	0.19±0.01	0.10±0.03	0.14±0.04	0.13±0.07	0.11±0.02	0.11±0.01	0.11±0.01	0.11±0.01	0.11±0.04	0.10±0.03
MAdM1	0.18±0.04	0.14±0.04	0.13±0.07	0.13±0.06	0.13±0.04	0.13±0.01	0.14±0.03	0.12±0.03	0.13±0.05	0.11±0.02
MIaM1	0.17±0.01	0.14±0.02	0.15±0.02	0.13±0.08	0.13±0.01	0.14±0.03	0.12±0.02	0.13±0.04	0.12±0.07	0.13±0.05
FIaM1	0.25±0.03	0.21±0.06	0.21±0.02	0.20±0.04	0.19±0.02	0.19±0.04	0.18±0.01	0.10±0.02	0.18±0.04	0.01±0.04
FIdM3	0.18±0.04	0.19±0.06	0.13±0.04	0.20±0.06	0.14±0.03	0.21±0.01	0.14±0.01	0.21±0.03	0.13±0.06	0.21±0.02
FAbM2	0.26±0.03	0.21±0.04	0.14±0.05	0.21±0.03	0.14±0.02	0.22±0.01	0.13±0.03	0.21±0.01	0.13±0.06	0.20±0.01

Each value in the table is the mean±standard deviation of triplicates

for 24 h–48 h at 37°C. The susceptibility and resistance of the strains were determined as per recommendation of NCCLS (NCCLS, 1997).

Hydrophobicity cell surface test

The degree of hydrophobicity of the strains was determined by the method used by Aswathy *et al.* (2008) with slight modification. This method was based on adhesion of cells. Cultures were grown in 10 ml of appropriate broth, centrifuged at 6,000×g for 5 min and the cell pellet was washed and re-suspended in 10 ml of Ringers solution (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$, and 0.01% $NaHCO_3$). The absorbance at 600 nm was measured (OD_A). Then 4 ml of cell suspension was mixed with an equal volume of n-hexadecane (apolar solvent), chloroform (acid solvent) and ethyl acetate (basic solvent). They were mixed thoroughly by vortexing for 2 min. The two phases were allowed to separate for 30 min and the absorbance of the aqueous phase (OD_B) was read at 600 nm.

$$\% \text{ Bacteria adhesion} = [(OD_A - OD_B) \times 100]/OD_A$$

Table 2. Effect of bile salts on the growth of the selected isolates (Each value in the table is the mean±SD of triplicates, OD at 620 nm)

Isolates	0 min			30 min			60 min			90 min			120 min		
	0.3%	0.5%	0.8%	0.3%	0.5%	0.8%	0.3%	0.5%	0.8%	0.3%	0.5%	0.8%	0.3%	0.5%	0.8%
MAbB4	0.21±0.02	0.23±0.01	0.22±0.05	0.23±0.07	0.26±0.02	0.24±0.01	0.27±0.04	0.29±0.01	0.27±0.02	0.32±0.01	0.34±0.10	0.32±0.02	0.38±0.02	0.39±0.02	0.37±0.02
MAdB1	0.08±0.03	0.20±0.04	0.15±0.06	0.16±0.04	0.17±0.01	0.15±0.01	0.15±0.02	0.17±0.04	0.15±0.01	0.16±0.03	0.17±0.03	0.16±0.03	0.16±0.02	0.18±0.02	0.15±0.02
MAdB2	0.08±0.02	0.04±0.01	0.08±0.03	0.12±0.03	0.13±0.02	0.12±0.03	0.19±0.01	0.20±0.06	0.18±0.04	0.25±0.11	0.23±0.04	0.24±0.05	0.24±0.02	0.23±0.02	0.21±0.02
MIaB1	0.09±0.01	0.03±0.01	0.03±0.01	0.12±0.02	0.11±0.03	0.10±0.01	0.13±0.05	0.12±0.01	0.12±0.05	0.09±0.01	0.07±0.01	0.06±0.01	0.06±0.02	0.05±0.02	0.03±0.02
MIaB2	0.17±0.04	0.15±0.02	0.13±0.02	0.19±0.02	0.18±0.05	0.15±0.03	0.27±0.06	0.20±0.02	0.17±0.06	0.16±0.02	0.15±0.02	0.13±0.05	0.14±0.02	0.12±0.02	0.11±0.02
MIeB1	0.21±0.06	0.13±0.02	0.12±0.06	0.19±0.04	0.15±0.02	0.14±0.01	0.35±0.06	0.31±0.05	0.27±0.12	0.62±0.15	0.54±0.14	0.53±0.17	0.64±0.02	0.59±0.02	0.47±0.02
MIeB4	0.22±0.06	0.14±0.02	0.15±0.02	0.18±0.01	0.14±0.01	0.12±0.01	0.16±0.02	0.12±0.02	0.12±0.01	0.16±0.01	0.13±0.03	0.12±0.03	0.15±0.02	0.12±0.02	0.11±0.02
FIaB1	0.28±0.09	0.13±0.01	0.09±0.03	0.20±0.01	0.19±0.06	0.13±0.01	0.33±0.01	0.30±0.09	0.21±0.04	0.53±0.13	0.47±0.17	0.33±0.11	0.54±0.02	0.48±0.02	0.32±0.02
FIaB3	0.18±0.01	0.12±0.01	0.10±0.01	0.13±0.06	0.12±0.06	0.11±0.02	0.13±0.02	0.15±0.01	0.12±0.01	0.10±0.01	0.11±0.05	0.09±0.02	0.09±0.02	0.10±0.02	0.08±0.02
FIcB1	0.25±0.03	0.15±0.01	0.14±0.01	0.15±0.05	0.12±0.05	0.12±0.02	0.14±0.03	0.11±0.01	0.10±0.02	0.08±0.02	0.06±0.01	0.05±0.02	0.07±0.02	0.06±0.02	0.05±0.02
FIdB3	0.22±0.04	0.14±0.01	0.11±0.04	0.19±0.06	0.16±0.04	0.18±0.03	0.20±0.01	0.18±0.03	0.14±0.03	0.22±0.04	0.18±0.01	0.13±0.05	0.23±0.02	0.18±0.02	0.11±0.02
FAaB1	0.23±0.03	0.13±0.02	0.13±0.01	0.18±0.06	0.15±0.05	0.15±0.04	0.20±0.02	0.16±0.02	0.16±0.02	0.19±0.06	0.17±0.05	0.14±0.04	0.17±0.02	0.16±0.02	0.10±0.02
FAbB2	0.14±0.05	0.17±0.02	0.08±0.01	0.24±0.01	0.19±0.06	0.20±0.06	0.32±0.04	0.30±0.05	0.32±0.05	0.35±0.07	0.39±0.15	0.39±0.15	0.37±0.02	0.40±0.02	0.46±0.02
FAbM3	0.13±0.06	0.05±0.02	0.05±0.01	0.19±0.04	0.16±0.02	0.17±0.02	0.28±0.04	0.25±0.02	0.24±0.03	0.34±0.09	0.33±0.08	0.30±0.01	0.35±0.02	0.35±0.02	0.33±0.02
PWaB3	0.14±0.06	0.06±0.02	0.07±0.02	0.19±0.04	0.17±0.01	0.18±0.06	0.31±0.06	0.29±0.04	0.29±0.04	0.41±0.12	0.40±0.12	0.39±0.03	0.42±0.02	0.41±0.02	0.40±0.02
MAdM1	0.29±0.02	0.24±0.02	0.21±0.05	0.37±0.03	0.37±0.08	0.28±0.08	0.36±0.07	0.32±0.07	0.31±0.11	0.39±0.14	0.39±0.16	0.36±0.05	0.39±0.02	0.38±0.02	0.31±0.02
FIdM3	0.32±0.03	0.17±0.02	0.14±0.01	0.51±0.07	0.25±0.09	0.29±0.08	0.32±0.10	0.24±0.07	0.25±0.02	0.32±0.11	0.21±0.09	0.25±0.03	0.29±0.02	0.19±0.02	0.19±0.02
FAbM2	0.20±0.04	0.21±0.02	0.21±0.03	0.25±0.07	0.21±0.02	0.20±0.07	0.32±0.09	0.25±0.04	0.24±0.04	0.32±0.09	0.27±0.12	0.26±0.03	0.35±0.02	0.31±0.02	0.29±0.02

Table 3. Overall ranking of isolated strains based on pH and bile tolerance

S.No	Isolates	Rank
1	MAbB4	1
2	FAbM2	2
3	FAbM3	2
4	FIdM3	4
5	FIdB3	5
6	MAdM1	6
7	MIeB1	7
8	FIaB1	7
9	FAaB1	9
10	FAbB2	10
11	MIaB2	11
12	FIaB3	12
13	MIeB4	13
14	FIaM1	13
15	PWaB3	15
16	FIcB1	16
17	MAdB1	17
18	MAdB2	18
19	MIaM1	19
20	MIaB1	20

Transit tolerance in gastrointestinal tract

Simulated gastric and small intestinal juices were prepared fresh daily. A simulated gastric juice was prepared by suspending 3mg/ml pepsin (1:3,000) in sterile saline and adjusted the pH to 3.0 with 1.0 M HCl. 1.0 ml of 24 h old cultures was subjected to centrifugation (10,000 rpm, 10 min) and washed twice with sterile saline before being re-suspended in simulated gastric juice. Resistance was assessed in terms of viable colony count and enumerated after incubation at 41°C for 2 h. After 120 min of gastric digestion, cells were harvested and suspended in simulated intestinal fluid which contained 1 mg/ml pancreatin and 7% fresh chicken bile at pH 8.0. The suspension was incubated at 41°C for 6 h and the viable count was determined (Musikasang *et al.*, 2005).

Identification of the isolates

The isolates with the greatest probiotic effect were identified by biochemical and molecular characterization. The DNAs were isolated, amplified by PCR and then the 16S rRNA genes in the PCR products were sequenced. The primers used were universal primers 8F 5′-AGAGTTTGATCCTGG CTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′. The sequences were submitted to the GenBank database and accession numbers were obtained.

Results

Fecal collection and isolation

One hundred and five bacterial strains were isolated from samples of 5 different categories of feces. Of these, 67 strains were aerobic and 38 were anaerobic. The strains that had low probiotic properties and the isolates unable to grow well at low pH were excluded. The best 20 isolates tolerant of pH 2.0 and 3.0 were selected based on their higher ranking and were used for further study (data not shown).

Screening of acid and bile tolerance

The effect of acidic conditions (pH 2.0 and 3.0) on the viability of the 20 isolates is shown in Table 1. About 75-97% survival was observed at low pH. After exposure for 120 min, strains FIdB3, FAaB1, FIaM1, FIdM3, and FAbM2 were found to survive at pH 2.0 at a higher rate compared to other isolates. This indicated that the tolerance to low pH was strain specific.

Bile tolerance has been described as an important factor in addition to to pH tolerance for survival and growth of microbes in the intestinal tract. The strains MAbB4, MIeB1, FIaB1, FAbB2, and PWaB3 showed tolerance to 0.8% of bile salts for 120 min (Table 2). However, MIaM1 and FIaM1 showed no growth at any of the bile concentrations. From an overall ranking (MS Excel, 2010), based on the data for survival rate and stability of the isolates for 2 h under various pH and bile conditions, the best four isolates were selected for further studies of their properties (Table 3).

Tolerance to other inhibitory substances

Assessments of probiotic potential also include the evaluation of tolerance to inhibitory substances such as NaCl and phenol. The top ranked 4 isolates from the pH and bile assays were assessed for their tolerance to these two inhibitory substances. The strains showed different degrees of resistance to phenol. Of the four isolates, FAbM2 and FIdM3 were able to tolerate 0.6% phenol. The others only tolerated 0.4% phenol and their numbers decreased after 24 h of incubation. With regard to salt tolerance, MAbB4, FAbM2, and FIdM3 were able to tolerate 12% NaCl with a slight decrease in their survival rate. Table 4 shows the growth tolerance towards these inhibitory substances as measured at 620 nm.

Table 4. Tolerance of the best strains towards growth inhibitory substances (OD at 620 nm)

Isolates	NaCl								Phenol					
	3%		6%		9%		12%		0.2%		0.4%		0.6%	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
MAbB4	0.37±0.06	1.58±0.12	0.37±0.13	1.43±0.17	0.37±0.06	0.99±0.05	0.39±0.03	0.86±0.03	0.29±0.01	1.16±0.08	0.31±0.05	1.60±0.15	0.27±0.07	0.4±0.03
FAbM2	0.19±0.02	1.28±0.13	0.22±0.05	0.84±0.07	0.21±0.05	0.31±0.03	0.21±0.03	0.20±0.04	0.22±0.02	0.83±0.09	0.27±0.04	0.77±0.06	0.31±0.06	0.39±0.04
FAbM3	0.25±0.04	1.77±0.02	0.23±0.06	1.49±0.14	0.25±0.03	1.38±0.13	0.29±0.01	0.98±0.06	0.28±0.04	1.21±0.11	0.24±0.02	0.20±0.01	0.24±0.03	0.11±0.03
FIdM3	0.19±0.03	0.64±0.12	0.19±0.01	0.55±0.02	0.20±0.01	0.26±0.03	0.21±0.02	0.13±0.01	0.23±0.04	1.10±0.14	0.22±0.03	0.21±0.02	0.36±0.03	0.32±0.04

Each value in the table is the mean±standard deviation of triplicates

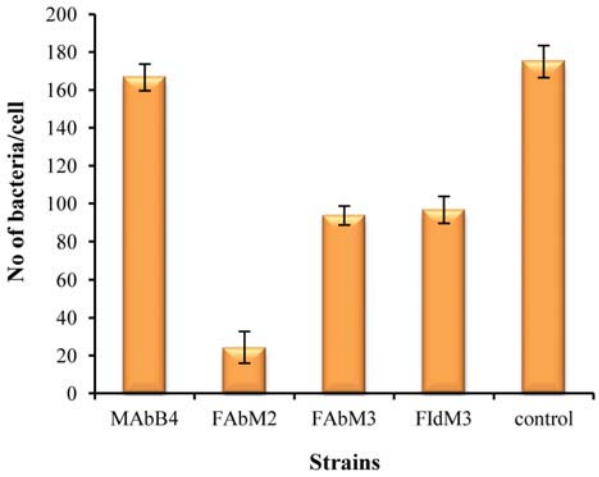

Fig. 1. The adhesion capacity of isolates to Caco-2 cells expressed as number of adhered bacteria (CFU/cell). Number of bacterial cells was counted from 20 random microscopic fields

Adhesion of strains to Caco-2 cells

High attachment was observed for MAbB4 was 166.66 CFU/cell, which was nearly that of the control strain *Bifidobacterium longum* (175 CFU/cell). Low attachment to the differentiated Caco-2 cells was observed for FAbM2 with an average of 24.33 CFU/cell and moderate attachment was observed with FIdM3 and FAbM3 at 96.66 and 93.66 CFU/cell respectively (Fig. 1). Since the adhesion property shows the closeness of host-microbe contact and the likelihood of competitive exclusion, the isolates with good attachment were used for further studies. The adhesions of the isolates were observed by light microscope using Gram stain (Fig. 2A). The isolate with highest adhesion (MAbB4) was also observed by scanning electron microscopy, which showed that this isolate binds to Caco-2 cells without any cell damage. It may use the brush borders of the Caco-2 cells for attachment (Fig. 2B).

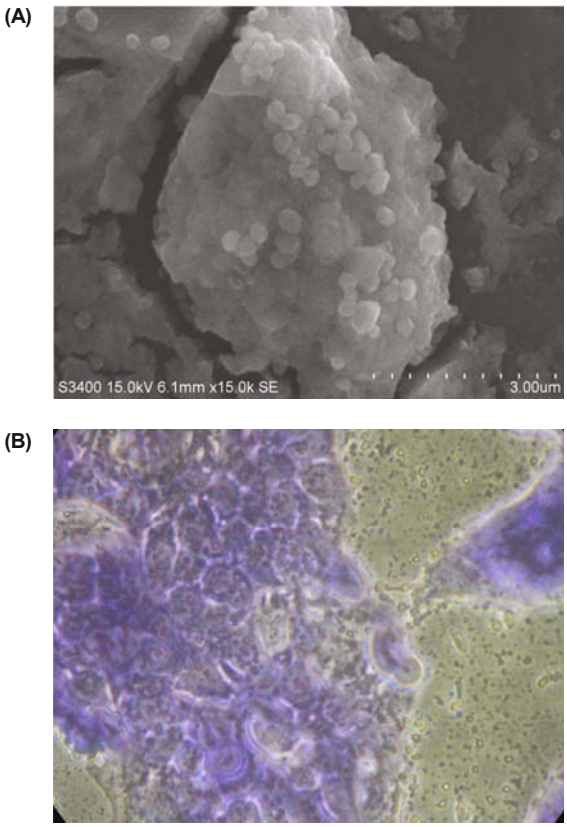

Fig. 2. Adhesion of MAbB4 strain to Caco-2 cells. (A) Light microscopy using the Gram staining method. (B) Scanning electron microscopy

Table 5. Adhesion of treated bacteria to Caco-2 cells

Isolates	Lipase	Trypsin	Sodium metaperiodate
MAbB4	+	+	+
FAbM2	+	-	+
FAbM3	+	+	+
FIdM3	+	+	-
Control	+	+	-

+, Denotes adhesion on Caco-2 cells after the treatment of bacterial cells
-, Denotes no adhesion or weak adhesion

To characterize the bacterial determinants involved in adhesion, bacteria were subjected to various treatments. Treatment of bacterial isolates with trypsin and pepsin lowered the adhesion of isolates FAbM2 and MAbB4. Similarly, lower attachment was found for FIdM3 subjected to sodium metaperiodate treatment (Table 5).

Antimicrobial activity assay

All the isolates showed a strong inhibition against *Salmonella* spp., but there was only a weak inhibition against other pathogens (Table 6). There was no inhibition against *Klebsiella* spp., similar to the strains isolated from human volunteers in China (Gu *et al*., 2008). No zone of inhibition was found in the control well.

Antibiotic resistance

The susceptibility patterns of the isolates against various antibiotics varied (Table 7). Two of the four isolates were resistant to both nystatin and methicillin, with the other two resistant to one but not both. There was no pattern of resistance to the other antibiotics tested, with most strains somewhat susceptible to most antibiotics, having a maximum of 4 cm and minimum of 0.2 cm clear zone.

Table 6. Antimicrobial activities towards pathogens

Microbes tested	Zone of clearance (mm)			
	MAbB4	FAbM2	FAbM3	FIdM3
Escherichia coli	-	-	-	5±0.6
Salmonella sp.	4±0.7	7±0.6	2±0.3	10±1.2
Enterococcus sp.	-	7±0.4	5±0.8	12±0.9
Klebsiella sp.	-	3±0.3	-	-
Staphylococcus aureus	5±0.6	-	-	4±0.2

Each value in the table is the mean±standard deviation of triplicates
-, represents absence of inhibition

Table 7. Antibiotic susceptibility test using the Kirby-Bauer method

Antibiotic disks	Diameter of zone of inhibition (cm)			
	MAbB4	FAbM2	FAbM3	FIdM3
Nystatin	-	-	-	0.8±0.2
Erythromycin	2.0±0.4	-	1.6±0.3	1.0±0.4
Streptomycin	1.6±0.6	0.6±0.1	1.0±0.2	1.0±0.1
Amikacin	1.9±0.5	0.7±0.1	1.2±0.2	0.9±0.1
Ciprofloxacin	2.9±0.5	1.8±0.5	4.0±0.8	-
Bacitracin	1.5±0.2	1.1±0.1	-	1.3±0.6
Gentamycin	2.8±0.9	1.6±0.2	3.0±0.4	1.7±0.5
Tetracyclin	2.6±0.8	2.8±0.4	2.1±0.4	1.2±0.6
Methicillin	1.8±0.2	-	-	-
Ceftrioxane	1.9±0.3	2.0±0.1	2.1±0.3	1.6±0.3

Each value in the table is the mean±standard deviation of triplicates

Hydrophobic cell surface test

The highest adhesion value for the microorganisms was obtained with chloroform with a maximum of 72% for FAbM2, which was higher than the results reported by Souza *et al.* (2007) showing only 47.99% (Fig. 3). The results reveal that hydrophobicity towards hexadecane was found in all the four isolates and comparatively there was less hydrophobicity towards ethyl acetate for all the isolates.

Transit tolerance in GI tract

The survival rate of the probiotics in the simulated GI transit is presented in Fig. 4. MAbB4 and FIdM3 were equally resistant to the pepsin and pancreatin treatment, which was more or less similar to that of the control. They showed a survival rate of 88% where the control was about 87%. The next highest survival was observed for FAbM3 with 71%, whereas the worst resistance was seen for FAbM2 with 47%.

Identification of the isolates

With regard to morphological and biochemical characteristics, all the strains were Gram-positive cocci, catalase and oxidase positive and H_2S negative except for FAbM3, which

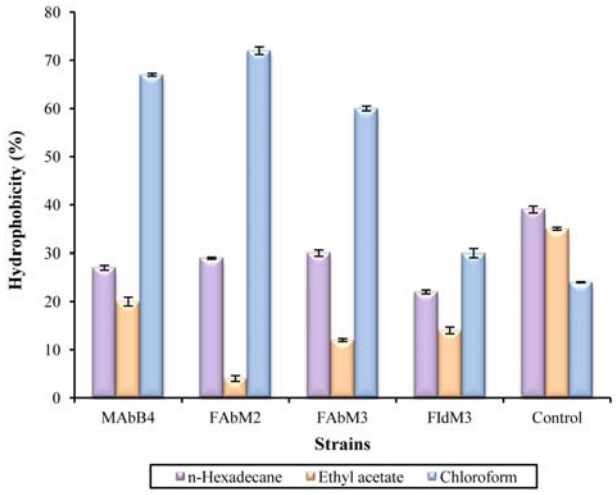

Fig. 3. Percent hydrophobicity of selected strains to hydrocarbons. Assay was done in triplicates and the bars represent the standard deviation

was a Gram-positive rod, and catalase and oxidase negative. The DNA sequences obtained were compared with database entries using BLASTN and the resulting phylogenetic trees were constructed using Phylip 3.6. (Figs. 5A, 5B, 5C, and 5D). From the results, the strains MAbB4 FAbM2, FAbM3 and FIdM3 were found to be *Staphylococcus succinus* (JF920302), *Enterococcus durans* (JF920299), *Lactobacillus plantarum* (JF920301), and *Enterococcus fecium* (JF920300), respectively. *S. succinus* MAbB4 is the second reported probiotic species in this genus, with *S. hominis* reported to be probiotic previously (Sung *et al.*, 2010).

Discussion

One of the baseline properties for probiotics is the ability to survive in the upper GI tract. Before reaching the distal part of the intestinal tract and exerting their probiotic effect,

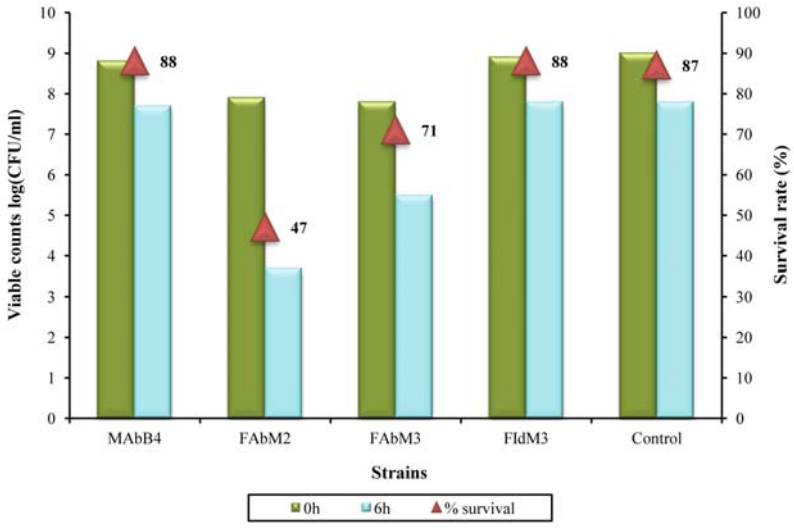

Fig. 4. Survival rates of the isolates on sequential incubation in simulated gastric juice and simulated intestinal juice.

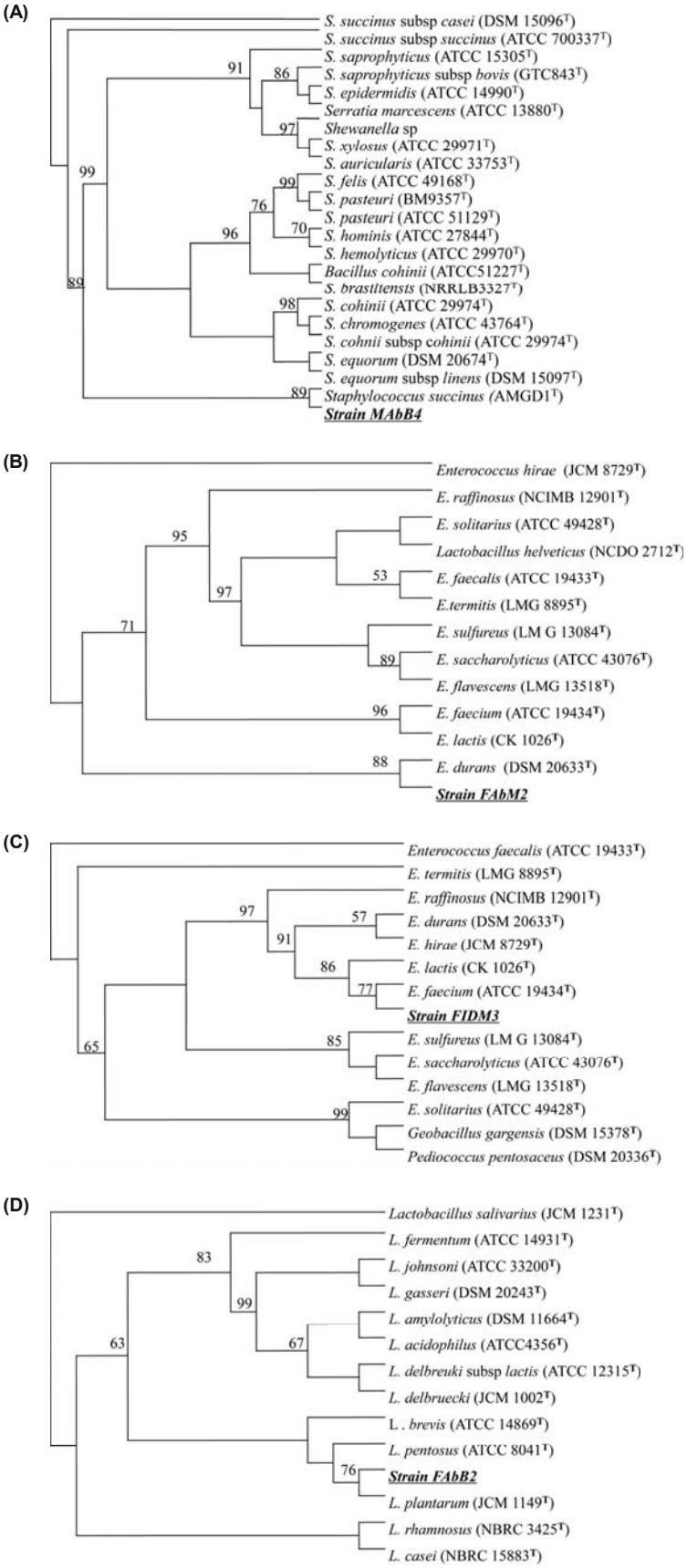

Fig. 5. Phylogenetic relationship of (A) MAbB4 (B) FAbM2 (C) FAbM3 (D) FIdM3. (A) shows the phylogeny between strain MAbB4 and members of the genus *Staphylococcus* based on 16S rRNA gene sequences. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis. The scale bar indicates the evolutionary distance value between sequences. (B) shows the phylogeny between strain FAbM2 and members of the genus *Enterococcus* based on 16s rRNA gene sequences. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis. The scale bar indicates the evolutionary distance value between sequences. (C) shows the phylogeny between strain FAbM3 and members of the genus *Lactobacillus* based on 16s rRNA gene sequences. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis. The scale bar indicates the evolutionary distance value between sequences. (D) shows the phylogeny between strain FIdM3 and members of the genus *Enterococcus* based on 16S rRNA gene sequences. The numbers are the estimated confidence levels, expressed as percentages, for the positions of the branches, determined by bootstrap analysis. The scale bar indicates the evolutionary distance value between sequences.

these bacteria must survive during transition through the stomach and upper part of the intestinal tract (Bao *et al.*, 2010). There was no loss of viability in pH 3.0, however, half of the isolates were not viable in pH 2.0. A survival rate of 96% for 90 min at pH 3.0 was observed in the strains isolated from human feces samples of volunteers inhabiting Bama longevity villages (Liu *et al.*, 2007). In comparison, some of our isolates showed a significant survival rate for 120 min. In some reported cases, none of the strains grew at pH 2.5 and only very few were reasonably acid tolerant (Jacobsen *et al.*, 1999; Aswathy *et al.*, 2008). In contrast, the strains isolated here did grow and some tolerated pH 2.0, similar to the strains isolated by Gu *et al.* (2008). Bile at 0.3% is the concentration used in selection of probiotic organisms for humans (Pancheniak and Soccol, 2005) who showed that a 0.3% bile tolerant organism is used as a probiotic for swine. The daily average biliary flow is around 2 L for a 40 kg swine, whereas a 70 kg adult human produces 400–800 ml of bile daily. Hence, 0.3% tolerable isolates could be potential probiotics for humans. A study by Gu *et al.* (2008) reported that 13 strains survived a 3 h exposure to 0.3% ox gall, which is in good agreement with our results, as our strains survived more than 2 h.

Lan-Szu and Bart (1999) have substantiated that strains selected as probiotic bacteria should tolerate acid and bile at least for 90 min, which is the time needed to cross the barrier; hence, in the present study the tolerance was checked for 120 min. In the case of strains isolated from humans of the Bama longevity village (Liu *et al.*, 2007), 0.1 and 0.2% bile salt had only a slight influence; however at 0.3% bile concentration, strains showed a critical effect. Different bifidobacterial strains showed distinct effects in their study. This is in accordance with the present data where the 20 strains showed varied effects. The resistance to phenol is an important probiotic property because phenol is formed in the intestine due to bacterial deamination of some aromatic amino acids derived from dietary and endogenous proteins. The strains were highly resistant to NaCl for 24 h. *L. plantarum* was also found to be tolerant to 0.4% phenol for 24 h but *L. johnsonii* BFE663 and BFE6128 strains were completely inhibited after 24 h (Pinto *et al.*, 2006). Higher salt concentration allows the bacteria to initiate metabolism, thereby producing acid, which in turn inhibits the growth of non-desirable organisms. Hence, tolerance to NaCl is also an important property for an efficient probiotic. The results were found to be in accordance with the findings of Aswathy *et al.* (2008) that an isolate from cabbage was tolerant to 12% NaCl and four other isolates were tolerant only up to 8%.

Adhesion of the probiotic microorganisms to the intestinal mucosa is a prerequisite feature for colonization and for antagonistic activity against enteropathogens. The adherence assay was carried out using the human intestinal cell line Caco-2, a well characterized cellular lineage established from human colonic adenocarcinoma (Fernandez *et al.*, 2009). The adhesion capacity of isolates is strain dependent and hence highly variable (Lahteinen *et al.*, 2010). Related results were given by the study observed by Chauviere *et al.* (1992) where the isolates from humans showed higher adherence to the intestinal cell line than the dairy strains. The host specificity for the adherence of lactobacilli is closely connected to the presence of some specific receptor molecules on the host cell, which can be recognized by specific molecules of the bacterial cell (Fuller *et al.*, 1978). The low attachment of MAbB4 suggests that the receptors for attachment might be a proteinaceous component. However, there was no significant difference in attachment when isolates were treated with trypsin and pepsin similar to that of Barrow *et al.* (1980). FIdM3 was observed to have no attachment after sodium metaperiodate treatment, indicating that the adhesion between a cell surface component and adhesion-promoting extracellular proteins might have been mediated by carbohydrates (Chauviere *et al.*, 1992). Lipase treatment did not show any effect on the adherence, as was also seen for the strain *Lactobacillus fermentum* 14 and *Streptococcus salivarius* 312 (Barrow *et al.*, 1980). Altogether these results suggest that there are quite significant differences in the mechanism of adhesion among the isolates; hence, a detailed study on the determinants involved in the interaction between cell surface components and the extracellular proteins is needed.

The antimicrobial activity is based on oxidative properties that result in irreversible changes in the microbial cell membrane. Many researchers and clinicians are interested in preventing or curing intestinal infections with probiotics, especially those caused by *E. coli*, *Shigella* sp., and *Salmonella* sp. The antibacterial activity of LAB is often due to the production of organic acids, diacetyl, hydrogen peroxide and bacteriocin or to bactericidal proteins formed during lactic acid fermentation. The isolates from the gastrointestinal tract of chicks were observed to have a higher inhibitory activity against *Salmonella* sp. than we observed, with a clear zone of 13–40 mm (Schillinger and Lucke, 1987).

The antibiotic susceptibility tests indicated that the four strains were resistant towards many of the antibiotics tested. A study observed by Ronka *et al.* (2003) was similar showing resistance to ciprofloxacin, methicillin, vancomycin and others with susceptibility. Isolates from yoghurt, sheep excreta and cabbage were found to be resistant to vancomycin and erythromycin (Chauviere *et al.*, 1992) whereas three of the four isolates studied here showed susceptibility to erythromycin.

Bacterial surface properties are associated with attachment to a variety of substrates, which in turn are associated with hydrophobicity. A study on *L. casei* by Mishra and Prasad (2005) revealed that there was higher hydrophobicity, about 40%, towards hexadecane, inferring that, *L. casei* possesses a hydrophobic character, which correlates in some cases with adhesion to epithelia. There was a high hydrophobicity with a maximum of 98.3% for *Bifidobacteria bifidum* A8; moreover, hydrophobicity was above 70% for most of the other *Bifidobacteria* sp. (Gueimondea *et al.*, 2005). It has been suggested that changes in bifidobacterium adherence may be related to metabolic changes leading to modification in the cellular membrane (Zavaglia *et al.*, 2002). As the hydrophobicity of the cell increases, the level of adhesion also increases (Rijnaarts *et al.*, 1993), although Conway and Reginald (1989) reported that there is a lack of correlation between the capacity for adhesion and hydrophobicity.

Transit tolerance of the isolates showed that the strains were tolerant to all stress conditions tested, with good sur-

vival rates. Similarly, the isolates taken from people of Bama village showed a maximum of 87.8%, 89.9%, and 89.4% survival and minimum of 48.9% (Liu *et al*., 2007). A maximum of only 43.68% survival was observed after sequential incubation in pepsin and pancreatin by Musikasang *et al*. (2005). In general, the acid tolerance of lactic acid bacteria has been linked to the induction of H^+ ATPase activity (Guo *et al*., 2009). It also depends on the composition of the cytoplasmic membrane. The tolerance is highly influenced by the type of bacterial strain, type of growth medium and the incubation conditions (Hood and Zotolla, 1988; Madureira *et al*., 2005). However, there was a tremendous decline in the viable counts after 24 h of incubation. Hence, those isolates with the most promising *in vitro* properties in the study were further subjected to *in vivo* studies using poultry trials and were found to be potential probiotics with health benefits.

Although the different strains vary in their probiotic properties, the findings suggest that the human flora could be an excellent source of probiotic microorganisms. Since human samples have not been explored extensively for probiotics, this study was designed with human fecal samples. Two isolates namely MAbB4 and FIdM3 showed all the essential probiotic properties including full tolerance to acid and bile, potent colonization and adherence to Caco-2 cell line. Moreover the isolates showed a strain specific inhibition against some intestinal pathogens and were resistant to simulated gastric juice and intestinal juice. The strains were identified as *Staphylococcus succinus* MAbB4, an unreported probiotic culture and *Enterococcus fecium* FIdM3. The other two strains were *Enterococcus durans* and *Lactobacillus plantarum*. Not all desirable probiotic characteristics were present in a single isolate, although many isolates displayed varying individual promising capabilities. Hence, these cultures might be useful for preventing intestinal infections. The *in vivo* study confirms the safety of the bacterial isolates for probiotic purposes, though additional research on their intrinsic genetic characteristics may be necessary.

References

Annan, N.T., Borza, A.D., and Hansen, L.T. 2008. Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. *Food Res. Int.* **41**, 184–193.

Aswathy, R.G., Ismail, B., John R.P., and Nampoothiri, K.M. 2008. Evaluation of the probiotic characteristics of newly isolated Lactic acid bacteria. *Appl. Biochem. Biotechnol.* **151**, 244–255.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. 2005. Host-bacterial mutualism in the human intestine. *Science* **307**, 1915–1920.

Bao, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y., and Zhang, H. 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Contr.* **21**, 695–701.

Barrow, P.A., Brooker, B.E., Fuller, R., and Newport, M.J. 1980. The attachment of bacteria to the gastric epithelium of the pig and its importance in the micro-ecology of the intestine. *J. Appl. Bacteriol.* **48**, 147–154.

Bauer, R.W., Kirby, M.D.K., Sherris, J.C., and Turck, M. 1966. Antibiotic susceptibility testing by standard single disc diffusion method. *Am. J. Clin. Path.* **45,** 493–496.

Chauviere, G., Coconnier, M.H., Kerneis, S., Fourniat, J., and Servin, A.L. 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. *J. Gen. Microbiol.* **138**, 1689–1696.

Collado, M.C., Meriluoto, J., and Salminen, S. 2007. *In vitro* analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. *Food Res. Int.* **40,** 629–636.

Conway, P.L. and Reginald, F.A. 1989. Role of erythrosin in the inhibition of adhesion of *Lactobacillus fermentum* strain 737 to mouse stomach tissue. *J. Gen. Microbiol.* **135**, 1167–1177.

Embleton, N.D. and Yates, R. 2008. Probiotics and other preventive strategies for necrotizing enterocolitis. *Semin. Fetal Neonatal Med.* **13**, 35–43.

Fernandez, M.F., Boris, S., and Barbes, C. 2009. Probiotic properties of human lactobacilli strains to be used in the gastro intestinal tract. *J. Appl. Microbiol.* **94**, 449–455.

Forestier, C., De Champs, C., Vatoux, C., and Joly, B. 2001. Probiotic activities of *Lactobacillus casei rhamnosus*: *in vitro* adherence to intestinal cells and antimicrobial properties. *Res. Microbiol.* **152**, 167–173.

Fuller, R., Barrow, P.A., and Brooker, B.E. 1978. Bacteria associated with the gastric epithelium of neonatal pigs. *J. Appl. Microbiol.* **35**, 582–589.

Gu, R.X., Yang, Z.Q., Li, Z.H., Chen, S.L., and Luo, Z.L. 2008. Probiotic properties of lactic acid bacteria isolated from stool samples of longevous people in regions of Hotan, Xinjiang and Bama, Guangxi, China. *Anaerobe* **14**, 313–317.

Gueimondea, M., Noriega, L., Margolles, A., de los Reyes-Gavilan, C.G., and Salminen, S. 2005. Ability of Bifidobacterium strains with acquired resistance to bile to adhere to human intestinal mucus. *Int. J. Food Microbiol.* **101**, 341–346.

Guo, Z., Wang, J., Yan, L., Chen, W., Liu, X., and Zhang, H. 2009. *In vitro* comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. *LWT - Food Sci. Technol.* **42**, 1640–1646.

Hood, S.K. and Zotolla, E.A. 1988. Effect of low pH on the viability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *J. Food Sci.* **53**, 1514–1516.

Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Perregaard, A., Sandstrom, B., Tvede, M., and Jakobsen, M. 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl. Microbiol.* **65**, 4949–4956.

Lahteinen, T., Malinen, E., Koort, J.M.K., Mertaniemi-Hannus, U., Hankimo, T., Karikoski, N., Pakkanen, S., Laine, H., Sillanpaa, H., Soerholm, H., and Palva, A. 2010. Probiotic properties of *Lactobacillus* isolate originating from porcine intestine and feces. *Anaerobe* **16**, 293–300.

Lan-szu, C. and Bart, W. 1999. Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci*. **82**, 23–31.

Liu, Z., Jiang, Z., Zhou, K., Li, P., Liu, G., and Zhang, B. 2007. Screening of bifidobacteria with acquired tolerance to human gastrointestinal tract. *Anaerobe* **13**, 215–219.

Madureira, A.R., Pereira, C.I., Truszkowska, K., Gomes, A.M., Pintado, M.E., and Malcata, F.X. 2005. Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. *Int. Dairy J.* **15**, 921–927.

Mishra, V. and Prasad, D.N. 2005. Application of *in vitro* methods for selection of *Lactobacillus casei* strains as potential probiotics. *Int. J. Food Microbiol*. **103**, 109–115.

Musikasang, H., Tani, A., Kittikun, A.H., and Maneerat, S. 2005. Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. *World J. Microbiol. Biotech*. **25**,

1337–1345.

NCCLS. 1997. Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.

Pancheniak, E.R. and Soccol, C.R. 2005. Biochemical characterization and identification of probiotic Lactobacillus for swine *B.CEPPA Curitiba* **23**, 299–310.

Park, Y., Lee, J., Kim, Y., and Shin, D. 2002. Isolation and characterization of lactic acid bacteria from feces of newborn baby and from Dongchimi. *J. Agric Food Chem.* **50**, 2531–2536.

Parvez, S., Malik, K.A., Ah Kang, S., and Kim, H.Y. 2006. Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.* **100**, 1171–1185.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van den Brandt, P.A., and Stobberingh, E.E. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *J. Pediatr.* **118**, 511–521.

Pinto, M.G., Charles, M.A.P., Schillinger, U., and Holzapfel, W.H. 2006. *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. *Int. J. Food Microbiol.* **109**, 205–214.

Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Lyklema, J., and Zehnder, A.J.B. 1993. Bacterial adhesion under static and dynamic conditions. *Appl. Microbiol.* **59**, 3255–3265.

Ronka, E., Malinen, E., Saarela, M., Rinta-Koski, M., Aarnikunnas, J., and Palva, A. 2003. Probiotic and milk technological properties of *Lactobacillus brevis*. *Int. J. Food Microbiol.* **83**, 63–74.

Schillinger, U. and Lucke, F.K. 1987. Identification of Lactobacilli from meat and meat products. *Food Microbiol.* **4**, 199–208.

Souza, M.R., Moreira, J.L., Barbosa, F.H.F., Cerqueira, M.O.P., Nunes, A.C., and Nicoli, J.R. 2007. Influence of intensive and extensive breeding on lactic acid bacteria isolated from *Gallus gallus domesticus ceca*. *Vet. Microbiol.* **120**, 142–150.

Sung, C., Kim, B.G., Kim, S., Joo, H.S., and Kim, P.I. 2010. Probiotic potential of *Staphylococcus hominis* MBBL 2–9 as anti-*Staphylococcus aureus* agent isolated from the vaginal micro biota of a healthy woman. *J. Appl. Microbiol.* **108**, 908–916.

Zavaglia, A., Kociubinski, G., Perez, P., Disalvo, E., and de Antoni, G. 2002. Effect of bile on the lipid composition and surface properties of bifidobacteria. *J. Appl. Microbiol.* **93**, 794–799.