CHAPTER 2

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *LACTOBACILLUS* STRAINS FROM HUMAN SOURCES

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2.1. Introduction

A complex microbiota of more than a thousand different bacterial species with a population of about 10^{14} cells inhabits the oral cavity, gastrointestinal tract (GIT), upper respiratory tract, vagina and skin, with a major part of this microflora residing in the human gut (Neish 2009). There is a general agreement on the important role of the gastro-intestinal (GI) microflora on the health status of men and animals. There are several reports available which provides evidences for the health promoting properties of lactobacilli (Pereira *et al*. 2003; LeBlanc *et al*. 2008; Nikolova *et al*. 2009). These organisms are known to favourably alter the intestinal microflora balance, promote intestinal integrity and mobility, inhibit the growth of pathogens and provide increased resistance to infection (Kim *et al*. 2008). They are able to survive in the gastrointestinal (GI) tract and are safe for the consumers (Tuomola *et al*. 2001; de Vries *et al*. 2006). The survivability and colonization in the digestive tract are considered critical to ensure optimal functionality and expression of health promoting physiological functions by probiotics (Kaushik *et al*. 2009). For survivability in the gut, the organism must tolerate acidic pH and bile toxicity of the digestive tract. In the context of their effective colonization, the ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of bacteria used as probiotics (FAO/WHO 2001). Therefore, adhesion is considered as a potential probiotic marker along with other

desirable attributes for screening of novel probiotic lactobacilli (Xu *et al*. 2009; Li *et al*. 2015). Functionally, they may neutralize the effect of pathogens by interfering with the downstream effects of toxins produced (Hugo *et al*. 2008). They may also express bacteriocin, lactic and acetic acid, and other antibacterial like substances against pathogens, besides competition for adhesion sites and inhibit the binding of pathogens to the mucosal surface (Neeser *et al*. 2000; Collado *et al*. 2006) although the nature of their binding and competition is still not very clear. They may also show antioxidative and immunomodulatory activities (Songisepp *et al*. 2004; Wells 2011). In a broad way, the isolation from human, capability to tolerate acidic pH and bile, antimicrobial activity and high adhesion ability are principle desirable properties in a potential probiotic strain (Dunne *et al*. 2001).

Lactobacillus rhamnosus strain GG (LGG) is a widely accepted standard probiotic strain (Sikorska and Smoragiewicz 2013; Damodharan *et al*. 2015), that has been extensively studied (Nawaz *et al*. 2011; Pisano *et al*. 2014). This has been shown to be resistant to acid and bile, have strong adhesion to cells, suppress bacterial enzyme activity, and produce antimicrobial substances (Lee *et al*. 2000). Earlier reports have confirmed that LGG is successful for the treatment of acute diarrhea and prevention of inflammatory bowel diseases (Bousvaros *et al*. 2005; Doron *et al*. 2005). In order to develop indigenous isolates that may be expected to fare better than non-indigenous isolates in view of local food habits, this study was aimed at isolating autochthonous lactobacilli from human faecal samples and check for their potential probiotic properties. These properties include ability to tolerate acid and high bile salt concentration, antimicrobial activity against various

pathogens and resistance to various antibiotics. The isolates were also checked for their ability to adhere to the intestinal epithelial cell-lines HT-29 and Caco-2 and also their ability to inhibit the adhesion of an enteropathogen to these cell-lines and compare the individual abilities with the established probiotic strain – *L. rhamnosus* GG.

2.2. Materials and methods

2.2.1. Standard *Lactobacillus* strain

Standard probiotic strain *L. rhamnosus* GG (LGG) was obtained as a kind gift from Dr. Shira Doron (MD, Department of Medicine, Tufts Medical Centre, Boston, Massachusetts, USA).

2.2.2. Isolation and identification of *Lactobacillus* strains

Isolation was carried out from human sources (human gut isolates) of six healthy human adults (more than seventy years of age). For isolation, 1 g of faecal sample was suspended in 10 mL sterile saline, mixed vigorously and allowed to settle. A loopful of the suspension was then streaked on Rogosa SL agar (a selective medium for *Lactobacillus* isolation; Himedia, Mumbai, India) plates containing 100 µg/mL cyclohexamide (SRL, Mumbai, India) to avoid the growth of yeast. The plates were then incubated at 37 ºC under static condition till sufficient growth was observed. About five to seven isolated colonies were then picked from each Rogosa SL agar plate. Isolated colonies were then transferred to MRS (deMan, Rogosa and Sharpe; Himedia) agar plates. The plates were then incubated at 37 ºC for 48 h. The isolates were then studied for their Gram's nature and biochemical

character like presence of catalase. The isolates which showed Gram positive nature and catalase negative phenotype were further analysed with the help of molecular techniques to identify them at species level.

Molecular identification of isolates was carried out by amplification of 16S–23S rRNA gene intergenic regions as reported by Tannock *et al*. (1999). For this, 16S–23S rRNA gene intergenic region of the isolates resuspended in sterile water was amplified from a single colony, using primers 16-1A (5'-GAATCGCTAGTAATCG-3') and 23-1B (5'-GGGTTCCCCCATTCGGA-3') by colony PCR (Table 2.1 and Table 2.2).

Table 2.1. Reaction system for 16S-23S rRNA gene intergenic region amplification.

Reaction Components	Volume (µL)
R.O water	16.9
10X Buffer for Taq DNA Polymerase	2.5
dNTP mix (2.5 mM each)	2.0
Forward primer (100 pmol/µL)	0.8
Reverse primer (100 pmol/µL)	0.8
Taq DNA polymerase (2.5 U/µL) (Sigma-Aldrich)	1.0
Colony suspension	2
Total volume	**25**

Table 2.2. Conditions for 16S-23S rRNA gene intergenic region amplification.

Steps	Temperature (°C)	Time	No. of cycles
Pre-cycle denaturation	94	5 min	1
Denaturation	94	45 sec	
Primer annealing	55	30 sec	30
Primer extension	72	1 min	
Post-cycle elongation	72	6 min	1

Agarose gel electrophoresis

The PCR amplified products were analyzed by electrophoresis on 0.8% agarose gel in 0.5X TBE followed by staining with ethidium bromide and viewed under UV light.

Composition of Tris Borate EDTA (5X; for 1 L solution)

Tris-Cl	54 g
Boric acid	27.5 g
EDTA (0.5 M)	20 mL
Distilled water (DW)	Make up volume to 1 L

2.2.3. Bile and acid tolerance

The method used for testing bile tolerance was that reported by Gilliland *et al*. (1984) with a few modifications. Briefly, 1×10^6 CFU/mL cells from overnight grown culture of each isolate was inoculated in MRS broth (control) as well as in MRS broth containing 1%, 2% and 3% (w/v) bile salts (sodium cholate and sodium deoxycholate; Sigma, USA) and incubated for 24 h at 37 ºC; following which the absorbance was measured at 600 nm to check their bile tolerance. To check their survival rate at different bile concentrations, the isolates were grown overnight in MRS broth, washed with PBS and 20 µL each of selected lactobacilli (1×10^8 CFU/mL) was transferred to 980 µL MRS broth containing 0.3% and 1% (w/v) oxbile and incubated at 37 ºC for 2 h. The samples were taken at 0 h and 2 h of incubation and cultures were plated on MRS agar plates after appropriate dilutions. The plates were incubated at 37 °C for 48 h and enumerated.

Further, for determining the survival rate of different isolates under acidic condition, the isolates were grown overnight in MRS broth, washed with PBS and 20 µL each of selected lactobacilli (1×10^8 CFU/mL) was transferred to 980 µL of acidic buffer (Casey *et al*. 2004) and incubated for 2 h at 37 ºC. The samples were taken at 0 min and 2 h of incubation, plated on MRS agar plates after appropriate dilutions and the enumeration was done following 48 h incubation at 37 ºC. The % survival rate of bile and acid tolerance was calculated from the mean of $\log_{10}$ CFU/mL of the cultures after 2 h treatment with respect to their mean of $\log_{10}$ CFU/mL before treatment (0 h).

Composition of acidic buffer (For 1L solution)

D-glucose	3.5 g
$CaCl_2$	0.11 g
NaCl	2.05 g
KH_2PO_4	0.6 g
KCl	0.37 g

pH was adjusted to 2.5 using HCl

2.2.4. Antimicrobial activity

To check the antimicrobial activity of the various lactobacilli, agar spot assay was used, as described by Schillinger and Lucke (1989) with minor modifications. The antimicrobial activity was determined against *Shigella dysenteriae* (*S. dysenteriae*), *Staphylococcus aureus* (ATCC 6538) (*S. aureus*), *Pseudomonas aeruginosa* (ATCC 25668) (*P.*

aeruginosa), *Salmonella typhi* (MTCC 733) (*S. typhi*), *Proteus vulgaris* (*P. vulgaris*) and *Escherichia coli* O26:H11 (*E. coli*) obtained from the culture collection facility of the department. Briefly, 2 µL each of lactobacilli (1×10^8 CFU/mL) were spotted on MRS agar plate and incubated for 24 h at 37 ºC. Following growth at these spots, 15 mL of Luria soft agar (0.6% w/v) containing 150 µL of overnight grown indicator bacteria was poured over the MRS agar plate. The plates were then incubated for 24 h at 37 ºC and the zone of inhibition was measured according to method of Baccigalupi *et al*. (2005).

2.2.5. Antibiotic susceptibility assay

Antibiotic susceptibility assay was done by disc diffusion method. Briefly, 100 µL of each of the lactobacilli (1×10^8 CFU/mL) grown overnight in MRS broth was spread evenly on the MRS agar plate and various antibiotic discs were placed on it. Commercially available antibiotic discs (Hi-media, Mumbai, India) containing Chloramphenicol (30 µg), Kanamycin (30 µg), Gentamycin (10 µg), Vancomycin (30 µg), Bacitracin (10 U), Ofloxacin (5 µg), Cephalothin (30 µg), Tobramycin (30 µg) and Cloxacillin (5 µg) were used. The plates were then incubated for 24 h at 37 °C and inhibition zone was measured inclusive of the diameter of the discs.

2.2.6. Cell culture

The human colonic adenocarcinoma cell lines, HT-29 and Caco-2 were obtained from National Centre for Cell Science (NCCS), Pune, India, and were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and maintained at 37 °C with 5% CO_2 and 95% air atmosphere. The

medium was supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, USA), 10 mM non-essential amino acids, 1 mM sodium pyruvate and 50 µg/mL gentamicin. The media lacked gentamicin whenever antibiotic free medium was used.

2.2.7. Adhesion to HT-29 and Caco-2

To check the adhesive ability of the isolates, HT-29 and Caco-2 cells were seeded at a density of 10^5 cells/well in 24-well standard tissue culture plates (Corning Inc., Corning, NY, USA) and incubated at 37 ºC in 5% CO_2/95% air atmosphere until confluency. The monolayers were then used for the adhesion assay. Before the assay, the monolayers were pre-incubated in Dulbecco's Modified Eagle Media (DMEM) (pH 6.5) without antibiotic for 2 h. For the assay, overnight grown *Lactobacillus* cells were harvested by centrifugation for 2 min at 10,000×*g* and 4 ºC, washed twice with Dulbecco's phosphate buffered saline (DPBS), pH 7.0 (Sigma-Aldrich, USA) and cell density was adjusted to desired level by measuring absorbance at 600 nm. Wells with the monolayers were treated separately with 1×10^8 viable cells of each bacterial cell suspension and incubated at 37 ºC for 150 min in 5% CO_2/95% air atmosphere. Un-adhered bacterial cells were then withdrawn from the wells and the HT-29 monolayers were washed twice with 1 mL DPBS each. The HT-29 and Caco-2 cells were lyse d by treatment with 0.5 mL 0.05% (v/v) Triton X-100 in DPBS for 20 min at 37 ºC. The cell lysate including bound lactobacilli were plated after appropriate dilutions on MRS agar plates and the enumeration was done following 48 h incubation at 37 ºC. At the end of each experiment, three randomly preselected unused wells were trypsinized and numbers of both HT-29 and Caco-2 cells were counted on

hemocytometer. The average value of both HT-29 and Caco-2 cells count were used for expressing the adhered bacteria per HT-29 and Caco-2 cell respectively.

2.2.8. Antagonistic effect of *Lactobacillus* isolates on adhesion of *E. coli* to HT-29 cells and Caco-2

Lactobacilli were also assayed for competitive inhibition (lactobacilli and pathogen were provided with an equal chance for binding at the same ratio), adhesion inhibition (investigate the lactobacilli ability to protect intestinal cells from being colonized by pathogen), and displacement inhibition (the ability of lactobacilli to displace colonized pathogen from intestinal epithelium) on HT-29 and Caco-2 cell lines using the isolates and enteropathogen *E. coli* O26:H11. For this, post confluent HT-29 and Caco-2 cells were pre incubated with antibiotic free medium for 2 h. Bacterial cells were processed as described above and cell density was adjusted to obtain 1×10^8 CFU/mL in sterile DMEM. For competitive adhesion assay, 100 µL each of lactobacilli and enteropathogen were added to the wells with HT-29 and Caco-2 monolayers at the same time, while in adhesion inhibition assay, lactobacilli cells were added before addition of the pathogen. To investigate the ability of lactobacilli to displace colonized pathogens from intestinal epithelium, the pathogen was first allowed to adhere to HT-29 or Caco-2 monolayers before lactobacilli adhesion and then co-incubated for 90 min. At the end of each assay, the adhered bacteria were released by treatment with 0.5 mL 0.05% (v/v) TritonX-100 in DPBS for 20 min at 37 ºC, and the both HT-29 and Caco-2 lysate including bound enteropathogen was plated after appropriate dilution on Luria agar plates. The enumeration was done after 18-24 h incubation at 37 ºC. Adhesion of enteropathogen alone was taken as control, and the

number of bacteria adhered to HT-29 and Caco-2 was considered as 100% to express percentage inhibition.

2.2.9. Statistical analysis

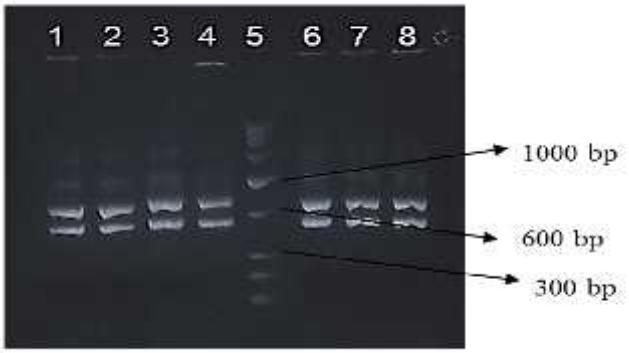
Values are given as mean values and standard deviations of triplicate independent experiments. Significant ANOVAs were followed by Dunnett's test in all the assays to compare with respect to positive control (LGG) ($P < 0.05$). All analysis was conducted using GraphPad Prism 6.01.

2.3. Results

2.3.1. Isolation and identification of *Lactobacillus* strains from human sources

Several lactobacilli were isolated from adult human faeces which were analyzed by microscopic observation, biochemical test and on the basis of 16S-23S rRNA gene intergenic spacer region. Microscopic and biochemical analysis revealed that, out of 127 isolates screened (14-17 per sample), 22 isolates were Gram positive rods and found negative for the production of catalase, and which grew on Rogosa SL and MRS agar plates. Further, these 22 isolates were selected for subsequent molecular identification on the basis of 16S–23S rRNA gene intergenic region amplification. The agarose gel profile of amplification products generated from the 16S-23S rRNA gene intergenic region of 9 isolates was compared and found similar to that of standard strain *L. rhamnosus* GG (LGG). Further the smaller fragment of amplified product was excised, eluted and re-amplified using the same set of primers. The smaller intergenic fragment of these nine

isolates was sequenced and based on sequence alignment with the NCBI database, only six were found to belong to the *Lactobacillus* genus. The 16S-23S rRNA gene intergenic region sequences were submitted to GenBank and their corresponding accession numbers were received as shown in Table 2.3.



Lane 1: *L. rhamnosus* GG
Lane 2: *L. salivarius* GPI-1(S)
Lane 3: *L. salivarius* GPI-4
Lane 4: *L. plantarum* GRI-2
Lane 5: Low range DNA ruler
Lane 6: *L. fermentum* GPI-1(B)
Lane 7: *L. fermentum* GPI-7
Lane 8: *L. fermentum* GPI-6

Figure 2.1. 0.8% Agarose gel stained with ethidium bromide for 16S-23S rRNA gene intergenic region PCR amplification.

Table 2.3. 16S-23S rRNA gene intergenic sequence analysis and GenBank submission of selected isolates.

No.	Isolate	Source (Human isolate of gut origin)	Accession number	16S–23S sequence based species identification	Similarity of 16S–23S sequence to that of reference strain in GenBank (%)
1	GPI-1(S)	Human gut isolate	JX118837	*L. salivarius*	86
2	GPI-4	Human gut isolate	JX118830	*L. salivarius*	91
3	GRI-2	Human gut isolate	JX118835	*L. plantarum*	99
4	GPI-1(B)	Human gut isolate	JX118836	*L. fermentum*	98
5	GPI-6	Human gut isolate	JX118833	*L. fermentum*	99
6	GPI-7	Human gut isolate	JX118831	*L. fermentum*	94

2.3.2. Bile tolerance

The above mentioned isolates were also inoculated in MRS media containing different concentration of bile salts to check their ability to survive in it. MRS medium lacking bile salt was used as a control where as 1%, 2% and 3% bile salt concentrations in MRS broth

were used to check the tolerance of the lactobacilli by measuring absorbance at 600 nm after 24 h incubation of these isolates (1×10^6 CFU/mL) at 37 ºC. Observations revealed that, 3% bile salt was the maximum tolerable concentration for the isolates (Fig. 2.2). The survival of lactobacilli was also examined by the difference in viable cell counts following 0 min and 2 h incubation in MRS containing 0.3% and 1% bile salts and the results were subjected to statistical analysis ($P < 0.05$). As given in Table 2.4, isolate *L. fermentum* GPI-6 showed best survival rate (91% at 0.3% bile salt, 89% at 1% bile salt) in contrast to standard strain LGG (81% at 0.3% and 1% bile salt). As evident from Table 2.4, there are other isolates that were intermediate between these two.

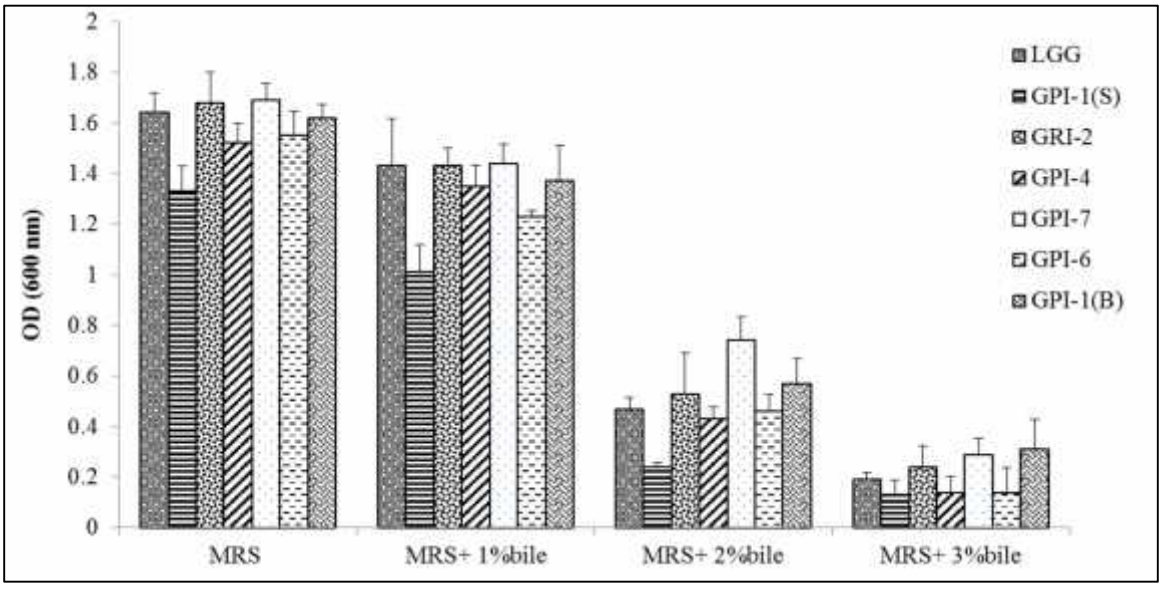


Figure 2.2. Bile salt tolerance: growth of lactobacilli under different bile salt concentrations. Each bar shows the mean value and error bar as standard deviation of three independent experiments. The strains were compared with the positive control (LGG).

Table 2.4. Survival rate of *Lactobacillus* strains in the presence of 0.3% and 1% bile salts.

	Pre- incubation *Lactobacillus* concentration (log_{10} CFU/mL)			**Bile tolerance (0.3%)† *Lactobacillus* concentration (log_{10} CFU/mL)**			**Bile tolerance (1%)† *Lactobacillus* concentration (log_{10} CFU/mL)**		
Cultures	**Mean**	**SD**	**Survival rate (%)**	**Mean**	**SD**	**Survival rate (%)**	**Mean**	**SD**	**Survival rate (%)**
LGG	8.36	0.01	100	6.78	0.03	81	6.76	0.16	81
GPI-1(S)	7.92	0.02	100	6.54	0.23	83	6.22	0.10	79
GPI-4	8.12	0.03	100	6.53	0.04	80	5.41*	0.2	67
GRI-2	8.43	0.03	100	7.21*	0.13	86	5.42*	0.18	64
GPI-1(B)	7.33	0.02	100	6.62	0.01	90	4.83*	0.65	66
GPI-6	7.79	0.01	100	7.08*	0.10	91	6.93	0.08	89
GPI-7	7.28	0.04	100	6.36*	0.12	87	6.26	0.11	86

Mean values (log_{10} CFU/mL) and standard deviations of *Lactobacillus* strains challenged to the presence of 0.3% and 1% bile concentrations. *Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). †Results were obtained from three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

2.3.3. Acid tolerance

Survival rate of lactobacilli in acidic buffer (pH 2.5) was examined by the difference in viable cell counts following 0 min and 2 h incubation, as shown in Table 2.5. All the isolates showed better tolerance to acidic pH of 2.5 as compared to standard strain LGG (73%), isolates *L. plantarum* GRI-2 and *L. salivarius* GPI-4 showed no reduction in viability while isolate *L. fermentum* GPI-1(B) showed 84% survival rate in acidic pH which was statistically higher than that of LGG.

Table 2.5. Survival rate of different *Lactobacillus* strains under acidic condition.

	Acid tolerance (pH 2.5)					
	***Lactobacillus* concentration (log_{10} CFU/mL) 0 h**			***Lactobacillus* concentration (log_{10} CFU/mL)† 2 h**		
Cultures	**Mean**	**SD**	**Survival rate (%)**	**Mean**	**SD**	**Survival rate (%)**
LGG	6.76	0.39	100	4.93	0.06	73
GPI-1(S)	6.52	0.09	100	5.12	0.08	79
GPI-4	6.25	0.32	100	6.53*	0.21	104
GRI-2	7.16	0.47	100	7.21*	0.13	100
GPI-1(B)	6.31	0.72	100	5.31*	0.12	84
GPI-6	6.52	0.14	100	4.84	0.02	74
GPI-7	6.92	0.16	100	5.10	0.08	74

Mean values (log_{10} CFU/mL) and standard deviations of *Lactobacillus* strains challenged to acidic condition. *Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). †Results were obtained from three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

2.3.4. Antimicrobial activity

Antimicrobial activity of the isolated lactobacilli was examined against Gram-negative *S. dysenteriae*, *P. aeruginosa*, *E. coli* O26:H11, *P. vulgaris* and *S. typhi* as well as Gram-positive *S. aureus*. The *L. fermentum* isolates GPI-7 and GPI-1(B) had the highest zone of inhibition against both Gram-positive and Gram-negative bacteria, followed by *L. salivarius* isolates GPI-1(S), GPI-4 and *L. plantarum* GRI-2. All the strains tested showed zone of inhibition towards *P. vulgaris* as shown in Table 2.

Table 2.6. Spectrum of antimicrobial activity exhibited by various lactobacilli.

Antimicrobial activity (Zone of inhibition in mm) † (Mean ± SD) *						
Cultures	***E. coli***	***P. aeruginosa***	***S. Typhi***	***S. aureus***	***S. dysenteraie***	***P. Vulgaris***
LGG	21.67±1.15 (+++)	16.33± 2.08 (++)	14.67± 0.58 (+)	21.33± 0.58 (+++)	17.00± 1.00 (++)	11.33± 0.58 (+/-)
GPI-1(S)	17.33±2.52* (++)	16.67±0.58 (++)	10.33± 0.58* (+/-)	23.33± 2.08 (+++)	19.67± 2.52 (++)	22.33± 2.08* (+++)
GPI-4	19.00±2.65 (++)	11.00± 3.61* (+/-)	19.00± 1.00* (++)	22.33± 1.15 (+++)	18.00± 2.00 (++)	22.00± 1.73* (+++)
GRI-2	21.67±1.15 (+++)	19.67± 2.08 (++)	10.67± 0.58* (+/-)	22.67± 2.08 (+++)	22.00± 2.65* (+++)	19.33± 1.53* (++)
GPI-1(B)	25.67±1.15* (+++)	17.67± 4.16 (++)	15.00± 1.73 (+)	14.67± 0.58* (+)	32.67± 0.58* (+++)	34.33± 1.53* (+++)
GPI-6	12.00.±1.73* (+/-)	11.33± 1.15* (+/-)	10.67± 0.58* (+/-)	12.00± 1.73* (+/-)	11.33± 1.15* (+/-)	13.33± 0.58 (+)
GPI-7	25.33± 0.58* (+++)	17.33± 1.15 (++)	21.00± 1.00* (+++)	19.67± 1.15 (++)	18.67± 1.53 (++)	24.33± 1.53* (+++)

†The inhibition zones ≤ 12 mm, 13-15 mm, 16-20 mm and more than 20 mm were classified as strains of no (+/-), mild (+), strong (++) and very strong (+++) inhibition, respectively. *Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). †Results were obtained from three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

2.3.5. Antibiotic susceptibility assay

In antibiotic susceptibility tests, commercially available antibiotic discs (Hi-media, India) containing Chloramphenicol (30 µg), Kanamycin (30 µg), Gentamycin (10 µg), Vancomycin (30 µg), Bacitracin (10 U), Ofloxacin (5 µg), Cephalothin (30 µg), Tobramycin (30 µg) and Cloxacillin (5 µg) were used. All the isolated strains of *Lactobacillus* were found to be resistant to Kanamycin and Ofloxacin antibiotic. However, *L. salivarius* isolates GPI-1(S) and GPI-4, *L. fermentum* isolates GPI-1(B) and GPI-6 were found to be resistant (no zone of inhibition) to all the tested antibiotics (Table 2.7).

Table 2.7. Spectrum of antibiotic susceptibility exhibited by various lactobacilli.

	Antibiotic susceptibility (Zone of inhibition in mm)[†] (Mean ± SD) [*]								
Cultures	**Ch**	**K**	**G**	**V**	**B**	**O**	**Ce**	**Cl**	**T**
LGG	26.33±1.53 (S)	10.00±1.00 (R)	20.00±1.00 (I)	10.67±1.15 (R)	17.67±0.58 (I)	19.00±1.00 (I)	21.67±1.53 (S)	20.67±2.52 (S)	25.33±2.52 (S)
GPI-1(S)	12.00 ±2.00 (R)[*]	11.67±1.53 (R)	12.33±2.08 (R)[*]	10.67±0.58 (R)	11.33±1.53 (R)[*]	11.33±1.15 (R)[*]	11.67±1.53 (R)[*]	10.67±0.58 (R)[*]	10.33±0.58 (R)[*]
GPI-4	11.33±1.53 (R)[*]	12.33±1.15 (R)	10.67±1.15 (R)[*]	12.00±1.00 (R)	12.33±1.15 (R)[*]	12.33±1.15 (R)[*]	10.67±0.58 (R)[*]	11.67±1.53 (R)[*]	11.67±0.58 (R)[*]
GRI-2	16.33±1.53 (I)[*]	11.67±1.53 (R)	11.00±1.00 (R)[*]	11.67±1.53 (R)	23.33±1.15 (S)[*]	14.33±0.58 (R)[*]	22.00±2.00 (S)	17.67±2.08 (I)	28.67±1.15 (S)
GPI-1(B)	12.33±2.52 (R)[*]	11.33±1.53 (R)	11.00±1.00 (R)[*]	10.67±0.58 (R)	11.67±1.53 (R)[*]	13.00±1.00 (R)[*]	11.33±1.53 (R)[*]	13.33±1.15 (R)[*]	10.33±0.58 (R)[*]
GPI-6	12.33±2.08 (R)[*]	11.67±2.08 (R)	11.33±1.53 (R)[*]	11.67±0.58 (R)	13.33±1.53 (R)[*]	12.67±1.15 (R)[*]	12.00±1.00 (R)[*]	11.67±1.53 (R)[*]	9.33±1.15 (R)[*]
GPI-7	23.00±1.00 (S)	12,67±2.08 (R)	13.67±1.53 (R)[*]	11.33±1.15 (R)	11.33±1.53 (R)[*]	13.67±0.58 (R)[*]	19.67±0.58 (I)[*]	17.33±0.58 (I)	14.67±2.52 (R)[*]

[†]The inhibition zones ≤ 15 mm, 16-20 mm and more than 20 mm indicates Resistant (R), Intermediate (I) and Sensitive (S) strains, respectively. [*]Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). [†]Results were obtained from three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

2.3.6. Adhesion of lactobacilli to intestinal epithelial cell lines

Two human colonic adenocarcinoma cell lines HT-29 and Caco-2 were used to study the adhesion potential of lactobacilli. The established probiotic strain, LGG was used to compare the adhesion ability.

Adhesion assay showed that isolates *L. salivarius* GPI-4 and *L. fermentum* GPI-7 showed significantly higher adhesion to HT-29 than that of the LGG (Fig. 2.3). Adhesion ability of *L. fermentum* GPI-6 was very poor among the isolates.

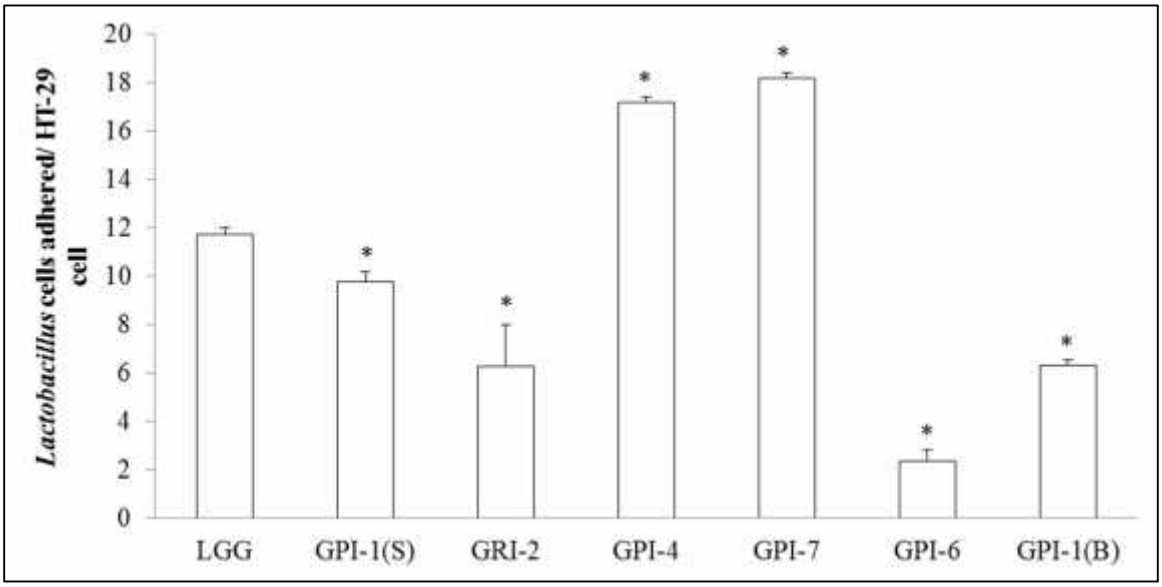


Figure 2.3. Adhesion of *Lactobacillus* isolates to HT-29 epithelial cell line compared with standard strains LGG. *Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). Each bar shows numbers of adhering bacteria are the mean value and error bar as standard deviation of three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

When the same study was done with Caco-2 cells, none of the isolates showed better binding to the Caco-2 than LGG ($P < 0.05$) (Fig. 2.4). Isolates *L. salivarius* GPI-4 and *L.*

fermentum GPI-7, were found to have similar adhesion to Caco-2 cells as seen with that of LGG.

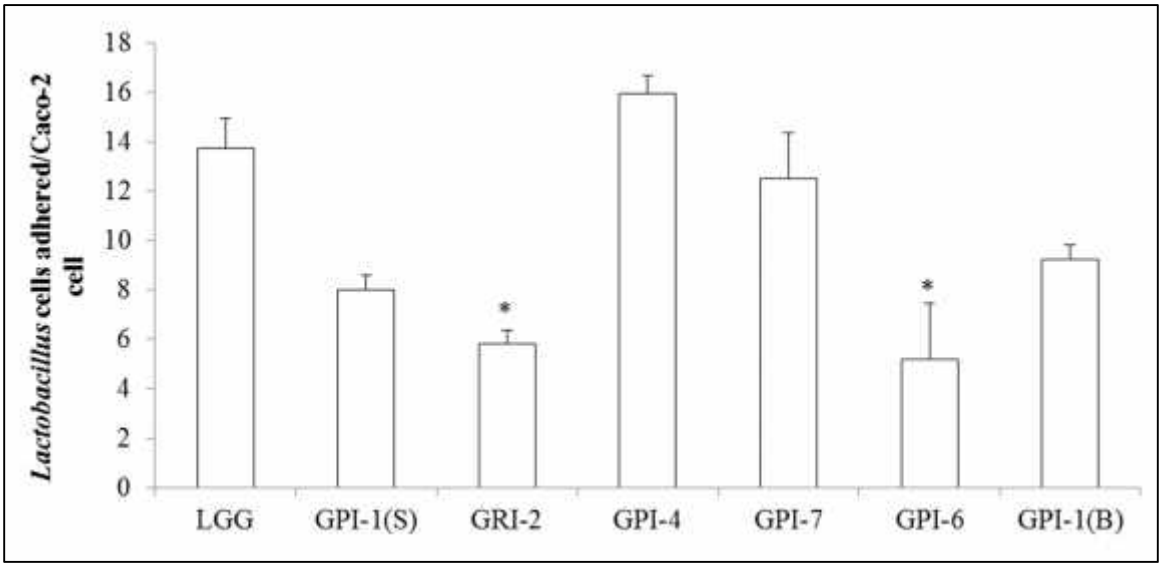


Figure 2.4. Adhesion of *Lactobacillus* isolates to Caco-2 epithelial cell line compared with standard strains LGG. *Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). Each bar shows numbers of adhering bacteria are the mean value and error bar as standard deviation of three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

2.3.7. Antagonistic effect of *Lactobacillus* isolates on adhesion of *E. coli* to intestinal epithelial cell lines (HT-29 and Caco-2)

The antagonistic effect of lactobacilli on *E. coli* adhesion was analysed on HT-29 and Caco-2 cells (Fig. 2.5 and 2.6, respectively). To mimic the *in vivo* condition, the assay was done in three ways, where: 1) lactobacilli were allowed to adhere first, followed by *E. coli* adhesion (Adhesion Inhibition assay), 2) *E. coli* was first incubated with the cell-line followed by *Lactobacillus* adhesion (Displacement Inhibition assay) and 3) both *Lactobacillus* and *E. coli* were simultaneously allowed to adhere (Competitive Inhibition assay). The numbers of enteropathogen bound to HT-29 cells under different adhesion assays are given in Fig. 2.5.

When compared to LGG, some of the isolates (*L. fermentum* strains GPI-7, GPI-6, GPI-1(B)) showed significantly higher reduction in *E. coli* adhesion to HT-29 cells in all the three assays ($P < 0.05$). *L. salivarius* GPI-4 reduced 77.93% and 87.32% *E. coli* adhesion to HT-29 with the adhesion inhibition and competitive inhibition assays, respectively. Only when allowed to adhere simultaneously isolate *L. plantarum* GRI-2 was able to reduce by 63.84% *E. coli* adhesion. Overall, all the strains were able to interfere with *E. coli* adhesion to HT-29 cells under three adhesion assays. The effects were strain specific and varied under different assays.

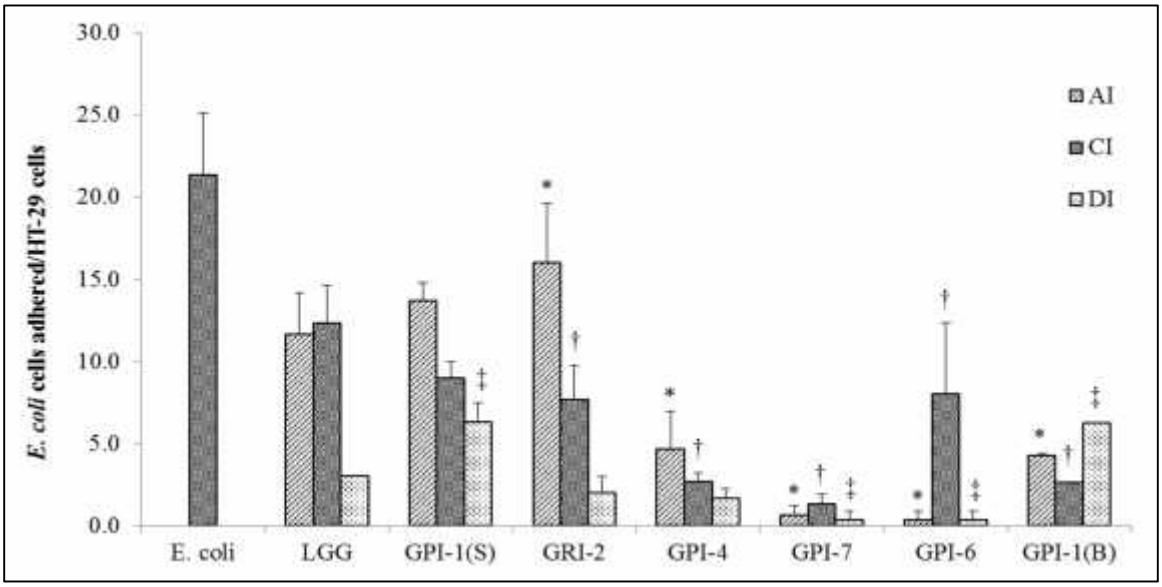


Figure 2.5. Adhesion of *Escherichia coli* to HT-29 cells following competition with, inhibition by, and displacement by various lactobacilli. Adhesion of *E. coli* in the absence of lactobacilli is denoted as control. AI- Adhesion Inhibition; CI- Competitive Inhibition; DI- Displacement Inhibition. *Mean value of adhesion inhibition of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). †Mean value of competitive inhibition of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). ‡Mean value of displacement inhibition of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). Each bar shows the mean value and error bar as standard deviation of three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

When the same study was done using Caco-2 cells (Fig. 2.6), all the isolates were able to reduce the *E. coli* adhesion significantly as compared to control. Similar results were observed with HT-29 cells. Compared to the standard strain LGG, significantly higher reduction in *E. coli* adhesion to Caco-2 cells was observed with *L. salivarius* GPI-1(S), GPI-6 and GRI-2 in all three assays ($P < 0.05$). *L. fermentum* GPI-7 displaced the *E. coli* by 90.90%.

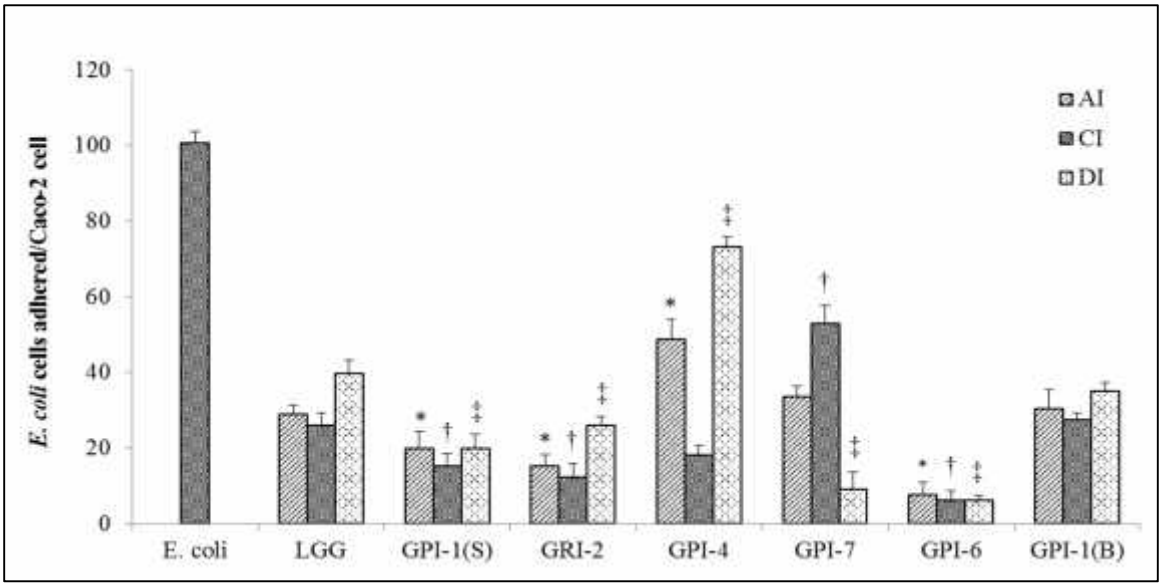


Figure 2.6. Adhesion of *Escherichia coli* to Caco-2 cells following competition with, inhibition by, and displacement by various lactobacilli. Adhesion of *E. coli* in the absence of lactobacilli is denoted as control. AI- Adhesion Inhibition; CI- Competitive Inhibition; DI- Displacement Inhibition. *Mean value of adhesion inhibition of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). †Mean value of competitive inhibition of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). ‡Mean value of displacement inhibition of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). Each bar shows the mean value and error bar as standard deviation of three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for multiple comparisons *vs.* the control group.

2.4. Discussion

In order to persist in the host, probiotics such as *Lactobacillus* must reach a suitable microhabitat and establish a proliferating population. Therefore, the ability to survive under

low pH conditions and high bile salt concentrations are desirable features for a successful passage though the gastrointestinal tract (Reid 2001). de Vries *et al*. (2006) discussed the safety and survival of *L. plantarum* in the human intestinal tract and the protective effects of this bacterium on the host. Several studies (Jain *et al*. 2009; Kaushik *et al*. 2009; Patel *et al*. 2012; Shah and Prajapati 2013) also demonstrated that their indigenous *Lactobacillus* strains exhibited high resistance against low pH and bile and possessed antibacterial, antioxidative and cholesterol lowering properties with a potential for exploitation in the development of indigenous functional food or nutraceuticals. In the present study, indigenous *Lactobacillus* isolates were checked for their resistance to acid (pH 2.5), bile tolerance (bile concentration at 0.3% and 1%), as well as having antibacterial activity. Besides this, adhesion to Caco-2 and HT-29 monolayers and antagonism against a selected pathogen was also checked.

The bile concentration of the human GI tract varies; the mean intestinal bile concentration is believed to be 0.3% w/v (Garcia-Hernandez *et al*. 2012). Hence this concentration was used in most studies screening for bile resistant strains (Hirano *et al*. 2003; Verdenelli *et al*. 2009). In the present study, bile concentrations of up to 1% w/v were used and most of the isolates survived the low pH and high bile conditions along with survival and normal growth at bile concentrations of up to 1% w/v. Isolate *L. fermentum* GPI-6 was found to have a better survival rate (91% at 0.3% bile salt, 89% at 1% bile salt) as compared to standard strain LGG (81% at 0.3% and 1% bile salt), there are other isolates that were intermediate between these two. The pH value of gastric acid varies in the range of about 1.5–4.5 in a period of 2 h, depending on the entering time and the type of gastric contents

(Verdenelli *et al*. 2009). In the present study, pH 2.5 was used as a representative gastric pH value which was also reported by Verdenelli *et al*. (2009). Strains *L. plantarum* GRI-2 and *L. salivarius* GPI-4 showed no reduction in viability while *L. fermentum* GPI-1(B) showed 84% survival rate in acidic pH 2.5. Earlier reports on lactobacilli at pH 2.5 showed similar survival as observed with the isolates used in this study (Saran *et al*. 2012; Tulumoglu *et al*. 2013).

Probiotics preferably should have antimicrobial activity, particularly against pathogens in the GI system. Antimicrobial activity and antibiotic susceptibility of lactobacilli are crucial criteria from the safety point of view for potential probiotics. This is because bacteria used as probiotics may serve as hosts of antibiotic resistance genes, which can be transferred to pathogenic bacteria (Sharma *et al*. 2014). However probiotics with known antibiotic resistance may also be used in patient suffering from antibiotic associated diarrhea (Siitonen *et al*. 1990). Additional knowledge of antibiotic resistance may be used to washout the probiotic once it outlives its utility as a delivery vehicle. Zhou *et al*. (2005) and Liasi *et al*. (2009) reported that most of the *Lactobacillus*, *Enterococcus* and *Pediococcus* strains used as probiotic were resistant to Gram-negative spectrum antibiotic and aminoglycoside antibiotics. In the present study, all the strains were found to be resistant (no zone of inhibition) to Ofloxacin and Kanamycin antibiotic. However, *L. salivarius* isolates GPI-1(S) and GPI-4, and *L. fermentum* isolates GPI-1(B) and GPI-6 showed no zone of inhibition to all the tested antibiotics. Barnby-Smith (1992) reported that research on antimicrobial substances produced by lactic acid bacteria has led to their potential use as natural preservatives to combat the growth of pathogenic microorganisms

in the food industry, and thereby to control the spread of infectious diseases. Further, Songisepp *et al*. (2004) demonstrated that Pikantne, the Estonian open-texture, soft cheese, proved to be an appropriate probiotic delivery vehicle for *L. fermentum* ME-3, which sustained antioxidative and antimicrobial activity. Antimicrobial assay of the isolates *L. fermentum* GPI-7 and GPI-1(B), and similarly *L. salivarius* GPI-4 and GPI-1(S) showed that although they were from the same species they possess different antimicrobial activity. This is in line with report of Flórez *et al*. (2005) which stated that the antimicrobial activity is strain specific. They have demonstrated maximum zone of inhibition against both Gram positive and Gram negative organisms, while zone of inhibition of most of the isolates is more or less similar to LGG against *E. coli*, *S. aureus* and *S. dysenteraie*, strain GPI-7 also showed zone of inhibition against *S. typhi* and GPI-4, GPI-7, GPI -1(S) and GPI-1(B) against *Pr. vulgaris* which is not the case with LGG.

One of the most significant properties of probiotic is their ability to adhere to the mucosal surfaces and to colonize the gastro-intestinal and urogenital tract (Bernet *et al*. 1994). Xu *et al*. (2009) demonstrated that *Bifidobacterium longum* B6 and *L. rhamnosus* GG strongly adhered to Caco-2 cells and effectively inhibited the adherence of pathogens to Caco-2 cells. The observation suggested that *in vitro* adhesion to Caco-2 cells is correlated with competitive inhibition, which is competitively excluding food borne pathogens. In addition, good adhesion of *L. plantarum* DJ-04 to Caco-2 cells was observed by Li *et al*. (2015), and suggested that this bacterium could significantly inhibit the adhesion of pathogenic bacteria to Caco-2 cells, with the inhibition percentage ranging from 52.37% to 90.33%. In the present study Caco-2 and HT-29 cells were used for their ability to mimic

the morphological and physiological function of the intestinal epithelial cell as they contain several receptors that recognize specific adhesion proteins on the surface of bacteria contributing to bacterial adhesion (Chauvière *et al*. 1992; Duary *et al*. 2011). Adhesion to HT-29 and Caco-2 was strain specific and varied within the same species. This was in agreement with results obtained from previous studies (Coconnier *et al*. 1993; Sarem *et al*. 1996). In the present study, it was observed that isolates GPI-4 and GPI-7 showed higher adhesion to HT-29 cells, while all isolates except for *L. fermentum* GPI-6 and *L. plantarum* GRI-2 showed adhesion to Caco-2 cells similar to LGG. Caco-2 cells have originated from human colon carcinoma and partially reproduce the characteristics of intestinal enterocytes as reported by Fogh *et al*. (1977), whereas the mucus-secreting goblet cells HT-29 represent the mucus layer as reported by Lesuffleur *et al*. (1991). The difference in binding seen therefore could be due to the surface characteristics of these two cell types. Many researchers have previously demonstrated protective effects against the attachment of a variety of enteric pathogens, including *E. coli* as the consequence of acidification with lactic acid (Ogawa *et al*. 2001), secreted non-acidic products (Coconnier *et al*. 1993) and interference with attachment to receptors or spaces, all of which may occur both directly and indirectly (Hirano *et al*. 2003). Hugo *et al*. (2006) reported that *L. delbrueckii* subsp *lactis* strain CIDCA 133 is able to antagonize the nitrate reductase activity of *E. coli*. It is known that *E. coli* O157:H7 is able to signal via protein kinase C, calmodulin and myosin light chain kinase (Philpott *et al*. 1998). Therefore Hugo *et al*. (2008) hypothesized that attachment of lactobacilli on to epithelial cells could antagonize signalling pathways involved in cytopathic effects related to *E. coli* O157:H7 infection. Neeser *et al*. (2000) investigated carbohydrate-binding specificities of the *L. johnsonii* La1 strain *in vitro*,

similar carbohydrate-binding specificities are known to be expressed on cell surface adhesins of several enteropathogens, enabling them to adhere to the host gut mucosa. These findings corroborated the hypothesis that selected probiotic bacterial strains could be able to compete with enteropathogens for the same carbohydrate receptors in the gut. Additionally, Lee *et al*. (2000) also showed that enterally-administered LGG decreases the frequency of *E. coli* K1A translocation in a neonatal rabbit model. The different lactobacilli in the present study were evaluated with regard to their ability to inhibit the adhesion of *E. coli* O26:H11 to HT-29 and Caco-2 cells. LGG was utilized as a positive control. *L. fermentum* strains GPI-6 and GPI-7 showed better inhibition of *E. coli* adhesion to both HT-29 and Caco-2 cells than LGG in all three inhibition assays. The other isolates showed variable adhesion inhibition in three inhibition assays tested. Gopal *et al*. (2001) showed 28% and 54% decrease of *E. coli* attachment to different epithelial cells with *L. acidophillus*, in case of *L. rhamnosus* the decrease observed was in the range of 17-23%. Kim *et al.* (2008) showed that in both the pre- and co-treatment experiments, their isolate *L. acidophilus* A4 gave the most profound attachment inhibitory effect for *E. coli* O157:H7 strain. Adhesion inhibition is an important aspect of the function of probiotic bacteria in protecting host gastrointestinal micro–environment from invading pathogens. It is generally believed that the resident gastrointestinal microflora *in vivo* provides protection to the host against possible colonization by pathogenic bacteria (Reid *et al*. 1990). Therefore the isolates used in this study may be used against possible colonization by pathogenic bacteria.