CHAPTER 3

Probiotic Attributes of Autochthonous *Lactobacillus rhamnosus* Strains of Human Origin

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Introduction

Human body lives in close harmony with a complex ecosystem that is composed of more than 1000 different bacterial species inhabiting the oral cavity, upper respiratory tract, gastrointestinal tract (GIT), vagina, and skin. This assortment known as the microbiota, is acquired soon after birth and persists throughout the life. However, the microbiota varies extensively among individuals, because it depends on many factors like diet, life style, genetic factors, etc. Lactobacilli is a diverse group of bacteria, to which we are exposed in our day-to-day life as they are commonly present in foods such as, fruits, vegetables, and fermented foods, etc. (Backhed *et al*., 2005). Some *Lactobacillus* species that reside in the gastrointestinal tract of mammals and vagina of humans and animals are associated with the well-being of the host (Hames and Vogel, 1995). Probiotics are "live microbes that when administered in adequate amount confer health benefit to the host" (FAO/WHO, 2001). The gut microbiota plays a key role in the host's overall health through its metabolic activities and physiological regulations such as digestion and assimilation of nutrients, protection against pathogen colonization, modulation of immune responses, and regulation of fat storage (Backhed *et al*., 2005). Alteration of the microbiota may cause some direct or indirect digestive pathology like infectious diseases, chronic inflammation, and metabolic disorders or atopic diseases. Factors that negatively influence the interaction between intestinal microorganisms, such as stress and diet, lead to detrimental health effects. Probiotic concept deals with the deliberate introduction of the new microbes beneficial to human host, as an attempt to change the indigenous microbial population equilibrium towards healthier composition (Charteris *et al*., 1998a). Allochthonous species may only confer short-term advantage, as they tend to get washed out. Therefore, we propose to explore indigenous autochthonous strains of lactobacilli as probiotics (Pithva *et al*, 2011). Lactobacilli, which often dominate the

healthy microbiota in the female urogenital tract, appear to protect against urogenital infections and bacterial vaginosis (Reid, 2001).

Several criteria used for the selection of novel probiotic strains are functional, safety, and technological aspects (Saarela *et al*., 2000). The functional aspects include (i) survival in the GIT and exertion of their beneficial influence (Dunne *et al*., 2001), (ii) antimicrobial activity, (iii) adhesion and colonization of gut epithelium, (iv) the ability to modulate immune response, and (v) influence on metabolic activities e.g., lactase activity, cholesterol assimilation, vitamin production (Tannock, 1995) etc. The safety aspect emphasizes the healthy human origin of the probiotic strains (Mattila-Sandholm *et al*., 2002). The technological aspect includes (i) the ability of probiotic strains to withstand production conditions, and (ii) survival in the final formulation of the appropriate food product.

Although, there are numerous probiotic strains available for commercial use, isolation and characterization of novel strains is still a fascinating research area particularly in India. One of the aims of the present work was to characterize autochthonous human origin *Lactobacillus* strains, and to evaluate their probiotic properties with reference to two standard strains *L. rhamnosus* GG (ATCC 53103), and *L. casei* DN 114 001 (Actimel strain, DANONE, France).

Materials and Methods

Bacterial strains and culture conditions

Lactobacillus strains were obtained from different sources, which include infant fecal isolate *Lactobacillus rhamnosus* Fb JX406746 and vaginal isolate of healthy female *Lactobacillus rhamnosus* Vc JX406745. Reference strains used were *L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* 231 (EF661653) and *L. casei* DN 114 001 (Actimel strain, DANONE, France). *Lactobacillus* strains were grown in de Man-Rogosa-Sharpe (MRS; Himedia, Mumbai, India) for 24 h at 37°C. The stock cultures were maintained in 10% (wt/vol) skim milk at 4°C.

The indicator strains *Enterobacter aerogenes*, *Salmonella typhi*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Staphylococcus epidermidis*, *Bacillus spp*., *Escherichia coli*, *Shigella sp*., *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Micrococcus leutus* were grown in Nutrient broth (Himedia, Mumbai, India) at 37°C. *Listeria monocytogenes*, *Streptococcus mutans*

and *Vibrio cholerae* were grown in Brain Heart Infusion and Luria Bertani broth (Himedia, Mumbai, India) respectively at 37°C for 18 h before assay. The *Candida* strains were cultured in Sabouraud Dextrose broth for 18 h at 37°C.

Functional aspects

Tolerance to pH, bile, NaCl, and phenol

Viability of *Lactobacillus* strains was evaluated as described by Jacobsen *et al*. (1999). *Lactobacillus* strains (10^7 cfu/ml) were used to inoculate 5 ml of MRS, MRS modified with bile salt (2, 4%), NaCl (8, 10%), pH (2, 3) adjusted with 0.1 M HCl, and skim milk with phenol (0.4, 0.6% phenol). Samples (0.1 ml) collected from these tubes after 0 and 4 h, were serially diluted, plated on MRS agar, and incubated at 37°C for 48 h for the determination of viability.

Preparation of lactobacilli cell suspension

Cell suspension used in various experiments described below, if not indicated otherwise, was prepared by growing *Lactobacillus* strains for 24 h in aerobic condition in MRS medium. The cells were pelleted by centrifugation (5000 rpm, 15 min, 4°C), washed twice with phosphate buffer saline (PBS – 0.1 M, pH 7.2, containing 0.85% (w/v) NaCl) and resuspended in phosphate buffer (0.1 M, pH 7) to get cell suspension having $A_{600} = 1$ and 10^9 cfu/ml.

Lysozyme tolerance

Viability of the cells in the presence of lysozyme was determined as described by Vizoso-Pinto *et al*. (2006). To simulate the *in vivo* dilution by saliva, 100 µl of bacterial suspension was mixed in a sterile electrolyte solution consisting of g/l of $CaCl_2$ (0.22), NaCl (6.2), KCl (2.2), $NaHCO_3$ (1.2) in the presence of 100 mg/l of lysozyme (Genei, Banglore). Bacterial suspension in electrolyte solution without lysozyme was included as control. Samples were incubated at 37°C and viable count after 10 and 120 min was determined on MRS medium. Survival was calculated as percentage of the cfu/ml after 10 and 120 min in comparison to the initial count (0 min).

Viability during simulated gastrointestinal transit

Viability during simulated gastric and intestinal fluid transit was evaluated as described by Charteris *et al*. (1998a). 100 µl of cell suspension was mixed with 1 ml of simulated gastric fluid (SGF) and incubated for 2 h at 37°C. The cells pelleted by

centrifugation were resuspended in 1 ml of simulated intestinal fluid (SIF) and incubated at 37°C for 3 h. SGF- and SGF-SIF-treated cells were serially diluted, and plated on MRS agar for the determination of cell viability. The control constituted of cells treated with phosphate buffer instead of SGF and SIF. The SGF comprised of 0.3% pepsin, 0.5% NaCl, and pH 2 adjusted with 1 M HCl. SIF comprised of 0.1% pancreatin, 0.5% bile salt, 0.5% NaCl, 0.4% phenol, and pH 8 adjusted with 1 M NaOH.

Hydrophobicity assay

Hydrophobicity of *Lactobacillus* strains was determined according to Rosenberg *et al*. (1980) with some modifications. Bacterial cells were pelleted by centrifugation from 24 h old cultures grown in MRS medium, washed twice with PBS, and resuspended in 3 ml of 0.1 M KNO_3 (pH 6.2) to approximately 10^9 cfu/ml and A_{600} (A_0) was measured. The two-phase system, obtained by mixing 1 ml of solvent (n-hexadecane, chloroform, and ethyl acetate) and 3 ml cell suspension, was pre-incubated for 10 min at room temperature, and vortexed for 2 min. The aqueous phase was removed after 20 min of incubation, and its A_{600} (A_1) was measured. The hydrophobicity of lactobacilli was calculated as $[(A_0-A_1)/A_0] \times 100$.

Mucin adhesion assay

Lactobacilli were evaluated for adhesion to immobilized porcine stomach mucin (Sigma-Aldrich, USA) in 96-well microtitre plates in sterile condition as described in Dhanani and Bagchi (2013). Microtitre plates were coated with 300 µl of mucin (0.5 mg/ml) in sterile Dulbecco's phosphate-buffered saline (PBS, pH 7; Sigma-Aldrich), at 4°C overnight. Wells were washed twice with sterile PBS to remove unbound mucin. 200 µl of bacterial cell suspension was added to the wells and incubated at 37°C for 90 min. Un-adhered bacterial cells were withdrawn, and wells were washed five times with 300 µl PBS. Adhered cells were released by treatment with 300 µl 0.05% (v/v) Triton X-100 in sterile PBS for 20 min at 37°C. The adhered bacterial cells were enumerated after appropriate dilution on MRS agar.

Autoaggregation assay

Autoaggregation assay was performed as described by Del Re *et al*. (1998) with certain modifications. Cell suspension (2 ml) was vortexed for 10 s, and incubated at 37°C. Aliquot of 0.1 ml collected from the upper surface at regular time interval was

mixed with 0.9 ml PBS and its A_{600} (UV 1601, Shimadzu, Japan) was recorded. The autoaggregation (%) is calculated as $[(A_0 - A_t)/A_0] \times 100$, where A_0 is A_{600} at 0 h and A_t represents the A_{600} of cell suspension at different time intervals (2, 4 and 24 h).

Coaggregation assay

Equal volumes of cells suspensions (1 ml=10^9 cfu/ml) of *Lactobacillus* and pathogen strains were mixed, and incubated at 37°C. The control contained 2 ml of pure bacterial or yeast cell suspension. A_{600} of these suspensions was measured at predetermined time intervals as described above. The coaggregation(%) was calculated using the equation of Handley *et al.* (1987), $100*[((A_{pat}+A_{Lacto})/2)-(A_{mix})]/[(A_{pat}+A_{Lacto})/2]$, where A_{pat} and A_{Lacto} represent A_{600} of control tubes and A_{mix} represents the A_{600} of the mixture of *Lactobacillus* and pathogen strains at predetermined time intervals.

Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity was evaluated according to Taranto *et al*. (2003). MRS agar was supplemented with 0.5% (w/v) bile salt (Himedia, Mumbai, India) and 0.37 g/l of $CaCl_2$. 10 µl of overnight grown culture in MRS broth was spot-inoculated on the modified MRS agar plates, and incubated in GasPak™ anaerobic jar (Himedia, Mumbai, India) at 37°C for 72 h. The formation of bile acid precipitations around the colony was considered as a positive result.

β-galactosidase activity

The mixture of 0.1 ml cell suspension and 2.7 ml permeabilization buffer was incubated on shaker at 37°C for 30 min. To this mixture, 0.6 ml of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) was added and the reaction was terminated after 1 h by adding 2 ml chilled 1 M Na_2CO_3. A_{420} was recorded against colorimetric blank. Permeabilization buffer consisted of 90 mM $Na_2HPO_4.2H_2O$, 35 mM $NaH_2PO_4.2H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 3.5 mM Sodium dodecyl sulphate, and 34 mM β-mercaptoethanol.

Antibacterial activity

The antibacterial activity of *Lactobacillus* strains was determined by the spot inoculation test (Schillinger and Lucke, 1989) with minor modifications. Briefly, 2 µl of *Lactobacillus* cell suspensions (untreated and SGF- and SGF-SIF-treated) were spot-inoculated on the surface of MRS agar plate and incubated for 24 h at 37°C. 100

µl (A_{600} = 0.2) of the indicator strains was mixed with 10 ml of 1% nutrient agar and overlaid on the MRS agar previously spot-inoculated with lactobacilli and incubated further for 24 h at 37°C. The indicator strains are listed in Table 4.

Antifungal activity

The antifungal activity of *Lactobacillus* strains was evaluated by overlay assay (Magnusson and Schnürer, 2001). Bacterial cell suspension was streaked (2 cm line) on MRS agar plates and incubated for 48 h at 37°C. 10 ml of glucose yeast extract and potato dextrose agar (1%) inoculated with 0.1 ml of yeast cultures or fungal spores (10^5 spores) respectively were poured on the previously inoculated MRS agar plates, and incubated at room temperature. The grading of antifungal activity is based on the size of inhibition zone around the bacterial growth.

Safety aspects

Antibiotic susceptibility test

Antibiotic susceptibility of *Lactobacillus* strains was evaluated using disc diffusion method described by Charteris *et al.* (1998b). 0.1 ml of 18 h old cultures (10^8 cfu/ml) of lactobacilli grown in MRS was inoculated in 25 ml molten MRS agar (1.5%), and poured in sterile Petri plates. Antibiotics Octadiscs (Himedia, Mumbai, India) were placed on MRS agar plates incubated at 37°C for 24 h and observed for the zone of inhibition.

Haemolytic activity

MRS agar plates (Himedia, Mumbai, India) containing 5% human blood were streaked with 18 h old *Lactobacillus* cultures growing in MRS medium at 37°C for 48-72 h. The plates were observed for haemolytic reaction.

Detection of biogenic amine production

The amino acid decarboxylase activity of *Lactobacillus* strains was determined as described by Bover-Cid and Holzapfel (1999). Amino acids used were L-tyrosine disodium salt, L-histidine monohydrochloride, L-ornithine monohydrochloride and L-lysine monohydrochloride (Himedia, Mumbai, India) for the detection of decarboxylase activity. Decarboxylase medium not containing amino acid served as negative control. *Enterococcus faecalis* served as positive control.

***Preparation and evaluation of the probiotic properties of the lyophilized powder of L. rhamnosus* Fb**

Cells were pelleted by centrifugation from 100 ml of 24 h old MRS grown *L. rhamnosus* Fb culture, washed thrice with PBS, stored-frozen for 18 h at -20°C and lyophilized using Micromodulyo 0230 (ThermoScientific, USA). Viable count of frozen and lyophilized cells was evaluated using MRS medium. Probiotic properties of lyophilized cells were evaluated by the methods described above.

Statistical analysis

Values are mean from three independent experiments ± standard deviation. Statistical differences in the viable count and other results were analyzed by one way analysis of variance (ANOVA) using Microsoft Excel 2010. *P* values of <0.05 were considered significant.

Results

Functional aspects

Tolerance to pH, bile, NaCl, and phenol

The isolate Vc showed survival of 81% in pH 2, followed by 231, Fb, reference strains Actimel and GG, showed survival of 72 and 67% respectively. *Lactobacillus* strains grow but slowly ($P<0.05$) in comparison to MRS control, with an average reduction of 1.74, 1.54 and 1.73 log cycles in 4% bile salt, 10% NaCl, and 0.6% phenol respectively (Table 1).

Lysozyme tolerance

Lactobacillus strains when incubated in the presence of lysozyme for 10 min did not alter the viability except Vc which showed 6% reduction (Fig. 1). Cells of *Lactobacillus* strains retained ≥84 viability when incubated for 120 min with lysozyme.

Table 1. Growth (log cfu/ml) of *Lactobacillus* strains, in MRS with/without bile salt (2, 4%), pH (2, 3), NaCl (8,10%), and phenol (0.4, 0.6%) after 4 h of incubation at 37°C

Media	Growth of *Lactobacillus* strains									
	Fb		GG		231		Vc		Actimel	
	(log cfu/ml)	(%)	(log cfu/ml)	(%)	(log cfu/ml)	(%)	(log cfu/ml)	(%)	(log cfu/ml)	(%)
Initial* (0 h)	7.80±0.14	-	8.15±0.04	-	8.04±0.06	-	7.97±0.06	-	7.93±0.04	-
MRS	9.69±0.13	100	11.1±0.03	100	9.69±0.30	100	9.84±0.09	100	9.69±0.13	100
MRS+bile salt (2%)	8.54±0.09	88	9.04±0.06	81	8.80±0.14	91	8.45±0.21	86	8.59±0.16	89
MRS+bile salt (4%)	8.15±0.21	84	8.84±0.09	80	8.15±0.21	84	8.00±0.00	81	8.15±0.21	84
MRS (pH 2)	7.30±0.01	75	7.39±0.55[a]	67	7.39±0.55[a]	76	7.95±0.07[a]	81	7.00±0.02	72
MRS (pH 3)	7.74±0.06	80	8.57±0.05[a]	77	8.02±0.09[a]	83	8.13±0.07[a]	83	7.39±0.13	76
MRS+NaCl (8%)	8.45±0.21	87	9.02±0.09	81	8.90±0.71	92	8.50±0.28	86	8.59±0.16	89
MRS+NaCl (10%)	8.15±0.21	84	9.47±0.06	85	8.24±0.34	85	8.00±0.00	81	8.45±0.21	87
Skim milk	10.21±0.01	105	9.56±0.03	86	10.40±0.01	107	9.61±0.04	98	9.59±0.01	99
Skim milk+phenol (0.4%)	9.02±0.02	93	9.19±0.04	83	8.97±0.04	93	9.12±0.02	93	8.71±0.09	90
Skim milk+phenol (0.6%)	8.60±0.04	89	8.25±0.07[a]	74	8.09±0.13	83	8.71±0.08	88	7.72±0.33	80

*viable count of *Lactobacillus* strains determined at 0 h; the results are representative mean±SD of three independent experiments, [a]not significantly different within same column, while the other values are significantly different (P<0.05).

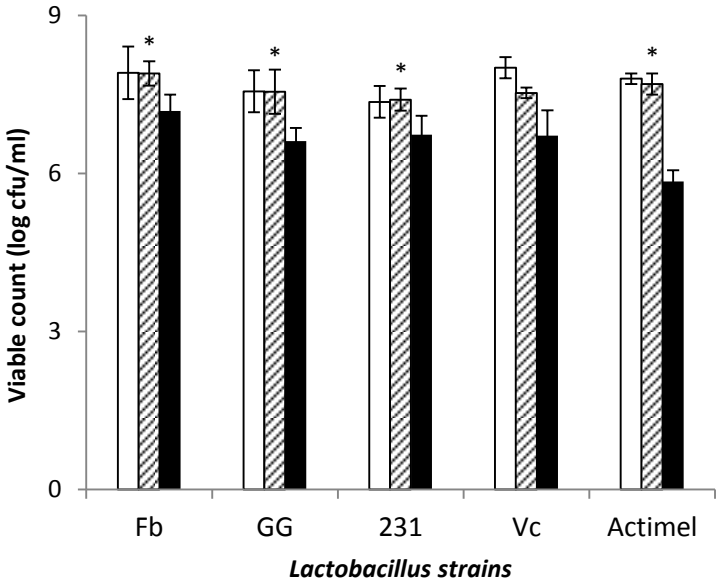

Fig. 1 Survival (log cfu/ml) of *Lactobacillus rhamnosus* strains Fb, GG, 231, Vc and *L. casei* Actimel in the presence of simulating saliva solution containing lysozyme (0.1 mg/ml) at □ 0 min, ▨ 10 min, ■ 120 min as determined by viable plate count method on MRS medium. Data are shown as mean viable count on MRS agar from three independent experiments and error bar indicates the standard deviation (SD), *not significantly different than initial count (P<0.05)

Viability during simulated gastrointestinal transit

The *Lactobacillus* strains Fb, Actimel and GG displayed reduction of 0.6, 1.7 and 1.9 log cycles respectively after 2 h of exposure to simulated gastric fluid; while 231 and Vc showed reduction of >2 log cycles (Fig. 2). The strain Fb showed survival of 93 and 78% after SGF and SGF-SIF transits, respectively, while in the case of reference strain GG, survival was 80 and 72%. The further reduction in the viability of *Lactobacillus* strains after simulated intestinal fluid transit, with an average reduction of 1.3 log cycles (0.8-1.9 log cycles) was significantly less (P<0.05) in comparison to simulated gastric fluid.

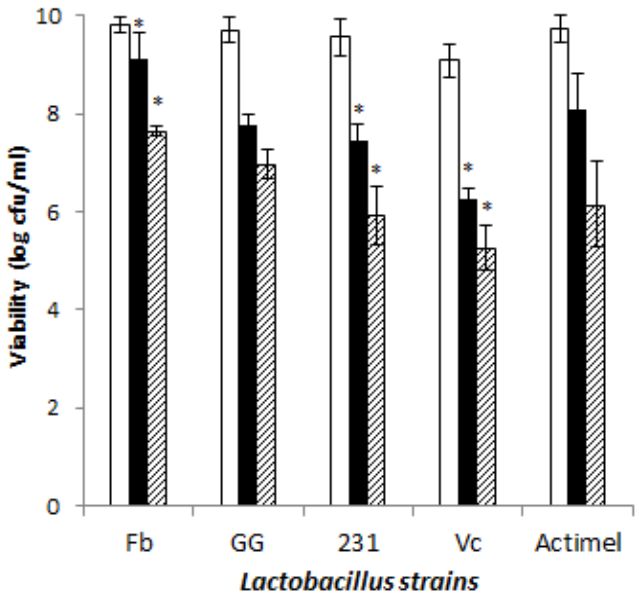

Fig. 2 Survival (log cfu/ml) of *Lactobacillus rhamnosus* strains Fb, GG, 231, Vc and *L. casei* Actimel before transit (□), and upon sequential exposure to simulated gastric fluid (■), simulated intestinal fluid (▨) as determined by viable plate count method on MRS medium. Data are shown as mean viable count on MRS agar from three independent experiments and error bar indicates the standard deviation (SD), *significantly different (P<0.05)

Hydrophobicity assay

Cell surface hydrophobicity and hydrophilicity were evaluated by the partitioning of cells between aqueous and organic phase at high ionic strength. The cell adherence of tested strains was 2-9%, 50-92% to n-hexadecane (apolar) and chloroform (acidic) solvents respectively. The adhesion to ethyl acetate was not detected among *Lactobacillus* strains except *L. rhamnosus* GG (Table 2).

Table 2. Cell surface hydrophobicity of *Lactobacillus* strains by Bacterial adhesion to hydrocarbon (BATH) assay

	% adhesion (±SD)		
Strains	n-hexadecane	chloroform	ethyl acetate
Fb	8.7±3.5	69.9±2.0	0
GG	5.7±2.1	91.6±2.0	11±4.5
231	6.3±1.8	66.9±1.0	0
Vc	9.1±7.4	51.0±6.0	0
Actimel	2.3±0.3	50.4±3.0	0

Values are mean±SD of three independent experiments

Mucin adhesion assay

All the strains adhere mucin albeit with varying capacity (Fig. 3). Strain Fb showed significant (P<0.05) adhesion, while strains Vc and Actimel showed moderate adhesion as compared to GG and 231.

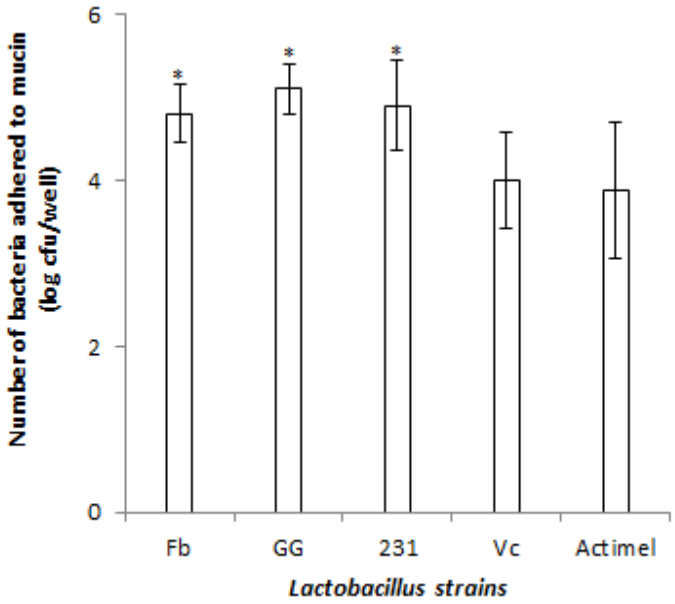

Fig. 3 Number of bacteria (log cfu/well) adhered to immobilized mucin in microtitre plate determined by viable count method using MRS medium. Error bar indicates the standard deviation and *not significantly different (P<0.05)

Autoaggregation assay

Autoaggregation increased significantly (P<0.05) with incubation time (Fig. 4). % autoaggregation after 2, 4, and 24 h was in the range of 5 to 40, 11 to 49, and 47 to 87 respectively. *L. rhamnosus* GG tended to aggregate rapidly reaching 87% after 24 h at 37°C.

Coaggregation assay

Lactobacillus strains showed coaggregation with all the tested pathogens listed in Table 3. The coaggregation was observed highest in GG followed by 231, Vc, Actimel and Fb. *Lactobacillus* strains coaggregated rapidly with *Candida spp*. than the enteropathogens. The coaggregation ability was strain-specific and time-dependent.

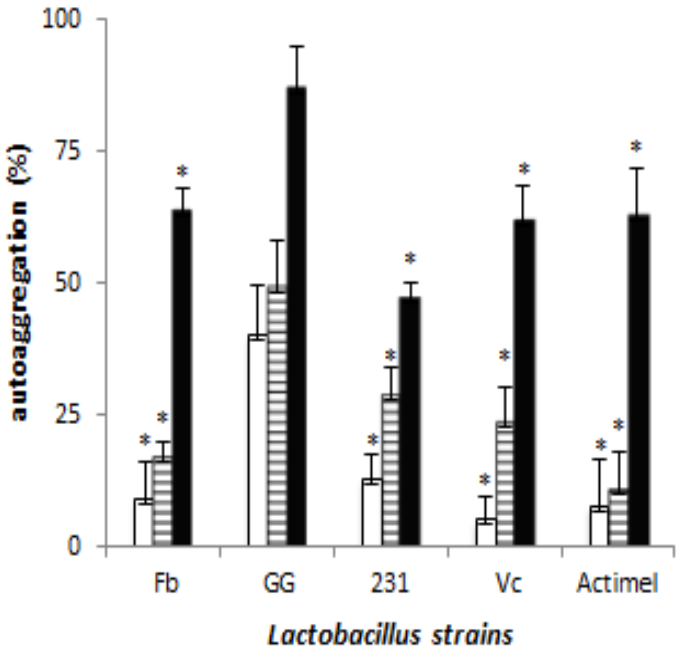

Fig. 4 Autoaggregation (%) of *L. rhamnosus* Fb, GG, 231, Vc and *L. casei* Actimel after 2(□) 4 (⊟) and 24 h (■) of incubation at 37°C. *L. rhamnosus* GG used as reference strain. Error bar indicates the standard deviation and *significantly increased with incubation time (P<0.05)

Bile salt hydrolase and β-galactosidase activity

Lactobacillus strains deconjugated bile salts as indicated by the white precipitation around the colonies developed on MRS medium containing bile salt. All the strains expressed β-galactosidase activity and extent of activity was not significantly different (P<0.05). The activity is expressed in Miller's unit±standard deviation are 40.33±0.04, 37.50±0.01, 41.00±0.25, 36.97±0.11 and 36.85±0.04, which accounts for the strains Fb, GG, 231, Vc and Actimel respectively.

Antibacterial activity

All the strains exhibited antimicrobial activity against the test pathogens (Table 4). The extent of antimicrobial action of lactobacilli varied with the test pathogens. The antimicrobial activity of *Lactobacillus* strains gradually decreased upon SGF and SGF-SIF transit against test pathogens. However, antimicrobial activity increased except strain 231 upon SGF transit against *Ent. aerogenes* and the activity of reference strains GG and Actimel against *E. coli.*

Table 3. % Coaggregation of *Lactobacillus* strains with enteropathogens and *Candida spp*. determined spectrophotometrically after 2 and 4 h of co-incubation

Strains	% Coaggregation (±SD)									
	Fb		GG		231		Vc		Actimel	
	2 h	4 h	2 h	4 h	2 h	4 h	2 h	4 h	2 h	4 h
Escherichia coli	22±9.3	36±1.4	37±7.4	53±6.2	18±1.1	37±4.5	9±5.9	39±3.9	29±6.0	45±3.5
Enterobacter aerogenes	11±7.7	30±1.9	25±0.4	51±6.3	22±9.5	44±4.1	8±5.8	43±7.5	20±6.9	36±9.8
Salmonella typhi	26±1.4	32±4.0	23±8.0	60±3.8	23±8.0	42±9.2	13±0.4	46±7.5	20±1.4	50±9.5
Shigella sp.	12±1.3	33±3.5	16±8.5	57±9.9	19±6.6	51±3.1	14±6.5	46±5.5	33±4.4	48±1.7
Proteus vulgaris	19±4.3	38±5.6	33±6.6	48±6.3	25±1.7	57±2.3	26±3.1	65±2.8	28±4.9	40±1.0
Candida albicans	31±5.4	52±1.0	32±1.0	67±5.5	27±0.1	63±0.4	15±1.1	52±1.3	47±1.4	58±3.0
Candida parapsilosis	23±4.2	60±3.7	45±2.0	74±1.0	32±1.1	68±2.7	29±0.3	67±0.5	25±0.5	67±0.5
Candida tropicalis	40±1.1	58±2.3	46±3.2	75±1.6	31±5.3	62±6.8	24±1.2	50±5.1	33±1.0	61±6.8

Values are the mean±SD from the three independent experiments

Table 4. Antibacterial activity of *Lactobacillus* strains determined food-borne and GIT-pathogens by spot overlay assay

Strains	Zone of inhibition (mm) ± SD*														
	L. rhamnosus Fb			*L. rhamnosus* GG			*L. rhamnosus* 231			*L. rhamnosus* Vc			*L. casei* Actimel		
	Control	SGF	SGF-SIF	Control	SGF	SGF-SIF	Control	SGF	SGF-SIF	Control	SGF	SGF-SIF	Control	SGF	SGF-SIF
*Escherichia coli**	28±2.9	25±5.8	24±7.8	27±5.8	32±4.0	30±5.5	23±2.9	18±3.8	19±3.1	29±2.3	25±8.9	28±6.7	27±5.8	29±8.1	29±7.8
Enterobacter aerogenes	15±0.6	18±5.9	13±2.6	21±3.5	20±4.0	23±7.8	13±5.2	11±1.5	10±1.5	14±3.8	15±4.6	12±2.6	14±1.2	19±8.1	19±4.6
Salmonella typhi	26±4.0	18±2.0	17±0.6	32±6.1	22±3.2	21±4.2	30±9.1	18±0.6	20±1.5	27±5.2	22±0.6	20±3.8	29±3.1	27±6.4	27±6.4
*Shigella sp.**	27±4.9	23±4.2	29±9.2	30±0.0	32±0.6	29±3.2	27±4.4	27±7.4	25±8.9	32±2.9	26±9.3	27±6.4	30±4.4	28±5.9	28±6.7
*Proteus vulgaris**	24±3.2	17±2.1	17±1.7	23±4.6	24±8.4	22±5.8	20±0.6	15±1.0	13±2.1	31±4.9	21±1.0	17±1.0	24±6.2	24±7.8	18±5.3
Serratia marcescens	32±2.9	27±3.8	25±7.8	31±2.3	26±5.3	27±1.7	26±0.6	26±8.9	27±9.7	31±1.2	30±2.0	29±3.1	28±1.5	24±3.2	26±5.5
*Klebsiella pneumoniae**	29±6.0	23±2.0	21±3.2	29±7.5	25±0.6	24±6.9	28±8.9	23±4.6	17±9.5	34±1.7	32±2.0	31±2.3	33±1.2	23±8.4	22±8.7
*Enterococcus faecalis**	15±4.0	11±0.6	13±2.6	18±4.9	15±2.1	12±3.6	18±2.5	11±1.0	11±1.0	17±4.7	12±1.5	10±1.5	18±2.0	11±0.6	11±1.0
Pseudomonas aeruginosa	29±9.2	21±2.1	15±8.4	31±2.0	23±7.8	17±9.8	33±2.3	20±4.0	15±7.5	19±1.0	14±2.0	15±1.0	33±4.0	20±4.9	16±7.5
Bacillus cereus	16±1.5	15±4.4	12±3.0	12±0.6	22±9.3	14±2.1	13±2.3	10±1.5	11±1.0	17±4.2	12±2.7	16±5.5	14±2.0	11±0.6	16±5.5
Bacillus megaterium	23±1.1	12±0.6	14±1.5	25±2.5	21±6.1	21±7.2	22±6.9	16±0.6	16±0.6	23±5.8	17±1.2	17±1.0	21±1.2	17±1.5	16±2.1
Bacillus subtilis	18±5.1	18±6.7	11±1.5	21±4.5	19±4.9	18±5.5	19±0.6	18±4.0	14±3.2	13±1.0	12±1.5	15±4.0	19±4.2	12±7.8	12±2.1
Vibrio cholerae	23±1.1	16±4.2	16±3.2	27±1.2	27±2.1	19±1.0	23±1.2	21±1.1	17±1.0	22±1.5	24±1.5	20±2.0	20±2.0	21±1.2	17±1.0
Listeria monocytogenes	17±1.1	16±3.8	14±2.0	17±1.5	17±0.6	19±0.6	19±2.1	12±0.6	14±1.5	19±4.2	20±6.1	14±3.5	21±1.2	15±1.0	13±0.6
Yersinia enterocolitica	35±3.5	26±5.9	24±3.2	32±0.6	29±3.6	26±1.0	27±4.9	28±7.2	23±2.3	30±1.5	23±9.7	23±6.7	24±2.0	24±2.0	25±2.6
Staphylococcus epidermidis	14±2.1	16±1.5	14±1.5	16±2.0	17±1.0	16±1.5	18±2.0	17±1.1	14±2.1	11±1.0	12±1.0	14±1.0	13±1.2	14±0.6	13±1.2
Streptococcus mutans	15±2.1	11±0.6	14±1.5	18±2.0	12±1.0	15±1.1	21±1.2	13±1.1	14±2.0	21±1.2	12±0.6	11±1.0	19±1.2	17±1.2	14±2.0
*Micrococcus leutus**	28±2.5	15±1.5	19±1.5	nd	nd	nd	31±1.0	22±1.5	19±1.1	33±2.3	19±1.2	21±1.2	32±3.2	21±1.2	18±1.0

Control indicates cells in phosphate buffer; SGF-treated cells and SGF-SIF-treated cells; *including 3-4 mm colony diameter of lactobacilli Values are mean±SD of three independent experiments, *indicates clinical strains were obtained from Government Hospital, Rajkot, India. Other strains were obtained from MTCC (Microbial Type Culture Collection Centre) Chandigarh, India.

Antifungal activity

Lactobacillus strains inhibited the growth of *Candida albicans* A, *C. tropicalis* B, *C. parapsilosis*, *Aspergillus niger*, and *A. flavus* but not *Saccharomyces cerevisiae*, and *C. albicans* E and F strains (Table 5).

Safety aspects

Antibiotic susceptibility test

Lactobacillus strains showed similar susceptibility pattern (Table 6). The strains are not susceptible to cell wall synthesis inhibitors (ampicillin, augmentin, carbenicillin, cefoperazone, vancomycin), antimicrobials of first generation (cephalothin), second generation (cephoxitin, cefuroxime), and third generation (cefpodoxime, ceftazidime, ceftriaxone, cephotaxime), cytoplasmic membrane disruptors (colistin), nucleic acid synthesis inhibitor (nalidixic acid), β-lactamase inhibitors (piperacillin/tazobactum, and ticarcillin/clavulanic acid), and protein synthesis inhibitors (fusidic acid and tobramycin). However, the same strains are sensitive to β-lactam antibiotics (oxacillin, penicillin, and ampicillin/sulbactum), protein synthesis inhibitors (amikacin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, netilin, and tetracycline), and nucleic acid synthesis inhibitors (ciprofloxacin, co-trimoxazole, nitrofurantoin and norfloxacin).

Table 5. Antifungal activity of *Lactobacillus* strains determined by overlay assay

Mold or yeast strains	Fb	GG	231	Vc	Actimel
	Inhibition*				
Candida albicans A*	+++	+++	+++	+++	+++
Candida tropicalis B*	+++	+++	+++	+++	+++
Candida albicans C*	-	-	-	-	-
Candida tropicalis D*	+	+	+	+	+
Candida albicans E*	-	-	-	-	-
Candida albicans F*	+++	+++	+++	+++	+++
Candida parapsilosis	+++	+++	+++	+++	+++
Saccharomyces cerevisiae	-	-	-	-	-
Aspergillus niger	+++	+++	+++	+++	+++
Aspergillus flavus	+++	++	+++	+++	+++

*Inhibition was graded as -, no visible inhibition; +, inhibition size per bacterial streak of 0.1 to 0.3% of the Petri plate; ++, inhibition size per bacterial streak of 3 to 8% of the Petri plate; or +++ inhibition size per bacterial streak of >8% of the Petri plate. * Clinical strains were obtained from the M. P. Shah Medical College, Jamnagar, India; while other strains were from MTCC, Chandigarh, India.

Table 6. Antibiotic susceptibility profile of *Lactobacillus* strains*

Antibiotics (µg)	Zone of inhibition (mm)				
	Fb	GG	231	Vc	Actimel
Amikacin (30)	17	10	18	15	16
Ampicillin/Sulbactum (10/100)	16	13	18	16	20
Chloramphenicol (30)	21	17	21	20	21
Ciprofloxacin (10)	14	16	18	16	13
Clindamycin (2)	20	18	12	13	15
Co-Trimoxazole (25)	10	11	10	10	12
Erythromycin (15)	16	18	14	11	19
Gentamicin (10)	17	12	19	16	15
Kanamycin (30)	12	10	12	13	11
Netilin (30)	13	NS	NS	10	15
Nitrofurantoin (300)	21	20	19	20	18
Norfloxacin (10)	11	14	11	11	10
Oxacillin (1)	11	10	9	10	12
Penicillin (1 unit)	18	16	15	18	14
Tetracycline (25)	20	20	17	18	19

NS - non-susceptible **Lactobacillus* strains are not susceptible to Amoxyclav (10), Ampicillin (10), Augmentin (30), Carbenicillin (100), Cefoperazone (75), Cefoperazone/sulbactum (75/30), Cefpodoxime (30), Ceftazidime (30), Ceftriaxone (30), Cefuroxime (30), Cephalothin (30), Cephotaxime (10 and 30) Cephoxitin (30), Colistin (25), Fusidic acid (10), Nalidixic acid (10), Piperacilin/Tazobactum (100/10), Piperacillin (75 and 100), Ticarcillin/ Clavulanic acid (75/10), Tobramycin (10) and Vancomycin (30)

Haemolytic activity

None of the *Lactobacillus* strains exhibited haemolytic activity on blood agar.

Biogenic amine production

None of the *Lactobacillus* strains produced purple coloration and zone of clearance on the plates containing histidine, lysine, ornithine and tyrosine respectively, suggesting that the amino acid decarboxylase activity was non-detectable, if not entirely absent. *Enterococcus faecalis* included as positive control produced clear zone around colonies on tyrosine plates providing evidence of the production of amino acid decarboxylase activity.

Probiotic properties of lyophilized cells of L. rhamnosus Fb

Fig. 5 shows that the viability of cells was not altered upon freezing (-20°C, 18 h), though 16% reduction was observed after lyophilization. Viability of lyophilized cells of *L. rhamnosus* Fb was not altered significantly (P<0.05) in the presence of 6% NaCl, 0.4% phenol and at pH 3. Cells retained >82% viability in 0.5% bile salt and at pH 2, while retained 80% viability upon sequential exposure to SGF and SGF-SIF transit. %viability of the lyophilized cells was calculated in comparison to MRS medium.

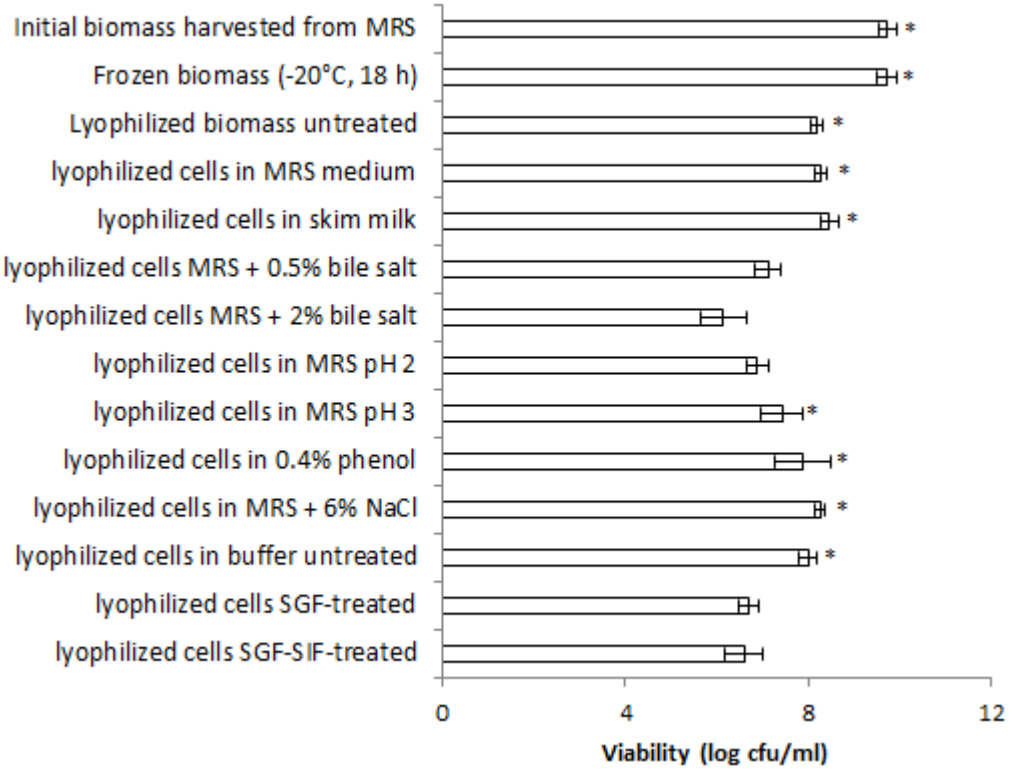

Fig. 5 Viability (log cfu/ml) of lyophilized cells of *L. rhamnosus* Fb in MRS medium and MRS modified with bile salt (0.5, 2%), pH (2, 3), NaCl (6%), and phenol (0.4%) after 4 h of incubation at 37°C, and after sequential exposure to simulated gastric fluid (SGF, 2 h) and intestinal fluid (SGF+SIF, 2, 3 h). Values are presented as mean viable count on MRS medium from two independent experiments, error bar indicates standard deviation. *values are not significantly different (P<0.05)

Discussion

Lactobacilli residing in human gastrointestinal tract (GIT) are non-pathogenic and adaptable strains forming an important biodefense factor. It is therefore worth

exploring the indigenous normal microflora of GIT as probiotics. It is necessary to screen and characterize numerous *Lactobacillus* strains in order to obtain ideal and novel probiotics, as all *Lactobacillus* strains, perhaps do not confer health benefits to the host. It is mandatory that the probiotic lactobacilli are of healthy human origin, non-pathogenic, and non-haemolytic, though the lactobacilli have a long history of being safe for humans and been conferred GRAS status.

An important step towards the selection of probiotic strain is to investigate the strain viability under conditions which mimic saliva and the GIT. For bacterial cells the stress begins in mouth, with lysozyme-containing saliva and continues in the stomach and upper intestine. Probiotic bacteria are first exposed in oral cavity to saliva that contains lysozyme and electrolytes. *L. rhamnosus* strains retained viability in the presence of lysozyme provides evidence of successful transit through oral cavity.

Prior to reaching the intestinal tract, probiotic bacteria must survive during transit through the stomach, where the secretion of gastric acid constitutes a primary defense mechanism against the ingested microbes. Gastric fluid is a crucial barrier to overcome prior to reaching the site of action (Dunne *et al*., 2001). The pH in human stomach is as low as 1-1.5 during fasting, to 4.5 after meal, and the food passage time through stomach is 90 min. *Lactobacillus* strains were evaluated under *in vitro* conditions for their probiotic properties *viz*. acid, bile, NaCl and phenol tolerance and also during simulated gastrointestinal transit. *L. rhamnosus* Vc, 231 and Fb have higher survival (>75%) when exposed to pH 2 for 4 h, than the reference strains *L. rhamnosus* GG and *L. casei* Actimel (≥72). Autochthonous *L. rhamnosus* strains of human origin exhibit better adaptation at pH 2 than reported previously for other *L. rhamnosus* strains isolated from traditional Maasai fermented milk products (Mathara *et al*., 2008). The results are promising in the light of the fact that the *L. rhamnosus* strains survived for 4 h, a much longer time than for the cells to pass through stomach, and exert the probiotic effects in intestine. The *L. rhamnosus* Fb, GG, 231, Vc and *L. casei* Actimel retain viability (69-93%) under conditions existing in the stomach. The decreased viability of *L. rhamnosus* Vc and 231 may perhaps be attributed to pepsin. The incubation time for treatment with gastric (120 min) and intestinal fluid (180 min) pretends the food ingestion and passage time from stomach to intestine during digestive processes (Gardiner *et al*., 2000). Probiotic bacteria are never exposed

directly to gastric fluid, as food or other carriers buffer the cells (Conway et al., 1987). Xanthopoulos *et al*. (2000) have also reported the high survival (>70%) of *L. rhamnosus* strains at pH 2. The acid tolerance of probiotic strains confer them the competitive advantage in gastrointestinal environment, and when used as starter cultures in food fermentation or as dietary adjuncts in acidic food preparation.

Bile tolerance is another essential criterion for the selection of a probiotic strains. Bile tolerance of *Lactobacillus* strains is evident from their high survival in the presence of 4% bile salt, in SIF, and their ability to produce bile salt hydrolase activity. Bile acids synthesized from cholesterol in the liver, are secreted in the conjugated form from gall bladder into the duodenum, where they play an important role in the digestion of fat. The bile salt concentration in the gut fluctuates from 1.5 to 2% (w/v) in the first hour of digestion, and decreases thereafter to 0.3% *ca*. (Noriega *et al*., 2004). Schillinger *et al*. (2005) reported that *L. acidophilus* produces bile salt hydrolase activity but not *L. paracasei* and *L. rhamnosus.* Our work presented here provides experimental evidence of the ability of *L. rhamnosus* strains Fb, GG, 231, Vc, and *L. casei* Actimel to produce bile salt hydrolase activity. Deconjugation of bile salts aids in the survival of *L. rhamnosus* in intestine, and moreover deconjugated bile salts being less soluble are efficiently re-absorbed from the intestinal lumen than their conjugated counterpart, resulting in excretion of larger amount of free bile acids in feces. Deconjugation of bile acids by lactobacilli could lead towards a reduction in serum cholesterol by (i) increasing the demand of cholesterol for *de novo* synthesis of bile acids to replace those lost in feces, and (ii) reducing cholesterol solubility and thereby, the absorption of cholesterol throughout the intestinal lumen (Pereira *et al*., 2003). Pancreatin may be responsible for the reduction in the viability of *Lactobacillus* strains in simulated intestinal fluid, as 0.5% bile salt does not alter the viability (data not shown).

L. rhamnosus strains tolerated 0.6% phenol, a toxic metabolite produced upon deamination of some aromatic amino acids during putrefaction by intestinal bacteria (Sathyabama *et al*., 2012). NaCl tolerance of the strains is also important for their survival during processing of fermented vegetables which is generally carried out in the presence of *ca*. 6-8% NaCl. It confers competitive edge over other undesirable organisms during food processing, and together with their antimicrobial activity against food-spoilage organisms, is of advantage for their use in food preservation.

The ability to adhere mucosal surface is mandatory for the pathogens to cause infection and for the probiotic organisms to exert their beneficial influence. The bacterial cell surface characteristics predominantly determine their ability to (i) adhere mucosal surfaces, (ii) autoaggregate and, (iii) coaggregate. Adhesion to mucosal surface is a desirable characteristic of probiotic bacteria for the colonization and persistence in the GIT. The bacterial adherence is in turn complex process and involves several mechanisms that depend on cell surface properties and extracellular protein profile of bacteria (Gibbons, 1996). The hydrophobicity of the cells has been associated with the initial interactions that are non-specific, weak and reversible, and the subsequent adhesion process mediated by specific-binding cell surface proteins and lipoteichoic acids (Conway *et al*., 1987). *L. rhamnosus* and *L. casei* characterized here displayed hydrophilic cell surface, the results observed by us are in accordance with the previously reported values (Kiely and Olson, 2000; Pelletier *et al*., 1997). In addition, *L. rhamnosus* cell surface is strong electron donor and weak electron acceptor, since it has high affinity for acidic solvent chloroform and low affinity for a basic solvent ethyl acetate. Hydrophobicity of cells has been correlated with the adhesion ability of lactobacilli (Ehrmann *et al*., 2002), whereas lack of correlation between hydrophobicity and bacterial adhesion has also been reported where strains with high hydrophobicity exhibited lower adhesion to human cell line (Vinderola *et al*., 2004; Schillinger *et al*., 2005; Mathara *et al*., 2008). Despite having hydrophilic cell surface, strain Fb is able to adhere mucin and is statistically comparable to *L. rhamnosus* GG. The extent of adhesion varied among the strains, which may be due to the different origins of the strains. In agreement with this, we also observed no association between hydrophobicity and adhesion ability of the strains studied here. The mucin adhesion results can be extrapolated for *in vivo* application. Thus, the hydrophobicity may be helpful in adhesion, but it is obviously not a prerequisite for a strong adherence capacity.

Autoaggregation and coaggregation abilities enable lactobacilli to form a barrier against colonization of mucosal surfaces by pathogens. This may be attributed to altered microenvironment of the coaggregated cell surface and antimicrobial metabolites of lactobacilli (Reid *et al*., 2004). Autoaggregation and coaggregation (i) help *Lactobacillus* cells to adhere and colonize gastrointestinal surfaces, (ii) provide protection in the hostile environment of the GIT and in turn (iii) extend their homing

time in the gut and, (iv) influence the host health by affecting the microbial homeostasis towards healthier composition. *L. rhamnosus* strains exhibited the ability to form autoaggregates but to varying extent; *L. rhamnosus* GG aggregated rapidly, which increased with incubation time. Autoaggregation and coaggregation abilities are strain-specific as reported for *L. plantarum* (Collado *et al*., 2007), dependent on incubation time, and related i.e. strains showing higher autoaggregation demonstrated higher coaggregation. It confers an additional advantage on probiotic lactobacilli, as it may serve as anti-infection mechanism in gastrointestinal tract as well as in urogenital tract (Reid *et al*., 2004).

β-galactosidase activity is an important property of probiotics. Lactose intolerance is found in people lacking the enzyme β-galactosidase, since lactose is not broken down in the upper regions of the small intestine and is thus used by the indigenous microbiota (De Vrese *et al*., 2001). All the *Lactobacillus* strains produced β-galactosidase activity and extent of activity did not vary with the strains, unlike *L. plantarum* strains as reported by Zago *et al*. (2011). These strains may therefore be useful to alleviate lactose intolerance in the gut and as digestive supplement.

L. rhamnosus strains exhibit broad antimicrobial spectrum against Gram-positive and Gram-negative organisms, which are major food spoilage organisms and gastrointestinal pathogens. *Lactobacillus* strains show varying degree of strain-specific antibacterial activity against tested pathogens. Strain-specific nature of the antimicrobial activity has also been reported earlier (Ambalam *et al*., 2009; Gaudana *et al*., 2010; Pithva *et al*., 2012). *L. rhamnosus* strains retain antimicrobial activity even after the passage through simulated gastrointestinal fluid, indicates the potential of these strains to provide protection against dysbiosis caused by various pathogenic microorganisms. Reduction in the extent of antimicrobial activity upon sequential exposure to simulated gastrointestinal and intestinal fluid indicates the activity is mainly due to presence of number of viable cells and its metabolites. Antimicrobial activity of *L. rhamnosus* confers on them the competitive advantage in controlling the proliferation of pathogenic bacteria in traditional fermented food or dairy products. Lactobacilli exert antimicrobial action through the production of organic acids (lactic and acetic acid), H_2O_2, and or other antibacterial molecules such as bacteriocins, low

molecular peptides, and antifungal peptides, phenyllactic acid and OH-phenyllactic acid, etc.

Fungal spoilage is the main cause of economic losses in bakery products and the source of mycotoxins, involving public health problems (Legan, 1993). Biopreservation, the use of microorganisms to preserve food and feed stuffs, is gaining increasing interest due to the consumers demand for reduced use of chemical preservatives (Prema *et al*., 2010). *L. rhamnosus* strains possess antifungal activity against *Aspergillus niger*, and *A. flavus*, anti-*Candida* activity against the major food spoilage organisms *C. parapsilosis*, and urogenital pathogens *C. albicans* and *C. tropicalis*. Gerbaldo et al. (Gerbaldo *et al*., 2012) reported antifungal activity of *L. rhamnosus* L60 against *A. flavus*. The antifungal metabolites of *L. rhamnosus* strains need to be identified and screened for their use as biopreservative against fungal spoilage.

The safety of the new strains intended to be used, as probiotics can be evaluated by determining strain-specific traits such as antibiotic resistance, biogenic amine production, and haemolytic activity. Verification of the presence of transferable resistance genes is an important constraint to be evaluated for the safety of probiotic strains. The non-susceptibility of *Lactobacillus* strains to certain cell wall synthesis inhibitors has been reported previously (Temmerman *et al*., 2003; Danielsen *et al*., 2003; Zhou *et al*., 2005). Considering these results, the non-susceptibility of *Lactobacillus* strains is innate and not a case of acquired resistance. The non-susceptibility of these strains towards certain antibiotics may be important for the antibiotic-probiotic simultaneous treatment in certain infectious conditions such as diarrhoea, urogenital tract infections, etc., may aid in rapid recovery of the healthier microbial balance. Our *L. rhamnosus* strains differ from other *L. rhamnosus* strains (Temmerman *et al*., 2003; Danielsen *et al*., 2003; Zhou *et al*., 2005) in being susceptible to protein synthesis inhibitors; amikacin, gentamicin, kanamycin, chloramphenicol, clindamycin, and nucleic acid synthesis inhibitors; co-trimoxazole and norfloxacin. The *L. rhamnosus* strains used in this study are susceptible to a broader range of antibiotics making them safer for use as probiotics.

Biogenic amines in food are mainly generated by microbial decarboxylation of the corresponding amino acids involving amino acid-specific decarboxylases (Brink *et al*.,

1990). None of the *Lactobacillus* strains produced detectable amine from histidine, lysine, ornithine and tyrosine. Histamine and tyramine have been recognized as the causative agent of histamine intoxication, whereas tyramine is related to food-induced migraines. In addition, amines are known to be potential precursors of carcinogenic nitrosamines, especially when nitrosable agents are present in food (Bover-Cid and Holzapfel, 1999). *L. rhamnosus* Fb, 231 and Vc, strains are safe as they neither harbour transferable antibiotic resistance genes, nor haemolytic and producer of biogenic amines such as tyramine, histamine, putrescine, and cadaverine.

Development of an efficient formulation of live bacterial cells is a major challenge in the commercialization of probiotic products. Probiotic formulations should retain predetermined dose of live bacterial cells during storage. Freeze-drying is a more convenient process and such cells can be used conveniently as a functional ingredient in a variety of probiotic food formulations. G-Allegria *et al*. (2004) reported *L. plantarum* strains originating from different sources to survive over 90% after lyophilization and freezing. 84% cells of *L. rhamnosus* Fb remain viable without losing probiotic properties after lyophilization. Such lyophilized *L. rhamnosus* Fb cells upon SGF, SIF transit exhibited 80% survival retaining acid-bile, NaCl, and phenol tolerance indicates the potential of strains to withstand the technological conditions.

In conclusion

This *in vitro* study demonstrates probiotic attributes including functional, safety, and technological aspects of autochthonous *L. rhamnosus* strains of human origin. Our work provides experimental evidences vindicating the strains *L. rhamnosus* Fb, *L. rhamnosus* Vc and *L. rhamnosus* 231 to possess potential probiotic properties according to the guidelines of WHO. Broad antimicrobial spectrum and their ability to coaggregate with food spoilage organisms and pathogens make these strains as potential biopreservative agents, and food additives in probiotic products. Work is in progress to identify the antimicrobial metabolites of *L. rhamnosus* strains, and to obtain biomass in various forms that would help in preparing diverse types of probiotic food formulations.