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ORIGINAL ARTICLE

In vitro probiotic evaluation of phytase producing *Lactobacillus* species isolated from *Uttapam* batter and their application in soy milk fermentation

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Abstract Probiotic lactic acid bacteria are health promoters and have been traditionally consumed without the knowledge that they have beneficial properties. These bacteria mainly involve in secreting antimicrobials, enhance immune-modulatory effects, and preserve the intestinal epithelial barrier by competitively inhibiting the pathogenic organisms. The aim of this study was to investigate the in vitro probiotic properties of *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus plantarum* ssp. *argentoratensis*, and *Lactobacillus plantarum* ssp. *plantarum* isolated from fermented *Uttapam* batter. The isolates produced bacteriocins that were effective against several pathogens. All the isolates exhibited tolerance to bile, gastric, and intestinal conditions. Beneficial properties like cholesterol assimilation and production of enzymes such as β-galactosidase, phytase and bile hydrolase varied among the isolates. Four isolates from each sub-species effectively adhered to Caco-2 cells and prevented pathogen adhesion. Using these strains, the soy milk was fermented, which exhibited higher antioxidant activity, 2,2-diphenylpicrylhydrazyl (DPPH) scavenging activity and decreased phytate content when compared to unfermented soy milk. Thus, these probiotic isolates can be successfully used for formulation of functional foods that thereby help to improvise human health.

Keywords Antioxidant activity · *Lactobacillus* · Phytase · Probiotic · Soy milk fermentation · *Uttapam* batter

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Introduction

Lactic acid bacteria (LAB) are a group of organisms that impose health benefits by live colonization in host cells or even by their antimicrobial metabolites through consumption of fermented food products. These bacteria are the normal flora of the gastrointestinal (GI) and urogenital tract and serve as potent probiotics to enhance health-modulating activities. World Health Organization (WHO) defined probiotics as "live microorganisms which when administered in adequate amounts confer health benefits on the host" (WHO/FAO 2002). Probiotic LAB confer several health benefits, which include synthesis of antimicrobial compounds, compete with pathogenic organisms for adhesion to intestinal epithelium, prevent infectious diseases and allergies (Sleator and Hill 2008), manage lactose intolerance, reduce serum cholesterol, provide anti-carcinogenic activity, and enhance immune function (Galdeano et al. 2007). They undergo a very hazardous journey through GI tract and colonize to jeopardize the survival of pathogenic microorganisms (Conway et al. 1987). Several preliminary in vitro tests are essential to qualify an organism to be probiotic such as resistance to gastric acidity, bile, and various antibiotics, and the ability to reduce pathogen adhesion to epithelium. Additional properties like cholesterol assimilation, absence of hemolytic activity, bile salt hydrolase, and β-galactosidase enzyme can expand the understanding of functional properties of these organisms. Nissen et al. (2009) proposed that the intake of functional foods (components include probiotics, prebiotics, polyunsaturated fatty acids (PUFA), antioxidants, vitamins, and minerals) could reduce the risk of several diseases. *Lactobacillus plantarum* has been widely used as a starter culture in fermentation of several food products like yogurt, cheese, pickles, kimchi, and sourdough (Hammes and Hertel 2006), the attribute of organisms from food extending to colonization in the intestine. Isolation and characterization of bacteriocinogenic (loss of activity after

protease treatment) lactobacilli species from fermented *Uttapam* batter, an indigenous South Indian fermented food source, was studied earlier where *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus plantarum* ssp. *argentoratensis*, and *Lactobacillus plantarum* ssp. *plantarum* were identified at sub-species level using molecular tools such as 16S rRNA and multiplex PCR (Saraniya and Jeevaratnam 2012). Soy milk is an economical protein and calorie-rich food consumed in several parts of the world. But unfermented soy milk contains high amount of phytate, an anti-nutrient factor that prevents absorption of minerals. Most of the earlier published works focused on the growth of LAB, pH and isoflavone content in soy milk but did not detail the reduction of phytate content in the soy milk (Chen et al. 2013; Li et al. 2012). Hence, an attempt was made in this study to evaluate the probiotic properties of *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus plantarum* ssp. *argentoratensis*, and *Lactobacillus plantarum* ssp. *plantarum* in vitro and the phytate content and antioxidant properties in fermented soy milk using these isolates as compared to unfermented soy milk.

Methodology

Strains and antibacterial assay

Potent isolates were isolated from fermented *Uttapam* batter and identified using 16S rRNA gene analysis and were deposited in GenBank, NCBI. Antibacterial activity assay using cell-free supernatant concentrate (CFSC) adjusted to pH 6.0 was carried out against intestinal pathogens (Table 1) by agar well diffusion assay (Saraniya and Jeevaratnam 2012). The pathogens used were obtained from the Microbial Type Culture Collection (MTCC), at Institute of Microbial Technology, Chandigarh, India, and maintained in MRS broth for LAB isolates and soya bean casein digest broth for non-LAB organisms and stored at −20 °C with 30 % glycerol.

Bile tolerance test

Bile tolerance test was carried out by growing the lactobacilli isolates in MRS broth containing 0, 0.1, 0.3 and 0.5 % of oxgall bile (Himedia, India) for 24 h at 37 °C. The growth of cells was monitored at 560 nm for every 1 h till 8 h and after 24 h. The delay in growth was calculated against time, which was used as a measure of bile tolerance (de Valdez and de Taranto 2001).

Screening of isolates for tolerance to various pH and simulated gastric and intestinal juice

Initial screening of isolates for tolerance to various initial pH (2.0, 3.0, 7.0) was done. The isolates were grown in MRS broth and the cells were pelleted in sterile condition. The cells of 10^8 colony forming units (cfu) were suspended in sterile saline solution, the pH of which was adjusted with 1 M HCl and incubated at 37 °C for 0, 2, and 4 h, respectively. The cell suspension was then plated on MRS agar, incubated at 37 °C, and the cfu was calculated. Further the isolates were subjected to in vitro simulated gastric and intestinal tolerance test. The cells were washed with saline, and the cells (10^8 cfu/ml) were suspended in saline adjusted to pH 2.0 with 3 mg/ml of pepsin for gastric tolerance and by addition of 0.3 % bile salt and 1 mg/ml pancreatin adjusted to pH 8.0 for intestinal tolerance. Cell suspension in saline with pH 7.0 serves as control. After 0, 2, 4, and 6 h of incubation at 37 °C, the cells were serially diluted and plated on MRS agar and the cfu was calculated (Osmanagaoglu et al. 2010).

Bacterial adhesion to hydrocarbons (BATH assay)

The in vitro bacterial cell adhesion to hydrocarbons was determined by the method of Doyle and Rosenberg (1995) with a few modifications. Isolates grown in MRS broth were centrifuged and the pellet was washed twice and suspended in phosphate buffer (pH 7.0) adjusted to 1.0 A_{560} using spectrophotometer (Shimadzhu, Japan). Equal volume of solvent (hexadecane and xylene) and bacterial suspension was added and vortexed for 2 min. The tubes were allowed to stand at 37 °C for 30 min for the separation of two phases. The aqueous phase (A) was carefully removed and the OD at 560 nm was measured. The percentage of cell surface hydrophobicity (%H) of the strain adhering to solvents was calculated using the formula,

$$\%\,Hydrophobicity = \frac{OD_I - OD_F}{OD_I} \times 100$$

where OD_I and OD_F are initial and final absorbance.

Auto-aggregation and co-aggregation property

For auto- and co-aggregation, cells were pelleted by centrifugation at 8,000*g* for 10 min and OD adjusted to 1.0 A_{560} using spectrophotometer and washed twice in 50 mM phosphate buffer (pH 7.0). The auto- and co-aggregation were performed as described by Osmanagaoglu et al. (2010). For co-aggregation studies, the isolates were incubated along with *Lactobacillus rhamnosus* (MTCC 1408), *Lactobacillus plantarum* (MTCC 6161), *Escherichia coli* (MTCC 728), and *Listeria monocytogenes* (MTCC 657). The percentage of auto-aggregation and co-aggregation was calculated using the formula,

$$\%\,Auto-aggregation\,or\,Co-aggregation = \frac{OD_I - OD_F}{OD_I} \times 100$$

where OD_I and OD_F are initial and final absorbance.

Adhesion to human epithelial carcinoma cell line Caco-2

The colonic adenocarcinoma cells (Caco-2) were procured from NCCS (National Centre for Cell Sciences), Pune, India. The cells were maintained in minimal essential medium (MEM) with 10 % fetal bovine serum (Sigma, India), 25 mM HEPES buffer, 20 U/ml penicillin, and 100 μg/ml of streptomycin. For adhesion assay, 10^5 cells were transferred to six-well plate to obtain confluence after a period of 15 days. The bacterial cells (10^8 cfu/ml) were suspended in supplement-free MEM, added to Caco-2 cells, and incubated for 90 min. After incubation, the cells were fixed with 2 % formaldehyde, stained with 0.1 % acridine orange, and visualized under fluorescence microscope. For pathogen exclusion assay, the Caco-2 cells were treated with isolates as described above, followed by addition of *Listeria monocytogenes* (10^8 cfu/ml in supplement-free MEM), incubated for an additional 60 min, and stained with 0.1 % acridine orange. The cells were collected and plated on either MRS agar, incubated anaerobically or Listeria enrichment broth (Himedia, Mumbai) for the selective enumeration of lactic acid bacteria and *Listeria monocytogenes* respectively. The exclusion percentage was determined using the formula,

$$\%\,Exclusion = \frac{A_I - A_F}{A_I} \times 100$$

where A_I is the initial cfu and A_F is the final cfu of *Listeria monocytogenes* after incubation (Osmanagaoglu et al. 2010).

Antibiotic susceptibility test

The isolates were checked for antibiotic susceptibility in MRS soft agar, inoculated using 10^8 cfu/ml with respective cultures that were allowed for solidification at room temperature. The commercial antibiotic disc (Himedia, India) was placed on to the agar and incubated at 37 °C for 24 h, and the resistance or sensitivity was noted according to the CLSI/NCCLS standard (CLSI 2011).

Cholesterol assay

MRS broth having 0.3 % bile oxgall was sterilized and to this a final concentration of 100 μg/ml of sterile cholesterol was added (MRS-CHO) and inoculated with 1 % (*v/v*) with an overnight culture of the respective isolates. Uninoculated MRS-CHO broth was also processed in the same way as the control. Cholesterol estimation was done spectrophotometrically by o-pthalaldehyde method (de Valdez and de Taranto 2001).

$$\%\,Assimilation = \frac{C - T}{C} \times 100$$

where C is the control (MRS-CHO without any inoculums) and T is the test (MRS-CHO inoculated with isolates).

Detection of bile salt hydrolase activity

The presence of bile hydrolase enzyme in the *Lactobacillus* strains was detected by culturing the bacteria on MRS agar containing 0.5 % (*w/v*) taurodeoxycholic acid (TDCA) and incubated under anaerobic conditions for 72 h. Deconjugation of taurodeoxycholate results in a white precipitate of deoxycholate in the area of bacterial colonies (Dashkevicz and Feighner 1989).

β-galactosidase activity

The isolates were evaluated for β-galactosidase activity by growing in MRS broth having lactose, and the cells were recovered by centrifugation at 8,000*g* for 10 min. The cells were washed with saline, suspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, and 50 mM β-mercaptoethanol pH 7.0), and permeabilized with toluene. The permeabilized cells were incubated with ortho-nitrophenyl-β-galactoside (4 mg/ml) at 37 °C for 15 min and the reaction was stopped by addition of 1 % sodium bicarbonate. The absorbance was read at 550 and 420 nm, respectively, and the enzyme unit was expressed in Miller units (Vinderola and Reinheimer 2003).

$$Miller\ Units = 1,000 * \frac{A_{420} - 1.75 \times A_{560}}{Time\ of\ incubation \times volume\ of\ culture \times A_{560}}$$

where the absorbance at 420 nm denotes the end product color, while the absorbance at 560 nm denotes the correction factor for cell turbidity.

Hemolytic activity in blood agar

Hemolysis in blood agar for the isolates was evaluated using blood agar base supplemented with 5 % of human blood as detailed by Gao et al. (2012). *Escherichia coli* for α-hemolysis and *Streptococcus pyogenes* for β-hemolysis were used as positive control.

Screening of phytase production

The isolates were screened initially for phytase production as described by Anastasio et al. (2010). The isolates were spotted in modified MRS agar containing phytate and incubated at 37 °C for 48 h. The clearing of zone around the spot after

incubation with 2 % cobalt chloride solution for 20 min indicates positive for phytase production.

Phytase assay

The isolate was grown in phytate-containing Chalmers broth for 24 h at 35 °C and the CFS was collected by centrifugation at 10,000*g* for 15 min. One milliliter of CFS was mixed with 0.6 ml of reaction mixture (3 mM phytate in 0.2 M sodium acetate buffer pH 4.5) and incubated at 50 °C for 15 min. The reaction was terminated by addition of 1 ml of 10 % TCA. The released inorganic phosphate was measured by addition of 0.75 ml of coloring reagent prepared freshly (4 volumes of ammonium molybdate in 5.5 % sulfuric acid and 1 volume of 2.7 % ferrous sulfate solution). The absorbance was measured at 700 nm. Phytase activity was measured in μmol of inorganic phosphate released from phytate. The Chalmers broth was used as negative control. One unit of phytase is defined as the amount of enzyme required to produce 1 μmol of inorganic phosphate per minute under assay conditions (Anastasio et al. 2010).

Preparation and fermentation of soy milk

Soybeans were soaked in three volumes of distilled water at ambient temperature for 10 h. The hydrated beans were dehulled manually and ground with distilled water with a ratio of 1:5 *w/v* and filtered through double layer cheesecloth. The resultant soy milk in the filtrate was sterilized at 121 °C for 15 min. The isolate was grown in MRS broth until the cells attained mid log phase and harvested by centrifugation at 8,000*g* for 10 min. After washing, the cells were suspended in sterile saline (0.85 % NaCl). Five milliliter of soy milk was inoculated with 2 % (*v/v*) of the isolates and incubated at 35 °C for 18 h. Uninoculated soy milk was used as control.

Estimation of phytate content

The phytate content in fermented and unfermented soy milk was extracted with trichloroacetic acid and precipitated with ferric salt. The iron content in the precipitate was determined using potassium thiocyanate and the phytate phosphorous was calculated assuming the constant 4Fe:6P molar ratio (Sadasivam and Manickam 2008).

Antioxidant activity of fermented and unfermented soy milk

2, 2-diphenylpicrylhydrazyl (DPPH) scavenging activity

An aliquot of 0.5 ml of sample was mixed with 1 ml of methanolic DPPH (final concentration: 0.1 mmol/L) and incubated in dark for 30 min. After incubation, the absorbance was read at 517 nm against reagent blank (Marazza et al. 2012). The DPPH scavenging activity was expressed in percentage using the formula given below:

$$\text{DPPH } scavenging\ (\%) = \left(1 - \frac{A_{517}\ of\ sample}{A_{517}\ of\ blank}\right) \times 100$$

Reducing power property

For evaluating the reducing power, 1 ml of PBS (phosphate buffered saline), 1 ml of 1 % (*w/v*) potassium ferricyanide, and 0.5 ml of sample were mixed together and incubated at 50 °C for 30 min. Then 1 ml of 10 % (*w/v*) TCA was added and the mixture was centrifuged at 8,000*g* for 10 min. To 2 ml of supernatant, 0.4 ml of 0.1 % (*w/v*) of ferric chloride wad added and vortexed. After incubation for 10 min at room temperature, the absorbance was measured at 700 nm. The higher the absorbance of reaction mixture, higher the reducing power (Marazza et al. 2012).

Statistical analysis

The results are expressed as mean and standard deviation of 3 independent experiments performed in duplicate. The statistical analysis was performed using OriginPro v8. The results were compared using analysis of variance (ANOVA) by Turkey's pot-hoc test and $p<0.05$ was considered as statistically significant.

Results and Discussion

The isolates from *Uttapam* batter were identified as *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus plantarum* ssp. *plantarum*, and *Lactobacillus plantarum* ssp. *argentoratensis* with accession numbers JN573616 to JN573623 (Saraniya and Jeevaratnam 2012). These isolates were evaluated for probiotic and beneficial properties to assess their suitability for the formulation of functional foods and biomedical applications. The present study also intends to establish the phytate content and antioxidant properties in fermented soy milk, studied using the *Uttapam* batter isolates. Thus identification of strains having antimicrobial, probiotic and other beneficial potential from different ecological niches and utilization of those collections of strains together for the formulation of functional foods would be advantageous to humans.

Antibacterial spectrum against LAB and pathogenic organisms

The antimicrobial activity (Table 1) of Uttapam batter LAB isolates was minimal or no activity against LAB indicators signifying that the isolates do not pose any threat to other LAB strains, while these isolates showed effective inhibitory activity against some intestinal Gram-positive and Gram-negative pathogens suggesting effective antimicrobial activity against pathogens. Earlier study showed that the eight isolates were bacteriocinogenic in nature as there was loss of activity after protease treatment (Saraniya and Jeevaratnam 2012).

Bile, simulated gastric, and intestinal tolerance test

Probiotic bacteria have to essentially prevail through stress environments, acidic stomach, and then through hydrolytic activity of enzymes including bile salt in the small intestine. Morelli (2000) proposed that the pH of stomach varies between pH 1.5 and 3.0. In cases of starvation and stress conditions, the pH usually declines to 1.5, while it increases to 3.0 after meal. Initial screening showed the eight isolates were tolerant to acidic and neutral pH. The isolates showed tolerance to simulated gastric and intestinal conditions with minimal decrease in cfu (Table 2). In addition, the isolates were resistant to 0.3 % bile exhibiting delay in growth of 1 to 7 h (Table 3), which was similar to the observations made by Morelli (2000) where the growth of *Lactobacillus* in the presence of oxgall is not species-dependent but strain-dependent. This study also showed that tolerance varied among the organisms, indicating that it was mostly strain-dependent.

Aggregation studies for the promising isolates

It is a desirable quality for a probiotic to adhere and aggregate on to the host cell to elicit the beneficial effects. Cesena et al. (2001) proposed that the isolates that have the ability to aggregate are generally hydrophobic in nature and can withstand to survive well in the intestinal tract. The isolates were evaluated for hydrophobicity using solvents xylene and hexadecane, and all isolates showed varying hydrophobicity (Table 3). Maximal hydrophobicity was shown by isolate SJ37. A correlation was observed between BATH assay and autoaggregation results wherein *Lactobacillus plantarum* ssp. *argentoratensis* SJ37 showed maximal hydrophobicity and aggregation. The hydrophobicity and the autoaggregation ability are two independent factors that are essentially required for adhesion. All the eight isolates showed effective co-aggregation against the viable pathogens *Listeria monocytogenes* and *Escherichia coli*, with >50 % aggregation property, while an earlier study on co-aggregation using *Lactobacillus rhamnosus* LC-705 and *Lactobacillus fermentum* ME-3 showed 29 and 25 %, respectively, against *Bifidobacterium vulgatus* whereas *Bifidobacterium longum* 46 and *Bifidobacterium lactis* 420 showed only 5 and 4 %, respectively, against *Bifidobacterium vulgates* (Collado et al. 2008).

Antibiogram for the eight isolates

Lactobacilli group of organisms naturally exhibits resistance to wide range of antibiotics, and the susceptibility testing of each strain is essential (Charteris et al. 1998). The eight isolates were studied for susceptibility/resistance to various groups of antibiotics, and the results are presented in Table 4.

Table 1 Antibacterial spectrum of the eight potent isolates against LAB and pathogens

Isolates	SJ65	SJ5	SJ22	SJ6	SJ37	SJ40	SJ33	SJ9
Lactobacillus rhamnosus MTCC 1408	10±1	12±1	12±1	11±1	10±1	12±1	11±2	12±2
Enterococcus faecalis MTCC 439	11±1	10±1	11±1	11±1	10±1	10±2	11±2	12±2
Staphylococcus aureus MTCC 737	18±3	17±2	17±3	16±2	16±4	16±3	15±3	18±3
Listeria monocytogenes MTCC 657	20±2	14±3	15±2	15±2	15±3	10±2	14±2	15±2
Bacillus cereus MTCC 1272	20±2	20±3	15±2	18±3	15±3	18±4	17±2	19±4
Vibrio parahemolyticus MTCC 451	17±3	16±3	15±2	16±2	14±4	15±2	16±2	16±3
Aeromonas hydrophila ssp. *hydrophilia* MTCC 1739	19±2	17±2	18±3	17±2	14±3	18±4	17±3	19±3
Escherichia coli MTCC 728	18±3	16±3	16±2	14±2	19±3	13±2	18±4	15±2

SJ65 – *Lactobacillus pentosus*, SJ5 and SJ22 – *Lactobacillus plantarum*, SJ6, SJ33, SJ37, SJ40 – *Lactobacillus plantarum* ssp. *argentoratensis*, SJ9 – *Lactobacillus plantarum* ssp. *plantarum*. Inhibition zone expressed in mm inclusive of well diameter 6 mm. Values are mean±SD of 3 independent experiments performed in duplicates

Table 2 Simulated gastric and intestinal tolerance of the eight isolates

Isolates	Conditions	Log cfu/ml			
		0	2	4	6 h incubation
SJ65	Control	9.30±0.39	9.31±0.37	9.19±0.15	ND
	Artificial gastric juice	9.52±0.50	7.53±0.47	6.88±0.82	ND
	Artificial intestinal juice	9.27±0.48	ND	8.66±0.33	8.12±0.41
SJ9	Control	9.44±0.28	9.26±0.36	9.17±0.36	ND
	Artificial gastric juice	9.37±0.49	7.64±0.29	7.14±0.56	ND
	Artificial intestinal juice	9.26±0.27	ND	8.15±0.20	7.76±0.28
SJ5	Control	9.44±0.61	9.62±0.21	9.00±0.64	ND
	Artificial gastric juice	9.34±0.50	7.94±0.14	6.65±0.39	ND
	Artificial intestinal juice	9.41±0.33	ND	8.08±0.26	7.05±0.34
SJ22	Control	9.46±0.37	9.15±0.57	9.17±0.47	ND
	Artificial gastric juice	9.11±0.49	7.33±0.39	7.31±0.32	ND
	Artificial intestinal juice	9.09±0.27	ND	8.34±0.39	8.47±0.53
SJ6	Control	9.34±0.39	9.20±0.24	9.21±0.40	ND
	Artificial gastric juice	9.79±0.15	7.51±0.33	6.87±0.09	ND
	Artificial intestinal juice	9.36±0.31	ND	7.91±0.16	8.07±0.64
SJ33	Control	9.40±0.42	9.52±0.39	9.26±0.40	ND
	Artificial gastric juice	9.64±0.29	7.45±0.30	7.14±0.40	ND
	Artificial intestinal juice	9.51±0.62	ND	8.52±0.28	7.41±0.32
SJ37	Control	9.16±0.37	9.14±0.59	9.34±0.49	ND
	Artificial gastric juice	8.61±0.22	8.34±0.38	7.56±0.21	ND
	Artificial intestinal juice	8.28±0.36	ND	8.01±0.25	7.80±0.17
SJ40	Control	9.69±0.19	9.24±0.53	9.51±0.33	ND
	Artificial gastric juice	8.30±0.38	8.68±0.29	7.30±0.72	ND
	Artificial intestinal juice	8.20±0.56	ND	8.06±0.36	8.02±0.44

Values are mean±SD of three independent experiments performed in duplicate. ND – not determined

Isolates SJ22, SJ37 and SJ65 were found to be resistant to most of the antibiotics, while the other isolates were susceptible to most of the protein synthesis inhibitors and some cell wall synthesis inhibitors. However, all isolates were resistant to nucleic acid synthesis inhibitors except Co-trimoxazole and Nitrofurantoin. Earlier reports have shown that LAB has intrinsic antibiotic resistance genes to antibiotics like tetracycline (*tet*), erythromycin (*erm*), chloramphenicol (*cat*), and vancomycin (*van*). These resistances are usually due to mutation at chromosomal or plasmid level and the genes are transferred either to the same group or to the pathogens by the mechanism of horizontal gene transfer (Ashraf and Shah 2011; Todorov et al. 2012). Resistance to aminogylcosides and nucleic acids inhibitors varied among the isolates, while all showed resistance to lipopolysaccharide inhibitor.

Beneficial properties of the isolates

Beneficial properties like the presence of β-galactosidase and bile salt hydrolase activity, cholesterol sequestering ability, and absence of hemolysis are presented in Table 3. Microbial β-galactosidase is currently of interest because it is utilized for bioprocess technology. β-galactosidase (lactase) is an enzyme that hydrolyzes glycosidic bonds, and the insufficiency of this enzyme leads to a condition called lactose intolerance with clinical symptoms like abdominal cramps and diarrhea. In this study, β-galactosidase production ranged between 835 and 2225 MU, where the maximal production was by *Lactobacillus pentosus* SJ65 followed by *Lactobacillus plantarum* ssp. *plantarum* SJ9. Earlier, Wang et al. (2010) showed maximal β-galactosidase production by *Lactobacillus paracasei* F08 and *Lactobacillus acidophilus* C11 isolated from infant feces and pickled cabbage. Bile acids are synthesized from cholesterol and conjugated with either glycine or taurine to form bile salts. The intestinal bacteria produce bile salt hydrolase enzyme and prevent bile salt toxicity (De Smet et al. 1995). The BSH-negative cells are more prone to bile salt toxicity through intracellular acidification (Begley et al. 2006). In this study, out of eight isolates *Lactobacillus pentosus* SJ65 and *Lactobacillus plantarum*

Table 3 Survival in intestinal condition, Cell surface properties and Adhesion to intestinal cells by the eight isolates from Uttapam batter

	SJ65	SJ9	SJ5	SJ22	SJ6	SJ33	SJ37	SJ40
Bile tolerance @	4 h	2 h	2 h	2 h	1 h	6 h	1 h	7 h
Cholesterol assimilation*	73±3[a]	71±2[a]	56±5[b]	60±4[b]	44±3[c]	48±5[c]	47±6[c]	44±4[c]
β-Galactosidase (MU)#	2225±153[a]	1574±92[b]	835±70[c]	1436±124[b]	1747±29[b]	1416±161[b]	1849±336[b]	1749±81[b]
Bile Salt hydrolase	+	–	–	–	+	+	+	+
Hemolysis	–	–	–	–	–	–	–	–
Hydrophobicity*								
Xylene	69±2[a]	77±4[b]	11±2[c]	94±3[d]	40±3[e]	40±6[e]	94±2[d]	32±4[e]
Hexadecane	61±3[a]	65±3[a]	33±2[b]	70±3[c]	35±3[b]	53±3[d]	92±3[e]	56±2[d]
Auto-aggregation*	63±1[a]	69±2[b]	48±4[c]	55±5[c]	55±4[c]	57±5[c]	76±3[d]	62±1[a]
Co-aggregation*								
Lactobacillus rhamnosus	57±3[a]	55±2[a]	69±2[b]	55±3[a]	50±3[c]	45±2[c]	57±4[a]	55±3[a]
Lactobacillus plantarum	61±5[a]	58±4[a]	49±3[b]	60±4[a]	46±4[c]	65±2[a]	59±2[a]	51±1[c]
Listeria monocytogenes	67±4[a]	61±5[a]	41±6[b]	63±4[a]	53±5[c]	56±5[a]	68±5[a]	39±3[b]
Escherichia coli	63±4[a]	65±5[a]	50±4[b]	57±3[a]	64±4[a]	61±3[a]	69±3[a]	48±3[b]
Caco-2 cell adhesion*	17.6±2.9[a]	15.6±3.4[a]	ND	14.9±2.8[a]	ND	ND	17.3±4.8[a]	ND
Pathogen exclusion*	84±4[a]	76±5[a]	ND	64±2[b]	ND	ND	79±3[a]	ND

Values are mean±SD of three independent experiments performed in duplicate. ND – not determined

@ Time delay for bile tolerance was calculated as time taken to increase OD_{560} by 0.3 units

* expressed in %

expressed in miller units

[a,b,c,d,e] Means within the same row bearing different superscripts differ significantly ($p<0.05$, ANOVA test)

ssp. *argentoratensis* SJ6, SJ33, SJ37, and SJ40 showed the presence of bile hydrolase in the medium containing TDCA, while *Lactobacillus plantarum* SJ5, SJ22 *and Lactobacillus plantarum* ssp. *plantarum* SJ9 did not show bile salt hydrolase activity.

Probiotics are known to have hypocholesterolemic effects by its ability to decrease cholesterol level in small intestine and help to prevent coronary heart disease. In this study, maximal cholesterol assimilation was observed by *Lactobacillus pentosus* SJ65, followed by *Lactobacillus plantarum* ssp. *plantarum* SJ9 with 73 and 71 %. Kimoto et al. (2002) have demonstrated the cholesterol-lowering ability by probiotics and the probable mechanisms involved. Cholesterol can be removed either by the process of assimilation or through deconjugation of the bile acids facilitating excretion or by cell surface binding with both live and dead cells of probiotic bacteria. They have also proposed that the incidence of cholesterol binding by dead cells was less compared to that of live cells. Although several debates still persist regarding the function of bile salt hydrolase activity and cholesterol assimilation by these bacteria, yet these assays are performed additionally to understand the functionality of these organisms to human health. The eight probiotic isolates were non-hemolytic, when grown in blood agar medium, and hence they can be used safely in animal and human applications that may lead to formulation of functional foods.

Caco-2 cell adhesion for the promising isolates

Adherence of bacteria to the hydrophobic surface is an essential property, which leads to aggregation on to the host cell. Adhesion to intestine is an effective property that allows colonization in GI tract. Based on the acid and bile tolerance, aggregation and beneficial properties, four potent isolates from each sub-species were chosen for cell adhesion analysis using Caco-2 cell lines (Fig. 1). In this study, adhesion of the isolates on to cell lines varied between 10 and 20 % (Table 3), while there was not any significant difference among isolates. An earlier study showed 6 to 8 % adhesion for *Lactobacillus plantarum* (Tuomola and Salminen 1998). In addition, the prevention of pathogen adhesion by the isolates showed 75 to 85 % exclusion by each sub-species, wherein maximal prevention of pathogen adhesion (84 %) was observed by *Lactobacillus pentosus* SJ65 against *Listeria monocytogenes*. Earlier studies also demonstrated that *Lactobacillus acidophilus* LB inhibited the adhesion of *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes*, while *Lactobacillus casei* ssp. *rhamnosus* Lcr35 against *Escherichia coli* and *Klebsiella pneumoniae* and also *Lactobacillus acidophilus* and *Lactobacillus casei* inhibited *Helicobacter pylori* NCTC 11637 using Caco-2 cell lines (Forestier et al. 2001; Midolo et al. 1995). Interactions between probiotics and intestinal mucus are very essential because they help to

Table 4 Antibiotic sensitivity/resistance properties for the eight LAB isolates

Antibiogram	SJ5	SJ22	SJ6	SJ33	SJ37	SJ40	SJ9	SJ65
Cell wall synthesis inhibitors								
Penicillin (10 mcg)	S	S	S	S	S	S	S	S
Amphicillin (10 mcg)	S	S	S	S	S	S	S	S
Cefuroxime (30 mcg)	S	S	S	S	S	S	S	S
Ceftriaxone (30 mcg)	S	S	S	S	S	S	S	S
Cephalexin (30 mcg)	S	R	R	S	R	R	S	R
Cepharadine (30 mcg)	S	R	R	S	R	R	S	R
Cephaloridine (30 mcg)	S	S	R	S	S	S	S	R
Cloxacillin (5 mcg)	S	R	R	S	S	R	S	R
Mecillinam (30 mcg)	R	S	R	S	R	R	R	R
Protein synthesis inhibitors								
Chloramphenicol (25 mcg)	S	S	S	S	S	S	S	S
Tetracycline (10 mcg)	S	S	S	S	S	S	S	S
Erythromycin (10 mcg)	S	S	S	S	S	S	S	S
Amikacin (10 mcg)	S	S	S	S	R	S	S	R
Gentamycin (30 mcg)	S	R	S	S	R	R	S	R
Lincomycin (10 mcg)	S	R	S	S	R	S	S	R
Streptomycin (10 mcg)	S	S	S	R	R	S	S	R
Nucleic acid inhibitors								
Ciprofloxacin (1 mcg)	R	R	R	R	R	R	R	R
Co-trimoxazole (25 mcg)	S	S	S	S	S	S	S	S
Nalidixic acid (30 mcg)	R	R	R	R	R	R	R	R
Norfloxacin (300 mcg)	R	R	R	R	R	R	R	R
Nitrofurantoin (300 mcg)	S	S	S	S	S	S	S	S
Lipopolysacharide inhibitor								
Colistin (10 mcg)	R	R	R	R	R	R	R	R

R Resistance, *S* Sensitive; *mcg* microgram

potentially prevent the adhesion of enteropathogenic organisms. An adherent probiotic bacterium effectively mediates several functions like competitive exclusion of pathogenic bacteria and modulation of immune function by interacting with gut-associated lymphoid tissue (GALT). The possible mechanisms by which pathogen adhesion is prevented could be by either specific blockage on cell receptors or steric interactions.

Phytase and antioxidant activity in unfermented and fermented soy milk by probiotic isolates

Phytate is a well-known anti-nutrient factor that prevents/reduces bioavailability of minerals like iron, calcium, magnesium, and zinc, and proteins in the human intestine. The phosphate in phytate cannot be absorbed by animals because they lack phytase enzyme. Phytase acts by hydrolyzing phytate into myoinositol and phosphate, thereby eliminating its anti-nutritional effects (Greiner and Konietzny 2006). The phytase produced by the microbial source, especially by LAB, is having more commercial applications. The isolates produced phytase at varied concentration; the isolate *Lactobacillus pentosus* SJ65 produced maximum (Table 5). The normal level of phytate content in soy beans varies from 9.2 to 16.7 mg/g of dry matter (Chen et al. 2013), which limits the usage of raw soy milk. Microbial fermentation of soy milk helps to degrade and enhance the nutritional value of soy milk. Among the four isolates, *Lactobacillus pentosus* SJ65 has decreased 66 % of phytate content in fermented soy milk as compared to the unfermented soy milk (Table 5), which was more than that produced by *Leuconostoc mesenteroides* KC51 isolated from *Kimchi*, which degraded only 50 % of phytate after 18 h of incubation (Oh and In 2009). Apart from the phytase activity removing anti-nutritional effects, these isolates also caused enhanced antioxidant activity in the final product (Table 5). The antioxidant-rich foods have shown highly beneficial effect on human health. Among the isolates, *Lactobacillus pentosus* SJ65 exhibited 87 % DPPH radical scavenging property and maximum reducing power (A_{700}: 1.74). Thus value addition can be achieved in the soy milk fermented with these LAB

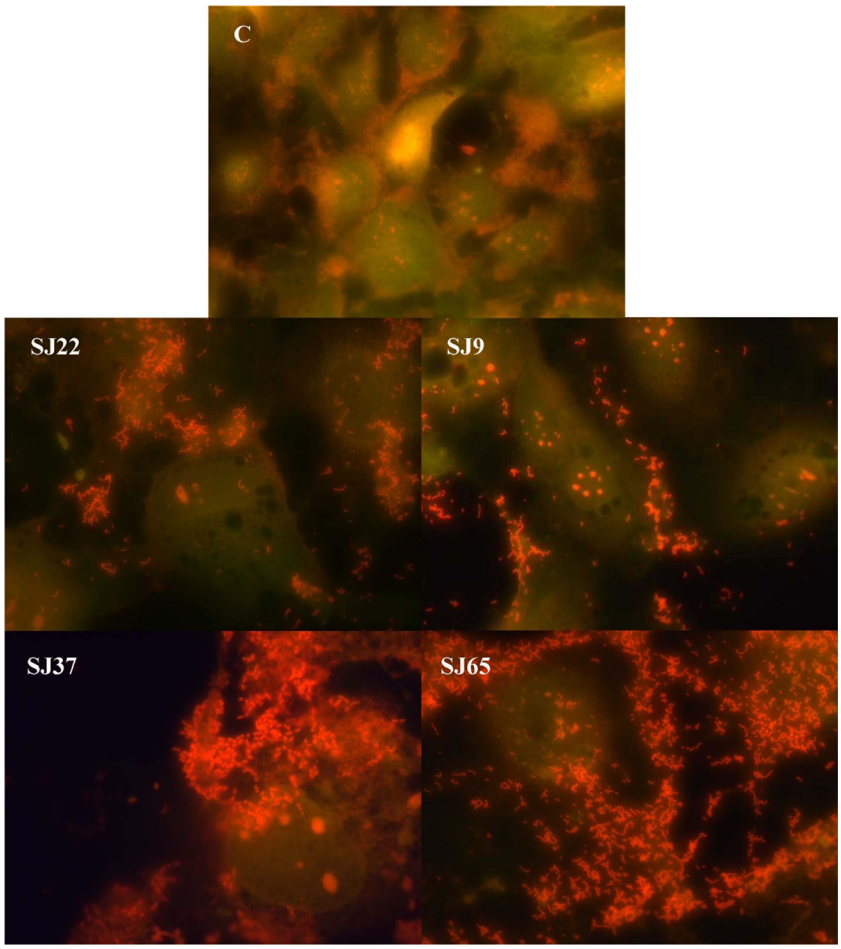

Fig. 1 Adhesion of *lactobacillus* on to cell lines stained with acridine orange and observed under fluorescent microscope. C – Control; SJ22 – *Lactobacillus plantarum*; SJ9 – *Lactobacillus plantarum* ssp. *plantarum*; SJ37 – *Lactobacillus plantarum* ssp. *argentoratensis*; SJ65 – *Lactobacillus pentosus*

Table 5 Phytase and antioxidant activity in unfermented and fermented soy milk by probiotic isolates

Isolates	Phytase production * (Units/ml)	Phytate content # (%)	DPPH scavenging$ (%)	Reducing property @ (A_{700})
Unfermented soy milk	NIL	100	71±5.64	1.15±0.04
Fermented soy milk				
SJ9	1.8±0.03	56±4.98	74±3.64	1.36±0.05
SJ22	1.8±0.07	58±4.26	76±4.65	1.52±0.06
SJ37	1.5±0.06	47±5.61	72±2.57	1.32±0.03
SJ65	2.1±0.08	66±2.27	87±3.68	1.74±0.04

Values are mean±SD of three independent experiments performed in duplicate

* expressed as units/ml phytase was defined as amount of enzyme required to produce 1 μmol of inorganic phosphate per minute under assay conditions

expressed as percentage. % of phytate=[(unfermented soy milk – fermented soy milk)/unfermented soy milk)] × 100

$ expressed as percentage % DPPH scavenging=[1 – (A517 of sample/A517 of control)] × 100

@ expressed as absorbing units at 700 nm

isolates. The enhanced antioxidant activity of fermented soy milk may be because of increased free phenolic content. The isoflavone, a predominant phenolic compound in soy milk, is metabolized by the bacteria and converted into health promoting compounds that have higher antioxidant activity (Chen et al. 2013). Telang et al. (2010) have also shown that approximately 50 % increased the concentration of daidzein and genistein, metabolic products of isoflavone, in fermented soy milk as compared to unfermented soy milk. Moreover, the fermentation by LAB leads to degradation of the soy milk protein into tripeptides and amino acids, which along with phenolic content exhibited synergistic antioxidant activity (Saito et al. 2003). As the probiotic isolate *Lactobacillus pentosus* SJ65 degrades phytate and exhibits greater antioxidant activity than other isolates, it can be used as the starter culture in cereal-based fermentation and supplied with diet.

Conclusion

In conclusion, evidence from in vitro evaluation of the *Lactobacilli* from fermented *Uttapam* batter, a south Indian

food source, could reckon in selection and the use of most efficient probiotic isolate *Lactobacillus pentosus* SJ65, which enhanced the nutritional status of fermented soy milk and needs to be further evaluated using animal models leading to formulations of functional foods beneficial to mankind.

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References

Anastasio M, Pepe O, Cirillo T, Palomba S, Blaiotta GA, Villani F (2010) Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. J Food Sci 75:28–35

Ashraf R, Shah NP (2011) Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. Review. Int Food Res J 18: 837–853

Begley M, Hill C, Gahan CGM (2006) Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72:1729–1738

Cesena C, Morelli L, Alander M, Siljander T, Tuomola E, Salminen S, Mattila-Sandholm T, Vilpponen-Salmela T, von Wright A (2001) *Lactobacillus crispatus* and its non-aggregating mutant in human colonization trials. J Dairy Sci 84:1001–1010

Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Prot 61: 1636–1643

Chen L, Madl RL, Vadlani PV, Li L, Wang W (2013) Value - added products from soybean: removal of anti-nutritional factors *via* bioprocessing. In: El-Shemy HA (ed) Soybean - bio-active compounds. InTech, Croatia, pp 161–180

CLSI (2011) Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. Clinical and Laboratory Standards Institute, Pennsylvania

Collado MC, Meriluoto J, Salminen S (2008) Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 226:1065–1073

Conway PL, Gorbach SL, Goldin BR (1987) Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J Dairy Sci 70:1–12

Dashkevicz MP, Feighner SD (1989) Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. Appl Environ Microbiol 55:11–16

De Smet I, Van Hoorde L, Vande Woestyne M, Christiaens H, Verstraete W (1995) Significance of bile salt hydrolytic activities of Lactobacilli. J Appl Bacteriol 79:292–301

de Valdez GF, de Taranto MP (2001) Probiotic properties of Lactobacilli: cholesterol reduction and bile salt hydrolase activity. In: Spencer JFT, de Spencer ALR (eds) Food microbiology protocols. Humana Press, New Jersey, pp 173–182

Doyle RJ, Rosenberg M (1995) Measurement of microbial adhesion to hydrophobic substrates. Methods Enzymol 253:542–550

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

Galdeano CM, LeBlanc M, Vinderola G, Bibas MEB, Perdigón G (2007) Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin Vaccine Immunol 14: 485–492

Gao Y, Li D, Liu S, Liu Y (2012) Probiotic potential of *Lactobacillus sake* C2 isolated from traditional Chinese fermented cabbage. Eur Food Res Technol 234:45–51

Greiner R, Konietzny U (2006) Phytase for food application. Food Technol Biotechnol 44:125–140

Hammes WP, Hertel C (2006) The genera Lactobacillus and Carnobacterium, in: Dworkin M, Falkow S, Rosenberg E, Schleifer K.-H, Stackebrandt E (Eds.). The Prokaryotes, New York, 4:320–403

Kimoto H, Ohmomo S, Okamoto T (2002) Cholesterol removal from media by Lactococci. J Dairy Sci 85:3182–3188

Li H, Yan L, Wang J, Zhang Q, Zhou Q, Sun T, Chen W, Zhang H (2012) Fermentation characteristics of six probiotic strains in soymilk. Ann Microbiol 62:1473–1483

Marazza JA, Nazarenob MA, de Gioria GS, Garroa MS (2012) Enhancement of the antioxidant capacity of soymilk by fermentation with Lactobacillus rhamnosus. J Funct Foods 4:594–601

Midolo PD, Lambert JR, Hull R, Luo F, Grayson ML (1995) *In vitro* inhibition of *Helicobacter pylori* NCTC 11637 by organic acids and lactic acid bacteria. J Appl Bacteriol 79:475–479

Morelli L (2000) *In vitro* selection of probiotic lactobacilli: a critical appraisal. Curr Issues Intest Microbiol 1:59–67

Nissen L, Chingwaru W, Sgorbati B, Biavati B, Cencic A (2009) Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. strains: a functional study in the small intestinal cell model. Int J Food Microbiol 135:288–294

Oh N, In M (2009) Phytate degradation by *Leuconostoc mesenteroides* KC51 cultivation in soymilk African. J Biotechnol 8:3023–3026

Osmanagaoglu O, Kiran F, Ataoglu H (2010) Evaluation of *in vitro* probiotic potential of *Pediococcus pentosaceus* OZF isolated from human breast milk. Probiotics Antimicrob Proteins 2:162–174

Sadasivam S, Manickam A (2008) Biochemical Methods, 3rd edn. New Age, New Delhi

Saito K, Jin D, Ogawa T, Muramoto K (2003) Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. J Agric Food Chem 51:3668–3674

Saraniya A, Jeevaratnam K (2012) Molecular characterization of bacteriocinogenic *Lactobacillus* species isolated from fermented *Uttapam* batter. Biosci Biotechnol Res Asia 9:417–421

Sleator RD, Hill C (2008) New frontiers in probiotic research. Lett Appl Microbiol 46:143–147

Telang AM, Joshi VS, Sutar N, Thorat BN (2010) Enhancement of biological properties of soymilk by fermentation. Food Biotechnol 24:375–387

Todorov SD, LeBlanc JG, Franco BDGM (2012) Evaluation of the probiotic potential and effect of encapsulation on survival for *Lactobacillus plantarum* ST16Pa isolated from papaya. World J Microbiol Biotechnol 28:973–984

Tuomola EM, Salminen SJ (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int J Food Microbiol 41:45–51

Vinderola CG, Reinheimer JA (2003) Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. Food Res Int 36:895–904

Wang C-Y, Lin P-R, Ng C-C, Shyu Y-T (2010) Probiotic properties of *Lactobacillus* strains isolated from the feces of breast-fed infants and Taiwanese pickled cabbage. Anaerobe 16:578–585

WHO/FAO (2002) Working group report on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada