

In *Vitro* Evaluation of Putative Probiotic Candidates Isolated from Various Origins

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To cite this article:

Nilofar Yunus Bhatti, Arati Savji Chavda, Bharatkumar Rajiv Manuel Vyas. In *Vitro* Evaluation of Putative Probiotic Candidates Isolated from Various Origins. *International Journal of Microbiology and Biotechnology*. Vol. 8, No. 1, 2023, pp. 1-9.

doi: 10.11648/j.ijmb.20230801.11

Received: August 12, 2022; **Accepted:** September 7, 2022; **Published:** January 13, 2023

Abstract: Present study describes isolation of potential probiotic lactic acid bacteria from chicken crop, human feces, buttermilk and chilly. The isolated *Lactobacillus* strains survive, tolerate and grow in MRS medium spiked with bile, salt and having acidic pH. The *Lactobacillus* isolates possess several probiotic properties, viz. (i) ability to bind gastrointestinal mucosa, up to $\geq 80\%$ cells adhered mucin, (ii) 50% cells retained viability during oro-gastro-intestinal transit, (iii) all the isolates exhibited broad anti-microbial spectrum against food spoilage and gastro-intestinal pathogens, *Limosilactobacillus fermentum* SBM showed maximum inhibition, (iv) ability to produce enzymatic activities like L-asparaginase, β -galactosidase and bile salt hydrolase (BSH) activities, *Limosilactobacillus fermentum* SBM showed maximum L-asparaginase activity (2.567 U/ml), and *Lactiplantibacillus pentosus* GCHI showed maximum β -galactosidase activity (296 \pm 0.1 Miller's Unit), (v) *Lactiplantibacillus pentosus* GCHI aggregated up to $\geq 92\%$ after 24 h, and (vi) the *Lactobacillus* isolates were susceptible towards nucleic acid synthesis inhibitors and cell wall synthesis inhibitor antibiotics. These *Lactobacillus* strains do not possess haemolytic, mucin degrading and DNase activities indicating their safety. Further characterization of these strains indicated potential probiotic properties and their suitability in food formulations as probiotics. The study presents an interesting illustration of mining of potential probiotic strains from nature exhibiting health benefits for human being and animals.

Keywords: Anti-Microbial Activity, BSH, *Lactobacillus* Strains, L-asparaginase, Probiotics, Safety Evaluation

1. Introduction

Lactic acid bacteria are diverse group of bacteria which we encounter on daily basis are mostly used as probiotics. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. They are found in fruits, vegetables, fermented foods and are part of normal flora of humans [2]. Lactic acid bacteria are most commonly used microorganisms as probiotics, especially *Lactobacillus* spp., because of the fact that they are desirable members of the intestinal microflora and that they have "Generally Recognized As Safe" (GRAS) status.

Probiotics must first overcome a number of physical and chemical barriers such as lysozyme and other hydrolytic enzymes in oral cavity, stomach acid, bile salts and enzymes

in the gastrointestinal tract (GIT). The exposure to gastric fluid is the crucial barrier to overcome prior to reaching the site of action, Kimoto et al. [3] and Begley et al. [4] found out that there is secretion of 2.5 l of gastric juice and 1 l of bile into GI tract. Human stomach pH can be as low as 1-1.5 during fasting and 4.5 after meal. *Lactobacillus* strains are capable of colonization of epithelial membrane and survival in hostile environment of GIT [5].

Lactic acid bacteria produce variety of antimicrobial metabolites viz. peptides, bacteriocins, organic acids, hydrogen peroxide, etc. [6] and therefore display broad antimicrobial spectrum against food-borne pathogens and food spoilage organisms.

There is a risk associated due to over-use and miss-use of antibiotics, thus administration of probiotics is an attractive strategy in improving health of living beings. The primary

clinical interest in the application of probiotics has been in the prevention of and treatment for GI infections and diseases [7]. Gut microbiota deviations have been associated with enhanced risk of specific diseases; therefore, modulation of an unbalanced indigenous microbiota forms the rationale of probiotic therapy [8]. Fungal spoilage is the main cause of economic losses in bakery and agricultural products and the source of mycotoxins, involving public health problems [9, 10].

Elevated levels of serum cholesterol is one of the major factors associated with coronary heart diseases [10]. Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in gall bladder for secretion in the gastrointestinal tract [11]. Bile salt hydrolase (BSH) catalyses- the hydrolysis of glycine or taurine-conjugated bile acids into the amino acid residues and the free bile acids. Thus, *Lactobacillus* are also important in lowering the serum cholesterol level [12].

Lactose intolerance is a common problem found in humans [13] and high content of lactose is not acceptable in dairy industry because it can cause grainy texture in frozen ice cream, condensed milk, etc. [14]. L-asparaginase is the key enzyme in the treatment of the lymphoblastic leukemia (ALL) and also used in pediatric regimes [15]. L-asparagine is an essential amino acid for the growth of the tumor cells. But cancer cells do not synthesis these amino acids. Thus, in the presence of L-asparaginase tumor cells cannot thrive. Thus, this enzyme is highly useful in cancer treatment [16].

In this report we are presenting data of evaluation of the functional *i.e.* anti-microbial activity, transit through oro-gastro-intestinal transit and safety aspects *i.e.* haemolytic activity, degradation of stomach mucin and DNase activity of *Lactobacillus* isolates. The LAB strains were subjected to safety characterization for making sure their safe use in human and animal consumption.

Lactocaseibacillus rhamnosus GG (ATCC 53103) was used as reference strain in the present study.

2. Materials and Methods

2.1. Isolation and Identification of *Lactobacilli*

Lactobacillus strains were isolated from chicken crop, buttermilk, chilli and human feces. Chicken crop was surface sterilized with phosphate buffer saline crushed with the help of spatula and forceps and used to inoculated (1 g) in de Man Rogosa & Sharpe (MRS) broth [17] containing 2% sugar (sucrose, lactose, glucose) and glycerol. Fecal samples from healthy infants were mixed with phosphate buffer saline (PBS 0.1 M, pH 7 containing 0.85% NaCl), and 0.1 ml aliquot was enriched in tube with 3 ml MRS containing various sugars mentioned above. The tubes were incubated at 15, 37 and 45°C for 48 h. Loopful of enriched culture was streaked on MRS agar, and incubated at 37°C till sufficient growth was observed. Individual chalky white colonies were randomly picked, cultured in MRS medium and subjected to microscopic and biochemical tests. Gram positive, catalase negative rods were further characterized for sugar fermentation, gas

production, and their ability to grow at 15 and 45°C. All the strains were maintained in 10% skimmed milk at 4°C.

2.2. Screening of Probiotic *Lactobacillus* Strains

Tubes containing 3 ml modified MRS was inoculated with 2% (v/v) 18-20 h cultures of lactobacilli. MRS was modified with bile salt (2 and 4%), pH (2, 3 and 4), NaCl (4 and 6%) and skim milk with phenol (0.4 and 0.6%) and the tubes were incubated at 37°C for 24 h. Loopful culture was streaked on MRS agar plates and observed for growth to determine the tolerance.

2.3. Functional Aspects

2.3.1. Viability During Simulated Oro-Gastro-Intestinal Transit

Viability of the *Lactobacillus* strains in the presence of lysozyme, simulated gastric and intestinal fluids was evaluated as described by Vizoso-Pinto *et al.* [18] and Charteris *et al.* [19] with few modifications [20].

2.3.2. Salt Aggregation Test

The cell surface hydrophobicity of lactobacilli was determined by salt aggregation test (SAT) according to Lindahl *et al.* [21]. Briefly 10 µl aliquot of fresh cell suspension (OD_{600} , $1/10^8$) in PBS was mixed on a glass slide with 10 µl of ammonium sulphate (pH 6.8) of various molarities (0.02, 0.2, 0.8, 1.6, 3.2 and 4.0 M). The formation of cell aggregates was observed after 1 min by visual reading. The lowest concentration of ammonium sulphate giving visible aggregation was scored as the SAT hydrophobicity value.

2.3.3. Autoaggregation

Autoaggregation assay was performed as described by Del Re *et al.* [22] with certain modifications according to Pithva *et al.* [20] 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 (1, 2, 4 and 24 h).

2.3.4. Mucin Adhesion Assay

Lactobacilli were evaluated for adhesion to immobilized porcine stomach mucin (Sigma-Aldrich, USA) by wielding the method given by Dhanani & Bagchi [23] in 96-well microtitre plates in sterile the adhered bacterial cells were enumerated after appropriate dilution on MRS agar plates.

2.3.5. Anti-bacterial Activity

The antibacterial activity of *Lactobacillus* strains was determined by modified protocol (incubation at 37°C for 24 h) of spot inoculation test as described by Schillinger and Lucke [24].

2.3.6. Anti-fungal Activity

The antifungal activities of *Lactobacillus* strains were determined against *Aspergillus niger* MTCC 3496, *Aspergillus flavus* MTCC 2798, *Penicillium roquefortii* MTCC 933 and *Rhizoctonia solani* (Sabouraud Dextrose Agar) were determined by overlay method as described by Magnusson and Schnurer [25].

2.4. Health Beneficial Activities

2.4.1. Bile Salt Hydrolase Activity

Bile salt hydrolase activity was evaluated according to Taranto et al. [26]. MRS agar was supplemented with 0.5% (w/v) bile salt (HiMedia) and 0.37 g/l of CaCl₂. Then 10 µl of 18-20 h culture in MRS broth was spot inoculated on modified MRS agar plates, and incubated at 37°C for 72 h. The formation of bile acid precipitation around the colony was considered as a positive result.

2.4.2. β-galactosidase Activity

The β-galactosidase activity in presence of glucose/lactose was performed according to Shekh et al. [27] and expressed in Miller's Unit. It was calculated as [(A_t-A_b)/t]*1000], where A_t is absorbance of test, A_b- absorbance of blank, t- time in min.

2.4.3. L-asparaginase Activity Production

L-asparaginase activity was determined in cell free culture and also associated with the biomass by measuring liberated free ammonia spectrophotometrically using Nessler's reagent as described by Imada et al. [28]. Protein content of CFC was determined by Bradford's method [29].

2.5 Safety Evaluation

2.5.1. Mucin Degradation

The ability of the *Lactobacillus* strains to degrade gastric mucin *in vitro* was evaluated using the method previously reported by Zhou et al. [30].

2.5.2. Haemolytic Activity

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

2.5.3. DNase Activity

DNase test was performed according to Shuhadha et al. [31]. DNase agar plates were spot inoculated with 18 h *Lactobacillus* culture and incubated at 37°C for 24 h and the plates were flooded with 1 N HCl held for 5 min and observed for the "halo" around the colonies.

2.5.4. Antibiotic Susceptibility Test

Susceptibility of *Lactobacillus* strains against antibiotics was assessed according to Charteris et al employing disc diffusion method with some modification. 0.1 ml of 18-20 h old culture was spread using sterile swab (HiMedia). Antibiotic dodeca universal discs (HiMedia) were placed upon MRS plates, incubated for 24 h at 37°C and observed for zone of inhibition.

2.6. Statistical Analyses

The experiments were reproduced at least twice and the values are mean of triplicates and results shown are of the representative experiment with ± standard deviation (SD). Statistical differences in the results were analysed by one-way analysis of variance (ANOVA) using Microsoft Excel 2010 at p <0.05 for the determination of significance.

3. Results and Discussion

3.1. Isolation and Identification of *Lactobacilli*

Well thought isolation and screening is mandatory to obtain novel probiotics therefore twenty-five Gram positive, catalase negative, non-spore forming, rod shaped bacteria were isolated and were tentatively identified as *Lactobacillus* on the basis of their phenotypic characterization. Five strains were further identified by 16S rDNA sequence analysis (Table 1).

Table 1. *Lactobacillus* strains isolated from various sources with NCBI accession numbers.

<i>Lactobacillus</i> strains	Sources		Genbank accession numbers
	Common name	Scientific name	
<i>Lactiplanti bacillus plantarum</i> SCH1	Chicken crop	Gallus gallus domesticus	MK246005
<i>Lactiplanti bacillus pentosus</i> GCHI	Chicken gut	Gallus gallus domesticus	MK245998
<i>Limosi lactobacillus fermentum</i> LF	Infant feces	-	MK245999
<i>Limosi lactobacillus fermentum</i> SBM	Buttermilk	-	MK246000
<i>Lacticaei bacillus rhamnosus</i> SCH	Chilli	Capsicum frutescens	MK246001

3.2. Screening of *Lactobacillus* Strains

Potential *Lactobacillus* strains were further characterized on the basis of their ability to survive in stress conditions which mimics GIT conditions Bile salt in the gut fluctuates between 1.5 to 2% during first hour of digestion, and thereafter decreases to 0.3% [32]. Tolerance and survival of *Lactobacillus* isolates was determined in the presence of bile, phenol, acid and salt (Table 2).

LAB isolates were able to grow in presence of 0.4 and 0.6% phenol a toxic metabolite produced upon deamination of aromatic amino acids during putrefaction by intestinal bacteria. NaCl tolerance of the *Lactobacillus*

strains is also important for their survival during processing of fermented food which is generally carried out in the presence of 6-8% NaCl. It confers competitive edge over other undesirable organism during food processing, and together with their antimicrobial activity against food-spoilage organism, is of advantage for their use in food preservation.

The count of *Lactobacillus* strains after growth in MRS at pH 3 was 4.8-5.8 log cfu/ml, in 4% bile salt 4.9-5.5 log cfu/ml, in 6% NaCl 5.9-7.1 log cfu/ml, in 0.6% phenol 6.4-7.5 log/ml and that in control was 6 to 9.35 log cfu/ml.

Table 2. Viable count of *Lactobacilli* after (i) 4 h incubation at 37°C in MRS (iii) 2 h in MRS modified with pH (2, 3), bile (2, 4%) and salt (6%) (iii) MRS modified with skim milk and skim milk phenol (0.6%).

MRS media	Viable count of <i>Lactobacillus</i> strains (log cfu.ml ⁻¹)					
	<i>Lacticasei bacillus rhamnosus</i> GG	<i>Lactiplanti bacillus plantarum</i> SCHI	<i>Lactiplanti bacillus pentosus</i> GCHI	<i>Limosi lactobacillus fermentum</i> LF	<i>Lacticasei bacillus rhamnosus</i> SCH	<i>Limosi lactobacillus fermentum</i> SBM
Control (0 h)*	7.0±0.2	8.1±1.2	8.9±0.5	8.3±1.1	7.5±1.0	8.4±1.1
(4 h)	9.4±1.0	10.0±1.1	9.6±1.3	9.8±1.2	8.8±1.0	9.7±1.2
pH 2	4.4±1.2	3.8±1.0	4.0±1.2	4.9 ^a ±1.0	5.1±1.3	5.1±1.2
3	5.4±1.0	5.8±0.9	5.5±0.7	4.8 ^a ±0.7	5.5±1.0	5.7±1.1
Bile 2%	5.8±1.3	6.2±1.2	5.8±0.4	5.7 ^a ±1.1	5.6±1.3	5.5±1.1
4%	5.1±1.1	5.4±1.3	5.2±1.4	5.6 ^a ±1.4	4.9±1.0	5.3±1.1
NaCl 4%	7.4±1.2	7.2±1.2	7.5±1.2	7.3±0.7	7.1±1.0	7.0±0.9
6%	6.5±1.2	6.5±0.8	5.9±0.9	7.0±1.4	6.7±1.0	7.1±1.3
Skim Milk	8.9±1.2	9.4±1.4	9.1±1.2	10.1±1.2	10.0±1.2	10.0±1.4
+ 0.4% Phenol	7.2 ^a ±1.2	7.5±1.2	6.3±0.9	7.9±1.2	7.7 ^a ±1.1	7.9±1.2
+ 0.6% Phenol	7.0 ^a ±1.4	7.2±1.2	6.8±1.3	7.5±1.3	7.6 ^a ±1.0	7.5±1.2

*Viable count of *Lactobacillus* strains determined at 0 h

^a not significantly different, while the other values significantly different (P<0.05).

3.3. Functional Aspects

3.3.1. Viability During Simulated Oro-gastro-Intestinal Transit

An important step towards the selection of probiotics strain is to investigate the strain viability under conditions which mimic saliva and the GIT. For bacterial cell the stress begins in mouth, with lysozyme-containing saliva and continues in the stomach and upper intestine. Gastric acid is a crucial barrier to overcome prior to reaching the site of action [33] the pH in human stomach is as low as 1-1.5 during fasting and upto 4.5 after meal, food passage time through stomach is 90 min. The acidic pH itself acts as a natural defence against sexually transmitted disease and AIDS [34, 35]. Isolated *Lactobacillus* strains exhibited admirable survival when they were evaluated for their tolerance to the environments mimicking oro-gastro-intestinal conditions. Survival of LAB decreased after the exposure towards lysozyme all the isolated showed ≥80%viability (Figure 1). The survival of *Lactobacillus* strains decreased 1.29 to 3.26 log cycles after the sequential exposure to lysozyme and SGF. Survival rate decreased further to 5.18 log cycles after exposure to SIF.

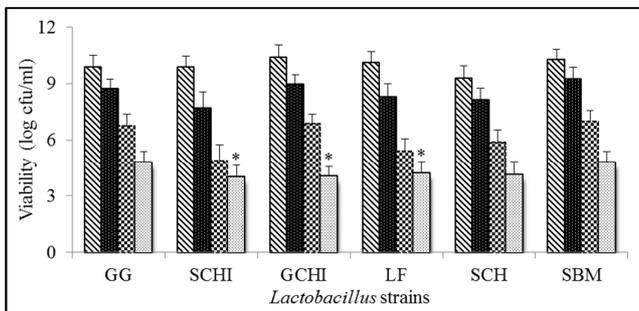


Figure 1. Survival (log cfu/ml) of *Lacticaseibacillus rhamnosus* strains GG, SCH, *Lactiplantibacillus plantarum* SCHI, *Limosilactobacillus fermentum* strains LF and SBM, *Lactiplantibacillus pentosus* GCHI before transit (▨) and upon exposure to Lysozyme (▩), Lysozyme-SGF (▧) and Lysozyme-SGF-SIF (▦) determined by viable count method on MRS medium *significantly different (P < 0.05).

Lacticaseibacillus rhamnosus SCH and *Limosilactobacillus fermentum* SBM showed the highest survival after the sequential transit (Figure 1).

3.3.2. Salt Aggregation and Autoaggregation Test

Adhesion to intestinal mucosal lining is considered one of the main features that indicate the beneficial effect of *Lactobacillus* strains. Adhesion is a complex process, and has been correlated with multiple factors like mucin-binding proteins, cell surface hydrophobicity and autoaggregation. The colonization potential is achieved by elevating the level of mucus production via regulation of cytoskeletal and tight junctional protein phosphorylation and the same can be estimated by salt aggregation and autoaggregation. *Lactobacillus pentosus* GCHI and *Lactobacillus fermentum* LF showed the lowest SAT value. While autoaggregation was found maximum at 24 h and it ranges between 7-46, 9-47, 21-73 and 33-92% for 1, 2, 4 and 24 h respectively. *Lactiplantibacillus pentosus* GCHI aggregated rapidly and maximum to 92% (Table 3, Figure 2).

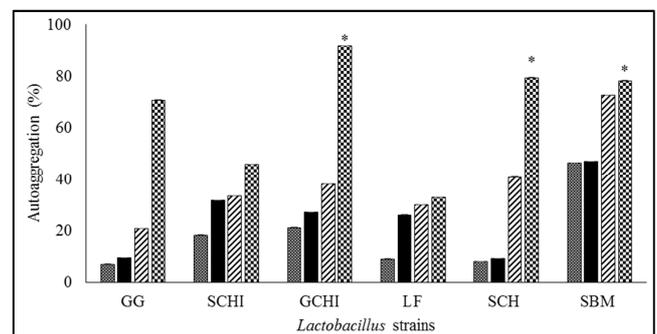


Figure 2. Auto-aggregation activities (%) produced by the *Lacticaseibacillus rhamnosus* GG, SCH, *Limosilactobacillus fermentum* LF, SBM, *Lactiplantibacillus plantarum* SCHI and *Lactiplantibacillus pentosus* GCHI after 1 h (▨), 2 h (▩), 4 h (▧) and 24 h (▦) grown in MRS medium at 37°C under static condition for 24 h *values are significantly different (P < 0.05).

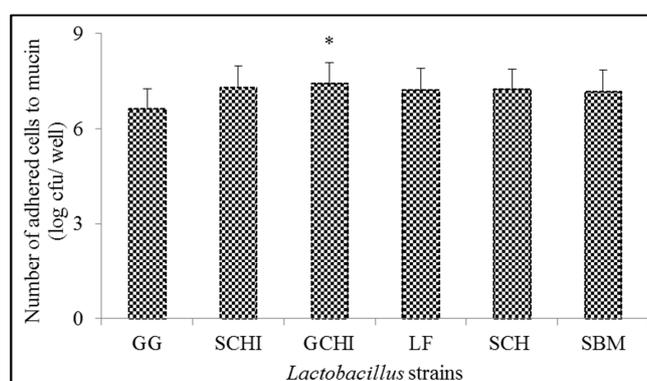
Table 3. Salt aggregation test (SAT) value of *Lactobacillus* strains at the lowest conc of ammonium sulphate giving visible aggregation after 1 min.

<i>Lactobacillus</i> Strains	SAT value (M)
<i>Lactocaseibacillus rhamnosus</i> GG	1.6*
<i>Lactiplantibacillus plantarum</i> SCHI	0.2
<i>Lactiplantibacillus pentosus</i> GCHI	0.02
<i>Limosilactobacillus fermentum</i> LF	0.02
<i>Limosilactobacillus fermentum</i> SBM	1.6*
<i>Lactocaseibacillus rhamnosus</i> SCH	0.02

*Values are significantly different ($P < 0.05$).

3.3.3. Mucin Adhesion Assay

Ability to compete with pathogens to adhere and colonize epithelial cells is one of the prime criteria to be selected as probiotic. *Lactobacillus* may exert health beneficial effects and modify the host immune system. The LAB strains tend to adhere coated mucin with varying degree. *Lactiplantibacillus plantarum* SCHI displayed 85% adhesion to intestinal mucin layer whereas other strains adhered up to $\geq 80\%$ (Figure 3).

**Figure 3.** Count of Lactic acid bacteria (log cfu/well) adhered to immobilized mucin in microtiter plate determined by viable count method using MRS medium *values are significantly different ($P < 0.05$).

3.3.5. Anti-fungal Activity

Table 4. Inhibition of gastrointestinal pathogens and food spoilage organisms by spot overlay assay of *Lactobacillus* strains grown in MRS medium under static condition at 37°C for 24 h.

Test Organisms	<i>Lactobacillus</i> Strains					
	<i>Lactocasei bacillus rhamnosus</i> GG	<i>Lactiplanti bacillus plantarum</i> SCHI	<i>Lactiplanti bacillus pentosus</i> GCHI	<i>Limosilacto bacillus fermentum</i> LF	<i>Lactocasei bacillus rhamnosus</i> SCH	<i>Limosi lactobacillus fermentum</i> SBM
Zone of inhibition (mm)						
<i>Escherichia coli</i> ^a	36±4	31±1	17±1	20±2	31±1	36±1
<i>Salmonella typhi</i>	26±1	32±1	13±1	27±3	26±2	30±1
<i>Klebsiella pneumoniae</i> ^a	31±1	39±1	21±1	33±1	10±1	36±2
<i>Enterococcus faecalis</i>	27±1	39±1	11±1	32±3	19±1	28±5
<i>Bacillus cereus</i>	23±1	35±1	28±1	27±1	27±1	27±1
<i>Bacillus megaterium</i>	20±1	35±6	26±1	26±3	20±1	31±1
<i>Bacillus subtilis</i>	27±1	27±2	15±2	31±1	37±1	37±1
<i>Staphylococcus epidermidis</i>	27±1	14±2	11±1	25±1	27±1	27±2
<i>Listeria monocytogenes</i>	22±1	12±1	19±1	32±1	24±2	35±1
<i>Yersinia enterocolitica</i>	24±1	23±1	14±1	37±2	19±1	33±2
<i>Pseudomonas aeruginosa</i>	29±1	37±1	17±1	-	36±1	-
<i>Proteus vulgaris</i> ^a	33±1	28±2	26±1	27±1	37±2	33±1
<i>Shigella sp.</i> ^a	29±2	39±1	16±1	36±1	28±1	37±1
<i>Micrococcus luteus</i> ^a	27±2	26±1	20±1	25±1	22±1	29±2

^a indicates clinical strains obtained from the Government Hospital, Rajkot, India. Other strains obtained from MTCC (Microbial Type Culture Collection Centre) Chandigarh, India.

3.3.4. Anti-bacterial Activity

Lactobacillus strains were examined for the antagonistic activity against Gram-positive and Gram-negative bacteria all the *Lactobacillus* strains exhibited broad antimicrobial spectrum against tested gastrointestinal pathogens and food spoilage bacteria as LAB strains inhibited the growth of different tested organisms. The antimicrobial activity was strain specific (Table 4). Strain-specific nature of the antimicrobial activity has also been reported earlier [36]. *Lactobacilli* exert antimicrobial action through the production of organic acids (lactic acid and acetic acid phenyl lactic acid and hydroxy-phenyl lactic acid), H₂O₂, antibacterial low molecular weight peptides and antifungal peptides,

Lactobacillus strains possess potential as probiotics since they display multiple health beneficial functions in addition to their safety and functional properties e.g. *Lactobacillus plantarum* produce γ -amino butyric acid, β -galactosidase and bile salt hydrolase having health promoting functions [27]. Further *Lactobacillus* strains are strong electron donors and weak electron acceptors, displaying strong basic and weak acidic characters [37]. They possess antigenotoxic and antimutagenic activities, broad antimicrobial spectrum, and therefore are also potential biopreservative agents. [36, 38].

Biopreservation, the use of microorganism to preserve food and feed stuffs, is gaining increasing interest due to the consumers demand for reduced use of chemical preservatives [39]. *Lactobacillus* strains inhibited the growth of *Aspergillus niger*, *A. flavus*, *Rhizoctonia solani*, *Penicillium roqueforti* and *Candida sp.* Spore germination was delayed as compared to control in *A. niger*, *A. flavus*, and *P. roqueforti* up to 4 days of incubation (Table 5). This inhibitory activity of *Lactobacilli* remains stable up to 8-10 days of incubation. None of the strain showed inhibitory activity against *Saccharomyces cerevisiae* (Table 5).

Table 5. Antifungal activity of *Lactobacillus* strains determined by overlay method for 72 h at RT.

Test Organisms	<i>Lactobacillus</i> Strains					
	<i>Lactocasei bacillus rhamnosus</i> GG	<i>Lactiplanti bacillus plantarum</i> SCHI	<i>Lactiplanti bacillus pentosus</i> GCHI	<i>Limosi lactobacillus fermentum</i> LF	<i>Lactocasei bacillus rhamnosus</i> SCH	<i>Limosi lactobacillus fermentum</i> SBM
	Zone of inhibition					
<i>Aspergillus niger</i>	+	++	++	+	+	+
<i>Aspergillus flavus</i>	+	+	+	+	++	+
<i>Rhizoctonia solani</i>	++	++++	+++	++++	++++	++++
<i>Penicillium roqueforti</i>	+++	++++	++++	++	++	+++
<i>Candida parasilopsis</i>	++	-	-	+	+	++
<i>Candida albicans A^b</i>	++	-	+	-	+	-
<i>Candida tropicalis B^b</i>	++	++	+	+	+	+
<i>Candida albicans C^b</i>	++	-	+++	-	++++	-
<i>Candida tropicalis E^b</i>	+	+	+	+	+	+
<i>Candida albicans F^b</i>	++	-	+	-	-	-
<i>Saccharomyces cerevisiae</i>	-	-	-	-	-	-

^a Inhibition grades: no visible inhibition (-); inhibition size < 0.5 cm (+); 0.5–2 cm (++); > 2 cm (+++).

^b Clinical strains obtained from the M. P. Shah Medical College, Jamnagar, India; other strains were from MTCC, Chandigarh, India.

3.4. Health Beneficial Activities

3.4.1. Bile Salt Hydrolase Activity

According to FAO/WHO, 2002 bile tolerance is another essential criterion for the selection of a probiotic strains. Bile acid is produced from cholesterol in the liver and secreted from gall bladder into the duodenum in conjugated form. Bile salts play an essential role in the digestion of fat in duodenum. BSH-positive organisms are considered for their use as probiotic because of their essential role in cholesterol removal [5, 40]. The broad distribution and abundance of BSHs in the GIT suggests that bile acid deconjugation is a selected adaptive trait of several bacterial species for symbiosis or pathogenesis within the host [41, 42]. BSH has been useful in detoxification of these harmful effects [43], lactobacilli have also been reported to reduce hyperlipidemia-associated lethal health-effects in animal model by decreasing serum cholesterol levels [44] whilst there are reports of some bile acids have toxic acidic or detergent like effects [4].

Bile salt hydrolase activity was detected by the precipitates formed surrounding the colony of strains. Degree of precipitation was indicated in terms of zone of precipitates observed. All isolates exhibited extensive bile salt hydrolase activity when grown in the presence of 1% bile salts (Table 6).

Table 6. Bile salt hydrolase activity produced by *Lactobacillus* strains growing in MRS medium at 37°C under static condition for 24 h.

<i>Lactobacillus</i> Strains	Bile salt hydrolase Zone of precipitation
<i>Lactocaseibacillus rhamnosus</i> GG	++
<i>Lactiplantibacillus plantarum</i> SCHI	++
<i>Lactiplantibacillus pentosus</i> GCHI	++
<i>Limosilactobacillus fermentum</i> LF	++
<i>Limosilactobacillus fermentum</i> SBM	+
<i>Lactocaseibacillus rhamnosus</i> SCH	+++

‘+’ Size of zone of precipitation 0.5-1.0 cm; ‘++’ Size of zone of precipitation 1.0-1.5 cm

‘+++’ Size of zone of precipitation 1.5-2.0 cm; ‘++++’ Size of zone of precipitation >2.0 cm.

3.4.2. β -galactosidase Activity

People lacking this enzyme activity exhibit lactose intolerance and it can lead to deficiency in vitamin D and calcium in infants and kids [45]. Other than that, β -galactosidase mediates transgalactosylation reaction for production of galacto-oligosaccharide (GOS) from lactose [46]. Thus, these strains can be potentially used for functional food production.

β -galactosidase activity is expressed in Miller’s Unit/ ml (\pm SD) are 2 ± 0.1 , 296 ± 0.1 , 45 ± 0.1 and 78 ± 0.0 , which accounts for *Lactocaseibacillus rhamnosus* GG, *Lactiplantibacillus pentosus* GCHI, *Limosilactobacillus fermentum* LF and SBM respectively (Table 7).

Table 7. β -galactosidase activities produced by the *Lactobacillus* strains growing in MRS medium at 37°C under static condition for 24.

<i>Lactobacillus</i> Strains	β -galactosidase activity Miller’s unit
<i>Lactocaseibacillus rhamnosus</i> GG	2 ± 0.1
<i>Lactiplantibacillus plantarum</i> SCHI	-
<i>Lactiplantibacillus pentosus</i> GCHI	$296\pm 0.1^*$
<i>Limosilactobacillus fermentum</i> LF	45 ± 0.1
<i>Limosilactobacillus fermentum</i> SBM	-
<i>Lactocaseibacillus rhamnosus</i> SCH	78 ± 0.0

*Values are significantly different (<0.05).

3.4.3. L-asparaginase Activity Production

L-asparaginase is the key enzyme in the treatment of the lymphoblastic leukemia (ALL) and also used in pediatric regimes [15]. Nausea, fever, diarrhoea and vomiting are the most common side effects from the L-asparaginase isolated from *Escherichia coli* (brand name Spectrila) and *Erwinia chrysanthemia* (brand name Erwinase) [47]. Due to GRAS status of *Lactobacillus* strains L-asparaginase production has become an added advantage apart from other health benefits. L-asparaginase activity was in range from 1.831 to 2.567 U/ml. *Limosilactobacillus fermentum* SBM produced maximum activity (Figure 4).

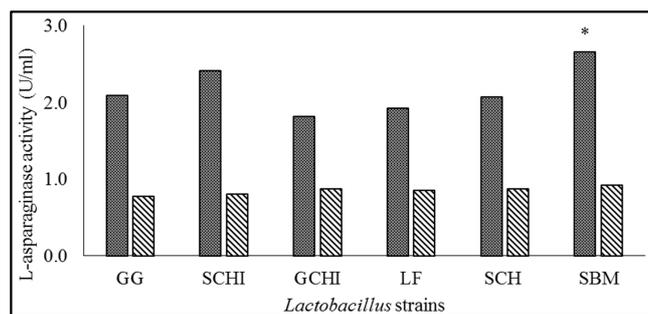


Figure 4. L-asparaginase activity produced by *Lactobacillus rhamnosus* GG and SCH, *Limosilactobacillus fermentum* LF and SBM, *Lactiplantibacillus plantarum* SCHI and *Lactiplantibacillus pentosus* GCHI using biomass (hatched) and cell free culture (solid) growing in MRS medium at 37°C under static condition for 24 h *values are significantly different ($P < 0.05$).

3.5 Safety Evaluation

3.5.1. Mucin Degradation

Lactobacillus strains did not show mucinolytic activity demonstrating inability of these strains to degrade gastrointestinal mucin *in vitro*, implicating non-invasive and non-toxic nature of these strains at the mucosal surface.

3.5.2. Haemolytic Activity

None of the *Lactobacillus* strains lysed heam protein nor reduced the iron in haemoglobin [20]. Thus, All *Lactobacillus* strains are generally regarded as safe because they do not harm the host thus can be used safely in food fermentations.

3.5.3. DNase Activity

The *Lactobacillus* strains did not produce DNase. DNase is a virulent factor for some pathogens and consequently the absence of this virulent factor indicated that these strains are safe.

3.5.4. Antibiotic Susceptibility Test

Antibiotic susceptibility was observed throughout all the LAB cultures. These strains showed non-susceptibility towards the nucleic acid synthesis inhibitors (norfloxacin, co-trimoxazole, gentamicin, netillin), cell wall synthesis inhibitor (augmentin), cytoplasmic membrane distributors (colistin). Whilst some of the strains were susceptible towards the same antibiotics and cell wall synthesis inhibitor (amoxicillin), antimicrobials of third generation (cephotaxime, ceftriaxone), protein synthesis inhibitor (amikacin) nucleic acid synthesis inhibitors (ciprofloxacin) (Table 8).

Table 8. Antibiotic susceptibility of *Lactobacillus* cultures determined by disc diffusion method.

Antibiotics	Conc (mg/disc)	<i>Lactobacillus</i> Strains					
		<i>Lactobacillus rhamnosus</i> GG	<i>Lactiplantibacillus plantarum</i> SCHI	<i>Lactiplantibacillus pentosus</i> GCHI	<i>Limosilactobacillus fermentum</i> LF	<i>Lactobacillus rhamnosus</i> SCH	<i>Limosilactobacillus fermentum</i> SBM
Ciproflaxacine	5	17	19	31	5	27	22
Ceftriaxone	10	40	21	40	10	38	24
Gentamicin	10	27	NS	19	23	28	24
Norfloxacin	10	NS	12	NS	18	NS	18
Augmentin	30	27	NS	18	19	29	37
Co-Trimoxazole	25	23	NS	15	NS	22	27
Amoxicilin	10	29	21	19	21	28	26
Furazolidone	50	23	22	17	25	15	21
Amikacin	30	17	10	20	20	18	18
Cefotaxime	30	26	17	40	25	36	26
Colistin	10	5	NS	NS	NS	14	15
Netillin	30	23	NS	12	NS	22	20

NS- non susceptible

Lactobacillus strains not susceptible to Gentamicin (10), Norfloxacin (10), Augmentin (30), Co-trimoxazole (25), colistin (10), Netillin (30).

4. Conclusions

Here five *Lactobacillus* strains isolated from various sources has been proven to be functionally potent and safe to be used as putative probiotics in functional foods. These five *Lactobacillus* isolates were able to grow in the presence of (i) low pH, bile, salt and phenol ii) remains viable after their exposure towards simulated gastro intestinal transit and mucin adherence iii) produces anti-microbial compounds which inhibits the growth of various gastro intestinal pathogens and food spoilage bacteria iv) produces health promoting enzymes like L-asparaginase, bile salt hydrolase, \square galactosidase v) do not possess any virulent traits and does not exhibit mucinolytic, haemolytic and DNase activity thus proving their safe use in various fermented foods and feed formulations for birds in poultry.

Acknowledgements

Ms. Nilofar Bhatti is thankful to University Grants Commission, New Delhi, India, for providing research assistance (Maulana Azad National Fellowship).

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