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Isolation and Characterization of Potential Probiotic *Lactobacillus gasseri* Strains Isolated from Different Sources

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Abstract

The present study was designed with a aim to isolate and select potential probiotic *L. gasseri* strains from breast fed human infant feces (0-3 months), saliva and breast milk samples (0-3 months old lactating mothers). A total number of 34 *L. gasseri* isolates isolated from these samples were initially screened to check their survival at pH-2. Out of these 34 isolates only nine isolates viz. Lg5, Lg6, Lg12, Lg23, Lg50b, Lg61b, Lg70, Lg8sf and Lg2sc were found to be most acid tolerant and selected for further *in vitro* evaluation. When tested for bile tolerance three isolates Lg70, Lg2sc, Lg8sf were found to resist 2% bile and showed highest viability among the tested nine strains. The nine isolates exhibited varying degree of hydrophobicity to three tested hydrocarbons and were also demonstrated to have adhesive properties on Caco-2 cell lines. Four *L. gasseri* strains viz. Lg50, Lg61b, Lg70 and Lg8sf were found to have strong BSH activity and were also found to assimilate cholesterol *in vitro*. The isolate Lg70 showed highest *in vitro* cholesterol assimilation following 24 h incubation in broth having cholesterol. Almost all the *L. gasseri* isolates showed activity against tested indicator strains. These nine isolates were susceptible to clinically used antibiotics. When tested for virulence traits none of the isolate were found positive for hemolysis, DNase and Gelatinase activity and hence were found to be safe. In this study the *L. gasseri* strain Lg70 was found as the most promising probiotic candidate.

Keywords: Probiotic, BSH activity, Caco-2 Cell Lines, *L. gasseri,* cholesterol assimilation

Lactobacilli are normal inhabitants of the gastrointestinal tract of human beings and are widely considered to exert a number of beneficial roles by maintaining a healthy intestinal microflora (FAO/WHO, 2002). Organisms having health promoting activities are defined as probiotics. According to FAO/WHO (2002) probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Historically, probiotic roles have been ascribed primarily to *Lactobacillus acidophilus*. Developments in molecular taxonomy have revealed six different *Lactobacillus* species that comprise the acidophilus group (Kullen *et al.*, 2000) and among this group *L. gasseri* is one of the most common homo-fermentative *Lactobacillus* species isolated from human intestine. *L. gasseri* demonstrates good survival in the GI tract (Pedrosa *et al.*, 1995) and has been associated with a variety of probiotic activities and roles including reduction of fecal mutagenic enzymes (Pedrosa *et al.*, 1995), stimulation of macrophages (Tejada-Simon and Pestka, 1999) and production of bacteriocins (Itoh *et al.*, 1995). *L. acidophilus* group has the ability to reduce serum cholesterol, possibly through binding of the dietary cholesterol with the cells or deconjugation of bile salts in the small intestine (Hosono, 2000). *L. gasseri* suppresses the reabsorption of bile acids into the enterohepatic circulation and enhances the excretion

of acidic steroids in feces of hypercholesterolemic rats thereby reducing the concentration of serum cholesterol in blood. The probiotic *L. gasseri* SBT2055 showed lowering effects on abdominal adipocity, body weight and other measures, suggesting its beneficial influence on regulation of obesity (Masao, 2008). *L. gasseri* OLL2809 had been proved effective in a clinical trial especially on the dysmenorrhea in endometriosis patients (Itoh, 2010). *L. gasseri* AM63T was found capable of degrading oxalate *in vitro* as well as under colon simulated condition suggesting its possible role in the management of kidney stone disease (Lewanika *et al.,* 2007). These evidences suggest that *L. gasseri* offers a wide range of health benefits and can be incorporated into functional dairy foods. Keeping in mind these health effects of *L. gasseri* this study was designed to select putative probiotic *L. gasseri* strains from breast fed human infant (0-3 months) feces, breast milk of lactating mothers (0-3 months old) and saliva.

MATERIALS AND METHODS

Bacterial Cultures, Media and Growth Conditions

The bacterial cultures used in this study are listed in Table 1. All the cultures used for study were maintained in their respective broths (Table 1) at 4ºC and sub-cultured after every 15–20 days. The reference strain of *L. gasseri* was obtained from ATCC. All the bacterial cultures were preserved in glycerol stocks at -20ºC as stock cultures. From these stocks cultures, working cultures were prepared and these were propagated twice prior to use by sub-culture in their respective broths (Table 1). All the chemicals used were procured from Sigma Aldrich Bangalore.

Isolation of *Lactobacillus gasseri* strains from different sources

A total number of 50 samples (30 fecal samples from breast-fed infants, who were less than 3 months old, 10 samples of breast milk and 10 samples of adult human saliva) were collected in sterile sample bottles from different localities of Karnal. One gram of human infant feces was transferred to 10 mL of sterile saline (0.85% sodium chloride) and mixed well. The serial dilutions were subsequently prepared in sterile saline. Appropriate dilutions of the samples were plated on MRS medium (De Man *et al.,* 1960). Plates were incubated at 37ºC for 24-48 h. Typical cream-colored colonies were picked up randomly and transferred to MRS broth. After growth at 37ºC for 24-48 h, the cultures were tested for purity and morphology by microscopic examination. All the isolates were maintained in MRS broth at 4ºC and sub-cultured after every 15-20 days. All the isolates were preserved in 25% glycerol at -20ºC. The cultures were grown prior to use by sub-culturing twice in MRS broth.

Table 1: Standard cultures used in this study

Salmonella typhi NCDC 113, Karnal, India	37°C	BHI
Escherichia coli NCDC 134, Karnal, India	37°C	BHI
Staphylococcus aureus NCDC 110 Karnal, India	37°C	BHI
Listeria monocytogenes ATCC15303	37°C	BHI
Enterococcus faecalis ATCC14506	37°C	BHI
Bacillus cereus ATCC 12504	37°C	BHI
Pseudomonas aerugenosa NCDC 1 0 4, Karnal, India	37°C	BHI
Pediococcus acidilactici LB 42, University of Wyoming, USA	37°C	MRS
Lactobacillus gasseri ATCC 19992	37°C	MRS

Molecular Characterization of Isolates

Isolation of Genomic DNA

DNA from all the 100 isolates and standard culture (*L. gasseri* ATCC19992) was isolated according to the standard procedure published by Pospiech and Neumann, (1995).

Genus- and Species-Specific PCR

The following genus- and species-specific PCR primers described by Dubernet *et al.* (2002) and Song *et al.* (2000) were used for this study: genus-specific LbLMA1-rev (CTC AAA ACT AAA CAA AGT TTC) and R16-1 (CTT GTA CAC ACC GCC CGT CA) (Dubernet *et al.,* 2002); species-specific F-Lgas 2 (TGC

TAT CGC TTC AAG TGC TT) and R-Lgas 3 (AGC GAC CGA GAA GAG AGA GA) (Song *et al.,* 2000). All the components (dNTP, buffer, Taq polymerase and primers) for the PCR were obtained from Sigma Aldrich Chemical Pvt limited, Bangalore. The reaction mixtures (25 μL) contained 10 pmol of each primer, 0.2 mM of each dNTP, 1 X PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100), 0.5 μL of DNA template (50-100 ng) and 0.5 U of Taq polymerase. The mixtures were overlaid with mineral oil. Amplification was carried out in a thermocycler (Eppendorf Master Cycler, Hamburg, Germany) as follows: initial denaturation at 95ºC for 5 min, followed by 35 cycles consisting of denaturation at 95ºC for 1 min, annealing for 1 min, and extension at 72ºC for 1 min, and a final 8-min extension step at 72ºC. The annealing temperatures used for LbLMA1-rev/R16-1 was 56 ºC and for F-Lgas 2/ R-Lgas 3 65ºC was used. The PCR products were stored at 4ºC. Aliquots of the PCR products were separated by 2% agarose gel electrophoresis in TAE buffer (pH 8.0). A 100-bp DNA marker (Sigma, Bangalore) was used as a reference. Gels were stained with ethidium bromide (1 g/mL) and visualized under UV light.

Screening of Isolates for Probiotic Attributes

Determination of Acid Tolerance

Resistance to acidic conditions was tested by the method of Clark *et al.,* (1997) with some modifications. The *L. gasseri* strains were grown overnight in MRS broth at 37ºC. The actively grown cells (10^8cfu/mL) were harvested by centrifugation (7,200Xg at 4ºC for 10 min) and resuspended in an equal volume of MRS broth with pH adjusted to pH 2.0 and pH 1.5. with 1 M HCl and simultaneously in MRS broth at pH 6.5 as control. Survival of the culture was evaluated by determining the viable counts of the samples (serially diluted in peptone water after 0 and 3h in acidic conditions) plated on MRS agar and incubated at 37ºC for 48 h.

Determination of Bile Tolerance

Tolerance to bile acids was tested according to the method of Gilliland *et al.,* (1984). The *L. gasseri* strains were grown overnight in MRS broth at 37ºC. The actively grown cells (10^8 cfu/mL) were harvested by centrifugation (7,200Xg at 4ºC for 10 min) and resuspended in an equal volume of MRS broth supplemented with 1% and 2% w/v Oxbile and without supplement as a control. Survival was evaluated by plate count on MRS agar after 0 and 6 h of incubation in MRS broth containing bile salts (reflecting the time spent by food in the small intestine), and subsequently, the plates were incubated at 37ºC for 48 h.

Determination of Cell Surface Hydrophobicity

Ability of the organisms to adhere to hydrocarbons is a measure of their adherence to the epithelial cells in the gut, that is, cell surface hydrophobicity. Cell surface hydrophobicity of selected isolates was determined according to the method described by Rosenberg *et al.,* (1980) using n-hexadecane.

% Hydrophobicity) calculated using the equation given below:

$$H\% = OD_{initial} - OD_{final} / OD_{initial} \times 100$$

Where OD initial and OD final are the absorbances (at 610 nm) before and after extraction with the three hydrocarbons.

Adhesion of Lactobacillus Strains to Caco-2 Cell Lines

Caco-2 cell lines were maintained in DMEM supplemented with 10% foetal bovine serum, 25 mM HEPES, 20 U/ml penicillin and 100 μg/ml of streptomycin. Cells from monolayers were detached by trypsinization. One mL 0.25% trypsin-EDTA solution (Sigma, USA) was added to each well of six-well plate and the plate was incubated for 15 min at room temperature. The detached cells were repeatedly but gently aspirated to make homogenous suspension. The cell suspension was then serially diluted with saline solution and plated on MRS agar. The plates were incubated for 24-48 h at 37°C and colonies were counted (B_1 cfu/mL). Bacterial cells initially added to each well of six-well plates were also counted (B_0 cfu/mL). The adhesion percentage

was then calculated as: % adhesion= (B_1 / B_0) ×100 where B_1= no of bacterial colonies after incubation and B_0= no of bacterial cells added initially.

Bile Salt Hydrolase Assay

The ability of the *L. gasseri* strains to deconjugate bile salts was determined according to the bile salt hydrolases assay of Taranto *et al.* (1995). *Enterococcus faecium* BFE 900 and *Pediococcus acidilactici* NCDC252 were used as a positive and negative control, respectively. The presence of the precipitated bile acids around colonies (white opaque halo) was considered as positive result.

Antibiotic Susceptibility of Lactobacillus gasseri isolates

Patterns of resistance/susceptibility to antibiotics of potential probiotic strains of *L. gasseri* were studied by a disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA).

A total number of 12 antibiotic discs of Vancomycin (30 μg), Streptomycin (10 μg), Erythromycin (15 μg), Gentamycin (10 μg), Tetracycline (10 μg), Penicillin (10 μg), Bacitracin (10 μg), Polymyxin B (300 units), Rifampicin (30 μg), Ciprofloxacin (10 μg) ampicillin (10 μg), Kanamycin (30 μg), were used. Mueller Hinton agar plates were poured and allowed to solidify. These were subsequently overlaid with 4 mL of Mueller–Hinton soft agar tempered at 45ºC and seeded with 200 μL of the active cultures. Petri plates were allowed to stand at room temperature for 15 min, and then, the antibiotic disks were dispensed onto the agar surface using forceps under aseptic conditions. The agar plates were incubated at 37ºC aerobically for 24 h.

Diameter (in mm) of the inhibition zone was measured using an antibiotic zone scale, and results were expressed in terms of resistance, moderate susceptibility or susceptibility by comparing with the interpretative zone diameters provided in the Performance Standards for Antimicrobial Disk Susceptibility tests (2009).

Screening of Lactobacillus Isolates for Antimicrobial Activity

Inhibitory spectra of *L. gasseri* strains were determined by using the agar spot test assay (Fleming *et al.,* 1975) against a broad range of Gram-positive and Gram-negative strains (Table 1). *L. gasseri* cultures for spot inoculation of agar surfaces were grown in MRS broth at 37ºC by three consecutive transfers at 24-h intervals. Indicator strains for seeding the soft agar for overlay were also grown in appropriate broths by three successive transfers at 24-h intervals at their optimum growth temperatures. The surface of the solidified and dried (1 day at 37ºC) tryptone glucose yeast extract (TGE) (Biswas *et al.,* 1991) agar (tryptone 10.0 g, glucose 10.0 g, yeast extract 10.0 g, magnesium sulfate 0.05 g, manganese sulfate 0.05%, Tween 80, agar 15 g and distilled water 1,000 mL) plates were spot inoculated with 5 μL of Lactobacillus cultures. The inoculated agar plates were incubated at 37ºC for 18–24 h and then overlaid with 7.0 mL of TGE (Biswas *et al.,* 1991) soft agar (0.7% agar) seeded with 30 μL of the indicator culture. A clear zone of 2 mm or greater, extending laterally from the border of the *Lactobacillus* colonies after incubation for 1 day at optimum temperature of indicator strains, was recorded as positive inhibition.

Evaluation of in vitro cholesterol assimilation ability

The efficiency of cultures to remove cholesterol from growth media was evaluated, in triplicate, by the method adopted by Gilliland and Walker, (1990). Freshly prepared MRS-Thio (MRS broth with 0.2% sodium thioglycolate) broth was supplemented with 0.2% sodium taurocholate. Sodium thioglycolate was as used as an oxygen scavenger. A filter-sterilized cholesterol solution (10 mg/mL in ethanol) was added to the broth to a final concentration of 70 μg/mL. The broth was inoculated with 1% of culture and incubated anaerobically by using GasPak Anaerobic System for 24 h at 37°C. After incubation, cells were removed by centrifugation for 7 min at 5,400 x g and 4°C. The method described by (Rudel and Morris, 1973) was used to determine the amount of cholesterol in the spent broth and in un-inoculated

broth. Briefly, the spent broth was collected in clean and dry tubes. From the collected spent broth, 0.5 mL was placed in a test tube and 3 mL of 95% ethanol was added followed by 2 mL of 50% KOH. After mixing thoroughly, the contents were heated at 60°C for 10 min in water bath and subsequently cooled. To each tube, 5 mL of n-hexane was added and mixed followed by a further 3 mL of distilled water which was also mixed. The tubes were allowed to stand for 15 min at room temperature to separate the phase. After phase separation, 2.5 mL of the upper hexane layer was separated and placed in a clean dry test tube. The hexane was evaporated at 60°C under the flow of nitrogen gas. To each tube, 4 mL of OPA (o-phthalaldehyde) reagent was added and allowed to stand at room temperature for 10 min. To each tube, 2 mL of concentrated sulphuric acid was added slowly to the side of tube, and mixed and allowed to stand for 10 min. The color developed in uninoculated broth and spent broth was read against blank at 550 nm. Percent reduction was determined in the spent broth by comparing values with uninoculated control.

$$\% \text{ cholesterol removal from media} = Cho_{uninoculated} - Cho_{inoculated} / Cho_{uninoculated} \times 100$$

Where, $Cho_{uninoculated}$ = uninoculated media containing cholesterol and $Cho_{inoculated}$ = inoculated media containing cholesterol.

DNase, Gelatinase and Hemolytic Activity

Hemolysis

The isolates of *L. gasseri* were grown overnight in MRS medium at 37ºC and then streaked onto blood agar base plates containing 5% of fresh calf blood obtained from Animal Health Complex of the Institute. The plates were incubated for 3 days at 37ºC. The hemolytic reaction was recorded by observation of a clear zone of hydrolysis around the colonies (b-hemolysis), a partial hydrolysis and greening zone (a-hemolysis) or no reaction (c-hemolysis).

DNase Test

The *L. gasseri* strains were streaked on DNase agar medium to check production of DNase enzyme. Plates were incubated at 37ºC for 48 h. A clear, pinkish zone around the colonies after incubation was considered positive for DNAse production (Gupta and Malik, 2007).

Liquefaction of Gelatin

Gelatinase production by selected *L. gasseri* isolates was studied by using tryptone neopeptone dextrose (TND) agar (tryptone 17.0 g, neopeptone 3.0 g, dextrose 2.5 g, NaCl 5.0 g, K_2HPO_4 2.5 g, agar 15 g, and distilled water 1,000 mL) containing 0.4% gelatin (Gupta and Malik, 2007). Petri plates containing the medium were spot inoculated with the test culture and incubated at 37 ºC for 3 days. The petri plates were then flooded with saturated ammonium sulfate solution. Development of clear zones around the spots against the opaque background indicated a positive reaction.

Screening of the L. gasseri Lg70 strain for compatibility with dahi cultures

L. gasseri strains Lg70 was screened for its compatibility with *dahi* cultures. The screening was done using the spot on- lawn method. MRS agar plates were overlaid with soft agar seeded with the *dahi* culture. Five µl spot of active, fresh culture of the Lg70 strain of probiotic *L. gasseri* were placed on the solidified agar lawn. The experiment was performed in duplicate separately for 9 mesophilic and 5 thermophilic *dahi* cultures. The agar plates were left undisturbed for an hour and transferred thereafter, to an incubator maintained at 37°C for 24 h incubation.

The plates were observed the next day for zones of inhibition and the results recorded. The *dahi* starter cultures that were inhibited by the *L. gasseri* Lg70 strain as observed through zone of inhibition. The five thermophilic cultures used were NCDC74, NCDC75, NCDC76, NCDC80, and NCDC158. The mesophilic cultures used for compatibility study were NCDC153, NCDC154, NCDC159, NCDC161, NCDC165, NCDC166, NCDC167, NCDC261 and NCDC265.

RESULTS AND DISCUSSION

Genus- and Species-Specific PCR

All the 100 isolates were subjected to PCR assay based on genus-specific primers LbLMA1/R161 targeted against 16S rRNA (Dubernet *et al.,* 2002). Fig. 1 shows the 250-bp PCR products obtained with the *L. gasseri* isolates of our study. We used the same genus-specific primers for the genus *Lactobacillus* as used by Dubernet *et al.* (2002) and Suja (2003). After ascertaining the identity of 50 isolates as *Lactobacillus* spp., further efforts were made to identify them at species level. The isolates were subjected to PCR-based assay using species-specific primer pairs Lgas 2/Lgas 3 as devised by Song *et al.* (2000).

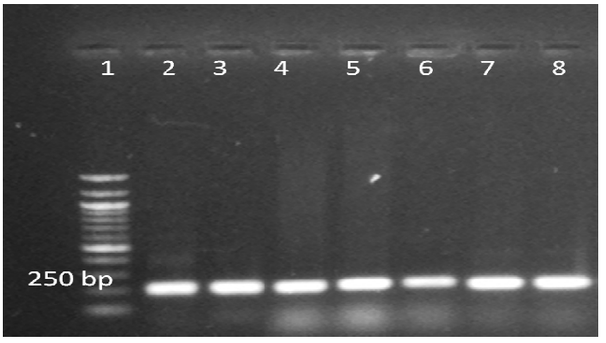

Fig. 1: Genus specific PCR (250bp)

Lane 1 = 100bp Marker, Lane 2 = Lg6, Lane 3 = Lg12, Lane 4 = Lg70, Lane 5 = Lg2sc, Lane 6= Lg50b, Lane 7 = Lg61b, Lane 8 = L. gasseri ATCC19993

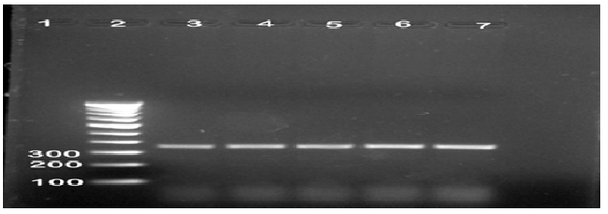

Fig. 2: Species specific PCR (360bp)

Lane 2 = 100bp marker, Lane 3 = Lg2sc, Lane 4 = Lg70, Lane 5 = Lg8sf, Lane 6 = Lg50b, Lane 7 = L. gasseri ATCC19993.

The results obtained (Fig. 2) showed a specific amplification product of 360 bp for the isolates of *L. gasseri.* Song *et al.* (2000) also successfully used the same species specific primers for the species *L. gasseri*. Out of 50 *Lactobacillus* isolates, only 34 were identified as *L. gasseri* using species-specific PCR.

Acid tolerance

Several factors have been claimed to affect the viability of probiotic bacteria including low pH and bile salts. The low pH is known to provide an effective barrier against the entry of bacteria into the intestinal tract. The pH of the stomach generally ranges from pH 2.5 to pH 3.5 (Singh *et al.,* 2012) so the viability of probiotic bacteria in the gut is the most important parameter for providing therapeutic functions. All the 34 isolates confirmed as *L. gasseri* were initially screened at pH-2 to select the highest acid tolerant strains. Out of thirty four isolates only nine demonstrated viability at acidic conditions of pH 2.0 after 3 h of exposure in MRS at 37ºC. These nine isolates were finally selected for further *in vitro* evaluation. At pH-2.0 the highest resistance to acidic conditions was observed for the *L. gasseri* isolates Lg50b and Lg70.

These isolates showed an increment of 0.4 log in their counts after 3 h incubation at pH-2. The isolate Lg8sf and Lg12 also showed good survival at pH-2 after 3 hours as they showed only 0.4 log to 1.2 log reduction after 3 h incubation. The nine *L. gasseri* isolates were also subjected to in vitro tolerance to acid levels of pH 3.0 for 3 h intervals at 37ºC. All the isolates showed complete acid tolerance towards pH-3.0 as no reduction in counts was observed. However at pH-1.5 none of the isolate was able to survive for 3 h (results not shown).

In the present investigation among all the tested nine isolates, Lg70, Lg50b, Lg8sf and Lg12 were found to be better survivors to acidic conditions. Similar results have been reported by others. Singh *et al*. (2012) in a study reported that out of 32 strains, 29 isolates demonstrated fairly high tolerance of *L. reuteri* strains to acidic conditions. In a similar study Kapila *et al.* (2014) reported that *Lactobacillus casei* spp. *casei* (log cfu per ml culture) showed viability in MRS broth at pH 2.0. A similar observation regarding

acid (low pH) tolerance of *Lactobacillus* K-7 was made by Bogovic and Rogelj, (2000).

Table 2: *In vitro* acid tolerance of selected *L. gasseri* isolates expressed in log cfu/ml

Isolates	pH-3.0	pH-2.0
	0h 3h	0h 3h
Lg 5	8.7±0.1 8.9±0.9	8.8±0.3 6.2±0.5
Lg 6	9.0±0.2 9.2±0.4	9.0±0.8 7.1±0.3
Lg 12	8.8±0.9 9.0±0.8	8.7±0.2 7.5±0.6
Lg23	9.0±0.3 9.3±0.6	8.9±0.5 6.3±0.2
Lg50b	8.7±0.7 8.9±0.9	9.1±0.1 9.5±0.5
Lg61b	9.1±0.4 9.3±0.8	8.7±0.12 6.3±0.2
Lg70	8.5±0.3 8.9±0.3	8.9±0.3 9.3±0.7
Lg 2sc	8.9±0.4 9.2±0.5	9.0±0.4 5.2±0.6
Lg8sf	9.0±0.3 9.4±0.7	9.1±0.8 8.7±0.9

values are presented as mean ± SD of three independent experiments performed in triplicate

Table 3: *In vitro* bile tolerance of selected *L. gasseri* isolates expressed in log cfu/ml

Isolates	1% Bile	2% Bile
	0h 3h	0h 3h
Lg 5	9.1±0.3 9.9.±0.7	8.8±0.6 4.2±0.9
Lg 6	8.9±0.5 9.2±0.3	8.7±0.7 6.3±0.7
Lg 12	9.2±0.2 9.7±0.7	9.2±0.8 7.2.±0.9
Lg23	8.7±0.5 9.2±0.5	9.1±0.4 6.4±0.6
Lg50b	9.0±0.6 8.5±0.6	8.5±0.5 6.2±0.7
Lg61b	8.8±0.7 9.5±0.7	8.8±0.8 5.5±0.4
Lg70	8.2±0.6 8.9±0.3	8.9±0.5 8.2±0.8
Lg 2sc	9.0±0.3 9.3±0.2	9.1±0.8 8.6±0.7
Lg8sf	8.5±0.5 9.2±0.5	8.7±0.3 7.5±0.7

values are presented as mean ± SD of three independent experiments performed in triplicate

Bile tolerance

Bile entering the duodenal section of the small intestine has been reported to reduce the survival of bacteria. Hence, tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar *et al.,* 1992). This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids which are very susceptible to destruction by bile salts that have detergent like characteristics (Vinderola and Reinheimer, 2003). The bile salt tolerance pattern of *L. gasseri* isolates are presented in Table 3. In the present investigation 1% concentration of bile was found to have no effect on the viability of the *L. gasseri* isolates. However, at a concentration of 2% bile, isolates Lg70, Lg8sf, Lg2sc and Lg12 were found to be most bile tolerant isolates as they showed only a slight reduction in their log counts. While Lg5 and Lg61b were found to be most bile sensitive isolates as they showed upto 4 log reduction in their counts. The 2% oxgall (bile salt) used for testing our strains represents the extreme concentration obtained in human intestine during the first hour of digestion (Gotcheva *et al.,* 2002). Afterwards the normal level of bile salt in intestine is around 0.3%. It is also mentioned that the resistance to bile salts varies a lot among the lactic acid bacteria species and even between strains themselves (Xanthopoulos, 1997).

Our results confirm observations of Mishra, (2005) who reported variations in the bile tolerance among their probiotic strains after different exposure times. In a similar study Singh *et al.,* (2012) reported that almost all the selected *L. reuteri* strains were able to tolerate 0.5% and 1% bile concentrations showing less than 2.2 log cycle reductions in their cell counts however, in the presence of 2% bile, 26 *L. reuteri* strains showed a survival with 3-3.5 log count reduction. Our results indicate that these *L. gasseri* isolates can survive bile conditions pertaining in the small intestine on account of their bile resistance ability.

Cell surface hydrophobicity

Adhesion to host gut epithelial cells and intestinal mucus is an important property of a probiotic strain for temporary colonization of the GI tract and stimulation of beneficial effects. The cultures exhibiting higher cell surface hydrophobicity could be better performers in terms of adherence to the intestinal epithelial cells, thus enhancing their useful property in competitive exclusion of pathogens (Schillinger *et*

al., 2005). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Schillinger *et al*., 2005). The cell surface hydrophobicity was evaluated using three hydrocarbons viz. n-hexadecane, n-octane and xylene. It is clear from Table 4, that strain Lg2sc showed highest affinity for n-hexadecane followed by Lg70 (70.68±1.03 and 65.59±0.89% respectively) in comparison with other strains. The isolate Lg70 (81.32±1.28% CSH) also showed highest affinity for xylene followed by isolates Lg2sc, Lg61b and Lg12 which was almost comparable. It is also evident from the table that there is a difference in behavior of nine test strains for n-octane as Lg2sc showed maximum affinity (77.19±1.24%) as compared to other isolates followed by Lg70 (72.23±1.12%).

Table 4: Cell surface hydrophobicities of *L. gasseri* isolates

	% Hydrophobicity		
Isolates	**Xylene**	**n-Hexadecane**	**Octane**
Lg 5	52.13±1.56	47.1±1.39	57.72±0.51
Lg 6	65.59±1.95	38.43±1.26	63.82±1.58
Lg 12	72.99±1.96	49.16±0.65	63.47±0.88
Lg 23	56.28±1.22	51.3±0.92	42.7±2.68
Lg 50b	57.22±2.21	44.46±1.19	62.1±0.76
Lg 61b	72.77±0.52	46.1±1.1	53.15±1.09
Lg 8sf	47.45±1.82	30.49±1.57	37.18±1.22
Lg 2sc	71.79±1.06	70.68±1.03	77.19±1.24
Lg70	81.32±1.28	65.59±0.89	72.23±1.12

Values are presented in triplicate, ±SEM

The percent hydrophobicities of Lg6, Lg12 and Lg50b for n-octane were almost comparable and ranged between 62.1±0.76 to 63.82±1.58%. It may be concluded from these results that the cultures had shown maximum adherence towards xylene while lowest towards octane. The high affinity of these isolates for three tested hydrocarbons demonstrate their adhesiveness. In a similar study Kaushik *et al*., (2009) had observed hydrophobicity of *L. acidophilus* LA7 in the presence of *n*-hexadecane or xylene which ranges between 57-58% whereas hydrophobicity of some strains of *L. acidophilus* has been reported as low as 2-5%. The variation in hydrophobicity to solvents has been reported in other probiotic bacteria also and has been explained by the fact that adhesion depends upon the origin of the strains as well as their surface properties (Morata *et al.,* 1998).

In vitro adhesion on Caco-2 cell lines

Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem (Freter, 1992; Alander *et al.,* 1997). The *in vitro* adhesion of all the nine *L. gasseri* isolates was tested using Caco-2 cell lines and results are presented in (Table 5).

Table 5: *In vitro* adhesion of *L. gasseri* strains on Caco-2 cell lines

***L. gasseri* isolates**	**Percent adhesion on caco-2 cell lines**
Lg 5	15±2.32
Lg6	17±3.05
Lg 12	10±4.56
Lg 23	8±1.59
Lg 50b	22±2.77
Lg 61b	12±3.59
Lg 70	25±2.67
Lg 8sf	13±4.75
Lg 2sc	12±3.23

Values are presented as mean ± SD of three independent experiments performed in triplicate

All the nine *L. gasseri* strains demonstrated good adhesion ability and the percent adhesion ranged from 12-25%. Lg70 was found to be highly adhesive on Caco-2 cell lines (25%). Thus Lg70 and Lg50b were found to be most adhesive strains on Caco-2 cell lines. Our results are in line with Mathara *et al*., (2008) who reported 98% adhesion for *L. acidophilus* BFE 6160. However Duary *et al.* (2011) in a study reported that based on per cent adhesion, *L. plantarum* Lp91, *L. plantarum* Lp9 and *L. plantarum* Lp77 were the most adhesive strains showing percent adhesion of 10.2±1.09, 7.4±1.34 and 7.3±0.28%, respectively. High adhesion of the strains tested in this study indicate a

high probiotic potential among the lactobacilli strains found in breast fed human infant feces.

Antibiotic susceptibility test

Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not contain transferable resistance. The antibiogram of nine potential probiotic *Lactobacillu gasseri* isolates was investigated using disc diffusion assay for a total number of twelve clinically important antibiotics according to Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007) and the results are shown in Table 6. All the nine cultures had shown variable response against different antibiotics.

Table 6: Antibiotic susceptibility of *L. gasseri* strains

Antibiotics	Lg 5	Lg 6	Lg 12	Lg 23	Lg 8sf	Lg 2sc	Lg 50b	Lg 61b	Lg 70
Riffampicin	S	S	S	S	S	S	S	S	S
Polymixin B	S	S	S	S	R	S	R	S	S
Gentamicin	S	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S	S	S	S
Ciprofloxacin	S	S	S	S	S	S	S	S	S
Vancomycin	R	R	R	R	S	R	S	R	R
Tetracycline	S	S	S	S	S	S	S	S	S
Penicilline	S	S	S	S	S	S	S	S	S
Ampicillin	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	R	R
Erythromycine	S	S	S	S	S	S	S	S	S
Bacitracin	S	S	S	S	S	S	S	S	S

(R)=resistant, (S)=sensitive

In this study it was found that all isolates were susceptible to the used antibiotics except Lg50b (polymixin resistant), Lg70 and Lg61b (kanamycin resistant). Most of the isolates were found to have resistance for vancomycin except Lg8sf and Lg50b. Our results in this regard are similar to those reported by others. Liasi *et al.* (2009) also demonstrated that, the 3 lactobacilli isolates tested (including one strain of *L. plantarum*) were susceptible to β-lactam group of antibiotic which include penicillin G, amoxicilline and ampicillin. Danielsen and Wind, (2003) found a high level of resistance to aminoglycosides and ciprofloxacin for all investigated *lactobacilli*. Similar aminoglycoside resistance results were also reported by Charteris *et al.* (2001) and Katla *et al.* (2001). Temmerman *et al.* (2003) in a study reported that the *lactobacilli* isolated from commercial products in Europe comprised strains resistant to tetracycline (29.5%), chloramphenicol (8.5%), and erythromycin (12%) and overall, more than 68% of the isolates exhibited resistance to two or more antibiotics. Considering these data, the antibiotic resistances that were observed for the strains in this study were considered to be intrinsic or natural resistances. The strains of *L. gasseri* did not contain any of the transferable, acquired resistances that are known to occur among LAB and include resistances toward chloramphenicol and erythromycin (Danielsen and Wind, 2003).

Table 7: Percent cholesterol removal from growth media

L. gasseri isolates	% Cholesterol assimilation
Lg 5	28±1.24
Lg 6	32±1.35
Lg 12	31±2.45
Lg 23	38±3.65
Lg 50b	42±1.46
Lg 61b	46±2.65
Lg70	52±3.74
Lg 2sc	50±1.73
Lg 8sf	43±2.56

Values are presented in triplicate, ±SEM

Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity is important for the bacteria to grow in and colonize the intestine (Moser and Savage, 2001) by deconjugating bile salts, which are readily excreted in the gastrointestinal (GI) tract. It has also been suggested that bile BSH enzyme might be a detergent shock protein that enables lactobacilli to survive the intestinal bile stress (De Smet *et al.*, 1995). In this investigation out of the nine isolates four isolates viz., Lg61b, Lg70, Lg6 and Lg50b

exhibited an intense level of BSH activity as indicated by intense precipitation of the bile salts Sodium taurocholate and Sodium taurodeoxycholate. None of the *L. gasseri* isolate showed deconjugation of sodium tauroglycocholate under the assay conditions. Singh *et al.* (2012) in a similar study also found that out of nine *L. reuteri* strains only LR20 exhibited an intense level of BSH activity as indicated by dense precipitation of the sodium taurocholate and sodium tauroglycocholate, while the rest of the 8 isolates showed only moderate levels of BSH activity.

Antibacterial spectra

The agar well diffusion method was used to study antimicrobial activity of all the nine selected isolates of *L. gasseri* against pathogenic organisms. The antimicrobial spectra of these nine *Lactobacillus gasseri* isolates was tested against *Enterococcus faecalis* ATCC14506, *Listeria monocytogenes* ATCC15303, *Pseudomonas aeruginosa* NCDC105, *Staphylococcus aureus* NCDC237, *Bacillus cereus* ATCC 12504, *Salmonella typhii* NCDN113, and *Escherichia coli* ATCC 25922. Antimicrobial activity was determined by measuring the diameter of zone of inhibition. All the strains were found to inhibit the growth of *Enterococcus faecalis* ATCC14506 and *Listeria monocytogenes* ATCC15303. These strains also inhibited the growth of *Pseudomonas aeruginosa* NCDC105 except Lg2sc. Lg12 and Lg23 gave >10mm zone of inhibition against *Staphylococcus aureus* NCDC 237 whereas Lg50b. Lg60b Lg2sc and Lg8sf did not show any zone of inhibition. All the isolates inhibited the growth of *Escherichia coli* ATCC 25922 and *Salmonella typhii* NCDC113 isolate with varying zones of inhibition. Except Lg12 and Lg50b all the strains were found to have antagonism for *Bacillus cereus* ATCC 12504.

Cholesterol Assimilation from Growth Media

The results obtained for cholesterol assimilation by *Lactobacillus gasseri* isolates are presented in Table 7. The percent cholesterol assimilation by different lactobacilli ranged from 28-52%. Out of nine *L. gasseri* isolates, Lg70 showed the highest cholesterol assimilation (52%) after 24 h incubation followed by Lg50b (50%). This is in agreement with earlier studies on *L. casei (Gilliland and Walker, 1990).* Ooi *et al*. (2010) had also reported assimilation of cholesterol by *L. acidophilus* by deconjugating bile salts under anaerobic conditions, which further support our findings. Also hypocholesterolaemic ability is likely due to the assimilation of cholesterol by *L. acidophilus cells* or/and attachment of cholesterol to the surface of *L. acidophilus* cells. In a study conducted using the *L. gasseri* isolate Lg70 Singroha *et al*. (2014) reported that feeding rats with milk fermented with Lg70 significantly reduced serum cholesterol level in rats fed on high cholesterol diet in comparison to control group. Tomaro-Duchesneau *et al.* (2014) evaluated the cholesterol assimilating property of *lactobacilli* strains and reported that all of the strains significantly (P<0.01) assimilated cholesterol. Lactic acid bacteria (LAB) with active bile salt hydrolase (BSH) activity have been suggested to lower cholesterol levels through interaction with host bile salt metabolism (De Smet *et al*., 1995). Critical analysis of our results revealed that these *Lactobacillus gasseri* isolates cultures exhibited an excellent hypocholesterolaemic effect and thus may be used as probiotics to prevent hypercholesterolemia in humans.

DNAse, Gelatinase and Hemolytic Activity

Absence of haemolytic and gelatinase activity is a selection criteria for probiotic strains, indicating that these bacteria are non-virulent. The mucoid lining constitutes the target across which many physiological substances are exchanged. Haemolysis activity would break down the epithelial layer while gelatinase activity would derange the mucoid lining. These impairments interfere with the normal functioning of these very important linings and would cause pathways for infections (De Vuyst *et al.,* 2003). Investigation of virulence potential was determined physiologically using the following tests namely gelatinase activity, hemolysis and DNase production. In this study none of the isolate was found to posses these virulent strains and thus considered as safe for use as probiotic. Earlier reports also reveal that lactic acid bacteria do not exhibit hemolysis and

gelatinases activity (Osmanagaoglu *et al*., 2010; Gao *et al*., 2012). Venkatasubramanian and Kadirvelu, (2013) in a study reported that none of lactobacilli exhibited hemolysis however, the positive strain *S. pyogens* MTCC 442 and *E.coli* MTCC 728 showed β- and α-haemolysis, respectively.

Screening of the dahi cultures for compatibility with probiotic *L. gasseri* strains

In the present study it can be interpreted from the experimental observations that *L. gasseri* Lg70 behaved as a promising potential probiotic strain. So it can be incorporated into products like dahi and in order to do so its compatibility study was done. All dahi cultures except NCDC 167 and NCDC 261 grew well in the presence of *L. gasseri strains*, demonstrating the absence of antagonism between them. The zone of inhibition around these two cultures was approximately 30 mm in diameter. These results indicate that *L. gasseri* strain Lg70 can be used to develop a product like *dahi* to exploit its health benefits since it is compatible with the *dahi* cultures.

CONCLUSION

With the present status of increasing drug resistant microorganism and the side effects caused by drugs, there is an ardent need for the development of alternative natural food products with health promoting properties. On the basis of *in vitro* evaluation Lg70 was found to be the most suitable strain for incorporation into functional dairy food. In this regard *L. gasseri* Lg70 strains can play a very important role. Food formulations with addition of such strain may be of significance with an immense health effect in humans.

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