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Molecular Insights into Bile Salt Hydrolase-Positive Lactobacilli: A PCR-Based Identification Approach

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Bile salt hydrolase (BSH)-positive *Lactobacilli* play a crucial role in gut health and have been associated with various physiological benefits. This study presents a comprehensive molecular investigation into the identification of BSH-positive *Lactobacilli* using a polymerase chain reaction (PCR)-based approach. The methodology involves the targeted amplification of specific genetic markers associated with BSH activity, enabling the precise discrimination of these beneficial bacteria within complex microbial communities. The study encompasses the design and validation of PCR primers specific to BSH-encoding genes, ensuring the accurate detection of BSH-positive *Lactobacilli* strains. Utilizing this molecular tool, we conducted extensive screenings across diverse environmental samples, revealing the prevalence and diversity of BSH positive strains in various ecosystems.

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1. INTRODUCTION

In recent times, there has been a wide spread change in consumer perception towards food. Food is being perceived not only as a source of nutrition but also as therapeutic agent. This shift has generated new concepts of functional foods and neutraceuticals. Lactic acid bacteria play a very important role as starters in production of such health foods as they are food grade organisms and are generally regarded as safe (GRAS). Included among them are special classes of beneficial organisms designated as probiotics which can promote the health of the consumers by expressing several desirable biological functions in the gut. There are numerous probiotic genera, species and strains that have been identified and characterized over the past one hundred years. Most notably, *Lactobacillus* and *Bifidobacteria* are most often considered in the probiotic category.

Probiotics are defined as “Live microbial food supplements beneficial to health and have a positive effect in the prevention and treatment of intestinal microbial balance [1]. A probiotic strain should be of host origin, non-pathogenic, technologically suitable for industrial processes, acid and bile-fast, adhere to the gut epithelial tissue, produce antimicrobial substances, modulate immune responses and influence the metabolic activities of the gut [2].

Strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile acids, attach to the epithelium and grow in the lower intestinal tract before they can start providing any health benefits. Cellular stress begins in the stomach, which has pH as low as 1.5. After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. After traveling through this harsh environment, the organism colonizes the epithelium of the lower intestinal tract.

Lactic acid bacteria traditionally used as starter cultures in the preparation of various fermented foods can also play a significant role as major ingredients in functional foods and neutraceuticals because of expression of health promoting functions. They have potential significance in fermentation, bio- processing, agriculture and health benefiting foods.

The probiotic properties of lactic acid bacteria are considered to be strain specific. Hence, the identification of probiotic *lactobacilli* at strain level by exploring appropriate analytical techniques has become extremely important step and is the need of the hour to enhance their credibility and commercial value.

Conventional phenotypic methods, however, are not suitable for identification of LAB, and are often ambiguous. Moreover, differentiation of these organisms at strain level is a difficult task. Genotypic methods and genetic analysis of genome sequences have paved the way for recent molecular techniques to be used for identification. The commonly used molecular techniques for identification of these organisms include Polymerase Chain Reaction with group specific primers, Dot Blot Hybridization [3]; FISH [4]; Terminal Restriction Fragment Length Polymorphism (TRFLP), Density Gradient Get Electrophoresis (DGGE) [5] and so on.

Among the aforesaid techniques, PCR is an effortless and reliable method for detection by targeting the particular gene responsible for probiotic characteristics. The recent exploration of complete genome sequences of probiotic organisms has been of immense value to develop the primers for assaying strain specific probiotic organisms for their use in functional foods, pharmaceuticals and neutraceuticals.

Probiotic properties of the *lactobacilli* strains are supposed to be strain specific and these functional properties are encoded by the specific genes located in the genomes of particular strains of *lactobacilli*. The knowledge of genome maps will provide a solid platform for comparative genomic analysis of these organisms that survive and establish in the hostile environment of the gastrointestinal tract of humans and protect them against the invasion of undesirable organisms.

Genome sequences and bioinformatics present volumes of information for rational selection of genes for identification, confirmation and characterization of functional roles of lactobacilli. The currently presumed roles associated with probiotic attributes important for colonization, survival and functionality include acid tolerance, bile tolerance, surface proteins, lipoteichoic acid, adherence factors and aggregation proteins etc**.** Some of the important genes that are encoded in genome of *L. acidophilus* NCFM are S layer protein (SlpA and –B), Mub-9, (Mucus binding

protein), BSH A and B (Bile salt hydrolases), PrLA-I and PrLA-II (phage ruminants), EPS cluster (endopolysaccharide) and Lactocin-B (bacteriocin). Out of these, BSH seems to be a potential probiotic marker that can be expressed for screening of new probiotic *lactobacilli* and the short listed cultures then can be further characterized for the additional functional attributes, directly related to secure human health progress.

Bile salt hydrolase activity has been reported to be expressed in several probiotic *lactobacilli*. The multiplicity of BSH encoding genes and bile transporters in *Lactobacillus johnsonii* NCC 533 and other *lactobacilli* species implies the potential importance of these gene sets for GIT survival and persistence.

By targeting the genes encoding BSH enzyme or targeting the genes responsible for other particular probiotic functions, appropriate specific primers can be designed to explore and develop suitable PCR techniques for identification of probiotic *lactobacilli*. This study is primarily focused to develop the primers for bile salt hydrolase genes in *lactobacilli* which will be helpful in mass screening of the probiotic *lactobacilli* with bile salt hydrolase activity. Incorporation of such genetic tools in probiotic research is expected to reveal the contribution of probiotics in general human health and well-being and will explicitly identify the mechanisms and corresponding host responses that provide the basis for their positive roles.

Keeping all the relevant issues in mind, this project was undertaken with the following objectives:

1) To develop PCR based techniques targeted against BSH for identification of BSH positive *lactobacilli*.
2) To apply the aforesaid techniques for screening of indigenous probiotic *lactobacilli*.
3) To clone and sequence BSH gene from selected indigenous BSH positive *lactobacilli* isolates to explore the possibility of developing strain specific PCR.

2. MATERIALS AND METHODS

2.1 Bacterial Cultures

The bacterial cultures used in this study are listed in Table 1 the *Lactobacillus* strains, non-lactobacilli as well as other non-lactic acid bacteria were obtained from the repository of National Collection of Dairy Cultures (NCDC), NDRI, Karnal, Molecular Biology Unit (MBU), NDRI, Karnal and Dr. K. J. Heller, federal Research Centre for Nutrition and Food, Kiel, Germany.

2.2 Culture Purity

The purity of all bacterial cultures was always ascertained prior to use by Gram's staining and cell morphology.

2.3 Maintenance and Propagation of Cultures

The bacterial cultures of Lactobacillus strains were propagated in MRS broth at 37°C for 16 to 18 h. One set was maintained as glycerol stocks at –70°C by mixing equal volume (500 µl) each of overnight grown bacterial culture and sterilized 50 percent glycerol. The cultures were stored at –70°C in ultra-low deep freezer (New Brunswick Scientific, USA), until further use. The cultures were always activated in MRS broth tubes prior to use by sub culturing. Another set of cultures were propagated at 37°C and preserved in litmus milk tubes and stored in refrigerator. The genomic DNA from non-lactic acid bacteria (LAB) used in this study was obtained from MBU

2.4 Molecular Weight Markers

The molecular weight markers, viz λ DNA/EcoR1 + HindIII double digest (Gibco BRL, USA, Bangalore Genei), 1kb ladder (Bangalore Genei) and 100 bp ladder were used in the study:

1. λ DNA / EcoR1 + HindIII double digest (0.125kb, .564kb, .831kb, .947kb, 1.275kb, 2.027kb, 3.530kb, 4.268kb, 4.973kb, 5.148kb, 21.226kb).
2. 1kb Ladder (1kb, 2kb, 3kb, 4kb, 5kb, 6kb, 7kb, 8kb, 9kb, 10kb).
3. 100bp ladder (0.1 to 1.0 kb)

2.5 Isolation of *Lactobacilli*

2.5.1 Collection of Samples

Lactobacilli cultures were isolated from mother's milk, human faecal samples, buffalo milk and probiotics capsules. The samples collected were enriched in MRS broth. For human faecal samples, sterile swabs were used and put in in MRS broth for enrichment. Incubation was

carried out at 37°C/2-3hrs and streaking was done on BCP-Lac-MRS Agar and typical Yellowish colonies were selected for morphological examination under microscope. Also pour plating was done with the dilutions 10^7 and 10^8 and the submerged colonies were selected for morphological examinations.

2.5.2 Microscopic examination

Gram staining and spore staining were performed by following standard methods. Then, the selected colonies were transferred into MRS broth and incubated for overnight at 37°C.

2.5.3 Catalase test

Catalase test was performed by growing the culture in MRS broth overnight at 37°C followed by addition of a drop of hydrogen peroxide. The production of effervescence indicated catalase positive reaction.

2.6 Isolation of Genomic DNA

The genomic DNA from all the cultures used in this study grown for 16 to 18 h in MRS broth at 37°C was extracted by the above method [6].

2.6.1 Procedure

The cells were harvested from 1.5 ml of overnight grown culture of *Lactobacilli* in a microcentrifuge for 10 min at 3000 g. The supernatant was discarded carefully without disturbing the pellet taking all necessary precautions. The pellet was resuspended in 0.5 ml of SET buffer (pH 7.5). Lysozyme was added to the above cell suspension at a concentration of 1 mg/ml and incubated at 37°C/1 hr. The above step was followed by addition of 1/10 (one-tenth) volume of 10% SDS and further incubated at 37°C with occasional inversion for 30 min. One third volume of 5 M NaCl and one volume of chloroform: isoamylalcohol (24:1) were then added to the above mix and incubated at room temperature for 30 min with frequent inversions for 30 min.

The samples were centrifuged at 4,000 rpm/15 min and the aqueous phase was transferred to a new tube. The DNA was precipitated by adding one and half volume of isopropanol and the tubes were inverted gently to mix and kept for 30 min at -20°C or kept at -20°C for overnight. The DNA pellet thus obtained was recovered after centrifugation at 12,000 rpm and washed with 70% ethanol and dried under vacuum in a speed vac system (Martin Christ, Germany) for 10 min. and finally dissolved in 50 μl TE buffer (pH 8.0). The extracted genomic DNA along with tracking dye were run on one percent agarose gel. Electrophoresis (mini/max submarine), (Hoeffer, USA) at 80 V for 30 min using 1 x TAE buffer. The gels were monitored on UV transilluminator (Fotodyne, USA) after staining with ethidium bromide (0.5 (μg/ml). The gels were subsequently photographed (MP-4 system, Fotodyne, USA).

2.7 Determination of DNA Concentration

2.7.1 Spectrophotometric analysis

The concentration of DNA samples used in the study was determined by the measurement of optical density. (OD) is a UV spectrophotometer (DU 640, Beckman, USA) at 260 and 280 nm. The purity of DNA was ascertained by measuring as ratios at 260 to 280 nm. The concentration and quality of DNA was calculated by the following equivalents as suggested by Sambrock et al. [7].

DNA mg/ml = A_{260} x dilution x 50.0
A_{260} = Absorbance in OD at 260 nm.
10D = 50 μg/ml (double standard DNA) and
10D = 30 μg/ml (Single standard DNA)

2.8 Identification of Isolates as *Lactobacilli* by PCR

The *lactobacillus* isolates were identified by PCR using primers LaLMA1 and R16-1.

2.8.1 PCR reaction

Before setting up of PCR, all the reagents were thawed except Taq DNA polymerase, mixed and spinned. Reagents were always kept on ice bath during the period of setting up of the reaction. The reaction mix comprising of 10x PCR butter (containing $MgCL_2$), dNTPs and primers was prepared and distributed to reaction tubes according to the requirements. The final volume of PCR mix was adjusted to 25μl.

2.8.2 PCR cycling steps

Template DNA was initially denatured at 95°C for 2 to 5 min. Next 30 to 45 cycles were

programmed based on annealing temperatures of primers used. The respective denaturation, annealing and extension temperatures used in this study for different sets of primers targeted against different *Lactobacillus* species have been explained under Results and Discussion.

Finally, an additional extension was given at 72°C for 5 to 10 min. After the run was over, the amplified PCR products were held at 4°C until further use.

2.8.3 Analysis of PCR products by agarose gel electrophoresis

PCR amplified products obtained with different templates were electrophoresed on the agarose gels (1.0 to 2.0%) by following the standard procedure as given by Sambrook et al. [7].

2.8.3.1 Agarose gel

Agarose of 1 to 2 percent concentration was prepared by dissolving the appropriate quantities of agarose in IX TAE buffer (pH 8.0) in a microwave oven or by keeping in boiling water bath. Ethidium bromide stock solution was added directly to molten agarose solution at the rate of 0.5 µg/ml before casting the gel (mini / midi / maxi).

2.8.3.2 Procedure

Molten agarose was cooled to 50°C and poured into respective moulds of minigel (50 ml) and midigel (100 ml) using appropriate comb (8 to 20). The surface was levelled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel plate was mounted on respective electrophoresis tanks (submarine mini, Hoeffer); Horizontal (midi, Bio-Rad). The respective electrophoresis tanks were filled with IX TAE electrophoresis buffer to cover the gel to a depth of about 1 min. The DNA samples were mixed with 5 µl of tracking dye and were loaded slowly into the slots of submarine gel using micropipette. Electrophoresis was carried out at 100 V (60 mA current) for one hour in mini gel electrophoresis apparatus and 1½ h in case of maxi gel system. After completion of electrophoresis, the gels were taken out of the chamber and examined under UV transilluminator (302 nm, Fotodyne) and photographed using a Polaroid camera with Polaroid type 55 sheet film (MP4 system).

2.9 Sugar Fermentation Patterns of *Lactobacillus* Isolates

The composition of the medium used for sugar fermentation is given below.

2.9.1 CHL medium

Component	Wt.
Polypeptone	:10.0 gm
Yeast extract	:15.0 gm
Tween 80	:1.0 ml
K_2HPO_4	:2.0 gm
Sodium acetate	:5.0 gm
Dipotassium Phosphate	:2.0 gm
$MgSO_4$, $7H_2O$	:0.2 gm
$MnSO_4$, $4H_2O$	:0.05 gm
Bromocresol purple	:0.17 gm
Distilled Water	:1000 ml
$_P$H	: 6.9

2.9.2 Procedure

Small test tubes (12x75 mm) were used for production of acid from different sugars. CHL medium was used as the basal medium. Four ml of the medium was taken in each tube and sterilized by autoclaving. One sugar disc (Hi-Media) was aseptically added to each tube. Each sugar tube was inoculated with 0.1 ml of inoculum and the tubes were incubated at 37^0C for 24- 48hrs and the result of colour change was recorded as positive and a control was used to compare the colour change.

List 1. List of reagents used for the study

Reagents	Volume	Conc.
Sterile milliq	Var	--
Reaction buffer, 10x with $MgCL_2$	2.5 µl	1x
dntp mix	2 µl	0.2 mM
Primer forward	1 µl	0.1 – 1 mM
Primer reverse	1 µl	0.1 – 1 mM
Taq DNA polymerase	0.5 – 1 µl	0.5 – 30
Template DNA	1-10 µl	100 ng – 1 µg

Table 1. Bacterial cultures used in the investigation

Sl. No.	Reference strains	Source
A)	**Lactobacillus cultures:**	
1.	*Lactobacillus acidophilus* (La), 195	NCDC
2.	Lactobacillus plantarum (Lpl), 201	NCDC
3.	*Lactobacillus johnsonii* La1	Kiel, Germany
4.	*Lactobacillus acidophilus* LA7	Kiel, Germany
5.	*Lactobacillus acidophilus*	Kiel, Germany
6.	*Lactobacillus acidophilus*	AIIMS, New Delhi
B)	**Other non-lactobacilli cultures:**	
1.	*Lactococcus lactis* ssp. *lactis*, 60	NCDC
2.	*Lactococcus lactis* ssp. *cremoris*, 81	NCDC
3.	*Lactococcus lactis* ssp. *diacetylactis*, 61	NCDC
4.	*Pediococcus pentosaceus*, 35	NCDC
5.	Bifidobacteria	MBU
6.	*Propionibacterium freudenreichii* ssp.*shermanii*, 139	NCDC
7.	*Leuconostoc mesenteroides* ssp. *cremoris,* 29	NCDC
8.	*Leuconostoc mesenteroides* ssp. *lactis*, 200	NCDC
C)	**Other non-LAB cultures**	
1.	*Enterobacter aerogenes* 106	NCDC
2.	*Shigella dysentriae* 107	NCDC
3.	*Salmonella typhi 113*	NCDC
4.	*Enterococcus faecalis* 114	NCDC
5.	*Klebsiella pneumoniae 138*	NCDC
6.	*Enterobacter aerogenes 173*	NCDC
7.	*Enterococcus faecium 211*	NCDC
8.	*Enterobacter aerogenes 248*	NCDC
9.	*Escherichia coli 0157*:H7	MBU
10.	*Listeria monocytogenes*	MBU
11.	*Bifidobacterium bifidum 203*	MBU
12.	*Bifidobacterium bifidum 228*	MBU
13.	*Bifidobacterium bifidum 228*	MBU

2.10 Screening of *Lactobacillus* Isolates for Probiotic Attributes

For selection of probiotic *lactobacilli*, the isolates were subjected to a battery of tests recommended as per WHO standards (FAO/WHO, 2002) as given below.

2.10.1 Acid tolerance

MRS broth was used to stimulate acidic conditions of gut. The broth was adjusted to pH 3.0, 2.5, 2.0 and 1.5 with 5 N HCl along with broth adjusted to neutral pH as a control. Overnight grown culture in MRS of *Lactobacillus* at 37^{o}C was inoculated at different pH values.

One ml of culture was taken from each tube immediately (0 hrs) and 10 fold dilutions were prepared in 0.1% peptone water. Pour plating was done using MRS agar. Then, one ml of culture was taken from each of these tubes after 1, 2, and 3 hrs respectively and plating procedure was repeated. The plates were incubated at 37^{o}C for 24 to 48 hrs and the results were recorded. Similarly, the optical density was measured at 600 nm. First the cell concentration was adjusted to 0.3 OD, then they were added to the pH adjusted tubes and OD was taken at 0 hr, 2 hr and 3 hr.

2.10.2 Bile tolerance

The cultures were grown on MRS agar and single colony was inoculated into broth after 18hr growth. The pH was adjusted to 4.5 with sterile 0.1 N HCl or 0.1 N NaOH. Bile salt solutions at 1.0%, 1.5% and 2.0% concentrations were prepared under sterile conditions with one control in MRS broth.

Overnight grown cultures were inoculated at different concentration of bile and immediately 1ml of culture was taken and 10 fold denial dilutions were prepared with 0.1% peptone water. Then the cultures were further incubated

at 37°C and pour plating was carried out using BCP-Lac MRS agar. The plates were incubated at 37°C/24-48hrs and colonies were counted. The procedure was repeated at 1, 2 and 3hrs respectively. Similarly, OD at 600 nm was measured at different bile concentrations by taking bile solution in MRS broth without culture as blank. The reading will taken at 1, 2 and 3 hrs.

3. RESULTS AND DISCUSSION

The main purpose of this investigation was to isolate indigenous and novel *lactobacilli* of human origin from the gut, having distinct probiotic functions and their subsequent characterization by developing appropriate PCR techniques based on selected probiotic markers. To achieve these objectives, a sizeable number of representative *lactobacilli* isolates were recovered on MRS agar from human faecal samples, probiotic preparations and raw buffalo milk. Based on the preliminary screening by colony characteristics and morphological factors on microscopic examination after Gram and spore staining as well as catalase test, a total of 100 isolates were selected as the subject for further studies. The results pertaining to isolation of typical *lactobacilli* of human origin from different niches are presented below.

3.1 Isolation of *Lactobacilli* from Different Niches

In the present investigation, a total of 25 human faecal samples from the subjects belonging to age group 23 – 30 including infant faecal samples, 5 human milk samples, 5 buffalo milk samples and two probiotic preparations (Prolac and Yakult) were used for the isolation of lactobacilli using BCP-Lac MRS agar as the growth medium. After plating the appropriate dilutions of the pre-enriched samples, (MRS broth) on BCP-MRS agar plates, typical yellow coloured colonies developed on the agar plates after 24 hrs. Initially, a sizable number of typical representative colonies from each sample were randomly picked from the agar plates and transferred to MRS broth and incubated at 37oC for overnight. The isolates grown in MRS broth were checked for purity under a microscope (Leica, Germany) after Gram and spore staining and the pure cultures appearing as typical thin, small and large rods and exhibiting Gram positive reaction on staining were subjected to catalase test. Based on these results, a total of 100 isolates typical of lactobacilli and catalase negative were selected as subject for further studies. A set of these isolates was preserved and maintained in litmus milk as well as glycerol stocks stored at -80°C in a deep freezer (New Bruinswick, USA).

3.2 Identification of Isolates as Lactobacilli by Genus Specific PCR

In order to ascertain the true identity of the aforesaid isolates as lactobacilli, all of them were subjected to PCR assay based on genus specific primers LbLMA1/R161 targeted against 16SrRNA developed previously in our lab. The PCR assay conducted with the template DNA obtained from the standard lactobacillus cultures and the lactobacillus isolates resulted into the amplification of a 250bp PCR products on the agarose gel which was specific for lactobacilli only. On the basis of our PCR results, 35 isolates out of 100 colonies along with three standard probiotic cultures namely *Lb. johnsonii La1, Lb. acidophilus LA7 and Lb. acidophilus P* showed the positive signal in the form of a distinct 250bp band on the gel, thereby, establishing their identity as lactobacilli. Our results with this regard are in complete agreement with these of Dubernet et al. [8] who also demonstrated amplification of 250bp product in the PCR assay with all the standard cultures as well as wild isolates of lactobacilli used in their study.

The breakup of these 35 isolates indicating the source of their recovery / origin has been recorded in Table 2. As can be evidenced from the table, majority of these isolates (24) were recovered from human faecal samples, 8 from raw buffalo milk, two from probiotic preparations and one from human milk.

3.3 Sugar Fermentation Profile of Lactobacillus Isolates

In this investigation, an attempt was also made to tentatively give the species status to the lactobacillus isolates confirmed by genus specific PCR on the basis of their sugar fermentation characteristics. The data pertaining to sugar fermentation profiles of 14 selected cultures has been presented in Table 3.

As is quite evident form the data presented therein, all the isolates were able to ferment Mannitol, Cellobiose and Sucrose, However, arabinose could not be fermented by any of the isolates. On the other hand, N-acetyl-glucosamine and melibiose were not fermented by only one isolate (Lb7) and two isolates (Lb2

and Lb10) respectively. Based on the typical sugar fermentation patterns in respect of our isolates vis-à-vis the standard fermentation profiles of different lactobacillus species as given in Bergey's Manual, 10 of the isolates namely Lb1, Lb3, Lb4, Lb5, Lb6, Lb8, Lb10, Lb14, Lb16 and Lb18 were tentatively identified as *Lb. plantarum*, one as *Lb. casei* (Lb2), two as *Lb. fermentum* (Lb7, Lb13) and one as *Lb. acidophilus* (Lb12). However, these results are just tentative and hence needs further substantiation by including some additional sugars such as Ribose, Dulcitol, Rhamnose, Xylose, Sorbitol etc. in the experiment to make them more realistic and conclusive. Our results in this regard are consistent with similar observations made by Marteau et al. [9]. However, the latter investigator also used API system for identification of species of Lactobacillus isolates used in his study.

3.4 Screening of Indigenous Lactobacillus Isolates for Probiotic Attributes

Since, the long term target of the present investigation was to find promising and novel indigenous probiotic lactobacilli for commercial application, all the 35 PCR positive Lactobacillus isolates that emerged from this study were subjected to a battery of standard tests recommended for determining their probiotic attributes as per WHO guidelines. The results pertaining to each of these tests will now be presented separately.

Table 2. Breakup of the isolates confirmed as lactobacilli by genus specific PCR

S. No.	Isolate	Source	Morphology	Catalase Reaction	Polymerase Chain Reaction
1	Lb1	Capsule	Gram +ve rods	-ve	+ve for Lb
2	Lb2	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
3	Lb3	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
4	Lb4	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
5	Lb5	Feacal	Gram +ve rods	-ve	+ve for Lb
6	Lb6	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
7	Lb7	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
8	Lb8	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
9	Lb9	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
10	Lb10	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
11	Lb11	Feacal	Gram +ve rods	-ve	+ve for Lb
12	Lb12	Feacal	Gram +ve rods	-ve	+ve for Lb
13	Lb13	Feacal	Gram +ve rods	-ve	+ve for Lb
14	Lb14	Capsules	Gram +ve rods	-ve	+ve for Lb
15	Lb15	Feacal	Gram +ve rods	-ve	+ve for Lb
16	Lb16	Feacal	Gram +ve rods	-ve	+ve for Lb
17	Lb17	Feacal	Gram +ve rods	-ve	+ve for Lb
18	Lb18	Feacal	Gram +ve rods	-ve	+ve for Lb
19	Lb19	Human milk	Short rods	-ve	+ve for Lb
20	Lb20	Feacal	Gram +ve rods	-ve	+ve for Lb
21	Lb21	Feacal	Gram +ve rods	-ve	+ve for Lb
22	Lb22	Feacal	Gram +ve rods	-ve	+ve for Lb
23	Lb23	Feacal	Gram +ve rods	-ve	+ve for Lb
24	Lb24	Feacal	Gram +ve rods	-ve	+ve for Lb
25	Lb25	Feacal	Gram +ve rods	-ve	+ve for Lb
26	Lb26	Feacal	Gram +ve rods	-ve	+ve for Lb
27	Lb27	Feacal	Gram +ve rods	-ve	+ve for Lb
28	Lb28	Feacal	Gram +ve rods	-ve	+ve for Lb
29	Lb29	Feacal	Gram +ve rods	-ve	+ve for Lb
30	Lb30	Feacal	Gram +ve rods	-ve	+ve for Lb
31	Lb31	Feacal	Gram +ve rods	-ve	+ve for Lb
32	Lb32	Feacal	Gram +ve rods	-ve	+ve for Lb
33	Lb33	Feacal	Gram +ve rods	-ve	+ve for Lb
34	Lb34	Feacal	Gram +ve rods	-ve	+ve for Lb
35	Lb35	Feacal	Gram +ve rods	-ve	+ve for Lb

3.4.1 Acid tolerance

Acid tolerance is perhaps one of the most important pre-requisites for the selection of probiotic lactobacilli as they must survive the harsh acidic environment in tehgut to remain there for a while in good number and express their health promoting functions (Conway et al, 1987). In the present investigation, the Lactobacillus isolates were subjected to *in vitro* tolerance to different acid levels of pH 1.0, 2.0 and 3.0 for various time intervals at 37^{o}C to simulate the conditions prevalent in the human gut. As is quite evident from the data presented therein, some of the isolates namely Lb10, Lb12 and Lb26 were able to survive even at pH 1.0 for 1-3hrs and their acid tolerance was comparable to that of standard probiotic cultures used in this study as indicated by relatively high OD values (0.3009, 0.3316 and 0.2963 for Lb10, Lb12 and Lb26 respectively) obtained at aforesaid pH after three hrs. Similarly, at pH 2.0 and 3.0, the aforesaid cultures particularly Lb10, the corresponding OD values were fairly high i.e. 0.2995 and 0.3152 after three hrs. Almost a similar trend in OD values was observed with teh remaining two cultures. Our results in this regard appear to be comparable with that of Mishra (2001) who also recorded high degree of acid tolerance in case of all the seven strains except NCDC-19 examined in his study even after 3hrs of incubation at pH 1.0, 2.0 and 3.0, although there were considerable variations in acid tolerance between strains. Almost similar observations were made by Goldin et al. [10] and Jacobsen et al. [11] who also recorded fairly high acid tolerance of their probiotic lactobacillus cultures such as Lactobacillus GG, C1 and Y strains respectively.

Our results on acid tolerance of Lactobacillus isolates are, however, inconsistent with the findings of Lankaputhra and Shah [12] who in general recorded a decrease in the number of survivors of *Lb. acidophilus* starins during 3hrs of incubations at all the pH conditions used in their study. This contradiction in results on acid tolerance could be attributed to varied tolerance of different Lactobacillus spp. and strains towards acidic conditions.

3.4.2 Bile tolerance

Bile tolerance is another important property used for selection of probiotic lactobacilli since human gastrointestinal tract (GIT) is studded with high concentrations of bile secretion in view of their potential role in food digestion. However, the rate of secretion of bile and the concentrations of bile in different regions of the GIT tract vary depending mainly on the type of food consumed. Bile concentrations in GIT can range from 0.5 to 2% at different regions and hence create an unfavourable environment in the gut for survival of micro-organisms due to toxicity of these salts. Hence, probiotic lactobacilli are required to be bile tolerant to get implanted and survive in the gut in presence of such a high concentration of bile salts prevalent therein. In the present study, the Lactobacillus isolates were subjected to *in vitro* bile tolerance test by examining effect of different levels of bile salts 1.0, 1.5 and 2.0 % on the growth and survival of Lactobacillus isolates used in the study.

As can be evidenced from these results, there was considerable variation in the bile tolerance of the Lactobacillus isolates examined in this investigation. However, some of these isolates exhibited fairly high bile tolerance at all three levels of bile concentration. In general, majority the isolates were able to survive at 1.0 %concentration of bile even after 3 hrs as can be revealed by fairly high OD_{600} values (more than 0.2) in almost all the cases. The survival however was relatively much lower at higher bile concentrations i.e. 1.5 and 2.0% during the corresponding period (3hrs). The maximum bile tolerance at 1% level (OD values 0.5287 and 0.5130) was recorded with Lb27 and Lb26 respectively after 3hrs of treatment. The corresponding values with 1.5 and 2.0 % bile concentrations with Lb27 were 0.3647 and 0.3126 which were comparable with those of the standard probiotic cultures used in the study as a positive control. The most bile sensitive isolate in this study was found to be Lb7 which could produce an OD_{600} value of only 0.0541. Our results in this regard are consistent with eth observations of several other investigators who also reported sizable variations in the bile tolerance among their probiotic strains after different exposure times [13-15,12].

3.4.3. Hydrophobicity of lactobacillus isolates

Another important *in vitro* test for studying the probiotic nature of lactobacilli is the hydrophobicity test based on the nature of their cell surface involved in interaction with phagocytes, adherence to non-wetable solid surfaces, partitioning at liquid:liquid and liquid: air interfaces. The hydrophobicity to hydrocarbons is an important feature of probiotic lactobacillus cell

Table 3. Sugar fermentation profile of selected lactobacillus isolates

Isolates	Arabi-nose	Mannitol	NAGA	Cello-biose	Melli-biose	Sucrose	Treha-lose	Tentative identify-cation at species level
Lb1	–	+	+	+	+	+	+	Lp
Lb2	–	+	+	+	–	+	+	Lc
Lb3	–	+	+	+	+	+	+	Lp
Lb4	–	+	+	+	+	+	+	Lp
Lb5	–	+	+	+	+	+	+	Lp
Lb6	–	+	+	+	+	+	+	Lp
Lb7	–	+	–	+	+	+	+	Lf
Lb8	–	+	+	+	+	+	+	Lp
Lb10	–	+	+	+	–	+	+	Lp
Lb12	–	+	+	+	+	+	+	La
Lb13	–	+	+	+	+	+	–	Lf
Lb14	–	+	+	+	+	+	+	Lp
Lb16	–	+	+	+	+	+	+	Lp
Lb18	–	+	+	+	+	+	+	Lp

Table 4. Cell surface hydrophobicity of lactobacillus isolates

Culture No.	Initial O.D.	Final O.D.	% Hydrophobicity
Lb3	0.7815	0.5254	32
Lb10	0.8134	0.4522	44.4
Lb19	0.5431	0.1712	68.4
Lb23	0.7652	0.5515	27.9
Lb25	0.6608	0.4256	35.5
Lb26	0.5654	0.4165	51.8
Lb27	0.8916	0.3265	63.0
Lb29	0.7851	0.4691	40.2
Lb.casei shirota	0.8608	0.3431	60.1
La P	0.7815	0.2163	72.3

surface. In this investigation, the hydrophobicity of our lactobacillus isolates was determined with one of the common hydrocarbons namely hexadecane. The results concerning the hydrophobicity of the test Lactobacillus cultures, the subject of this study are presented in Table 4.

From the data presented therein, it can be evidenced that maximum hydrophobicity (68.4%) towards hexadecane was recorded with Lb19 followed by Lb27 (63.0%) which were almost comparable to the values obtained with standard probiotic cultures *Lb. casei* Shirota and LaP (60.1 and 72.3%) respectively. Our results in this regards are in agreement with those of Morata De Ambrosini et al. [16] who also observed very high hydrophobicity of *Pediococcus pentosaceus* and *Propionibacterium acidopropinici* and *Lb. casei*. However, Conway and Reginold [17] reported lack of correlation between capacity for adhesion and hydrophobicity. Hence, utmost care needs to be taken to draw any conclusive inference from such studies.

4. CONCLUSION

From the foregoing results, it can be concluded that bsh based PCR assays developed in this study could be very useful not only in Lactobacillus identification at species or strain level but also can provide a relatively simple, quick and realistic solution for mass *in vitro* screening of bsh positive lactobacilli of human origin A total of 25 human faecal samples including infant faecal samples, 5 human milk samples, 5 buffalo milk samples and two probiotic preparations were sued for the isolation of Lactobacilli. A total of 100 isolates based on Gram and spore staining and catalase negative reaction were used in the study. Out of a total of 100 isolates, 35 along with three standard probiotic cultures namely *Lb. johnsonii La1, Lb. acidophilus LA7 and Lb. acidophilus P* showed the positive signal in the form of a distinct 250 bp band when subjected to genus specific PCR assay using LbLMA1/R16-1. On the basis of suagr fermentation profiles of few selected

isolates, 10 of the isolates namely Lb1, Lb3, Lb4, Lb5, Lb6,Lb8, Lb10, Lb14, Lb16 and Lb18 were tentatively identified as *Lb. plantarum*, one as *Lb. casei* (Lb2), two as *Lb. fermentum* (Lb7, Lb13) and one as *Lb. acidophilus* (Lb12). All the 35 PCR positive Lactobacillus isolates that emerged from this study were subjected to a battery of standard tests recommended for determining their probiotic attributes as per WHO guidelines. Some of the isolates namely Lb10, Lb12 and Lb26 were able to survive even at pH 1.0 for 1-3 hrs as indicated by relatively high OD values of 0.3009, 0.3316 and 0.2963 respectively. The maximum bile tolerance at 1% level (OD values 0.5287 and 0.5130) was recorded with Lb27 and Lb26 respectively after 3 hrs of treatment. The most bile sensitive isolate in this study was found to be Lb7 which could produce an OD_{600} value of only 0.0541. Maximum hydrophobicity (68.4%) towards hexadecane was recorded with Lb19 followed by Lb27 (63.0%).

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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