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In Vitro Screening of Indigenous *Lactobacillus* Isolates for Selecting Organisms with Better Health-Promoting Attributes

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Abstract Lactobacilli have several attributes that provide health benefits to the host. The aim of this study was to screen indigenous lactobacilli from human gut and fermented foods for such attributes as production of β- and α-galactosidase and also their ability to reduce serum cholesterol. Lactobacilli were cultured on MRS broth and β-galactosidase activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Three isolates *Lactobacillus fermentum* GPI-3 and *L. fermentum* GPI-6 and *Lactobacillus salivarius* GPI-1(S) showed better β-galactosidase activity than the standard strains *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus plantarum* ATCC 8014. The isolates showed variability in assimilating cholesterol during growth. Several isolates showed excellent cholesterol-lowering ability compared to standard strains LGG and *L. plantarum* ATCC 8014. Isolate *L. rhamnosus* SCB being the highest acid producer (pH 4.38) also showed the highest cholesterol reduction compared to other strains including standard strains. The ability of these isolates to produce α-galactosidase was also studied and the maximum α-galactosidase activity was found in isolate *L. salivarius* GPI-1(S) followed by *L. fermentum* FA-5 and *Lactobacillus helveticus* FA-7. This study therefore reports *Lactobacillus* isolates that have superior probiotic properties when compared to the standard strains; hence, they could be considered as potential probiotic strains, which can provide health benefits to the Indian population.

Keywords α-Galactosidase · β-Galactosidase · Cholesterol removal · Lactobacilli · Probiotics

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Introduction

Lactic acid bacteria (LAB) possess various health-promoting properties useful for both humans and animals [1–3]. β-Galactosidase deficiency causes lactose intolerance [4, 5] and amelioration of this situation by β-galactosidase from LAB [6, 7] involves conversion of lactose into easily metabolizable glucose and galactose. The symptoms of lactose intolerance decrease the quality of life and daily activities. The addition of lactobacilli-producing β-galactosidase as probiotic in dairy products can thus be used for improving lactose digestion.

Consumption of LAB also reduces serum cholesterol levels, as suggested by human and animal studies [1, 8]. This is also corroborated by in vitro experiments using growth medium containing bile salts. Similarly, in vitro uptake of cholesterol from culture media has also been shown for many strains of lactobacilli [9–11]. Bile salt hydrolase plays a significant role in cholesterol removal by deconjugating the bile salts [12]. Deconjugated bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterparts [13]. Additionally, free bile salts are less efficient in the solubilization and absorption of lipids in the gut [13, 14], eventually leading to less uptake of cholesterol. Lactobacilli may also remove cholesterol by bringing about co-precipitation of cholesterol with free bile salts, bacterial assimilation of cholesterol, or attachment of cholesterol to the surface of *Lactobacillus* cells [15, 16]. Furthermore, it was also demonstrated by Kumar et al. [17] that the amount of cholesterol removed from the broth was variable, depending on the culture and the pH, during growth.

Consumption of soybean and pulses is limited because of intestinal disturbances caused by α-D-galactosides such as melibiose, raffinose and stachyose, as well as branched polysaccharides such as galactomannans and galactoglucomannans [18, 19]. α-Galactosidase which cleaves the α-1,6-linked galactose residues from such carbohydrate complexes is therefore used for the hydrolysis and release of such oligosaccharides present in food substances. Studies have shown a reduction in gastrointestinal discomfort due to gas, after addition of probiotics to pulse and soybean meal containing diets [20]. Earlier studies have established that *Lactobacillus rhamnosus* GG (LGG) has cholesterol removing ability and *Lactobacillus plantarum* ATCC 8014 has both α-galactosidases and β-galactosidase activities [21, 22].

Due to the above attributes, lactobacilli have been used as active ingredients in probiotic food such as bio-yoghurt, dietary adjuncts, and health-related products. Therefore, in the present study, lactobacilli were assessed for these attributes and several strains were found to perform better than the standard probiotic strains *L. rhamnosus* GG (LGG) and *L. plantarum* ATCC 8014 and therefore could be considered for further studies.

Materials and Methods

Bacterial Strains and Culture Conditions

A total of 20 different lactobacilli strains from different sources were used in this study as given in Table 1. They were analyzed for their probiotic properties in an earlier study that includes bile and acid tolerance, adhesion to Caco-2 and HT-29 cells and antimicrobial activity against test pathogens [23, 24]. Prior to being used, they were serially propagated three times in the appropriate medium. Lactobacilli were cultivated in de Man, Rogosa, and Sharpe (MRS)

Table 1 Strains used in the present study

No.	Isolate	Source	Accession number	16S–23S sequence based species identification
1	GPI-1(S)	Adult human gut origin	JX118837	*Lactobacillus salivarius*
2	GPI-4	Adult human gut origin	JX118830	*Lactobacillus salivarius*
3	GRI-2	Adult human gut origin	JX118835	*Lactobacillus plantarum*
4	FA-7	Fermented rice (Nyogrin)	KT337436	*Lactobacillus helveticus*
5	GPI-1(B)	Adult human gut origin	JX118836	*Lactobacillus fermentum*
6	GPI-3	Adult human gut origin	JX118834	*Lactobacillus fermentum*
7	GPI-6	Adult human gut origin	JX118833	*Lactobacillus fermentum*
8	IIS11.2	Child gut origin	KT337437	*Lactobacillus fermentum*
9	FA-5	Fermented soybean seeds (Agya)	KT337435	*Lactobacillus fermentum*
10	FA-1	Fermented bamboo shoot (Iku)	KT337434	*Lactobacillus fermentum*
11	GKI-1	Adult human gut origin	JX118832	*Lactobacillus fermentum*
12	GPI-7	Adult human gut origin	JX118831	*Lactobacillus fermentum*
13	M	Curd of buffalo milk	FJ899641	*Lactobacillus delbrueckii*
14	ASt1	Adult human gut origin	FJ899642	*Lactobacillus fermentum*
15	CS23	Child gut origin	FJ899639	*Lactobacillus plantarum*
16	CS25	Child gut origin	FJ899640	*Lactobacillus rhamnosus*
17	CS24.2	Child gut origin	FJ870560	*Lactobacillus plantarum*
18	SCA	Child gut origin	JX118842	*Lactobacillus rhamnosus*
19	SCB	Child gut origin	JX118841	*Lactobacillus rhamnosus*
20	CS5.2	Child gut origin	FJ899643	*Lactobacillus casei*

broth (MRS; Himedia, Mumbai, India). A 1.0% inoculum was used and incubated at 37 °C for 24 h in static conditions. Seed cultures of each strain were taken at the end of the exponential phase of growth at cell densities of ca. 10^8 CFU/mL. Standard probiotic strain *L. rhamnosus* GG (LGG) and standard dairy strain *L. plantarum* American Type Culture Collection (ATCC) 8014 were obtained as kind gifts from Dr. Shira Doron (MD, Department of Medicine, Tufts Medical Centre, Boston, MA, USA) and Food and Drugs Laboratory (FDL; Vadodara, India), respectively.

β-Galactosidase Production

For qualitative assay, an overnight grown culture was streaked on MRS agar plate containing 0.01% X-gal (5-bromo-4-chloro-2-indolyl-β-D-galactopyranoside) and 0.1 mM IPTG (iso-propyl-thio-β-D-galactopyranoside) as an inducer. The plates were incubated for 24 h to 3 days at 37 °C in microaerobic environment and observed for the appearance of blue colonies. This was followed by quantitative assay where intracellular β-galactosidase activity in whole cells was determined according to the method of Miller [25] with slight modifications. Overnight grown cultures were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS) pH 7.0, and inoculated 1% (*v*/*v*) in MRS-lac broth (containing lactose). Cultures were incubated at 37 °C for 24 h (microaerobic environment). Cells were then harvested, washed twice with PBS, and A_{560} was adjusted to 1.0 with the same buffer. One milliliter of the cell suspension was permeabilized with 50 μL of toluene:acetone (1:9, *v*/*v*) solution, vortexed for 7 min and immediately assayed for β-galactosidase activity. To 100 μL of the permeabilized cell suspension, 900 μL of phosphate buffer and 200 μL of o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) (4 mg/mL) were added. Tubes were then incubated at 37 °C for 15 min, and the reaction stopped by the addition of 0.5 mL of 1 mol/L Na_2CO_3. Absorbance at both 420 and 560 nm was

then recorded for each tube and β-galactosidase activity was calculated in Miller units (MU) as follows:

$$1000 \text{ x } [(A_{420} - 1.75 \times A2_{560})/(15 \text{ min} \times 1 \text{ mL} \times A1_{560})]$$

where $A1_{560}$ was the absorbance just before assay and $A2_{560}$ was the absorbance of the reaction mixture.

Cholesterol Removal by Different Lactobacilli and by *Lactobacillus*-Fermented Curd

Bacteria grown overnight in MRS broth were washed with PBS (pH 7.0) following which 1×10^8 cells were suspended in 1 mL of 0.3% ox-bile MRS broth (Himedia, Mumbai, India) containing cholesterol (150 mg/dL). Cells were allowed to grow for 24 h at 37 °C in microaerobic environment and then pelleted down and the supernatant was used for cholesterol estimation by colorimetric assay. Cholesterol reagent was added to 10 μL of supernatant and incubated for 10 min at 37 °C following which absorbance was taken at 505 nm. This assay was done with the help of cholesterol estimation kit (Reckon Diagnostics, Baroda, India). Cholesterol concentration (in mg/dL) and cholesterol reduction (%) were calculated, using the formula [(Absorbance of test)/(Absorbance of standard)] × 200 and [(150 – mean of residual cholesterol conc. in the supernatant)/150] × 100, respectively.

Cholesterol removal from broth was also checked for *Lactobacillus*-fermented curd (1×10^8 cells of the each culture were inoculated in to 10 mL of milk individually and incubated overnight at 37 °C under static condition (microaerobic environment)), for which, the same procedure as described above was used. Furthermore, pH and whey protein concentration of this *Lactobacillus*-fermented curd were also checked.

α-Galactosidase Production

α-Galactosidase activity was assessed as per method described by Donkor et al. [26] with a few modifications. To summarize it, all organisms were used following three successive propagations in sterile MRS broth at 37 °C for 24 h. Subsequently, 1×10^8 cells of the culture were inoculated into 1 mL of sterile MRS broth and incubated at 37 °C for 24 h in microaerobic environment. Following this, the cells were harvested and the cell pellet was washed twice with cold 50 mM sodium phosphate buffer (pH 5.5). Cells were finally resuspended in 1 mL of the same buffer, placed in an ice bath for 10 min followed by sonication for 10 min. The above steps of cooling and sonication were repeated twice to ensure that the bacterial cells were completely lysed. The cell debris was removed by centrifugation and the resultant supernatant was used as a crude enzyme extract. α-Galactosidase assay was carried out according to the method of Scalabrini et al. [27] with some modifications. Briefly, a 150-μL aliquot of enzyme extract was mixed with 300 μL of 5 mM p-nitrophenyl-α-D-galactopyranoside (PNPG) and incubated at 37 °C for 30 min, following which 300 μL of cold 0.2 mol/L sodium carbonate solution was added to stop the reaction. The α-galactosidase activity was determined by the rate of hydrolysis of PNPG. The amount of p-nitrophenol released was measured at 420 nm. A standard calibration curve was prepared using known concentrations of p-nitrophenol (Sigma-Aldrich, Steinheim, Germany). One unit of enzyme activity was defined as the amount of enzyme that released l.0 μM of p-

nitrophenol from PNPG per milliliter per min under the assay conditions. The specific activity was expressed as units (U) of α-galactosidase activity per milligram of protein. The protein concentration of the crude enzyme extracts was determined using the method of Bradford [28].

Statistical Analysis

Values are given as mean values and standard deviations of triplicate independent experiments. Significant ANOVAs were followed by Dunnett's test in all the assays to compare with respect to positive controls (LGG and *L. plantarum* ATCC 8014) ($P < 0.05$). All the analysis was conducted using Graph pad Prism 6.01.

Results

β-Galactosidase Production

Lactobacillus isolates were grown on MRS-X-gal agar plate for determining their ability to produce β-galactosidase. Most of the cultures excepting strains *L. delbrueckii* M, *L. fermentum* ASt-1, *L. rhamnosus* CS25, *L. rhamnosus* SCA, and *L. rhamnosus* SCB gave blue colored colonies, indicating their ability to produce β-galactosidase enzyme (Table 2). In the case of strains, *L. fermentum* GPI-7, *L. fermentum* GKI-1, *L. fermentum* GPI-1(B), *L. fermentum* IIS11.2, *L. fermentum* GPI-3, *L. salivarius* GPI-4, *L. salivarius* GPI-1(S), *L. plantarum* ATCC 8014, *L. casei* CS5.2, and *L. plantarum* CS23, blue-colored colonies appeared within 24 h while for LGG, *L. fermentum* GPI-6, *L. fermentum* FA-5, *L. fermentum* FA-1, *L. plantarum* GRI-2, *L. helveticus* FA-7, and *L. plantarum* CS24.2, colonies turned blue after 48 h of incubation. Furthermore, following enzyme assay, β-galactosidase activity was found significantly higher than both standard strains LGG and *L. plantarum* ATCC 8014, for most of the cultures ($P < 0.05$, Table 2). Excellent levels were found for *L. salivarius* GPI-1(S), *L. fermentum* GPI-6, and *L. fermentum* GPI-3 which were about twofold compared to LGG and *L. plantarum* ATCC 8014.

Cholesterol Removal by Different Lactobacilli

In the present study, lactobacilli were examined for their ability to reduce cholesterol by inoculating lactobacilli directly in MRS broth as well as MRS broth inoculated with starter culture from various lactobacilli fermented curd. The cholesterol reduction by these methods in MRS broth containing oxgall and cholesterol following 24 h for growth of various lactobacilli at 37 °C was determined (Tables 3 and 4 respectively). Uninoculated sterile broth was used as control.

Cholesterol Removal from Broth by Different Lactobacilli

Residual cholesterol concentration was determined in the supernatants from growth media and the results are given in Table 3. Most of the cultures showed good cholesterol removal in supernatant than both standard strains LGG and *L. plantarum* ATCC 8014, excepting *L. rhamnosus* CS25, *L. fermentum* IIS11.2 and *L. fermentum* GKI-1. However, strain *L. rhamnosus* SCB (78.76%) showed significant ($P < 0.05$) and best cholesterol lowering ratio

Table 2 β-Galactosidase activity of different lactobacilli isolates

Cultures	Growth in MRS-X-gal plate (h)	Appearance of blue colony (h)	β-Galactosidase activity (Miller units)[a]	
			Mean	SD
LGG	24 h	48 h	251.49	1.45
ATCC 8014	24 h	24 h	238.80	0.26
GPI-7	24 h	24 h	370.25***	1.22
GPI-4	24 h	24 h	289.21***	1.99
GPI-6	24 h	48 h	421.81***	1.70
GKI-1	24 h	24 h	295.18***	2.93
GPI-1(B)	24 h	24 h	152.01***	1.80
GPI-1(S)	24 h	24 h	438.04***	1.87
GRI-2	24 h	48 h	378.32***	0.39
FA-5	24 h	48 h	305.85***	0.82
GPI-3	24 h	24 h	444.62***	1.29
FA-1	24 h	48 h	268.11***	1.58
FA-7	24 h	48 h	298.54***	1.92
IIS11.2	24 h	24 h	276.53***	3.26
CS24.2	24 h	48 h	201.97***	2.06
ASt-1	24 h	No color after 72 h	Nil	Nil
M	24 h	No color after 72 h	Nil	Nil
CS5.2	24 h	24 h	258.40***	0.75
CS23	24 h	24 h	374.54***	1.80
CS25	24 h	No color after 72 h	Nil	Nil
SCA	24 h	No color after 72 h	Nil	Nil
SCB	24 h	No color after 72 h	Nil	Nil

ATCC American Type Culture Collection *L. plantarum* ATCC 8014; LGG *L. rhamnosus* GG; GPI-1(S), GPI-4: *L. salivarius* strains; GRI-2, CS24.2, CS23: *L. plantarum* strains; FA-7: *L. helveticus*; GPI-1(B), GPI-3, GPI-6, IIS11.2, FA-5, FA-1, GKI-1, GPI-7, ASt-1: *L. fermentum* strains; M: *L. dulbrueckii* M; CS5.2: *L. casei* CS5.2; CS25, SCA, SCB: *L. rhamnosus* strains

[a] Results were obtained from three independent experiments. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons vs. the positive control group

***Mean value of isolates was significantly different from that of both *L. rhamnosus* GG ($P < 0.05$) and *L. plantarum* ATCC 8014 ($P < 0.05$)

among all, while strains *L. plantarum* CS24.2 (50.21%), *L. plantarum* CS23 (45.42%), *L. salivarius* GPI-1(S) (45.35%), and *L. delbrueckii* M (45.43%) were better than LGG (21.13%) and *L. plantarum* ATCC 8014 (30.90%).

Cholesterol Removal by Lactobacillus-Fermented Curd

Residual cholesterol concentration was also determined in the supernatant of growth media inoculated with starter culture from various lactobacilli fermented curd and the results are given in Table 4. It was observed that strain *L. rhamnosus* SCB (76.50%) had excellent cholesterol reducing ability from growth medium as compared to both standard strains LGG (30.54%) and *L. plantarum* ATCC 8014 (40.18%). The strains *L. casei* CS5.2 (56.34%), *L. plantarum* CS23 (49.57%), *L. delbrueckii* M (46.17%), *L. salivarius* GPI-1(S) (44.84%), and *L. fermentum* GPI-6 (45.28%) also showed significant ($P < 0.05$) and better cholesterol reduction ability compared to both the standard probiotic stains. The results showed that more cholesterol reduction was observed in case of *Lactobacillus* fermented curd as compared to use

Table 3 Cholesterol removal using different lactobacilli directly in MRS broth

Cultures	Residual cholesterol conc. in the supernatant (mg/dL)[a]		Cholesterol reduction (%)
	Mean	SD	
Control	150	0.00	–
LGG	118.30	1.56	21.13
ATCC 8014	103.65	1.83	30.90
GPI-7	106.86*	2.09	28.76
GPI-4	103.39*	1.36	31.07
GPI-6	103.32*	1.87	31.12
GKI-1	146.32***	0.82	2.45
GPI-1(B)	131.84***	2.20	12.10
GPI-1(S)	81.97***	1.27	45.35
GRI-2	128.37***	1.47	14.42
FA-5	110.61***	0.75	26.26
GPI-3	89.12***	1.19	40.59
FA-1	103.32*	1.87	31.12
FA-7	117.66**	1.39	21.56
IIS11.2	146.40***	0.75	2.40
CS24.2	74.68***	1.65	50.21
ASt-1	103.32*	1.87	31.12
M	81.85***	2.15	45.43
CS5.2	88.06***	1.24	41.29
CS23	81.87***	2.00	45.42
CS25	142.75***	0.72	4.83
SCA	99.84*	1.13	33.44
SCB	31.86***	2.08	78.76

Control: MRS + oxbile (0.3%) + cholesterol without lactobacilli

[a] Results were obtained from three independent experiments. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons vs. the positive control group

*Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$); **mean value of isolates was significantly different from that of *L. plantarum* ATCC 8014 ($P < 0.05$); ***mean value of isolates was significantly different from that of both *L. rhamnosus* GG ($P < 0.05$) and *L. plantarum* ATCC 8014 ($P < 0.05$)

of *Lactobacillus* cultures directly in MRS broth except for the strains *L. rhamnosus* SCA, *L. plantarum* CS24.2, *L. fermentum* FA-5 and *L. fermentum* GPI-3 where more of cholesterol reduction was seen in case of *Lactobacillus* culture in broth, while the medium inoculated with *L. fermentum* GKI-1 (5.52%) and *L. rhamnosus* CS25 (4.44%) showed no significant decrease in cholesterol content.

pH of Curd Prepared with Different Lactobacilli

Deconjugation of bile salts by BSH takes place at acidifying and pH-controlled conditions. The pH of curd fermented by various lactobacilli was determined. It was observed (Table 4) that the strains producing more acidic curd showed better cholesterol reduction. Strain *L. rhamnosus* SCB (pH 4.38) being most acidic showed highest cholesterol reduction. Similarly, strains *L. plantarum* CS23 (pH 4.91), *L. salivarius* GPI-1(S) (pH 4.92), *L. casei* CS5.2 (pH 4.89), and *L. delbrueckii* M (pH 4.93) also showed acidic pH with significant reduction of cholesterol content ($P < 0.05$, Fig. 1).

Table 4 Cholesterol lowering assay using *Lactobacillus* fermented curd

Cultures	Residual cholesterol conc. in the supernatant (mg/dL)[a]		Cholesterol reduction (%)
	Mean	SD	
Control	150	0.00	–
LGG	104.19	2.49	30.54
ATCC 8014	89.72	7.10	40.18
GPI-7	93.07*	3.45	37.95
GPI-4	86.17*	3.45	42.55
GPI-6	82.08***	1.18	45.28
GKI-1	141.72***	2.71	5.52
GPI-1(B)	106.86**	3.45	28.76
GPI-1(S)	82.74*	2.00	44.84
GRI-2	130.08***	1.98	13.28
FA-5	123.74***	0.70	17.51
GPI-3	93.07*	3.45	37.95
FA-1	88.99*	1.15	40.67
FA-7	103.05**	0.70	31.30
IIS11.2	89.59*	6.90	40.27
CS24.2	110.12**	0.40	26.59
ASt-1	103.46**	0.23	31.03
M	80.74***	2.02	46.17
CS5.2	65.48***	3.45	56.34
CS23	75.65***	0.37	49.57
CS25	143.34***	1.48	4.44
SCA	106.86**	3.45	28.76
SCB	35.25***	0.78	76.50

Control: MRS + 0.3% oxbile + cholesterol without lactobacilli fermented curd

[a] Results were obtained from three independent experiments. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons vs. the positive control group

*Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$); **mean value of isolates was significantly different from that of *L. plantarum* ATCC 8014 ($P < 0.05$); ***mean value of isolates was significantly different from that of both *L. rhamnosus* GG ($P < 0.05$) and *L. plantarum* ATCC 8014 ($P < 0.05$)

Protein Concentration in Whey

Protein levels in whey from curd fermented by various lactobacilli were determined and the results are given in Fig. 2. Whey of strain *L. rhamnosus* SCB fermented curd (0.63 μg/μL) showed the lowest protein concentration as compared to both standard strains LGG (0.86 μg/μL) and *L. plantarum* ATCC 8014 (1.28 μg/μL) fermented curd. However, *L. salivarius* GPI-1(S) (0.74 μg/μL), *L. plantarum* CS23 (0.71 μg/μL), *L. plantarum* CS24.2 (0.78 μg/μL), *L. fermentum* GPI-6 (0.75 μg/μL), *L. fermentum* GPI-7 (0.75 μg/μL), *L. delbrueckii* M (0.73 μg/μL), and *L. casei* CS5.2 (0.71 μg/μL) fermented curd showed significantly ($P < 0.05$) low protein concentration in their whey. It was observed that strains having less protein concentration in the whey are better fermenters and thus form better curd.

Based on the above data, strains were categorized as strong, moderate, and weak fermenting strains: *L. rhamnosus* SCB, *L. plantarum* strains CS24.2, CS23, *L. fermentum* strains GPI-7, GPI-6, *L. dulbrueckii* M, *L. casei* CS5.2, and *L. salivarius* GPI-1(S) were strong fermenting strains. *L. rhamnosus* CS25, *L. fermentum* strains GPI-3, GKI-1,

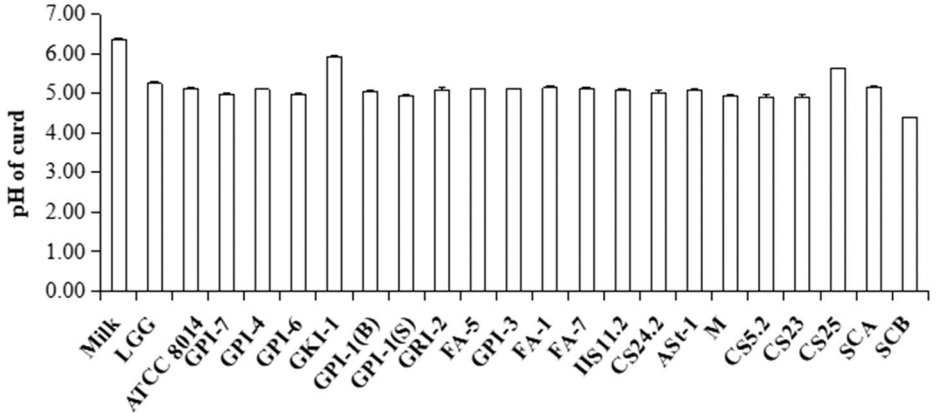

Fig. 1 pH of curd prepared using different lactobacilli. Values are means of three independent experiments, with standard deviations represented by vertical bars. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons vs. the positive control group. *Mean value of isolates was significantly different from that of *L. rhamnosus* GG ($P < 0.05$). ***Mean value of isolates was significantly different from that of both *L. rhamnosus* GG ($P < 0.05$) and *L. plantarum* ATCC 8014 ($P < 0.05$)

IIS11.2, ASt-1, and FA-5 were moderately fermenting strains. The strains *L. fermentum* FA-1, GPI-1(B), *L. rhamnosus* SCA, *L. helveticus* FA-7, *L. plantarum* GRI-2 and *L. salivarius* GPI-4 were weakly fermenting strains. Strains *L. salivarius* GPI-1(S) and *L. plantarum* CS23 were categorized as strong fermenting strains and also showed higher β-galactosidase production. They also performed equally well in cholesterol removal when *Lactobacillus* culture was used directly in broth as well as when *Lactobacillus* fermented curd was used as inoculum.

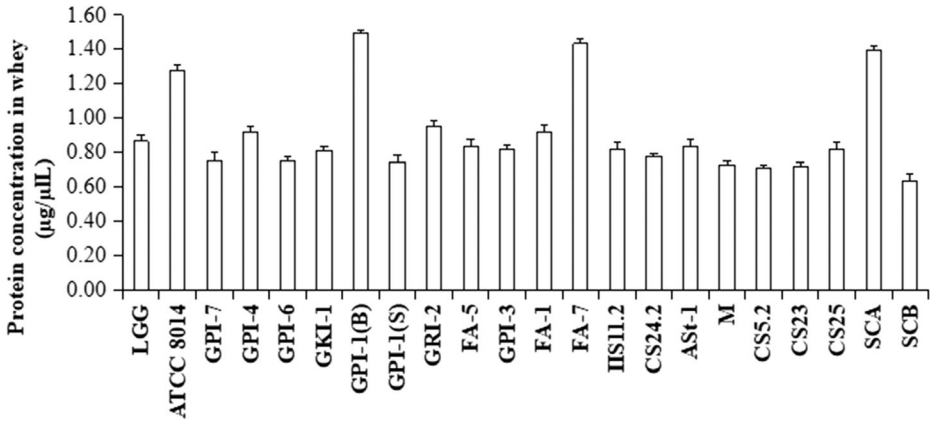

Fig. 2 Protein concentration of whey. Values are means of three independent experiments, with standard deviations represented by vertical bars. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons vs. the positive control group. **Mean value of isolates was significantly different from that of *L. plantarum* ATCC 8014 ($P < 0.05$). ***Mean value of isolates was significantly different from that of both *L. rhamnosus* GG ($P < 0.05$) and *L. plantarum* ATCC 8014 ($P < 0.05$)

α-Galactosidase Production

Isolates were also screened on the basis of their ability to produce α-galactosidase in order to select those with potential for digestion of complex oligosaccharides. The strains exhibited different levels of α-galactosidase activities, which are given in Table 5. Most of the cultures showed better α-galactosidase activity as compared to both standard strains LGG (0.074 U/mg protein) and *L. plantarum* ATCC 8014 (0.157 U/mg protein). *L. salivarius* GPI-1(S) (12.939 U/mg protein) showed significantly ($P < 0.05$) highest level of α-galactosidase activity followed by *L. fermentum* FA-5 (9.627 U/mg protein) and *L. helveticus* FA-7 (8.150 U/mg protein).

Discussion

Lactobacilli are frequently associated with health-promoting effects in human and animal intestines. Lactose intolerance, the impaired ability to digest lactose, has been recognized as a problem in many children and most adults throughout the world [29, 30]. In the present study, different lactobacilli were checked for β-galactosidase, since it is the enzyme that hydrolyses lactose into easily metabolisable glucose and galactose. Inclusion of β-galactosidase

Table 5 α-Galactosidase activity of different lactobacilli

Cultures	Activity (U)		mg protein		Specific activity[a] (U/mg protein)	
	Mean	SD	Mean	SD	Mean	SD
LGG	0.003	0.0002	0.043	0.001	0.074	0.003
ATCC 8014	0.006	0.0002	0.038	0.001	0.157	0.002
GPI-7	0.017	0.0002	0.023	0.001	0.735***	0.033
GPI-4	0.158	0.0003	0.043	0.001	3.664***	0.044
GPI-6	0.038	0.0000	0.040	0.002	0.949***	0.044
GKI-1	0.006	0.0003	0.040	0.001	0.152	0.007
GPI-1(B)	0.130	0.0025	0.028	0.001	4.619***	0.138
GPI-1(S)	0.373	0.0002	0.029	0.000	12.939***	0.006
GRI-2	0.286	0.0002	0.048	0.001	6.011***	0.178
FA-5	0.373	0.0003	0.039	0.001	9.627***	0.131
GPI-3	0.002	0.0002	0.014	0.001	0.172	0.019
FA-1	0.154	0.0002	0.029	0.001	5.262***	0.101
FA-7	0.364	0.0003	0.045	0.000	8.150***	0.007
IIS11.2	0.005	0.0002	0.027	0.001	0.185	0.006
CS24.2	0.003	0.0002	0.037	0.001	0.081	0.005
ASt-1	0.001	0.0002	0.022	0.001	0.068	0.009
M	0.001	0.0002	0.039	0.001	0.038	0.005
CS5.2	0.038	0.0005	0.035	0.002	1.072***	0.068
CS23	0.002	0.0002	0.025	0.001	0.089	0.008
CS25	0.003	0.0003	0.046	0.001	0.061	0.007
SCA	0.002	0.0002	0.033	0.000	0.068	0.005
SCB	0.002	0.0003	0.032	0.001	0.057	0.010

[a] Results were obtained from three independent experiments. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons vs. the positive control group

***Mean value of isolates was significantly different from that of both *L. rhamnosus* GG ($P < 0.05$) and *L. plantarum* ATCC 8014 ($P < 0.05$)

producing lactobacilli as probiotics in milk and cheese and other dairy products could help overcome lactose intolerance symptoms in humans [31]. Our study showed that most of the cultures had higher β-galactosidase activity than both standard strains *L. rhamnosus* GG (LGG) and *L. plantarum* ATCC 8014. The highest levels of this enzyme were nearly twofold in *L. fermentum* strain GPI-3, followed by *L. salivarius* GPI-1(S) and *L. fermentum* strain GPI-6 compared to both standard strains. The values found for the tested lactobacilli were in the range of values previously reported by Meira et al. [32] and Belicová et al. [33].

Several studies have shown a direct relationship between consumption of cultured dairy products and a reduction of serum cholesterol levels in humans and animals [34–36], although the exact mechanism of cholesterol reduction by lactobacilli is unclear. Several mechanisms have been proposed, which include assimilation of cholesterol into bacterial cell membranes [16, 37], co-precipitation of cholesterol with deconjugated bile [38], cholesterol binding to the bacterial cell walls [39], incorporation of cholesterol into the cellular membranes of lactobacilli during growth [40], conversion of cholesterol into coprostanol [41], production of short-chain fatty acids (SCFAs) during the growth of bacteria [42], and enzymatic deconjugation of bile acids by bile-salt hydrolase (BSH) of lactobacilli [11, 43]. Moreover, deconjugated bile salts being less soluble are efficiently reabsorbed from the intestinal lumen than their conjugated counterparts, resulting in excretion of larger amount of free bile acids in feces. Therefore, the deconjugation of bile acids by lactobacilli could lead towards a reduction in serum cholesterol either by increasing the demand of cholesterol for formation of new bile acids to replace those lost in feces or by reducing cholesterol solubility and thereby absorption of cholesterol throughout the intestinal lumen [13, 44]. In addition, Gilliland et al. [45] reported that cholesterol was partially removed from the medium after culturing of *Lactobacillus acidophilus* RP32 in the presence of oxbile as the source of bile salts. Liong and Shah [46] reported that the precipitation of cholesterol in culture fluids appears to be related to deconjugation of bile salts due to BSH activity of lactobacilli and their subsequent precipitation at low pH. In our study, the extent of cholesterol removal was from 2.40 to 78.76% in case of *Lactobacillus* directly used in 0.3% oxbile containing MRS broth and ranged from 4.44 to 76.50% when *Lactobacillus* was used from fermented curd. Among the strains tested, *L. rhamnosus* SCB achieved the highest removal in both types of cholesterol removal studies, using lactobacilli directly in MRS broth and as inoculation from fermented curd, compared to both standard strains. In the present study, our isolated LAB showed excellent cholesterol removal (up to 78.76%) similar to earlier reports by Kuda et al. [47] (up to 61%) and Miremadi et al. [48] (up to 65%). Kumar et al. [17] revealed that the amount of cholesterol that was removed from the growth media was variable, depending on the culture and the pH, during the growth of lactobacilli. pH is an important parameter for the assimilation and reduction of cholesterol. Although some studies have shown that the optimal pH for bile salt deconjugation by lactobacilli is lower than 6.0 [49, 50], others have suggested that the high BSH activity of some *Lactobacillus* species can be partially attributed to the low pH of the medium. In our study, it was also seen that strains which produced more acidic curd showed better cholesterol reduction. *L. rhamnosus* SCB being most acidic (pH 4.38) showed the highest cholesterol removal compared to other strains including standard strains. Isolates *L. plantarum* CS23 (pH 4.91), *L. salivarius* GPI-1(S) (pH 4.92), and *L. casei* CS5.2 (pH 4.89) also showed acidic pH with significant removal of cholesterol in MRS broth.

Protein concentration in whey of curd produced by different lactobacilli was also determined. It was observed that strains having less whey protein concentration had better curd fermenting ability (data not shown). Result showed that curd containing strains *L. rhamnosus*

SCB, *L. plantarum* CS24.2, *L. plantarum* CS23, *L. fermentum* GPI-7, *L. fermentum* GPI-6, *L. delbrueckii* M, *L. casei* CS5.2, and *L. salivarius* GPI-1(S) showed less protein concentration in their whey. Hence, we conclude that these isolates were good fermenting strains.

α-Galactosidase hydrolyses α-D-galactosidic bonds present in oligosaccharides like raffinose and stachyose. It is not synthesized by humans and thus the presence of these oligosaccharides could hinder digestion and cause flatulence, since these sugars are then utilized by the gas generating intestinal microorganisms. These enzymes can be used to digest these oligosaccharides and upgrade the nutrition of legume food [51, 52]. In the past, α-galactosidase was considered as an effective food additive to remove these anti-nutrient oligosaccharides, which occurred in soybean meal containing diets [19, 53]. Hence, in the present study, α-galactosidase activity of these isolates was also checked and the specific activity for each was calculated. It is seen that most of the cultures showed better α-galactosidase activity as compared to both the standard strains LGG (0.074 U/mg protein) and *L. plantarum* ATCC 8014 (0.157 U/mg protein). *L. salivarius* GPI-1(S) (12.939 U/mg protein) showed the highest value of α-galactosidase activity compared to other isolates including both standard strains followed by *L. fermentum* FA-5 (9.627 U/mg protein) and *L. helveticus* FA-7 (8.150 U/mg protein). Some of the isolates showed better α-galactosidase activity than that reported by Liu et al. [20] in case of *L. rhamnosus* and *L. casei*.

Conclusions

This study has therefore been able to select several lactobacilli with better health promoting attributes than standard probiotic strains LGG and *L. plantarum* ATCC 8014 in terms of production of β-galactosidase and α-galactosidase, in addition to ability to reduce cholesterol levels.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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