CHAPTER 3

STUDY PRODUCTION OF β-GALACTOSIDASE AND ~-GALACTOSIDASE AND CHOLESTEROL REMOVAL BY *LACTOBACILLUS* STRAINS

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3.1. Introduction

Lactic acid bacteria (LAB) possess various health-promoting properties useful for both humans and animals (Kaushik *et al*. 2009). β-galactosidase deficiency causes lactose intolerance (Vasiljevic and Jelen 2001) and amelioration of this situation by β-galactosidase from LAB (Corral *et al*. 2006) is a result of converting lactose into easily metabolisable glucose and galactose. The symptoms of lactose intolerance decrease the quality of life and daily activities (LeBlanc *et al*. 2008). The addition of lactobacilli producing β-galactosidase as probiotic in dairy products, can thus be used for improvement of lactose digestion.

Earlier studies on human and animal also showed that consumption of LAB reduces serum cholesterol levels (Pereira and Gibson 2002; Pereira *et al*. 2003). 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 (Gilliland *et al*. 1985; Gilliland and Walker 1990). Bile salt hydrolase plays a significant role in cholesterol removal by deconjugating the bile salts (Klaver and van der Meer 1993). Deconjugated bile salts are less soluble and less efficiently reabsorbed from

the intestinal lumen than their conjugated counterparts (de Rodas *et al*. 1996). Additionally, free bile salts are less efficient in the solubilisation and absorption of lipids in the gut (Reynier *et al*. 1981). Pereira *et al*. (2003) stated that 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. The growing cells of *Lactobacillus helveticus* assimilated cholesterol and showed high intracellular cholesterol oxidase like activity, which has not been previously reported (Ahire *et al*. 2012). Study demonstrated the assimilation and intracellular degradation as a cholesterol-lowering mechanism in lactobacilli. Furthermore, it was also demonstrated by Brashears *et al*. (1998) 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 are restricted 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 (Naumoff 2004). 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 (Kidd *et al*. 2001; LeBlanc *et al*. 2008). Earlier reports revealed that *Lactobacillus rhamnosus* GG (LGG) has cholesterol removing ability and *Lactobacillus plantarum* ATCC 8014 have both α-galactosidases and β-galactosidase activities (Silvestroni *et al.* 2002; Kumar *et al.* 2013).

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, the isolated 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.

3.2. Materials and methods

3.2.1. Bacterial strains and culture conditions

Lactobacilli used in this study were adult human gut isolates – *L. fermentum* GPI-7, *L. fermentum* GPI-6, *L. fermentum* GKI-1, *L. fermentum* GPI-1(B), *L. fermentum* GPI-3, *L. fermentum* ASt-1, *L. salivarius* GPI-1(S), *L. salivarius* GPI-4, *L. plantarum* GRI-2, child gut isolates – *L. fermentum* IIS11.2, *L. casei* CS5.2, *L. plantarum* CS23, *L. plantarum* CS24.2, *L. rhamnosus* CS25, *L. rhamnosus* SCA, *L. rhamnosus* SCB, fermented food (of North East India) isolates – *L. fermentum* FA-5, *L. fermentum* FA-1, *L. helveticus* FA-7 and curd isolate *L. delbrueckii* M. Prior to being used they were serially propagated three times in the appropriate medium. Lactobacilli were cultivated in de Man, Rogosa and Sharpe (MRS) broth (MRS; Himedia, Mumbai, India). A 1.0% inoculum was used and incubated at 37 ºC 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.

3.2.2. -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 ºC 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 (1972) with slight modifications. Overnight grown cultureswere harvested by centrifugation, washed twice in phosphate buffered saline (PBS) pH 7.0 and inoculated 1% (v/v) in MRS-lac broth. Cultures were incubated at 37 ºC for 24 h. 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 ºC 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) 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.

3.2.3. 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 containing cholesterol (150 mg/dL). Cells were allowed to grow for 24 h at 37 ºC 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 ºC following which absorbance was taken at 505 nm. This assay was done with the help of cholesterol estimation kit (Reckon Diagnostics, Vadodara, India) and cholesterol concentration was calculated (in mg/dL) as follows:

[(Absorbance of test) / (Absorbance of standard)] x 200

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

Cholesterol removal from broth was also checked for the cell-free supernatant (CFS was sterilized by using a 0.45μm filter) produced from different lactobacilli (50% CFS was diluted with fresh MRS broth containing 0.3% ox-bile and cholesterol (150 mg/dL)), for which, the same procedure as described above was used.

3.2.4. α-Galactosidase production

α-Galactosidase activity was assessed as per method described by Donkor *et al*. (2007) with a few modifications. To summaries it, all organisms were used following three successive propagations in sterile MRS broth at 37 ºC for 24 h. Subsequently, 1×10^8 cells of the culture were inoculated into 1 mL of sterile MRS broth and incubated at 37 ºC for 24 h. 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 then the vials were 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*. (1998) 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 ºC for 30 min, following which 300 μL of cold 0.2 M per litter 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 (1976).

3.2.5. 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 analysis was conducted using GraphPad Prism 6.01.

3.3. Results

3.3.1. -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* strains CS25, SCA and SCB gave blue coloured colonies, indicating their ability to produce β-galactosidase enzyme (Table 3.1). In the case of strains, *L. fermentum* GPI-7, GKI-1, GPI-1(B), IIS11.2, GPI-3, *L. salivarius* GPI-4, GPI-1(S), *L. casei* CS5.2, *L. plantarum* ATCC 8014 and CS23, blue coloured colonies appeared within 24 h while for *L. rhamnosus* LGG, *L. fermentum* GPI-6, FA-5, FA-1, *L. plantarum* GRI-2, CS24.2 and *L. helveticus* FA-7 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 3.1). Excellent levels were found for *L. salivarius* GPI-1(S), *L. fermentum* GPI-6 and GPI-3 which were about 2 folds compared to standard strains LGG and *L. Plantarum* ATCC 8014.

Table 3.1. 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)***	
			Mean	**SD**
LGG	24 h	48h	251.49	1.45
ATCC 8014	24h	24h	238.80	0.26
GPI-7	24h	24h	370.25†‡	1.22
GPI-4	24h	24h	289.21†‡	1.99
GPI-6	24h	48h	421.81†‡	1.70
GKI-1	24h	24h	295.18†‡	2.93
GPI-1(B)	24h	24h	152.01†‡	1.80
GPI-1(S)	24h	24h	438.04†‡	1.87
GRI-2	24h	48h	378.32†‡	0.39
FA-5	24h	48h	305.85†‡	0.82
GPI-3	24h	24h	444.62†‡	1.29
FA-1	24h	48h	268.11†‡	1.58
FA-7	24h	48h	298.54†‡	1.92
IIS11.2	24h	24h	276.53†‡	3.26
CS24.2	24h	48h	201.97†‡	2.06
ASt-1	24h	No colour after 72 h	ND	ND
M	24h	No colour after 72 h	ND	ND
CS5.2	24h	24h	258.40†‡	0.75
CS23	24h	24h	374.54†‡	1.80
CS25	24h	No colour after 72 h	ND	ND
SCA	24h	No colour after 72 h	ND	ND
SCB	24h	No colour after 72 h	ND	ND

*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. ND = Not determined. †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 ($P < 0.05$).

3.3.2. Cholesterol removal by different lactobacilli

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 and by inoculating CFS from various lactobacilli. The cholesterol reduction by these methods in MRS broth containing oxgall and cholesterol following 24 h of anaerobic growth of various lactobacilli at 37 ºC was determined. Uninoculated sterile broth was used as control.

3.3.2.1. 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 3.2. 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 GKI-1. However, strain *L. rhamnosus* SCB (78.76%) showed significant ($P < 0.05$) and best cholesterol lowering ratio amongst all, while strains *L. plantarum* CS24.2 (50.21%), 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%).

It was also observed that the extent of cholesterol removal was from 2.76% to 42.53% when CFS produced from lactobacilli was used as inoculum in MRS broth. CFS of *L. rhamnosus* SCB (42.53%) demonstrated best cholesterol lowering ratio amongst all (data not shown).

Table 3.2. Cholesterol removal using different lactobacilli directly in MRS broth.

Cultures	Residual cholesterol conc. in the supernatant (mg/dL)*		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. *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. †Cholesterol reduction (%) = [(150 – mean of residual cholesterol conc. in the supernatant)/150] x 100. ‡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 ($P < 0.05$).

3.3.2.2. 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 3.3. 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 of *Lactobacillus* cultures directly in MRS broth. However this result was changed when the strains *L. rhamnosus* SCA, *L. plantarum* CS24.2, *L. fermentum* FA-5 and GPI-3 were used, where more of cholesterol reduction was observed in case of *Lactobacillus* culture in broth. The medium inoculated with *L. fermentum* GKI-1 (5.52%) and *L. rhamnosus* CS25 (4.44%) showed no significant decrease in cholesterol content.

Table 3.3. Cholesterol lowering assay using *Lactobacillus* fermented curd.

Cultures	Residual cholesterol conc. in the supernatant (mg/dL)*		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. *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. †Cholesterol reduction (%) = [(150 – mean of residual cholesterol conc. in the supernatant)/150] x 100. ‡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 ($P < 0.05$).

3.3.2.3. pH of curd prepared with different lactobacilli

Deconjugation of bile salts by BSH takes place at acidifying and pH controlled conditions and the pH of curd fermented by various lactobacilli was determined. It was observed (Table 3.3) 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. 3.1).

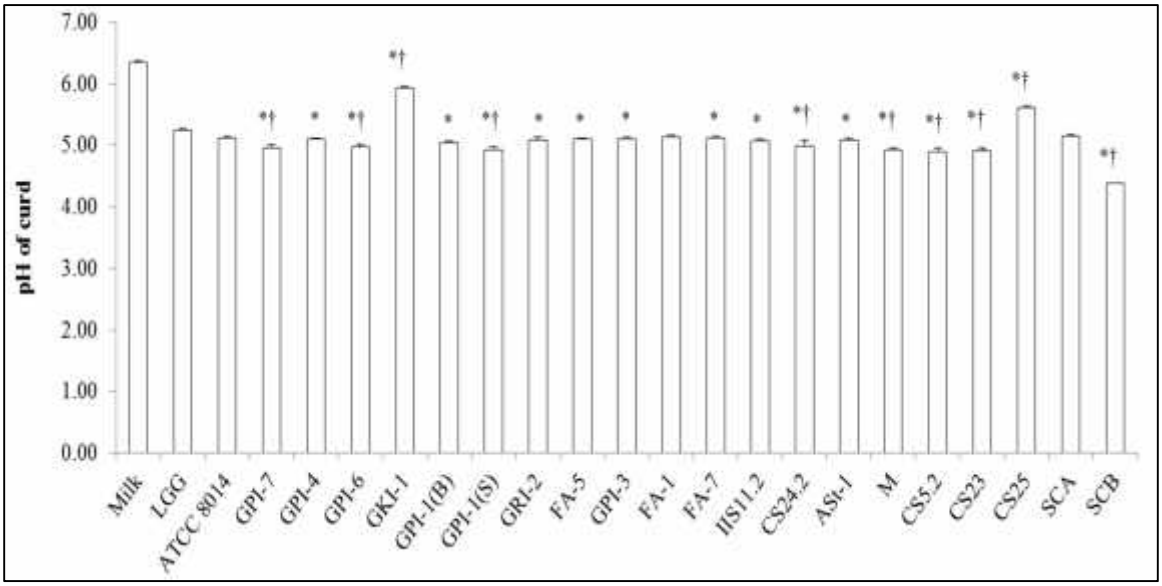

Figure 3.1. pH of curd prepared using different lactobacilli. Values are means of three independent experiments, with standard deviations represented by vertical bars. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent 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 *L. plantarum* ATCC 8014 ($P < 0.05$).

3.3.2.4. Protein concentration in whey

Protein levels in whey from curd fermented by various lactobacilli were determined and the results are given in Fig. 3.2. Whey of strain *L. rhamnosus* SCB fermented curd (0.63 μg/μL) showed 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), CS24.2 (0.78 μg/μL), *L. fermentum* GPI-6 (0.75 μg/μL), 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 more fermentable and thus form better curd (data not shown).

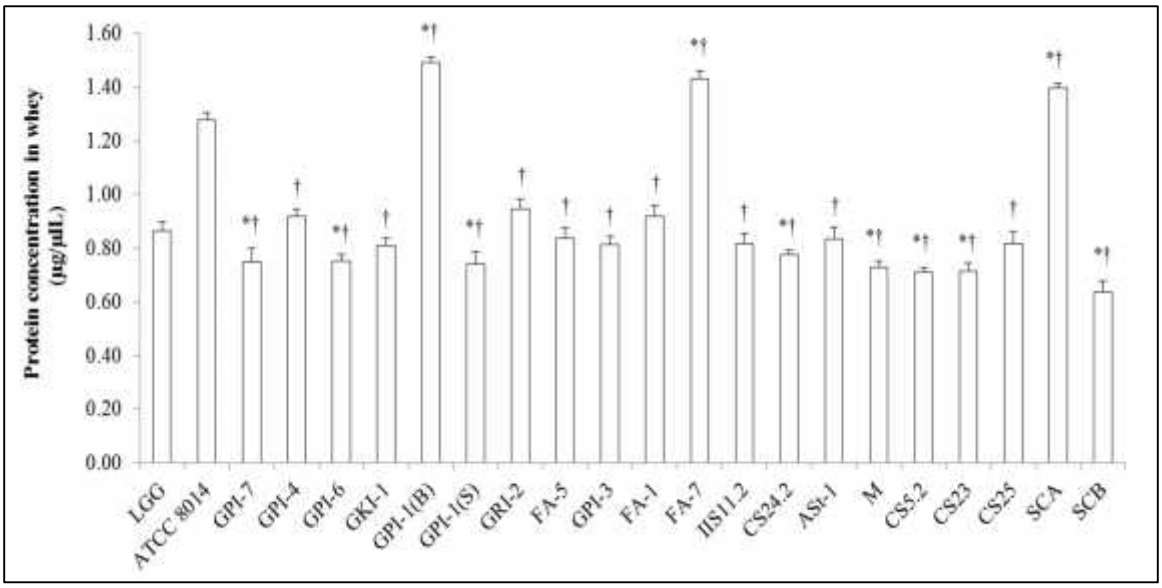

Figure 3.2. Protein concentration of whey. Values are means of three independent experiments, with standard deviations represented by vertical bars. The strains were compared with two different positive controls (*L. rhamnosus* GG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. 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. rhamnosus* GG ($P < 0.05$). †Mean value of isolates was significantly different from that of *L. plantarum* ATCC 8014 ($P < 0.05$).

Based on the above data, strains were categorized as strong, moderate and weak fermentable strains: *L. rhamnosus* SCB, *L. plantarum* CS24.2 and CS23, *L. fermentum* GPI-7 and GPI-6, *L. dulbrueckii* M, *L. casei* CS5.2 and *L. salivarius* GPI-1(S) were categorized in strong fermentable strains. *L. rhamnosus* CS25, *L. fermentum* strains GPI-3, GKI-1, IIS11.2, ASt-1 and FA-5 were moderately fermentable strains. Whereas strains *L. fermentum* FA-1 and GPI-1(B), *L. rhamnosus* SCA, *L. helveticus* FA-7, *L. plantarum* GRI-2 and *L. salivarius* GPI-4 were weakly fermentable strains. Strains *L. salivarius* GPI-

1(S) and *L. plantarum* CS23 were categorized as strong fermentable strains and 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.

3.3.3. ~-Galactosidase production

Isolates were also screened on the basis of their ability to produce α-galactosidase in order to select for those with potential for digestion of complex oligosaccharides. The strains exhibited different levels of α-galactosidase activities, which are given in Table 3.4. 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).

Table 3.4. α-Galactosidase activity of different lactobacilli.

Cultures	Activity Units		mg protein		Specific activity* (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

*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 ($P < 0.05$).

3.4. Discussion

Lactobacilli are frequently associated with health-promoting effects in human and animal intestines. The effect of sugar like lactose, on the small intestinal transit time is not well documented (He *et al*. 2006) but this plays a major role in conditions like lactose

intolerance. Lactose intolerance; the impaired ability to digest lactose has been recognized as a problem in many children and most adults throughout the world (Heyman 2006). 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 producing lactobacilli as probiotics in milk, cheese and other dairy products could help to overcome lactose intolerance symptoms in humans (de Vrese *et al*. 2001). This 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 was nearly 2 folds 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*. (2012) and Belicová *et al*. (2013).

Several studies have shown a direct relationship between consumption of cultured dairy products and a reduction of serum cholesterol levels in humans and animals (Grunewald 1982; Agerbaek *et al*. 1995). 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 (Tomaro-Duchesneau *et al*. 2014; Aquino *et al*. 2017), co-precipitation of cholesterol with deconjugated bile (Liong and Shah 2006), cholesterol binding to the bacterial cell walls (Liong and Shah 2005), incorporation of cholesterol into the cellular membranes of lactobacilli during growth (Lye *et al*. 2010a), conversion of cholesterol into coprostanol (Lye *et al*. 2010b), production of short-chain fatty acids (SCFAs) during the growth of bacteria (de Preter *et al*. 2007) and enzymatic

deconjugation of bile acids by bile-salt hydrolase (BSH) of lactobacilli (Lambert *et al*. 2008; Mahmoudi *et al*. 2017). 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 faeces. 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 faeces or by reducing cholesterol solubility and thereby absorption of cholesterol throughout the intestinal lumen (Pereira *et al*. 2003; Costabile *et al*. 2017), transformation of cholesterol to 4-cholesten-3-one by cholesterol oxidase. Cholesterol oxidase (3-β hydroxysterol oxidase, EC 1.1.3.6) are secreted bacterial enzymes that catalyze the first step in the degradation of cholesterol. Cholesterol oxidase is traditionally used as a key enzyme for detecting and quantifying cholesterol present in serum and food (Ahire *et al*. 2012; Kumari and Shamsher 2015). Ahire *et al*. (2012) reported that the cholesterol reduction of *L. helveticus* cultured was attributed to the production of cholesterol oxidase. In addition, Gilliland *et al*. (1985) 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. Klaver and van der Meer (1993), reported that 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 this study, the extent of cholesterol removal was from 2.40% to 78.76% when *Lactobacillus* was directly used in 0.3% oxbile containing MRS broth, ranged from 4.44% to 76.50% when *Lactobacillus* was used from fermented curd and was ranged from 2.76% to 42.53% (data not shown) when CFS produced from lactobacilli was used in MRS broth.

Among the strains tested, SCB achieved the highest removal in all three types of cholesterol removal studies, using lactobacilli directly in MRS broth and as inoculation from fermented curd, and also as inoculation from CFS produced from lactobacilli, compared to both standard strains. In the present study isolated LAB showed excellent cholesterol removal (up to 78.76%) as similar to earlier reports by Kuda *et al*. (2013) (up to 61%) and Miremadi *et al*. (2014) (up to 65%). Brashears *et al*. (1998) showed that the amount of cholesterol that was removed from the growth media was variable, depending on the culture and the pH, during growth of lactobacilli. pH is an one of the 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 (Klaver and van der Meer 1993; Brashears *et al*. 1998), others have suggested that the high BSH activity of some *Lactobacillus* species can be partially attributed to the low pH of the medium. In this study also it was observed that the strains which produced more acidic curd showed better cholesterol reduction. *L. rhamnosus* SCB being most acidic (pH 4.38) showed the highest cholesterol removal from 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 and 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 and CS23, *L. fermentum* GPI-7 and 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 fermentable 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 (Garro *et al*. 2004).

In the past, α-galactosidase has been considered as an effective food additive to remove these anti-nutrient oligosaccharides, which occurred in soybean meal containing diets (Kidd *et al*. 2001). Hence in the present study, α-galactosidase activity of these isolates was also checked and the specific activity for each was calculated. It was observed 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 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*. (2014) in case of *L. rhamnosus* and *L. casei*. This study has therefore been able to select several lactobacilli that have been demonstrated to behave better 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.