

Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillus plantarum* strains in Sprague–Dawley rats

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Abstract

The purpose of the present study was to evaluate the anti-hypercholesterolaemic effects of two putative probiotic bile salt hydrolase (Bsh)-producing *Lactobacillus plantarum* strains, i.e. Lp91 and Lp21, in rats. *L. plantarum* Lp91 exhibited excellent tolerance to low pH and high bile salt concentrations as well as showed potential Bsh activity, cholesterol assimilation and cholesterol co-precipitation ability along with *L. plantarum* Lp21 and NCDO82 strains. Furthermore, the potential effect of *L. plantarum* Lp91 on plasma cholesterol level was evaluated in Sprague–Dawley rats. Five treatment groups of rats (n 6) were fed experimental diets: normal diet, hypercholesterolaemic diet (HD), HD plus *L. plantarum* Lp91 (HD91) at $\geq 1.0 \times 10^8$ colony-forming units (cfu)/g, HD plus microencapsulated *L. plantarum* Lp91 (HDCap91) at $\geq 1.0 \times 10^8$ cfu/g and HD plus *L. plantarum* Lp21 (HD21) at $\geq 1.0 \times 10^8$ cfu/g for 3 weeks. Feed intake and feed efficiency differed significantly among the five groups. After 21 d of dietary treatment, comparative analysis revealed 23.26, 15.71 and 15.01% reduction in total cholesterol, 21.09, 18.77 and 18.17% reduction in TAG, 38.13, 23.22 and 21.42% reduction in LDL-cholesterol, and the corresponding HDL-cholesterol values increased at the rate of 18.94, 10.30 and 7.78% in treated groups HD91, HDCap91 and HD21, respectively. Faecal excretion of cholic acid and faecal lactobacilli counts were significantly higher in the probiotic treatment groups than in the control groups. In conclusion, these results suggest that the indigenous *L. plantarum* Lp91 strain has the potential to be explored as a probiotic in the management of hypercholesterolaemia.

Key words: *Lactobacillus plantarum*; Microencapsulation; Bile salt hydrolase; Plasma lipids

An elevated serum cholesterol level is considered to be one of the major risk factors associated with atherosclerosis and CHD^(1,2) – the major cause of morbidity and mortality around the world^(3,4). The WHO predicts that by the year 2020, up to 40% of all deaths will be related to CVD. Although drug-based therapy (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors or drugs) is currently being used to treat this condition⁽⁵⁾, it is often suboptimal, expensive and suffers from unwanted side effects^(6–8). Another natural cost-effective and safe alternative approach recently being explored to manage cholesterol-related problems is based on probiotic intervention. Probiotics particularly belonging to the genera *Lactobacillus* and *Bifidobacterium* as biotherapeutics have been investigated for reducing the level of cholesterol in the serum by virtue of bile salt hydrolase (Bsh) activity through direct impact on host bile salt metabolism^(9–12). Bsh-producing

lactobacilli have the selective advantage of surviving and colonising the lower small intestine where the enterohepatic cycle takes place and, therefore, Bsh activity could be considered as one of the important colonisation factors⁽¹³⁾. Bile salts are secreted as bile into the duodenum in the form of N-acyl compounds conjugated with glycine or taurine⁽¹⁴⁾, and enhance the emulsification of lipids and aid in the absorption of lipid nutrients⁽¹⁵⁾ apart from undergoing enterohepatic circulation⁽¹⁶⁾. During this process, deconjugation of bile salts by Bsh results in the liberation of the amino acid residue and the formation of deconjugated bile acids. Deconjugated bile acids are excreted more rapidly from the intestinal tract than the conjugated ones. Since free bile acids are excreted from the body, the synthesis of new bile salts from cholesterol can reduce the total cholesterol concentration in the body⁽¹⁷⁾. Furthermore, deconjugated bile salts are known to co-precipitate

Abbreviations: AI, atherogenic index; Bsh, bile salt hydrolase; cfu, colony-forming unit; HD, hypercholesterolaemic diet; HD21, HD with *Lactobacillus plantarum* Lp21; HD91, HD plus *L. plantarum* Lp91; HDCap91, HD with microencapsulated *L. plantarum* Lp91; HDctrl, high-cholesterolaemic diet control; MRS, de Man, Rogosa and Sharpe; NDctrl, normal diet control; SD, Sprague–Dawley.

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cholesterol at pH values lower than 5.5⁽¹⁸⁾ and bind to bacterial cells and dietary fibre, which enhance their faecal excretion⁽¹⁹⁾. Free bile salts are less effective detergents than conjugated bile salts and less efficient in forming mixed micelles⁽²⁰⁾. Therefore, the deconjugation of bile salts can decrease the solubility of cholesterol^(9,21). In recent years, the possibility of using bile salt deconjugation by intestinal micro-organisms to lower serum cholesterol levels *in vivo* has received considerable attention^(10,22,23). However, in order to bring these beneficial effects, the probiotic lactobacilli have to overcome the acidity of the gastric juices, numerous digestive enzymes, bile acid, intestinal peristaltic movement, immune response and low surface tension⁽²⁴⁾. Therefore, to be effective as probiotics, lactic acid bacteria must survive the low pH of the stomach and arrive at the intestines as live microbes⁽²⁵⁾. Although the cholesterol-lowering ability of probiotics and probiotic-based dairy foods has been investigated by different groups across the world^(11,26–30), there is very little information available on direct or indirect involvement of Bsh activity associated with such cultures in this process^(10,22,23,31). Moreover, whatever information that is available on this subject is mainly limited to probiotic strains of Western origin, which may not be as effective in expressing their bioactive functions in Indian subjects as in their own native population due to different gut ecology and food habits. The cholesterol-lowering functions of indigenous probiotic strains of lactobacilli have not been worked out as yet. Hence, the present study was primarily focused to tap the functional properties of putative probiotic *Lactobacillus plantarum* strains isolated from an Indian gut in a rat model with the long-term objective of using them as biotherapeutics for the management of high cholesterol in the Indian population.

Experimental methods

Bacterial strains

L. plantarum Lp91 and Lp21, the subject of the present study, were laboratory isolates recovered from an Indian gut whose identity and probiotic attributes were established previously in our laboratory as per FAO/WHO guidelines⁽³²⁾. *L. plantarum* NCDO82 (also designated as CSCC5276 or VTTE-71 034) used as a reference culture in the present study was procured through the courtesy of Dr N. P. Shah from Victoria University, Australia. Stock cultures were maintained in 15% glycerol at -70°C . Before their use, *Lactobacillus* cultures were subcultured three times in de Man, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.2% sodium thioglycolate (Sigma Chemical Company, St Louis, MO, USA) and incubated anaerobically (10% H_2 , 10% CO_2 and 80% N_2) in a MK3 Anaerobic workstation (Don Whitley Scientific Limited, Shipley, West Yorkshire, UK) at 37°C for 18–22 h.

In vitro tolerance to pH 2.5 and 2 % bile

Initially, the cell suspension of each of the test cultures, namely Lp91, Lp21 and NCDO82, was prepared by pelleting the overnight grown cultures (37°C) followed by washing and resuspension in peptone saline at the rate of 10^9 colony-forming units (cfu)/ml. MRS medium preadjusted to pH 2.5 and 6.8 was inoculated individually with approximately 10^8 cfu/ml of Lp91 and Lp21 as well as NCDO82 followed by incubation at 37°C for 3 h. Viability was determined using the plate count method. An aliquot of 1 ml sample was drawn from each tube at 0, 1, 2 and 3 h, pelleted and washed, and tenfold serial dilutions were made using peptone saline diluent and plated on MRS-Thio agar followed by anaerobic incubation of plates at 37°C for 24 h.

Bile salt mix (Hi-Media, Mumbai, India) was added at 2% to MRS-Thio broth for the determination of bile tolerance of *Lactobacillus* strains. Overnight grown cultures were pelleted (4300 g for 10 min at 4°C), washed, resuspended in peptone saline and inoculated (approximately 10^8 cfu/ml) into MRS broth with bile salt (2%) and without bile salt, and their viability was determined using the plate count method. The cultures were incubated in the medium at 37°C for 0, 1, 2 and 3 h. The samples were collected, pelleted and washed, and cfu of the samples were determined by serially diluting in peptone saline water followed by plating onto the MRS agar medium and incubating at 37°C under anaerobic conditions for 24 h.

Bile salt hydrolase activity

Direct plate assay. Initially, the Bsh activity of the test cultures was assessed by the qualitative direct plate assay. Bacterial cells were streaked on MRS-Thio agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (filter sterilised) and 0.37 g of CaCl_2 /litre. The plates were incubated anaerobically at 37°C for 72 h, while MRS agar plates without supplementation of taurodeoxycholic acid sodium salt were used as controls. The presence of precipitated bile acid around colonies (opaque halo) or the formation of opaque granular white colonies with a silvery shine was considered as a positive reaction^(33,34).

Resting cell bile salt hydrolase assay. The Bsh assay was performed by measuring the release of amino acids from hydrolysis of conjugated bile salts by *Lactobacillus* strains used in the study as described by Liong & Shah⁽³⁵⁾, Tanaka *et al.*⁽³⁶⁾ and Grill *et al.*⁽³⁷⁾ with some modifications. Briefly, the test cultures were inoculated at 1% into freshly prepared MRS-Thio broth and incubated at 37°C for 16–18 h. Exponentially grown cells were harvested (12 000 g for 30 min at 4°C), washed twice with peptone saline and resuspended in 0.1 M sodium acetate buffer (pH 5.0) to give an optical density of 5.0 at 600 nm. The bacterial suspensions were incubated at 37°C with 10 mM (final concentration) sodium

glycocholate. The reaction mixture (200 μ l) contained 100 μ l cell suspension and 100 μ l of 20 mM-conjugated bile salt (sodium salt of glycocholic acid). Reaction mixes were incubated at 37°C for 30 min. The enzymatic reaction was terminated by the addition of an equal volume (200 μ l) of 15% TCA (w/v) followed by centrifugation at 18 000 **g** for 15 min. The amount of amino acids present in the supernatant was measured as described by Liong & Shah⁽³⁵⁾. An aliquot of 0.2 ml of supernatant obtained after centrifugation was added to 1 ml of distilled water and 1 ml of ninhydrin reagent (0.5 ml of 1% ninhydrin in 0.5 M-citrate buffer, pH 5.5, 1.2 ml of 30% glycerol and 0.2 ml of 0.5 M-citrate buffer, pH 5.5). The preparation was vortexed vigorously and kept in a boiling water-bath for 14 min. After subsequent cooling, absorbance was determined at 570 nm using glycine or taurine as standards. Bsh activities were quantified from the standard curve prepared for glycine or taurine. One unit of Bsh activity (U/ml) was defined as the amount of enzyme that liberated 1 nmol of amino acid from substrate per min per OD₆₀₀.

Bile salt deconjugation. MRS-Thio broth supplemented with 6 mM-sodium glycocholate was inoculated with overnight grown culture at 1% and incubated anaerobically at 37°C for 20 h. Bile salt deconjugation ability was measured as per the protocol given by Liong & Shah⁽³⁵⁾ based on the release of free cholic acid. After incubation, 10 ml culture of each organism was adjusted to pH 7.0 with NaOH (1 M) and centrifuged at 10 000 **g** at 4°C for 10 min. The pH of the supernatant thus obtained was adjusted to 1.0 with HCl (10 M). The assay reaction was set up by adding 1 ml of the supernatant into 2 ml of ethyl acetate followed by vortexing for 1 min. Then 2 ml of the ethyl acetate layer was transferred into a glass tube and evaporated under N₂ at 60°C. The residue was immediately dissolved in 1 ml of NaOH (0.01 M). After complete mixing, 1 ml of furfuraldehyde (1%) and 1 ml of H₂SO₄ (8 M) were added, and the mixture was vortexed for 1 min before heating at 65°C in a water-bath for 10 min. After cooling, 2 ml of glacial acetic acid was added, and the mixture was vortexed for 1 min. Absorbance was read at 660 nm. The amount of cholic acid released was determined from the cholic acid standard curve (Sigma Chemical Company).

Cholesterol assimilation

Water-soluble cholesterol (polyoxyethanyl-cholesteryl sebacate, Sigma) was dissolved in 50% ethanol (5 mg/ml), filter sterilised and added to MRS-Thio broth supplemented with 0.3% ox-bile at a final concentration of 80–100 μ g/ml. Medium was inoculated with each test culture at the 1% level and incubated anaerobically at 37°C for 20 h. After the incubation period, cells were centrifuged (10 000 **g** at 4°C for 10 min), and the remaining cholesterol concentration in the broth was determined using a colorimetric method as described by Liong & Shah⁽³⁵⁾. Then 1 ml of the aliquot was added with 1 ml of KOH (33%, w/v) and

2 ml of absolute ethanol, vortexed for 1 min and left at 37°C for 15 min. After cooling, 2 ml of distilled water and 3 ml of hexane were added followed by vortexing for 1 min. Then 1 ml of the hexane layer was transferred into a glass tube and evaporated under N₂. The residue was immediately dissolved in 2 ml of *o*-phthalaldehyde reagent (Sigma). After complete mixing, 0.5 ml of concentrated H₂SO₄ was added, and the mixture was again vortexed for 1 min. Finally, the absorbance was read at 550 nm after 10 min. The amount of cholesterol removed from broth was determined by subtracting the amount in each broth sample (μ g/ml) from the amount present in the uninoculated control.

Co-precipitation of cholesterol with deconjugated bile

Freshly prepared sterile MRS-Thio broth was supplemented with 6 mM-sodium glycocholate. Filter-sterilised water-soluble cholesterol was added to the broth at a final concentration of 80–100 μ g/ml. Co-precipitation of cholesterol with cholic acid formed was determined by the difference between cholesterol level in the control (inoculated MRS broth without bile) after the incubation period and the final cholesterol level in the inoculated MRS broth supplemented with sodium glycocholate.

Microencapsulation of probiotic lactobacilli

The probiotic bacterial cells were microencapsulated in sodium alginate matrix as described by Sheu & Marshall⁽³⁸⁾. A 4% sodium alginate solution was prepared, sterilised by autoclaving (120°C for 15 min) and cooled to 38–40°C. Then 20 ml of sodium alginate solution and 4 ml of cell suspension (approximately 10¹¹ cfu/ml) were transferred into a centrifuge tube (40 ml), and the contents were mixed homogeneously. The alginate cell mixture was added drop wise to 100 ml of soyabean oil containing 0.2% Tween 80 (emulsifier) with continuous magnetic stirring. Within 5 min, a uniformly turbid emulsion was obtained into which 0.1 M-CaCl₂ (100 ml) was added quickly to break the emulsion and for the hardening of alginate microcapsules. The capsules were then harvested by gentle centrifugation at 1000 rpm for 10 min at 4°C and washed with 0.1 M-CaCl₂. The beads were separated by decantation and stored in a refrigerator (7 \pm 1°C).

Experimental animals

A total of thirty adult male Sprague–Dawley (SD) rats (mean body weight 150 (SEM 10) g) were procured from the National Institute of Pharmaceutical Education and Research, Mohali, India, and were kept at the small animal house maintained at the Institute. The animals were divided into six homogeneous groups comprising of six animals each, housed in individual AI cages and maintained under a constant 12 h light–12 h dark cycle.

Table 1. Composition of experimental high-cholesterolaemic diet*

Constituents	g/100 g
Starch	50.0
Casein	20.0
Refined oil (sunflower)	10.0
Vitamin mixture†	1.01
Mineral mixture‡	4.00
Choline chloride	0.20
Sodium cholate	0.12
Met	0.30
Cholesterol	0.50
Sucrose	10.0
Cellulose powder	3.87

* Normal diet = hypercholesterolaemic diet – cholesterol.

† AIN-76 vitamin mixture.

‡ AIN-76 mineral mixture.

Room temperature was controlled at 22–25°C with about 56–60% relative humidity. Before conducting the animal trial, prior approval of the Institute's Animal Ethics Committee was obtained, and the mice were maintained in accordance with the National Institute of Nutrition, India guidelines for the care and use of laboratory animals.

Diet and experimental design

The base composition of the experimental diet is recorded in Table 1. Animal diets were formulated based on AIN-76. Table 2 provides the detailed formula of experimental diets used in the present study. Five experimental diets included a normal diet, a hypercholesterolaemic diet (HD), a HD with *L. plantarum* Lp91 (HD91), a HD with microencapsulated *L. plantarum* Lp91 (HDCap91) and a HD with *L. plantarum* Lp21 (HD21). The HD contained 0.5% (w/w) supplemental cholesterol.

Preparation of diets

Overnight grown test cultures were pelleted at 5000 rpm for 30 min at 4°C and washed two times with saline. The cell pellet of each culture was mixed with the HD to

Table 2. Experimental diets of different treatment groups

Serial number	Groups	Experimental diets
1.	NDCtrl	Normal diet
2.	HDCtrl	High-cholesterol diet
3.	HD91	Hypercholesterolaemic diet containing <i>Lactobacillus plantarum</i> Lp91 (approximately 10 ⁸ cfu/g)
4.	HDCap91	Hypercholesterolaemic diet containing microencapsulated <i>L. plantarum</i> Lp91 (approximately 10 ⁸ cfu/g)
5.	HD21	Hypercholesterolaemic diet containing <i>L. plantarum</i> Lp21 (approximately 10 ⁸ cfu/g)

NDCtrl, normal diet control; HDCtrl, hypercholesterolaemic diet control; HD91, HD containing *L. plantarum* Lp91; cfu, colony-forming units; HDCap91, HD containing microencapsulated *L. plantarum* Lp91; HD21, HD containing *L. plantarum* Lp21.

achieve the final cell concentration of > 10⁸ cfu/g of diet. Microencapsulated *L. plantarum* Lp91 was mixed with the HD to achieve the desired cell concentration, and the cfu/g of diet was determined by suspending 1 g of diet in 9 ml of phosphate buffer saline (137 mM-NaCl, 2.7 mM-KCl, 10 mM-Na₂HPO₄ and 2 mM-KH₂PO₄, pH 7.4), and then the subsequent dilutions were made in peptone saline and plated on MRS-Thio agar to determine the exact cfu/g of diet.

Feeding schedule

Rats were fed a rodent chow diet for 7 d to remove the effect of stress experienced by the animals due to separation from the main stock and to become accustomed to the testing regimen. At the end of the adaptation period, all the groups were fed on their assigned experimental diets for the next 21 d. After 21 d of the treatment period, all the animals were fed on the rodent chow for a further 7 d, which was considered as the post-treatment period. During the entire course of the experiment, the rats had free access to water and to the group-specific diet (20 g/100 g body weight per d). Feed intake was recorded daily and body weight was measured weekly.

Blood sampling and analytical procedures

The overnight fasted rats were bled from the tail vein at the 21st day for plasma lipid analysis. The blood samples collected were placed in heparinised sterile microfuge tubes and centrifuged at 2000 g for 15 min at 4°C. The samples were analysed for total cholesterol, TAG and HDL-cholesterol using commercial enzymatic kits (Autopak, M/s Siemens Diagnostics Limited, Gujarat, India). Friedewald's equation⁽³⁹⁾ was applied to analyse the following other plasma lipid fractions:

- (1) LDL-cholesterol = total cholesterol – HDL-cholesterol – (TAG/5). All the concentrations are given in mg/l.
- (2) VLDL-cholesterol = The quotient (TAG/5) is used as an estimate of VLDL-cholesterol.
- (3) Atherogenic index (AI) = The AI was calculated as (total cholesterol – HDL-cholesterol)/HDL-cholesterol.

Determination of faecal cholic acid

The faecal droppings of the experimental animals were collected over a 2 d period (days 19 and 20) using disposable wooden chopsticks to avoid contamination by feed or rodent hair. The samples were dried in an oven (55°C) for 2 d, and any remaining hair or dust contaminants were blown away using an air blower; the remaining material was finely ground using an analytical mill for further analysis. Faecal cholic acid levels were measured as per the published methods^(40–43) with some modifications. Dried faeces (0.1 g) were extracted twice with 3.5 ml ethanol at

80°C for 1 h. After two extractions, the ethanol was evaporated under N₂ gas at 50°C, and the residue was dissolved in 2.5 ml ethanol. Aliquots of these alcoholic extracts were evaporated to dryness in a water-bath, and the residue was dissolved in 2.5 ml of glacial acetic acid (60%). Then 1 ml portions of the resulting solution were pipetted into each of two small test tubes, to which 6 ml of 8 M-H₂SO₄ was added. Then 1 ml of 1% furfural solution (Hi-Media) in 60% glacial acetic acid was added to one of these tubes, and 1 ml of 60% glacial acetic acid was added to other tubes that served as a blank. The content of these tubes was mixed thoroughly, heated in a water-bath at 67°C for 15 min and cooled to room temperature. The absorbance of the resulting solutions was monitored at 610–690 nm with that of a suitable cholic acid (sodium salt) standard. Cholic acid standards were prepared as follows: 5 ml portions of two stock alcoholic solutions containing 0.25 and 0.5 mg of sodium cholate were evaporated to dryness and treated exactly as were the unknowns. The cholic acid concentration in faecal samples was calculated by deducting the colour equivalent of the blank tube from that of the tube containing furfural:

$$\begin{aligned} &\text{Cholic acid concentration } (\mu\text{mol/l}) \\ &= \frac{\text{ASF} - \text{ASB}}{\text{ACF} - \text{ACB}} \times \text{cholic acid standard } (\mu\text{mol/l}), \end{aligned}$$

where ASF is the absorbance of the sample with furfural, ASB is the absorbance of the sample blank, ACF is the absorbance of the cholic acid standard with furfural, and ACB is the absorbance of the cholic acid standard blank.

Microbiological analysis of faecal samples

Faecal samples from the experimental SD rats were collected weekly in separate sterile tubes for microbial analyses. Samples for microbial analyses were processed within 1 h of collection. Each sample was homogenised with a stomacher using sterile PBS and peptone saline diluents. Subsequent tenfold serial dilutions of each sample were plated in triplicate. All the enumeration media were obtained from Hi-Media Pvt Limited (India). Nutrient agar was used for total plate count, whereas eosin methylene blue agar was used for *Escherichia coli*. Violet red bile agar was used for coliforms, and MRS agar was used for total lactobacilli. Plates of total lactobacilli were incubated anaerobically at 37°C for 48 h in an anaerobic workstation (Don Whitley Scientific Limited, Shipley, West Yorkshire, UK), while plates for the enumeration of total plate count, *E. coli* and coliforms were incubated at 37°C for 48 h.

Statistical analysis

Data analysis was carried out with SPSS, Inc. software (version 10.0). One-way ANOVA was used to study any significant difference between means with a significance level

of $P \leq 0.05$. Critical difference values were used to perform multiple comparisons between means. All the data are presented as means with their standard errors (n 6).

Results

The main focus of the present study was to assess the role of two putative Bsh-producing indigenous probiotic *L. plantarum* strains Lp91 and Lp21 on their survival at high bile salt concentration and their cholesterol-lowering effects both under *in vitro* and *in vivo* conditions. However, before evaluating the hypocholesterolaemic effect of these strains in SD rats, the survivability of the strains at acidic pH and high bile salt concentrations was checked under *in vitro* conditions along with the quantification of Bsh production, bile salt deconjugation, cholesterol assimilation and co-precipitation of cholesterol with deconjugated bile salts. The results obtained from these experiments are presented later.

Survivability of probiotic *Lactobacillus* strains at low pH and high bile concentration

The effect of acidic pH (2.5) on the survivability of *Lactobacillus* strains at different incubation periods has been recorded in Table 3. All the three strains exhibited adequate survival at pH 2.5 for 3 h despite marginal variations in the degree of viability. However, *L. plantarum* Lp91 exhibited the highest survival rate of 86.89 (SEM 0.23) % after 1 h followed by *L. plantarum* Lp21 and NCDO82, which showed 85.07 (SEM 0.68) and 84.71 (SEM 0.61) % survival rates, respectively, after the same period. After 2 h, the survival rates of *L. plantarum* Lp91, Lp21 and NCDO82 were recorded as 74.87 (SEM 0.66), 73.87 (SEM 0.72) and 73.72 (SEM 0.99)%, respectively. The highest decrease in survivability appeared after 3 h of incubation in respect of all the three strains of *L. plantarum* Lp91, Lp21 and NCDO82 examined in the present study. In general, *L. plantarum* Lp91 was more acid tolerant than *L. plantarum* Lp21 and NCDO82, which showed almost similar level of tolerance. However, statistical analysis of results obtained in this experiment did not reveal any significant differences ($P > 0.05$) in the percentage survivability of the three *Lactobacillus* strains.

The presence of bile salts in the environment of bacterial cultures under *in vitro* conditions is much more detrimental than the effect of low pH. The concentration of bile salts used in this experiment was very high as a strong selective factor, while the *in vivo* bile concentration in the intestine is much lower. Data presented in Table 3 indicate that the most bile-tolerant strain was *L. plantarum* Lp91, which survived after 0, 1, 2 and 3 h of incubation in 2% bile up to 96.82 (SEM 0.81), 88.34 (SEM 0.25), 76.23 (SEM 0.39) and 62.5 (SEM 0.71)%, respectively. On the other hand, *L. plantarum* Lp21 and NCDO82 survived bile treatment at the rate of about 61.93 (SEM 0.35) and 60.16 (SEM 0.98)%,

Table 3. Probiotic attributes of selected *Lactobacillus* strains (Mean values with their standard errors, *n* 6)

Probiotic attributes	Unit	Time (h)	Strain					
			Lp91		Lp21		NCDO82	
			Mean	SEM	Mean	SEM	Mean	SEM
% Survival at pH 2.5		0	98.59	0.23	98.82	0.44	98.10	0.20
		1	86.89	0.23	85.07	0.68	84.71	0.61
		2	74.87	0.66	73.87	0.72	73.72	0.99
% Survival at 2% bile		3	65.21	1.29	63.32	1.36	63.86	0.92
		0	96.82	0.81	94.99	0.66	95.96	0.69
		1	88.34	0.25	88.53	0.35	86.02	0.45
	2	76.23	0.39	74.83	0.71	73.44	1.60	
	3	62.50	0.71	61.93	0.35	60.16	0.98	
Bile salt hydrolase activity	nmol/ml per min*		99.29	4.46	88.63	2.50	59.79	1.79
Bile salt deconjugation	mM		3.87	0.12	3.29	0.19	2.99	0.28
Cholesterol assimilation	µg/ml		68.97	0.63	68.54	0.71	64.94	1.37
Cholesterol co-precipitation	µg/ml		3.77	0.64	3.12	0.75	2.39	0.61

Lp91, *Lactobacillus plantarum* Lp91; Lp21, *L. plantarum* Lp21; NCDO82, *L. plantarum* NCDO82.

* One unit of bile salt hydrolase activity has been defined as the liberation of 1nmol amino acid from substrate per min per OD₆₀₀.

respectively, after 3 h. All *Lactobacillus* strains exhibited resistance against high concentrations of bile salts, which is a normal characteristic of commensal gut flora. However, statistical analysis did not prove the significance of differences ($P > 0.05$) observed between the survival of lactobacilli strains.

Bile salt hydrolase activity

The Bsh activity of the test cultures was estimated both by qualitative and quantitative assays.

Qualitative taurodeoxycholic acid sodium salt plate assay. The ability of *Lactobacillus* strains to hydrolyse taurodeoxycholic acid sodium salt was indicated by the precipitation of the bile salt along with the formation of opaque granular white colonies with a silvery shine around them on the medium plates (Fig. 1). The three strains differed in their taurodeoxycholic acid sodium salt hydrolysing activity as can be evidenced from the intensity of the precipitated opalescent zones, which was

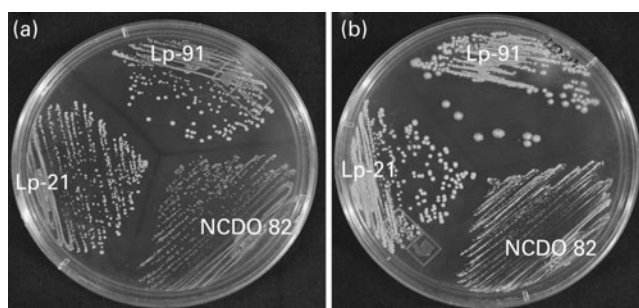


Fig. 1. Bile salt hydrolase activity of *Lactobacillus plantarum* Lp91, Lp21 and NCDO82 on solid de Man, Rogosa and Sharpe-Thio medium. Plates were incubated anaerobically for 72 h at 37°C. The control medium plate is shown in (a); the assay medium plate containing 0.5% taurodeoxycholic acid sodium salt is shown in (b). The precipitation or the formation of opaque granular colonies with a silvery shine in the agar is indicative of bile salt hydrolase activity.

relatively higher in *L. plantarum* Lp91 as compared to the *L. plantarum* Lp21 and NCDO82 strains.

Quantitative ninhydrin assay. All the three *Lactobacillus* strains, when subjected to the resting cell ninhydrin assay (Table 3) to quantify their ability to hydrolyse glycocholic acid, showed that *L. plantarum* Lp91 and Lp21 possessed significantly ($P \leq 0.05$) higher Bsh activity (99.29 (SEM 4.46) and 88.63 (SEM 2.50) nmol/ml per min, respectively) in comparison to NCDO82 (59.79 (SEM 1.79) nmol/ml per min). By expressing strong Bsh activity, both Lp91 and Lp21 were expected to play a positive health-promoting role specifically by lowering serum cholesterol levels.

Bile salt deconjugation

L. plantarum Lp91, Lp21 and NCDO82 demonstrated deconjugation activity at different levels (Table 3). There were significant differences ($P \leq 0.05$) among the *Lactobacillus* strains in the deconjugation of sodium glycocholate from suspension in the growth medium during 20 h of incubation. *L. plantarum* Lp91 and Lp21 deconjugated the highest level of sodium glycocholate as indicated by the release of 3.87 (SEM 0.12) and 3.29 (SEM 0.19) mM-cholic acid, respectively, in comparison to 2.99 (SEM 0.28) mM-cholic acid released by NCDO82.

Cholesterol assimilation

The test strains were also able to remove cholesterol from MRS-Thio broth supplemented with 0.3% ox-bile plus cholesterol during growth (Table 3). *L. plantarum* Lp91 and Lp21 could assimilate cholesterol appreciably as indicated by high cholesterol removal values, i.e. 68.97 (SEM 0.63) and 68.54 (SEM 0.71) µg/ml, which were significantly different ($P \leq 0.05$) from *L. plantarum* NCDO82 (64.94 (SEM 1.37) µg/ml).

Co-precipitation of cholesterol with deconjugated bile

Cholesterol was co-precipitated concurrently with deconjugation of sodium glycocholate at varying levels ($P > 0.05$), ranging from 2.39 to 3.77 $\mu\text{g/ml}$ by all the three cultures (Table 3). Deconjugation of sodium glycocholate by *L. plantarum* Lp91 brought about a relatively higher amount of cholesterol co-precipitation (3.77 (SEM 0.64) $\mu\text{g/ml}$) compared to *L. plantarum* Lp21 (3.12 (SEM 0.75) $\mu\text{g/ml}$) and *L. plantarum* NCDO82 (2.39 (SEM 0.61) $\mu\text{g/ml}$).

Cholesterol-lowering ability of bile salt hydrolase-positive Lactobacillus strains in Sprague–Dawley rat model

L. plantarum Lp91 and Lp21 were assessed for their cholesterol-lowering ability under *in vivo* conditions using the SD rat as the model system. *L. plantarum* Lp91 cells in encapsulated and non-encapsulated formats were separately mixed with experimental diets to achieve the final concentration of approximately 10^8 cfu/g. The final concentration of bacterial cells in the individual diets was $\geq 10^8$ cfu/g at 0 h and 10^7 cfu/g after 24 h.

Body weight and feed efficiency

All the experimental rats used in the study remained healthy, and their body weight gain, feed intake and feed efficiency after 21 d were calculated and recorded for all the groups as indicated in Table 4. Statistical analysis revealed that mixed groups had a significant ($P \leq 0.05$) effect on feed intake and feed efficiency ratio, while a non-significant ($P > 0.05$) effect was found on initial body weight, final body weight and gain in weight compared to single groups. However, after 3 weeks of treatment, final body weight (216.83 (SEM 6.08) g) of the normal diet control group (NDCtrl) and the HDCap91 group was lower in comparison to other groups (219.67 (SEM 7.15) to 230.67 (SEM 7.50) g) and did not differ significantly ($P > 0.05$). The hypercholesterolaemic diet control (HDCtrl) group animals, which were fed on a cholesterol-enriched

diet, showed a relatively higher body weight (238.33 (SEM 9.22) g) in comparison to other experimental groups. The highest body weight gain was recorded in the HDCtrl group (90.67 (SEM 2.43) g), while the body weight gain of the NDCtrl group was slightly lower than that of the other groups and did not differ significantly ($P > 0.05$). Similarly, feed intake was higher in the HDCtrl group (174.35 (SEM 2.78) g) than in probiotic treatment groups and the NDCtrl group. The feed efficiency in rats fed HD91 (54.42 (SEM 5.20)) was greater than the HDCtrl (52.84 (SEM 1.33)), NDCtrl (51.49 (SEM 2.38)), HDCap91 (46.89 (SEM 1.86)) and HD21 (41.99 (SEM 1.50)) groups.

Plasma lipid profile

The effect of dietary treatments on plasma lipid profile (plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, TAG and AI) has been recorded in Table 5 and Fig. 2. Total plasma cholesterol values for each dietary treatment group were recorded as 768.29 (SEM 41.01), 1037 (SEM 39.9), 795.7 (SEM 75.9), 874.1 (SEM 59.2) and 881.3 (SEM 62.2) mg/l for the NDCtrl, HDCtrl, HD91, HDCap91 and HD21 dietary groups, respectively. Groups HD91, HDCap91 and HD21 showed decreases in plasma cholesterol levels of 23.26, 15.71 and 15.01%, respectively, compared with the HDCtrl group after 21 d of dietary treatment. These three groups also demonstrated a lowering of LDL-cholesterol at a significant level. LDL-cholesterol values for each group were recorded as 293.7 (SEM 46.7), 607.8 (SEM 50.7), 376.1 (SEM 83.2), 466.7 (SEM 51.2) and 477.6 (SEM 72.5) mg/l for the NDCtrl, HDCtrl, HD91, HDCap91 and HD21 groups, respectively. The reduction in LDL-cholesterol was 38.13, 23.22 and 21.42% in the HD91, HDCap91 and HD21 groups, respectively, while HDL-cholesterol showed the opposite trend. HDL-cholesterol values for all the five groups were recorded as 317.8 (SEM 31), 202.3 (SEM 11.5), 240.6 (SEM 13.5), 223.1 (SEM 14.2) and 218 (SEM 10.1) mg/l, respectively, and the corresponding increases for HDL-cholesterol in the probiotic treatment groups, i.e. HD91,

Table 4. Body weight, weight gain and feed efficiency of rats fed control and experimental diets (Mean values with their standard errors, $n = 6$)

Component	Treatment groups									
	NDCtrl		HDCtrl		HD91		HDCap91		HD21	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Initial wt (g)	150.0 ^{NS}	5.84	147.67	9.49	148.33	4.86	146.67	7.71	147.50	6.10
Final wt (g)	216.83 ^{NS}	6.08	238.33	9.22	230.67	7.50	216.83	7.24	219.67	7.15
Wt gain (g)	66.83 ^{NS}	2.86	90.67	2.43	82.33	7.59	70.17	2.24	72.17	2.65
Feed intake (g)	130.04 ^a	2.54	174.35 ^c	2.78	151.83 ^b	3.39	149.94 ^b	2.22	171.89 ^c	2.11
FER*	51.49 ^b	2.38	52.84 ^b	1.33	54.42 ^b	5.20	46.89 ^{a,b}	1.86	41.99 ^a	1.50

NDCtrl, normal diet control; HDCtrl, hypercholesterolaemic diet control; HD91, HD containing *L. plantarum* Lp91; HDCap91, HD containing microencapsulated *L. plantarum* Lp91; HD21, HD containing *L. plantarum* Lp21; FER, feed efficiency ratio.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

NS $P > 0.05$.

* FER = wt gain (g)/feed intake (g) \times 100.

Table 5. Plasma total cholesterol, TAG, HDL-, LDL- and VLDL-cholesterol concentrations (mg/l) in rats fed experimental diets (Mean values with their standard errors)

Group	Plasma lipids											
	Total cholesterol		TAG		HDL-cholesterol		LDL-cholesterol		VLDL-cholesterol		AI*	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NDCtrl	768.2 ^a	41.0	783.1 ^a	60.2	317.8 ^a	31.0	293.7 ^a	46.7	156.6 ^a	12.0	1.51 ^a	0.23
HDCtrl	1037.0 ^b	39.9	1134.1 ^b	121.4	202.3 ^a	11.5	607.8 ^c	50.7	226.8 ^b	24.3	4.24 ^b	0.45
HD91	795.7 ^a	75.9	894.9 ^a	30.8	240.6 ^a	13.5	376.1 ^{a,b}	83.2	179 ^a	6.1	2.41 ^{a,b}	0.47
HDCap91	874.1 ^a	59.2	921.2 ^a	57.8	223.1 ^a	14.2	466.7 ^{a,b,c}	51.2	184.2 ^a	11.5	2.93 ^{a,b}	0.20
HD21	881.3 ^{a,b}	62.2	928.0 ^a	45.8	218.0 ^a	10.1	477.6 ^{b,c}	72.5	185.6 ^a	9.1	3.10 ^{a,b}	0.36

AI, atherogenic index; NDCtrl, normal diet control; HDCtrl, hypercholesterolaemic diet control; HD91, HD containing *Lactobacillus plantarum* Lp91; HDCap91, HD containing microencapsulated *L. plantarum* Lp91; HD21, HD containing *L. plantarum* Lp21.

^{a,b,c} Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* AI = (total cholesterol – HDL-cholesterol)/HDL-cholesterol.

HDCap91 and HD21, were 18.94, 10.30 and 7.78%, respectively. On the basis of statistical analysis, it was found that plasma HDL-cholesterol concentrations differed significantly among all the experimental groups ($P \leq 0.05$). Similarly, plasma TAG and VLDL-cholesterol concentrations also differed significantly ($P \leq 0.05$) among all the groups throughout the experiment. After dietary treatment, TAG and VLDL-cholesterol levels in the HD91, HDCap91 and HD21 groups were reduced by 21.09, 18.77 and 18.17%, respectively. The AI of the dietary treatment groups of rats also differed significantly ($P \leq 0.05$). The AI of treatment groups increased sharply after feeding on the hypercholesterolaemic diet in comparison to that recorded with the NDCtrl group (1.514 (SEM 0.232)). However, the AI of the probiotic treatment groups was found to decrease after 21 d of treatment as compared with the HDCtrl group (4.247 (SEM 0.458)). The maximum decline in AI was recorded in the HD91 group (2.415 (SEM 0.472)) in comparison to the HDCap91 (2.937 (SEM 0.203)) and HD21 groups (3.104 (SEM 0.364)).

Faecal cholic acid excretion

As shown in Fig. 3, faecal cholic acid levels differed significantly among the various groups ($P \leq 0.05$). Supplementation of the HD with Bsh-positive *L. plantarum* Lp91 and Lp21 led to considerable increases in the excretion

of cholic acid in the faeces. After 21 d of feeding, the maximum faecal cholic acid excretion (5.489 (SEM 0.09) $\mu\text{mol/g}$ faeces) was recorded in the HDCap91 group, whereas in the other probiotic treatment groups HD91 and HD21, the corresponding increase in faecal cholic acid was 5.084 (SEM 0.09) and 5.183 (SEM 0.13) $\mu\text{mol/g}$, respectively. However, in animals fed on the normal diet, the faecal cholic acid content was found to be 1.084 (SEM 0.09) $\mu\text{mol/g}$, which was lower than the HDCtrl group (1.703 (SEM 0.04) $\mu\text{mol/g}$).

Faecal microbial analysis

The overall mean (\pm SEM) faecal bacterial counts at 0, 7, 14, 21 and 28 d obtained from different groups have been recorded in Table 6. Rats supplemented with the probiotic treatment diets showed relatively higher total bacterial counts (8.991–9.629 cfu \log_{10}/g) in the faecal samples as compared to the control groups. However, total bacterial counts decreased progressively in all the treatment groups during the post-treatment period (28 d). Although the presence of faecal lactobacilli was consistently recorded in all the groups with some variation ($P \leq 0.05$), the rats fed on *Lactobacillus* strains as a dietary adjunct maintained a high level of lactobacilli in the faecal samples throughout the experiment compared with control groups. The maximum faecal *Lactobacillus* count was observed in

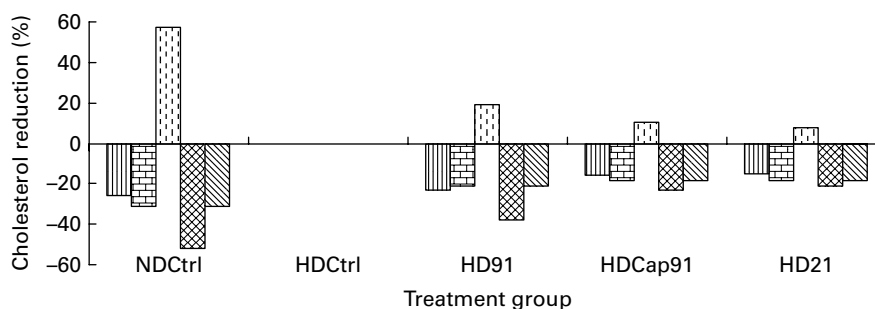


Fig. 2. Percentage decrease in plasma lipids (total cholesterol (□), TAG (▨), HDL (▩), LDL (▧) and VLDL (▮)-cholesterol) of dietary treatment groups. NDCtrl, normal diet control; HDCtrl, hypercholesterolaemic diet control; HD91, HD containing *Lactobacillus plantarum* Lp91; HDCap91, HD containing microencapsulated *L. plantarum* Lp91; HD21, HD containing *L. plantarum* Lp21.

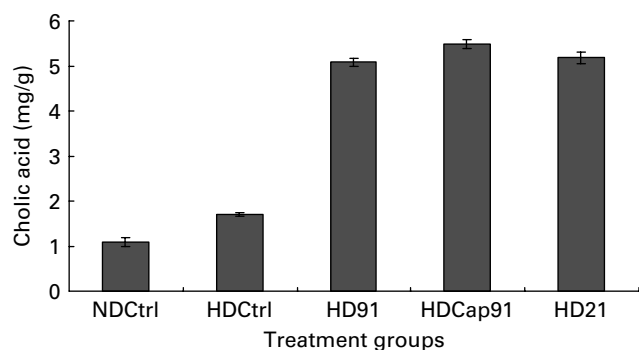


Fig. 3. Faecal cholic acid concentration for rats fed experimental diets. NDCtrl, normal diet control; HDCtrl, hypercholesterolaemic diet control; HD91, HD containing *Lactobacillus plantarum* Lp91; HDCap91, HD containing microencapsulated *L. plantarum* Lp91; HD21, HD containing *L. plantarum* Lp21.

HDCap91 group rats compared with HD91 and HD21 group rats. After the post-treatment period (28 d), faecal lactobacilli counts were significantly higher in rats fed on *L. plantarum* Lp91 and Lp21 than those in the HDCtrl and NDCtrl groups. Total lactobacilli ranged from 8.782 to 9.717 cfu log₁₀/g of faeces in all the experimental groups. These findings clearly indicate that feeding of Bsh-active lactobacilli as a dietary adjunct resulted in a more stable shift of the intestinal lactobacilli population than feeding of the high-cholesterol and normal diet alone. However, discontinuation of *Lactobacillus* feeding in all probiotic treatment groups resulted in a consistent decrease in faecal lactobacilli. Rats supplemented with the diet containing *L. plantarum* Lp21 showed a

marginal decrease in faecal *E. coli* counts compared with the HDCtrl group during the dietary treatment and post-treatment period. Total coliforms ranged from 7.707 to 7.966 cfu log₁₀/g among all the treatment groups.

Discussion

It is now well documented that hypercholesterolaemic conditions are the predisposing factors associated with an increased risk of CHD. Lowering of total cholesterol and LDL-cholesterol in the serum/plasma of hypercholesterolaemic patients reduces the incidence of CVD. One of the possible strategies that could be promising and cost effective in lowering cholesterol levels in serum is by modulating diets through probiotic interventions. This approach could be very effective in the management of CVD, including strokes. Probiotic lactobacilli are considered as normal components of the intestinal microflora in both humans and animals, and have been invariably associated with various health-promoting functions. It is in this context that probiotics are gaining prominence and are used as dietary adjuncts in the development of novel probiotic foods for health applications. Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant to survive in the human gastrointestinal tract. The transit time from entrance to release from the stomach has been estimated to be approximately 90 min, with further digestive processes requiring longer residence time⁽⁴⁴⁾. Stresses to organisms begin in the stomach with a pH between 1.5

Table 6. Effect of probiotic feeding on faecal bacterial population (Mean values with their standard errors, *n* 6)

Log CFU/g of faeces	Days	Treatments									
		NDCtrl		HDCtrl		HD91		HDCap91		HD21	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total aerobes	0	9.18 ^{a,b}	0.07	9.23 ^b	0.03	9.25 ^b	0.03	9.04 ^a	0.02	9.09 ^{a,b}	0.03
	7	8.99 ^a	0.02	9.19 ^a	0.04	9.61 ^b	0.02	9.61 ^b	0.03	9.55 ^b	0.03
	14	9.10 ^a	0.03	9.15 ^a	0.02	9.46 ^b	0.04	9.62 ^b	0.03	9.51 ^b	0.02
	21	9.06 ^a	0.02	9.20 ^a	0.04	9.61 ^b	0.01	9.59 ^b	0.04	9.51 ^b	0.04
	28	9.10 ^a	0.04	9.22 ^{a,b}	0.02	9.46 ^{b,c}	0.03	9.59 ^c	0.02	9.35 ^{a,b,c}	0.03
<i>Lactobacillus</i>	0	8.94 ^{NS}	0.04	9.01 ^{NS}	0.04	9.02 ^{NS}	0.04	8.91 ^{NS}	0.05	8.90 ^{NS}	0.04
	7	8.78 ^a	0.04	8.92 ^a	0.04	9.52 ^b	0.02	9.65 ^b	0.01	9.60 ^b	0.03
	14	8.97 ^a	0.05	8.97 ^a	0.05	9.45 ^b	0.02	9.71 ^b	0.01	9.62 ^b	0.03
	21	8.88 ^a	0.05	9.04 ^a	0.05	9.64 ^b	0.03	9.59 ^b	0.03	9.58 ^b	0.04
	28	8.98 ^a	0.05	9.10 ^a	0.04	9.39 ^b	0.02	9.55 ^b	0.02	9.25 ^b	0.02
Coliforms	0	7.91 ^{NS}	0.04	7.95	0.02	7.96	0.02	7.89	0.02	7.84	0.02
	7	7.70 ^a	0.04	7.89 ^b	0.02	7.86 ^b	0.02	7.88 ^b	0.02	7.82 ^b	0.04
	14	7.91 ^a	0.03	7.90 ^a	0.03	7.77 ^a	0.04	7.93 ^a	0.02	7.86 ^a	0.03
	21	7.77 ^a	0.02	7.95 ^b	0.02	7.95 ^b	0.02	7.87 ^{a,b}	0.03	7.85 ^{a,b}	0.03
	28	7.85 ^a	0.03	7.96 ^a	0.03	7.83 ^a	0.04	7.92 ^a	0.03	7.77 ^a	0.03
<i>Escherichia coli</i>	0	7.93 ^{a,b}	0.03	7.98 ^b	0.02	7.96 ^b	0.02	7.88 ^a	0.02	7.88 ^a	0.02
	7	7.77 ^{NS}	0.03	7.90	0.02	7.87	0.02	7.87	0.03	7.85	0.03
	14	7.96 ^a	0.02	7.87 ^a	0.02	7.79 ^a	0.02	7.91 ^a	0.02	7.83 ^a	0.02
	21	7.77 ^a	0.03	7.99 ^b	0.01	7.93 ^{a,b}	0.02	7.88 ^{a,b}	0.02	7.86 ^{a,b}	0.03
	28	7.88 ^{a,b}	0.02	8.02 ^b	0.03	7.86 ^{a,b}	0.01	7.93 ^{a,b}	0.03	7.75 ^a	0.02

CFU, colony-forming unit; NDCtrl, normal diet control; HDCtrl, hypercholesterolaemic diet control; HD91, HD containing *Lactobacillus plantarum* Lp91; HDCap91, HD containing microencapsulated *L. plantarum* Lp91; HD21, HD containing *L. plantarum* Lp21.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

NS $P > 0.05$.

and 3·0, and in the upper intestine that contains bile^(45,46). The survival and tolerance of probiotic cultures are considered essential for expressing their physiological functions optimally⁽⁴⁷⁾. The present study was initially aimed at investigating the role of two Bsh-positive *L. plantarum* strains Lp91 and Lp21 in their survival under high bile concentration along with cholesterol-lowering ability under *in vitro* conditions before assessing their functional efficacy *in vivo* in a rat model. Strains of lactobacilli used in the present study showed varying levels of viability at pH 2·5 and 2% bile salts after 3 h. Many authors have also investigated the effect of bile on survival of lactic acid bacteria with varying degree of survivability/inhibition^(48–50).

In the present study, all the three *Lactobacillus* strains could deconjugate and hydrolyse glycocholic acid and assimilated cholesterol micelles in their cellular membrane along with co-precipitation of cholesterol in the presence of deconjugated cholic acid. There was a good correlation between bile salt deconjugation and Bsh activity in the three *Lactobacillus* strains. The present results in this regard are in agreement with those of Klaver & van der Meer⁽¹⁸⁾ who theorised that while bile salts were deconjugated and the pH of the fermentation media was dropped due to natural acid production by culture, cholesterol micelles destabilised and cholesterol co-precipitated with free bile acids. Although the precise mechanism of the cholesterol-lowering activity of lactic acid bacteria has not yet been worked out completely, probiotics may alter serum cholesterol by two possible mechanisms: (1) directly binding dietary cholesterol into the small intestine before cholesterol can be absorbed into the body^(51–53) and (2) bile salt deconjugation by Bsh to produce free bile acids^(17,23,35). Free bile acids thus formed by the deconjugation of conjugated bile salts are less soluble and are less likely to be reabsorbed by the intestinal lumen compared to bile salts, and are lost from the human body through faeces^(54,55). This could lead to a higher metabolism of cholesterol and, subsequently, could result in the reduction of serum cholesterol⁽²¹⁾. However, the development of any therapy, even those using biological products, requires methodological research of the potential side effects to ensure their safety for health applications. Since large amounts of deconjugated bile salts may have undesirable effects for the human host, concern may arise over the safety of administering a Bsh-active probiotic strain. However, the positive feature that goes in their favour is that bacterial genera that would most likely to be used as probiotics (*Lactobacillus* and *Bifidobacterium*) are not capable of dehydroxylating deconjugated bile salts as reported by Takahashi & Morotomi⁽⁵⁶⁾, Ahn *et al.*⁽³⁴⁾ and Begley *et al.*⁽⁵⁵⁾, and hence the majority of the breakdown products of Bsh activity by probiotic strain may be precipitated and excreted out in the faeces.

The Bsh activity of probiotics has often been correlated with their *in vivo* cholesterol-lowering effect^(10,22,23). To establish the role of Bsh in cholesterol lowering, the

effect of potential Bsh-producing *L. plantarum* Lp91 strain in its microencapsulated and non-encapsulated form was examined in the present study in a rat model along with *L. plantarum* Lp21 (non-encapsulated). During the last decade, many studies have been conducted on experimental animal models and human subjects to establish the cholesterol-reducing ability of probiotic cultures, i.e. *Lactobacillus* spp. and *Bifidobacterium* spp.^(27,57). However, there are only very few specific reports available on the effects of Bsh-positive *L. plantarum* on serum lipid profiles in either animal models or human subjects⁽²²⁾. In the present study, experiments were designed specifically to elucidate the role of Bsh in cholesterol lowering in SD male rats. The present study shows that Bsh-active *L. plantarum* strains could reduce plasma total cholesterol and LDL-cholesterol in rats fed a diet high in cholesterol. Supplementation of the diet with *L. plantarum* Lp91 (HD91) resulted in a significant reduction in plasma total cholesterol, LDL-cholesterol and TAG by 23·26, 38·13 and 21·09%, and a significant enhancement of 18·94% in HDL-cholesterol in hypercholesterolaemic rats. Similarly, plasma total cholesterol, LDL-cholesterol and TAG values obtained from HDCap91 and HD21 probiotic treatment groups were also significantly lower than for the hypercholesterolaemic control group, and HDL-cholesterol values of these groups were higher than in the hypercholesterolaemic control group. Although encapsulated Lp91 did reduce total cholesterol, LDL-cholesterol and TAG along with increased HDL-cholesterol in plasma, these values were on the lower side than those recorded with Lp91 as such. This variability in results with regard to Lp91 under encapsulated *v.* non-encapsulated conditions could be attributed to the release of fewer cells in a free form from the encapsulated preparation in the lower gut, thereby restricting the availability of Bsh for bile acid deconjugation and also due to the loss of encapsulated Lp91 in faeces. Nguyen *et al.*⁽³¹⁾ investigated the hypocholesterolaemic effects of Bsh-active *L. plantarum* PH04 in a mouse model, and recorded 7 and 10% reduction in serum cholesterol and TAG. In a similar study, Ha *et al.*⁽²²⁾ used a Bsh-producing *L. plantarum* strain, which had been isolated from human faeces, to reduce serum cholesterol levels in SD male rats, and recorded 27·7 and 28·66% reductions in total cholesterol and LDL-cholesterol. The AI for the probiotic treatment groups decreased significantly ($P \leq 0\cdot05$) when compared with the hypercholesterolaemic control group. These observations are consistent with the findings of previous reports carried out on similar lines⁽⁵⁷⁾, indicating a significant decrease in AI of probiotic treatment groups. De Smet *et al.*⁽¹⁰⁾ had reported the use of enhanced Bsh activity of the *L. reuteri* strain to reduce serum cholesterol levels in pigs fed on a high-cholesterol diet. However, only a few reports are available on the use of the Bsh-active *L. plantarum* strain^(9,22) to reduce serum cholesterol levels in rats or other animal models. The use of microencapsulated Bsh-active *L. plantarum* in

reducing plasma cholesterol levels in rats had never been reported to the best of our knowledge. This is perhaps the first study wherein the anti-hypercholesterolaemic effect of microencapsulated *L. plantarum* Lp91 has been demonstrated in rats fed on a HD. However, the *in vitro* bile acid deconjugation ability of isogenic microencapsulated *L. plantarum* 80 strain was studied, and it was found that microencapsulated *L. plantarum* 80 cells could efficiently break down glycodeoxycholic acid and taurodeoxycholic acid⁽⁵⁸⁾. It was shown in the present study that faecal cholic acid excretion increased throughout the experimental period in the probiotic treatment groups, while significantly higher excretion was observed in groups fed on diets containing Bsh-active *L. plantarum* strains than control groups of rats (Fig. 3). Higher excretion of cholic acid through faeces in the HD91, HD91 and HD21 groups indicated that changes in bile salt metabolism had been brought about by ingested Bsh-active *L. plantarum* strains. This clearly points to a shift towards a more Bsh-active *Lactobacillus* population in the rat gut, which might influence the bile salt metabolism to reduce the amount of bile salts available for resorption. Consequently, the feedback inhibition mechanism of bile salt synthesis is decreased, and the synthesis of bile salts from cholesterol increased, yielding reduced serum cholesterol levels.

Although the effects of probiotic lactobacilli on different bacterial counts in faeces had been investigated previously by different groups^(27,29,59), the literature specifically on the effect of Bsh-active *Lactobacillus* strains on faecal bacterial counts in animal models is quite scanty. In this investigation, we had specifically examined the effects of experimental diets containing potentially Bsh-active Lp91 and Lp21 strains on faecal bacterial population. Surviving passage through the gastrointestinal tract is considered to be important for probiotics to function effectively in the intestine⁽⁵¹⁾. In the present study, faecal microbial analysis revealed significantly higher faecal lactobacilli counts in probiotic treatment groups compared to that with the control groups. This could be attributed to their ability to survive at low pH and high bile concentration as described previously in *in vitro* experiments. Occurrence of high lactobacilli counts during the post-treatment period (28 d) in groups fed on Bsh-active *L. plantarum* strains clearly suggests possible involvement towards the contribution of Bsh in gut colonisation as reported previously by Dussurget *et al.*⁽⁶⁰⁾. However, no significant effect on faecal coliforms and *E. coli* counts was recorded in groups fed on *L. plantarum* strains, while a significant decrease in faecal *E. coli* and coliform counts has been reported previously due to probiotic feeding in rats^(27,29). The outcome of the present study demonstrates that consumption of *L. plantarum* Lp91 as a probiotic dietary adjunct might be useful in reducing human serum cholesterol level. However, well-designed placebo-controlled clinical trials need to be conducted to validate the efficacy

and safety of the strain and its use in the management of high cholesterol in humans. The alteration in bile salt metabolism through enhanced bile salt hydrolase activity might also affect cholesterol lowering more directly by influencing its solubility and intestinal absorption^(21,61) and by enhanced faecal excretion of deconjugated bile acids⁽²³⁾. Based on the 1–2 rule^(55,62), which states that a 1% reduction in serum cholesterol causes a 2% lowering of the risk for CHD, a significant positive effect for patients suffering from elevated cholesterol might be obtained by ingesting lactobacilli to improve hydrolysis of bile salts. In order to further elucidate the precise role of Bsh in cholesterol lowering, our next target is to assess the effect of microencapsulated recombinant Bsh and *Lactobacillus* clone in animal models for establishing its bioactive functionality, which forms the subject of a separate communication.

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