



Hypocholesterolemic Effect of Potential Probiotic *Lactobacillus fermentum* Strains Isolated from Traditional Fermented Foods in Wistar Rats

Pooja N. Thakkar^{1,2} · Ami Patel³ · Hasmukh A. Modi¹ · Jashbhai B. Prajapati²

Published online: 23 November 2019

© Springer Science+Business Media, LLC, part of Springer Nature 2019

Abstract

The current research project was undertaken to explore the therapeutic potential of two potent probiotic *Lactobacillus fermentum* strains, i.e., PD2 and PH5 in a hyperlipemic healthy adult Wistar rat model, with a particular focus as biotherapeutics for the management of high cholesterol in Indian population. Rats fed on cholesterol-enriched diet supplemented with potential probiotics strain *Lactobacillus fermentum* PH5 significantly affected serum lipid profile by reducing serum cholesterol (67.21%), triglycerides level (66.21%), and LDL cholesterol level (63.25%) in comparison to rats that received cholesterol-enriched diet (Model) only. Both the strains decreased the cholesterol levels in liver compared with Model group, but PH5 was found to be more effective (30.65% reduction) in liver total cholesterol (TC) lowering action. In addition, the fecal coliforms were significantly reduced besides increased LAB in feces of rats receiving probiotic curd having *Lactobacillus fermentum* PH5. Our results demonstrated that supplementation with either of the two strains was efficient in reducing serum cholesterol, LDL-cholesterol and TG concentrations in rats compared to those fed the same high-cholesterol diet but without LAB supplementation.

Keywords Hypocholesterolemic effect · Probiotics · *Lactobacillus fermentum* · Bile salt hydrolase activity · Wistar rats

Introduction

The incidence of hypercholesterolemia is increasing swiftly with the improvements of people's living standards and amendments in lifestyles. Elevated serum cholesterol level is usually considered to be the most imperative risk factor for the development of cardiovascular diseases (CVDs), such as atherosclerosis, hypertension, coronary heart disease, and stroke. Both drug therapy and non-pharmacological approaches, including dietary interventions, behavior modification, and regular exercise, are common strategies to lower cholesterol levels. Current drug therapies (i.e., statins), despite the proven cholesterol lowering ability, with their high relative costs and

associated side effects, are not viewed to be optimal long-term answers [1, 2]. It is more attractive to develop possible strategies and safer alternative therapies by modulating diet through probiotic interventions that could be promising and cost effective in lowering cholesterol [3].

Previous in vitro screening experiments revealed that strain PD2 (*dosa* batter isolate) and strain PH5 (*handva* batter isolate) were able to tolerate maximum bile concentration, produce bile salt hydrolase (BSH) enzyme, and could deconjugate bile salts like sodium taurocholate (ST) [4, 5]; they were identified as *Lactobacillus fermentum* by molecular typing methods. These potential probiotic strains were investigated for probiotic candidatures using obligatory tests as well as evaluated for safety aspect by performing amino acid decarboxylating activity (production of biogenic amines), hemolysis activity, antibiotic resistance, and gelatinase activity [4–6]. Both the strains showed negative response for all these tests indicating possible safe use for further investigations. Further, with respect to direct cholesterol assimilation (in vitro), PH5 showed a maximum reduction of 76.85% followed by PD2 69.66% compared to control by utilizing human plasma as a source of cholesterol [4, 5]. Thus, the study was undertaken to explore the therapeutic potential of

✉ Ami Patel
amiampatel@yahoo.co.in; ami@midft.com

¹ Department of Life Sciences, Gujarat University, Ahmedabad, India

² Department of Dairy Microbiology, SMC College of Dairy Science, Anand Agriculture University, Anand, India

³ Division of Dairy Microbiology, Mansinhbhai Institute of Dairy & Food Technology-MIDFT, Mehsana, Gujarat State, India

probiotic dahi made from two potent probiotic *Lact. fermentum* strains, i.e., PD2 and PH5 in a hyperlipemic healthy adult Wistar rat model, with a particular focus as biotherapeutics for the management of high cholesterol in Indian population.

Materials and Methods

Lact. fermentum PD2 [gene accession no.KR612224] and PH5 [gene accession no.KR612226] were isolated previously from the batter of traditional Indian fermented nondairy products [4–6]. Further, both these strains having potential probiotic attributes were selected for current investigation on the basis of their better abilities to lower cholesterol in in vitro trials. For the preparation of probiotic (curd) diet, overnight grown test cultures, i.e., *Lact. fermentum* PD2 and PH5 were pelleted at 5000 rpm for 30 min at 4 °C and washed twice with saline. The cell pellet of each culture was inoculated into sterile reconstituted skim milk and incubated at 37 °C to achieve curdling. The curd samples from each culture PD2 and PH5 were stirred and diluted to attain the final concentration 10^7 and 10^9 CFU/g as two define doses, respectively. The concentrations of the bacterial strains were maintained constant and checked by plating on selective (MRS) agar media over the fermentation time during each trial.

Experimental Animals

Healthy adult Wistar albino rats (Either sex), 4–6 weeks old weighing 200–250 g, were used for the present study. The animals were housed under well-controlled conditions of temperature (22 ± 2 °C) and humidity ($55 \pm 5\%$) for 12:12 h light-dark cycle. Animals had free access to conventional laboratory diet (purchased from Pranav Agro Pvt. Ltd.) and tap water ad libitum throughout the adaptation period. The protocol of the experiment in this thesis was approved by Institutional Animal Ethical Committee (IAEC) of Anand Pharmacy College (Protocol No: APC/2015-IAEC/1504).

Experimental Design and Diet

At the end of the adaptation period, animals were randomly selected, weighed, and divided into seven different groups with six animals in each group with their assigned diet as shown in Table 1. Adult Wistar albino rats of Group B (model), Group C (Standard) and other experimental groups (Groups D, E, F and G) were made hyperlipidemic by the oral administration of high-fat diet (Atherogenic diet) by mixing with regular pellet diet. The base composition of the atherogenic (hyperlipidemic) diet included cholesterol, cholic acid, coconut oil, sucrose, and normal laboratory diet 2%, 1%, 10%, 40%, and 47%, respectively.

Rats of the experimental groups (Groups D, E, F, and G) were also fed with 2 ml probiotic (stirred curd) in two doses once daily in the morning through oral feeding/gastric incubation for 28 consecutive days. Control and Model groups received an equivalent amount of normal saline. Rats of Group C were treated with markedly available standard drug atorvastatin (10 mg/kg of their body weight) only to compare effect of treatment with our probiotic solutions. 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 day). The experiment was carried out for 4 weeks. Food intake of the animal was observed daily whereas the body weight was determined weekly. After the feeding period, the rats were euthanized and the weight of the visceral organs (Liver, Kidney, Spleen, Lungs and Heart) and Fat pad (mesenteric, perirenal, and epididymal white adipose tissues) was measured.

Assay for Serum Lipid

Blood samples of the animals of each group were collected at 0 day and the end of the experiment on 28th day. The overnight-fasted animals were given light anesthesia and blood was collected from the retro orbital plexus with capillary tubes. Each sample of serum was analyzed for serum total cholesterol (TC), serum high-density lipoprotein cholesterol (HDL-C), serum low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) using commercially available kits (Coral Clinical Systems, Goa) based on the enzymatic oxidation of these molecules. Atherogenic indexes (TC/HDL-C and LDL-C/HDL-C ratios) were then calculated [1, 2].

Assay for Liver Cholesterol and Triglycerides

After euthanasia, the livers from all groups were collected, perfused, rinsed with physiological saline solution, weighed, and homogenized. Liver tissues were extracted following standard procedure of Shakuto et al. [7] with slight modifications. An aliquot extract was supplied for lipids determinations by observing absorbance in UV–Visible spectrophotometer (UV-1601 Shimadzu, Japan) using standardized commercial kit (Coral Clinical Systems, Goa).

Histopathological Analysis

Rat livers from each group were dissected out and frozen rapidly to about -20 °C till use. All the tissues are then placed in neutral formaldehyde (10%), embedded in paraffin and processed into 5 μ m sections for light microscopy according to the routine procedures. The sections were stained with hematoxylin-eosin (HE) for histological assessment [8].

Table 1 Experimental groups with their respective diet

Group name	Diet and dose
A Normal control	Regular standard diet pellet
B Model control	Atherogenic diet (hyperlipidemic diet)
C Standard control	Atherogenic diet + standard antihyperlipidemic drug (atorvastatin 10 mg/kg)
D Test dose 1- PD2	Atherogenic diet + curd having 10 ⁷ probiotic strain PD2
E Test dose 2- PD2	Atherogenic diet + curd having 10 ⁹ probiotic strain PD2
F Test dose 3- PH5	Atherogenic diet + curd having 10 ⁷ probiotic strain PH5
G Test dose 4- PH5	Atherogenic diet + curd having 10 ⁹ probiotic strain PH5

Analysis of Fecal Samples for Fecal Sterol, Fecal Water, and Fecal Microflora

Fecal droppings were collected during the last 2 days of life of rats, and fecal neutral and acidic sterols were extracted with slight modifications as suggested by Xie et al. [2]. In the middle of the 4th weeks, rat feces were amassed, weighed, and dried at 80 °C in a vacuum drying oven until a constant weight was achieved within 24 hours, and then reweighed. Fecal water content was calculated as suggested by Lee et al. [9]. Fecal water content (%) = [(weight before drying-weight after drying)/weight before drying] × 100.

Each sample was homogenized using a mortar and pestle using sterile PBS. Subsequent tenfold serial dilutions of each sample were plated in duplicate as per the method of Xie et al. [2]. Eosin methylene blue (EMB) agar was used for *Escherichia coli*, Violet red bile agar was utilized for coliforms, and MRS agar was used for total lactobacilli. Plates of total lactobacilli were incubated anaerobically at 37 °C for 48 h, while plates for the enumeration of *E. coli* and coliforms were incubated aerobically at 37 °C for 48 h. The numbers of colony-forming units (CFU) are expressed as log₁₀ CFU per g.

Results and Discussion

The in vivo effect of two nondairy origin potent probiotics *Lact. fermentum* (PD2 and PH5) was analyzed to determine how their cholesterol metabolism would affect hyperlipidemic Wistar albino rats as the model system in current approach.

Effect of Potential Probiotic on Anthropogenic Parameters

All the experimental rats utilized in this study appeared healthy throughout the whole feeding period of 28 days. Their body weight, fat pad, and food consumption were calculated and recorded for all the groups during the experimental period as indicated in Table 2.

The initial body weight of rats showed no significant difference among groups. After 28 days of experimental period, the rats in group B (Model) exhibited an increasing trend in body weight (289.78 ± 13.9 g), probably as a consequence of their greater calorie intake, due to the greater energetic density of the high cholesterol diet compared with group A (Normal) which fed a regular diet. While feeding probiotic 10⁷ and 10⁹ CFU/ml of *Lact. fermentum* PD2 and PH5, other experimental groups (groups labeled D, E, F, G) showed an increase in the final body weight compared to their initial weight. Vijayendra and Gupta [10] reported *Lact. acidophilus* fermented dahi helped to improve body weight gain in rats. Food consumption was found significantly less (80.39 ± 4.16) in group B (Model) compared to group A (normal) (100.57 ± 10.29). All probiotic fed groups (D, E, F and G) exhibited equal food consumption efficiency ratio as shown in Table 3. Food efficiency % was calculated on the basis of weight gain (g) and mean food consumption.

After the rats were killed, fat pad (mesenteric, perirenal, and epididymal white adipose tissues) was separated and weighed. The weight of fat pad was observed to be significantly raised in group B (Model) 3.42 vs. 2.97 g. Group C (Standard) and group G (PH Test dose 2) demonstrated similar weight of fat pad ($p > 0.05$), 2.84 and 2.92 g respectively. Lowest fat pad weight was found to be observed in case of PD Test dose 1 (2.06 g).

Effect of Potential Probiotic on Weights of Various Organs

After the rats were killed, the visceral organs (liver, kidney, spleen, heart, lungs) were collected and weighed as shown in Table 3. The rats had the lowest liver weight in group A (Normal) while significantly highest liver weight in group B (Model). Although administering probiotic dahi alters the liver weight. Group D (*Lact. fermentum* PD2 dose 1) significantly lowered liver weight as compared with the group B (Model). Group E, F, and G also lowered the liver weight, but without statistical significance.

There were no significant difference in the weight of hearts, lungs, and kidneys among the seven groups. Significant

Table 2 Anthropogenic parameters of rats during 28 day

Groups	Weight gain (g)	Food consumption (g/day)	Food efficiency %	Fat pad (g)
A	20.58 ± 2.86 ^d	100.57 ± 10.29 ^a	20.66 ± 2.95 ^e	2.97 ± 1.17 ^b
B	45.48 ± 7.5 ^a	80.39 ± 4.16 ^c	56.47 ± 12.74 ^a	3.42 ± 0.45 ^a
C	14.02 ± 3.46 ^e	79.51 ± 4.17 ^c	17.89 ± 1.16 ^f	2.84 ± 0.71 ^b
D	30.65 ± 2.71 ^b	87.69 ± 3.84 ^b	34.96 ± 3.44 ^b	2.06 ± 0.30 ^e
E	26.77 ± 1.19 ^c	86.19 ± 3.36 ^b	31.50 ± 1.19 ^c	2.70 ± 0.38 ^c
F	24.74 ± 6.72 ^c	86.82 ± 4.44 ^b	28.48 ± 4.56 ^d	2.34 ± 0.41 ^d
G	29.69 ± 4.69 ^b	86.48 ± 3.49 ^b	34.38 ± 7.52 ^b	2.92 ± 0.48 ^b

Values are expressed as Mean ± S.E.M; *Values with different superscripts differ significantly ($p < 0.05$) in each columns

differences between spleen organs weight of group A (Normal) and group B (Model) control was observed, with insignificant changes in treatment groups.

Effect of Potential Probiotic Feeding on Serum Lipid Profile

Reduction of serum total cholesterol (TC) and LDL-C and increase in HDL-C may be fundamental treatment opinion for CVDS. The effect of dietary treatments (probiotic curd) on serum lipid profile (serum total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol) has been recorded in Table 4.

Serum Total Cholesterol (TC)

Blood serum total cholesterol (TC) levels in the seven groups are shown in Fig. 1. Before the experiment, TC showed no significant difference among groups. High-cholesterol diet could significantly increase serum TC levels (462.74 ± 5.62 vs. 72.17 ± 3.2 mg/dl) in group B (Model) compared to group A (Normal). Group C (Standard) administrated with atorvastatin averted the rise in cholesterol levels by bringing 22.95% reduction as compare to group B (Model).

Probiotic test solution (Groups D, E, F and G) showed varying degrees of cholesterol lowering abilities in vivo. At the end of 4 weeks, compared with the group B (Model), both

the strains showed significant ($p < 0.05$) reduction in serum TC levels as 58.58, 57.74, 55.6, and 67.21% in Groups D, E, F, and G, respectively. Thus, groups D, E, and F (PD Test dose 1, 2 and PH dose 1) showed comparable effect on cholesterol reduction, while group G exhibited maximum total cholesterol lowering potential (151.77 mg/dl vs. 462.74 mg/dl of model). In a study of Mohania et al. [11] concerning lipid profile by feeding LaVK2 Dahi on hypercholesterolemic rats, plasma total cholesterol level was decreased by 22.6% when compared to rats fed with buffalo milk.

Serum Triglycerides (TG)

High-fat diet administered group B (Model) showed significant ($p < 0.05$) rise in serum triglyceride levels, 122.35% compared to group A (Normal). Treatment with standard and potential probiotics significantly reduced the triglyceride levels, 66.3%, 65.26%, 50.5%, 70.69%, and 66.21%, respectively, in groups C, D, E, F, and G; compared to group B (Model) as depicted in Fig. 1. There were no significant differences ($p > 0.05$) among groups C (Standard), D (PD test dose 1) F (PH test dose 1), and G (PH test dose 2) as they showed nearly similar effects on serum triglyceride level of rats. Thus, based on the results we can assume, probiotics might be able to lower serum triglyceride level akin to standard cholesterol lowering drugs available in the market (i.e., atorvastatin).

Table 3 Effect of probiotic PD2 and PH5 on weight of visceral organs of rats

Groups	Liver	Kidney	Spleen	Lungs	Heart
A	6.95 ± 0.22	0.93 ± 0.05	0.5 ± 0.04	1.54 ± 0.08	0.86 ± 0.07
B	11.23 ± 0.54#	0.98 ± 0.09	0.8 ± 0.06#	1.6 ± 0.11	0.91 ± 0.04
C	10.73 ± 0.04	0.86 ± 0.02	0.68 ± 0.03	1.66 ± 0.14	0.9 ± 0.07
D	8.03 ± 1.96*	0.83 ± 0.17	0.58 ± 0.12	1.25 ± 0.26	0.7 ± 0.14
E	10.72 ± 0.48	1 ± 0.10	0.9 ± 0.08	1.61 ± 0.18	0.85 ± 0.08
F	9.88 ± 0.63	0.78 ± 0.03	0.76 ± 0.07	1.2 ± 0.04	0.83 ± 0.08
G	10.7 ± 0.08	0.81 ± 0.04	0.7 ± 0.07	1.53 ± 0.02	0.9 ± 0.03

Values expressed are Mean ± S.E.M; * – significantly different from model (B); # – significantly different from normal (A)

Table 4 Serum lipid profile (mg/dl) of rats at the end of feeding period

Groups	Total cholesterol	Triglycerides	HDL-C	LDL-C
A	72.17 ± 3.20 ^e	127.27 ± 11.55 ^b	43.92 ± 1.44 ^b	19.8 ± 4.09 ^f
B	462.74 ± 5.62 ^a	283.37 ± 7.71 ^a	41.95 ± 3.32 ^b	276.74 ± 7.42 ^a
C	356.57 ± 5.83 ^b	95.5 ± 7.89 ^c	66.74 ± 3.33 ^a	184.76 ± 9.91 ^b
D	191.67 ± 9.16 ^c	98.47 ± 7.44 ^c	41.42 ± 2.35 ^b	135.42 ± 7.72 ^c
E	195.59 ± 9.36 ^c	140.27 ± 11.24 ^b	34.96 ± 3.17 ^c	139.14 ± 4.87 ^c
F	205.47 ± 7.41 ^c	83.06 ± 3.9 ^c	45.95 ± 2.64 ^b	116.36 ± 6.34 ^d
G	151.77 ± 4.06 ^d	95.8 ± 5.34 ^c	42.71 ± 2.60 ^b	101.72 ± 2.41 ^e
Anova table				
S.E.M.	4.77	5.86	1.96	4.63
F-Test	*	*	*	*
CD	14.48	17.78	5.94	14.04
%CV	3.54	7.69	7.48	5.76

Values expressed are Mean ± S.E.M.; *Values with different superscripts differ significantly ($p < 0.05$) in each rows and columns

Batish et al. [12] reported 23.26% and 21.09% reduction in serum TC and TG, respectively, while evaluating potential of probiotic strains in SD (Sprague-Dawley)

rats. Further, Singhora et al. [13] reported 20.69% reduction in serum TG level by feeding fermented milk with *Lact. gasseri* Lg70.

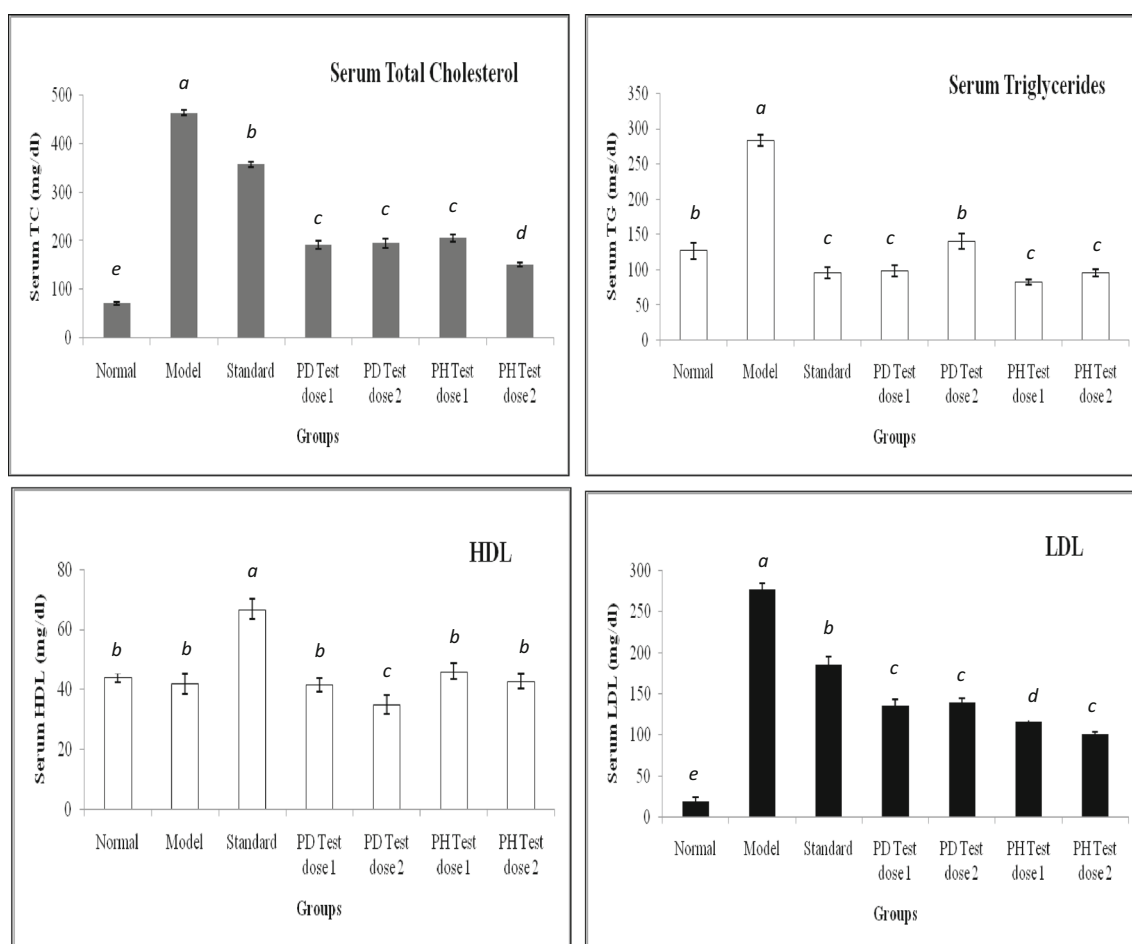


Fig 1 Effect of probiotic on (i) Serum total cholesterol (TC) level; (ii) Serum triglyceride (TG) level; (iii) HDL-C level; and (iv) LDL-C level. *Values with different superscripts differ significantly ($p < 0.05$)

High-Density Lipoproteins (HDL-C)

High-density lipoprotein accumulates surplus cholesterol that cholesterol metabolizing cells cannot utilize. Thus, it's an important factor to control arteriosclerosis. Atorvastatin administered in the group C (Standard) raised the HDL level in serum by 51.95% (highest compared to each group) compared to group B (Model). Moreover, group B (Model) showed 4.49% reduction ($p > 0.05$) when compared with group A (Normal). Furthermore, three groups, D, F, and G, exhibited ($p > 0.05$) no effect compare to group B (Model). Whereas, group E fed with *LACT. fermentum* PD2 showed significant decrease compared to Model and other probiotics (Fig. 1). Thus, treatment with probiotic did not show obvious influences on HDL levels in this study.

Similarly, Tamai et al. [14], Ibrahim et al. [15] and St-Onge et al. [16] did not observe any variation in the level of blood HDL cholesterol in rats or human by feeding fermented milk, milk yogurt, and soy yogurt. *Lact. plantarum* MA2 isolated from Tibetan kefir grains did not influence serum HDL level in rats [17].

Low Density Lipoproteins (LDL-C)

LDL-C is the main component of serum cholesterol inducing hypercholesterolemia. The elevated serum LDL cholesterol levels induced by feeding high-cholesterol diets were reduced in the PD2 and PH5 strains treated groups. Atorvastatin showed significant reduction ($p < 0.05$) 33.24% (184.76 ± 9.91 vs. 276.74 ± 7.42) in LDL level as compared to group B (Model). Potential probiotics (treatment groups D, E, F, and G) demonstrated significant ($p < 0.05$) decline in the LDL levels, 51.07%, 49.73%, 57.96%, and 63.25%, respectively, as compared to the group B (Model). As shown in Fig. 1, we can postulate that both PD Test dose 1 and 2 treated groups (D and E) exhibited comparable effect in LDL reduction 135.42 and 139.14 mg/dl, respectively. PH Test dose 1 (Group F) significantly diminished LDL (116.36 mg/dl vs. 276.74 of Model). Among all probiotic fed groups, PH Test dose 1 (Group G) was able to reduce maximum LDL (101.72 mg/dl) compared to group B (Model) 176.74 mg/dl.

Similarly, supplementation of diet with *Lact. plantarum* LP91 and LP21 resulted in significant reduction in LDL-C values by 38.13 and 21.42%, respectively [12]. In a recent study by Singhora et al. [13], test group which was fed on milk fermented with *Lact. gasseri* Lg70, a 49.54% significant reduction in LDL-C was observed compare to positive control (HD diet) group.

Atherogenic Index

TC/HDL-C and LDL-C/HDL-C ratios are recognized as two important signs of CVDs risk and were reduced in rats

supplemented with LAB [1, 18]. The higher ratios are, the greater the CVD risk is. Therefore, reduction in serum TC, LDL-C, and increase in HDL-C may be an imperative treatment alternative for CVDs.

In the present study, as shown in Fig. 2, AI of the group B (Model) showed sharp increase after 4 weeks of feeding on the hyperlipidemic diet in comparison with that of group A (Normal control). All the four groups fed with probiotic dahi (D, E, F and G) including group C (Standard) exhibited significantly lower TC/HDL-C and LDL-C/HDL-C ratios when compared with hypercholesterolemic control group (Model) proposed their potentiality in cholesterol metabolism. Among all probiotic fed groups, *Lact. fermentum* PH5 fed groups (F and G) exhibited maximum potential to bring down atherogenic index by affecting TC/HDL-C and LDL-C/HDL-C ratios compared to PD2 fed groups (E and F). PH5 Test dose 2 has significantly high ability to reduce TC/HDL-C (3.58 vs. 11.18) and LDL-C/HDL-C (2.35 vs. 6.59). Batish et al. [12] also reported that AI for the probiotic (*Lact. plantarum*) treatment groups [HD91 (2.41) and HD21 (3.10)] decreased significantly when compared with the hypercholesterolemic control group (4.24).

Effect of potential Probiotic feeding on Liver lipid profile

Liver total cholesterol (Liver TC)

Liver TC (Table 5) levels differed significantly among all the seven groups. The liver TC levels of rats fed a high-cholesterol diet group B (Model) had significantly increased ($p < 0.05$) compared to 163.39% with group A (Normal). Standard drug atorvastatin prevented the rise in liver cholesterol levels with reduction of 33.88% (235.4 ± 7.72 vs. 356 ± 9.73) as oppose to the group B (Model).

PD test dose 1 and 2 (Group D and E) administration resulted in decreasing the liver cholesterol level ($p < 0.05$) by 24.97 and 16.34%, respectively. PH Test dose 1 (Group F)

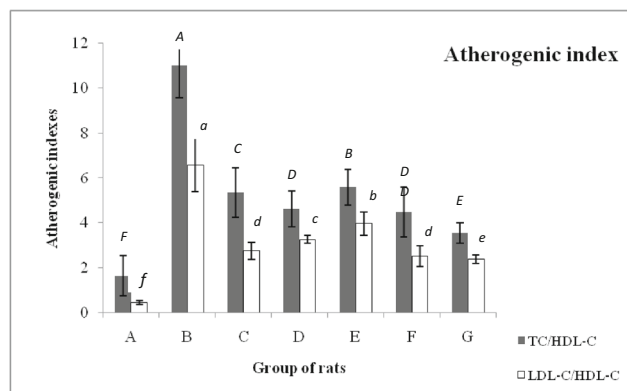


Fig 2 Effect of probiotic on atherogenic index. *Values with different superscripts differ significantly ($p < 0.05$)

Table 5 Liver lipid profile (mg/dl) of rats at the end of feeding period

Groups	Liver TC	Liver TG
A	135.16 ± 8.86 ^e	56.71 ± 7.08 ^d
B	356.00 ± 9.73 ^a	274.07 ± 12.16 ^a
C	235.40 ± 7.72 ^d	135.46 ± 9.5 ^c
D	267.12 ± 15.49 ^c	187.55 ± 8.3 ^b
E	297.85 ± 6.25 ^b	180.04 ± 11.94 ^b
F	273.10 ± 8.01 ^c	159.48 ± 3.77 ^{bc}
G	246.90 ± 7.44 ^d	150.86 ± 11.58 ^c
Anova table		
S.E.M.	6.72	6.81
F-Test	*	*
CD	20.38	20.65
%CV	4.50	7.21

Values expressed are Mean ± S.E.M.; *Values with different superscripts differ significantly ($p < 0.05$) in each column

showed comparable effect with PD Test dose 1 (Group D) as shown in Fig. 3. While, Group C (Standard) treated with atorvastatin and PH Test dose 2 (Group G) showed maximum besides comparable potential to diminish liver cholesterol in rats. Similarly, fermented dairy product isolate NS12 (*Lact. delbrueckii* sp. *bulgaricus*) was reported effective to reduce 34.58% liver cholesterol [8]. The effect of dietary treatments (probiotic curd) on liver lipid profile (serum total cholesterol, triglycerides) has been recorded in Table 5.

Liver Triglycerides (Liver TG)

The group A (Normal) displayed the lowest liver TG level and Model group (B) displayed the highest liver TG level. Atorvastatin treatment resulted in significant decline of 50.58% in the liver triglycerides value compared to group B (Model). All the four experimental groups (HC + PD2) and (HC + PH5) had lower levels of liver TG than HC group ($p <$

0.05), and there was no obvious differences among the four groups. All four experimental groups D, E, F, and G, fed with probiotic dahi, showed decrease in liver triglyceride values 31.57, 34.31, 41.82, and 44.96%, respectively, as shown in Fig. 3. Group C (Standard), and probiotic *Lact. fermentum* PH5 Test dose 2-treated group G demonstrated comparable results in terms of liver triglyceride reduction. The lower level of hepatic cholesterol and triglyceride content could reduce the rate of conversion of LDL to LDL-C particles, and result in the decline in serum LDL-C concentration, that might be another reason for the effects of our two strains to lower LDL-C level.

Supplementation with either of the two strains was effective in reducing serum TC, LDL-C, and TG concentrations in rats compared to those fed the same high-cholesterol diet but without LAB supplementation. In particular, these effects were more evident with *Lact. fermentum* PH5 fed groups. Our results confirm those described in previous reports [1, 8, 11, 12, 19].

Histopathology Analysis

Fig. 4 illustrated the effects of *Lact. fermentum* strains PD2 and PH5 on hepatic steatosis. Hematoxylin-eosin (HE) staining, a semi-quantitative method showed the differences in liver tissue structures and lipid accumulation of all groups.

The rat liver of group A (Normal) had a well-organized structure as there was no fatty vacuolization in liver cells. The liver tissue in the group B (Model) had a moderate degree of vacuolization and increased lipid deposition in the cytoplasm. Hepatocyte steatosis was obviously alleviated by feeding probiotic dahi containing PD2 and PH5 strains compare to group B (Model). The LAB-treated rats exhibited overall normal gross liver appearances but high fatty vacuolization in Model group. Similarly, liver lipid deposition was evaluated in a semi quantitative manner by Xie et al. [2].

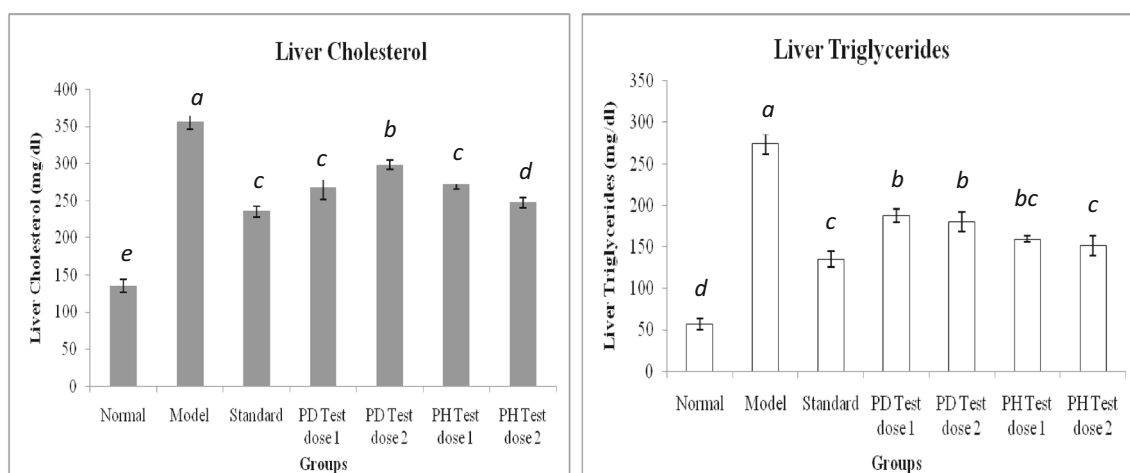
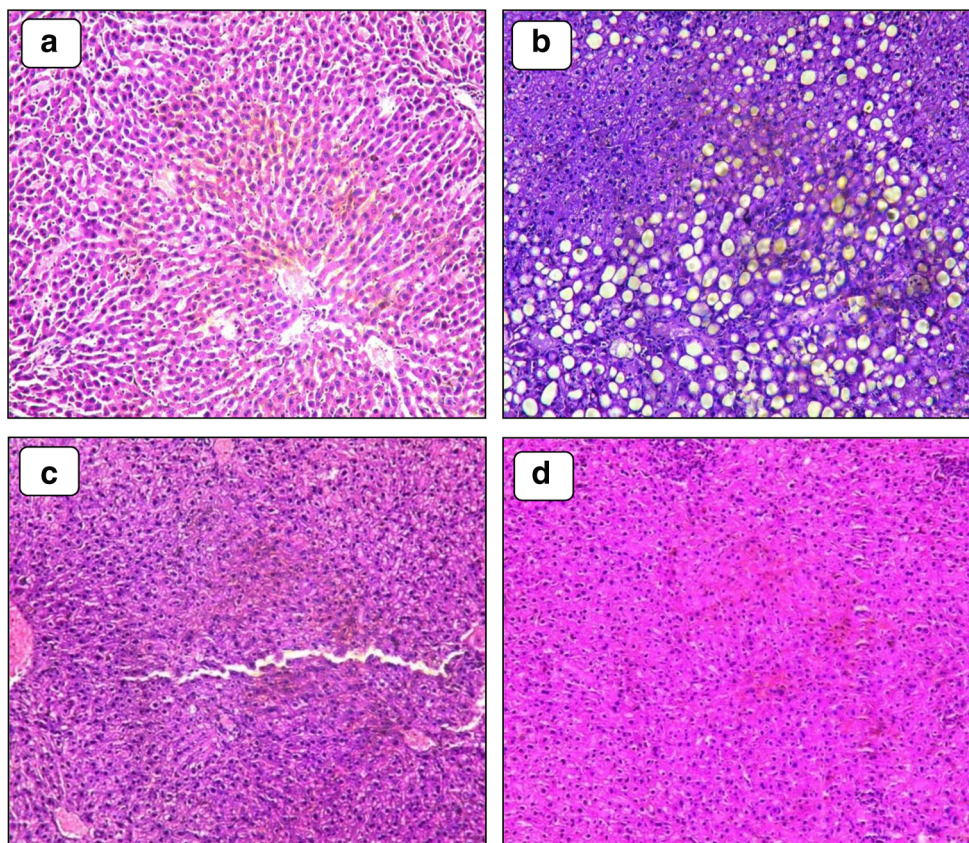


Fig. 3 Effect of probiotic on (i) Liver-TC level, and (ii) Liver-TG level. *Values with different superscripts differ significantly ($p < 0.05$)

Fig 4 Histopathology of liver A: group A (Normal diet); B: group B (Model group-high cholesterol diet); C: High-cholesterol diet + PD2 probiotic dahi; D: High-cholesterol diet + PH5 probiotic dahi



Fecal Sterol Analysis

Many probiotic strains could augment fecal elimination of bile acids through bile acid deconjugation, and this may alter the cholesterol synthesis pathways and resulted in a decrease of serum cholesterol concentration [20]. We found more bile acids released in the feces as well as highest fecal cholesterol especially in those rats fed with PH5 groups (F and G).

PH5 Test dose 1 released highest fecal cholesterol (52.26 mg/day) among other probiotic fed groups (D, E, and F) as well as group C (Standard). Moreover, both PH5-treated groups (F and G) liberated highest bile acid in the feces of rats 30.05 and 30.43 mg/day, respectively followed by group E (23.39 mg/day) and group D (20.18 mg/day). The lower fecal cholesterol and bile acids in the feces of rats of group B (Model) might be due to deposition or absorption of lipids in

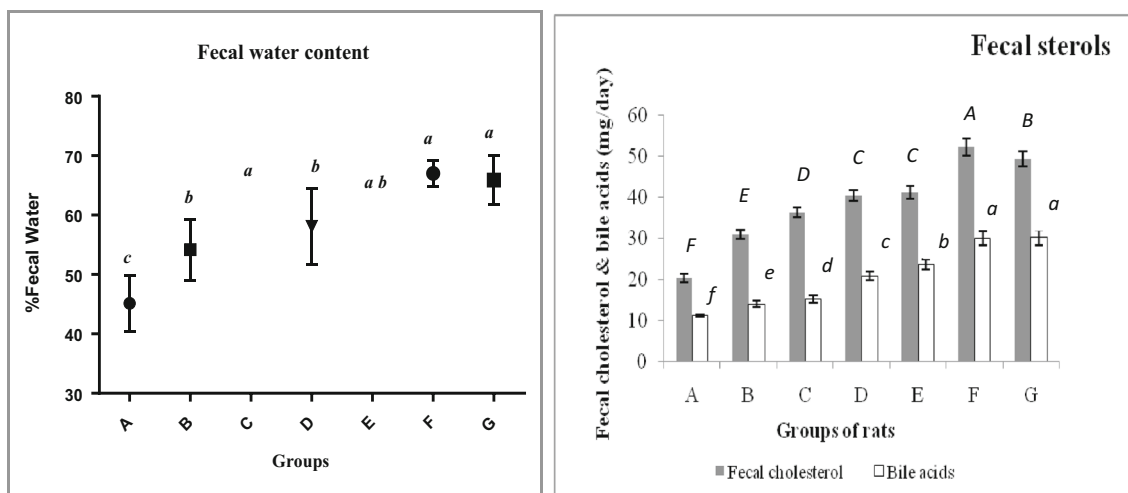


Fig 5 (i) Analysis of fecal water content, (ii) Effect of probiotic on fecal cholesterol and bile acid (4th week). *Values with different superscripts differ significantly (p < 0.05)

tissues as well as blood (Fig. 5). Overall, *Lact. fermentum* PH5 seems to be more effective in fecal cholesterol reduction and liberation of free bile acids through effective bile deconjugation mechanism. Similar experiment was performed by Xie et al. [2] using *Lact. fermentum* M1-16 feeding in hyperlipidemic rats; and reported 30.2% (37.33 mg/day) fecal cholesterol with 19% (19.29 mg/day) fecal cholic acid. Similar observation was reported by Batish et al. [12] as fecal cholic acid excretion increased throughout the experimental period in the probiotic treatment groups HDCap91, HD91 and HD21.

Fecal Water Content Analysis

Since the fecal water content can be exploited as an index of fecal elimination, our observations recommend that these two LAB strains have laxative potential and may stimulate bowel movements. As a result, the transit time for cholesterol absorption in the intestine might also be reduced.

Fecal water content data collected at the fourth week is shown in Fig. 5. However, at the end of the feeding period, highest fecal moisture content was found in *Lact. fermentum* PH5 fed groups (F and G) as 67.01 and 65.88, respectively ($p < 0.05$). The results were comparable ($p > 0.05$) with group C (Standard) who demonstrated 62.1% moisture content in the feces of rats. PD2 Test dose 2 also exhibited 59.72 percent moisture in feces of rats. In a similar study by Xie et al. [2], probiotic solutions having *Lact. plantarum* 9-41-A and *Lact. fermentum* M1-16 exhibited 63% and 62% fecal moisture content, respectively, compare to their Model (53%).

Fecal Microbiological Analysis

Intestinal microbiota plays an important role in modulation of host energy and lipid metabolism [21]. The effects of probiotic lactobacilli on different bacterial counts in feces had been investigated previously by different research groups [22, 23]. The overall mean fecal bacteria counts at 0 and 28 days obtained from different experimental groups are recorded in Table 6 revealed the effects of the different diets and LAB supplementation on the rat intestinal bacterial flora.

The rats fed on *Lactobacillus* strains as a dietary adjunct maintained a high level of lactobacilli (probiotic *Lact. fermentum* PD2 and PH5) in the fecal samples throughout the experimental period compared with the control groups. Total lactobacilli ranged from 8.47 to 9.94 CFU/g of feces in all the experimental groups. We also found Coliforms were significantly increased in high-cholesterol diets fed rats, and PD2 and PH5 strains restored the changes. Total coliforms count ranged from 6.03 to 7.24 CFU/g among all the treatment groups. Thus, increase in the number of fecal lactobacilli indicates their effective colonization in rat intestinal tract besides decrease in coliforms. However, rats supplemented with the diet containing PH dose 1 and 2 (Groups F and G) showed a marginal decrease in fecal *E. coli* counts

Table 6 Effects of probiotic feeding on fecal bacterial population

Treatment groups	Days	Lactobacilli	Coliforms	<i>E. coli</i>
A (Normal)	0	8.47 ± 0.15	6.93 ± 0.05	7.14 ± 0.14
	28	8.95 ± 0.13	6.68 ± 0.08	7.09 ± 0.21
B (Model)	0	8.97 ± 0.03	7.08 ± 0.09	7.74 ± 0.13
	28	8.76 ± 0.12	7.23 ± 0.12	7.82 ± 0.16
C (Standard)	0	8.81 ± 0.13	7.24 ± 0.08	7.76 ± 0.07
	28	9.03 ± 0.24	7.08 ± 0.03	7.56 ± 0.11
D (PD test dose 1)	0	9.29 ± 0.03	6.26 ± 0.13	7.75 ± 0.17
	28	9.94 ± 0.78	6.12 ± 0.02	7.66 ± 0.21
E (PD test dose 2)	0	9.50 ± 0.15	6.10 ± 0.05	7.56 ± 0.05
	28	9.84 ± 0.27	6.04 ± 0.13	7.23 ± 0.13
F (PH test dose 1)	0	9.06 ± 0.04	6.63 ± 0.15	7.48 ± 0.08
	28	9.86 ± 0.09	6.07 ± 0.19	6.89 ± 0.11
G (PH test dose 2)	0	9.20 ± 0.10	6.19 ± 0.07	7.77 ± 0.12
	28	9.45 ± 0.06	6.03 ± 0.04	6.57 ± 0.19

*Values expressed are Mean ± S.E.M

compared with their counts on 0 day. Comparable fecal bacterial counts were obtained by Batish et al. [12] while studying hypercholesterolemia in SD rats using BSH producing *Lact. plantarum*. Hu et al. [8] reported relatively similar data while studying lipid metabolism in *Lactobacillus* strains, i.e., NS5 and NS12.

Conclusion

Present study demonstrated that animals fed with probiotic dahi (i.e., fermented with potential probiotic strains PD2 and PH5) might be useful in reducing hypercholesterolemia induced by the consumption of the atherogenic diet. At the outset, the probiotic dahi reduced plasma cholesterol and triglycerides suggesting that the effects of the probiotic dahi might have commenced at the level of the GI tract, inhibiting absorption of these lipids. In addition to reducing hepatic cholesterol and hepatic triglycerides, aortic tissue lipids were also decreased by ingestion of the probiotic dahi. Furthermore, the indigenous strains have also shown to possess bile salt hydrolase (BSH) activity in vitro and thereby suggesting that this BSH activity could be responsible for lowering the plasma total cholesterol concentration. Our study had some limitations, we used Wistar rats, and the effects observed in animal model may not be exactly the same in human. 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.

Compliance with Ethical Standards

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

Ethical Approval This work was approved by Institutional Animal Ethical Committee (IAEC) of Anand Pharmacy College, Anand, Gujarat, India (Protocol No: APC/2015-IAEC/1504).

References

- Aminlari L, Shekarforoush SS, Hosseinzadeh S, Nazifi S, Sajedianfard J, Eskandari MH (2018) Effect of probiotics *Bacillus coagulans* and *Lactobacillus plantarum* on lipid profile and feces bacteria of rats fed cholesterol-enriched diet. *Probiotics Antimicrob Proteins* 27:1–9
- Xie N, Cui Y, Yin YN, Zhao X, Yang JW, Wang ZG, Fu N, Tang Y, Wang XH, Liu XW, Wang CL (2011) Effects of two *Lactobacillus* strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet. *BMC Complement Altern Med* 11(1):53
- Ashar MN, Prajapati JB (2001) Role of probiotic cultures and fermented milks in combating blood cholesterol. *Indian J Microbiol* 41:75–86
- Thakkar PN (2016) Screening, characterization and probiotic significance of indigenous bacterial isolates. Doctoral dissertation thesis submitted to Department of Life Sciences, University School of Sciences, Gujarat University, Ahmedabad, Gujarat State, India
- Thakkar P, Modi H, Prajapati J (2015) Isolation, characterization and safety assessment of lactic acid bacterial isolates from fermented food products. *Int J Curr Microbiol App Sci* 4(4):713–725
- Thakkar P, Modi H, Dabhi B, Prajapati J (2014) Bile tolerance, bile deconjugation and cholesterol reducing properties of *Lactobacillus* strains isolated from traditional fermented foods. *Int J Fermented Foods* 3(2):157–165
- Shakuto S, Oshima K, Tsuchiya E (2005) Glimepiride exhibits prophylactic effect on atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* 182(2):209–217
- Hu X, Wang T, Li W, Jin F, Wang L (2013) Effects of NS *Lactobacillus* strains on lipid metabolism of rats fed a high cholesterol diet. *Lipids Health Dis* 12:67–78
- Lee DK, Jang S, Baek EH, Kim MJ, Lee KS, Shin HS, Chung MJ, Kim JE, Lee KO, Ha NJ (2009) Lactic acid bacteria affect serum cholesterol levels, harmful fecal enzyme activity, and fecal water content. *Lipids Health Dis* 8:21–29
- Vijayendra SVN, Gupta RC (2012) Assessment of probiotic and sensory properties of dahi and yogurt prepared using bulk-freeze dried cultures in buffalo milk. *Ann Microbiol* 62:939–947
- Mohania D, Kansal VK, Shah D, Nagpal R, Kumar M (2014) Therapeutic effect of probiotic dahi on plasma, aortic and hepatic lipid profile of hypercholesterolemic rats. *J Cardiovasc Pharmacol Ther* 18:490–497
- Batish VK, Kumar R, Grover S (2010) Hypocholesterolemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillus plantarum* strains in Sprague-Dawley rats. *Br J Nutr* 105:561–572
- Singhora G, Neha P, Ravinder KM, Santosh KM (2014) Hypocholesterolemic effect of a *Lactobacillus gasseri* strain Lg70 isolated from breast fed human infant feces. *J Inno Biol* 1(2):105–111
- Tamai Y, Yoshimitsu N, Watanabe Y, Kuwabara Y, Nagai S (1996) Effect of milk fermented by culturing with various of lactic acid bacteria and yeast on serum cholesterol level in rats. *J Ferment Bioeng* 82:181–182
- Ibrahim A, El-sayed E, El-zeini H (2005) The hypocholesterolemic effect of milk yogurt and soy-yogurt containing bifidobacteria in rats fed on a cholesterol-enriched diet. *Int Dairy J* 15:37–44
- St-Onge MP, Farnworth ER, Savard T, Chabot D, Mafu A, Jones PJ (2002) Kefir consumption does not alter plasma lipid levels or cholesterol fractional synthesis rates relative to milk in hyperlipidemic men: A randomized controlled trial. *BMC Complement Altern Med* 2:1–7
- Wang Y, Xu NV, Xi A, Ahmed Z, Zhang B, Bai X (2009) Effects of *Lactobacillus plantarum* MA2 isolated from Tibet kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. *Appl Microbiol Biotechnol* 84:341–347
- Lampka M, Grąbczewska Z, Jendryczka-Maćkiewicz E, Hołyńska-Iwan I, Sukiennik A, Kubica J, Halota W, Tyrakowski T (2010) Circulating endothelial cells in coronary artery disease. *Pol Heart J* 68(10):1100–1105
- Nguyen T, Kang JH, Lee MS (2007) Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *Int J Food Microbiol* 113:358–361
- Park YH, Kim JG, Shin YW, Kim SH, Whang KY (2007) Effect of dietary inclusion of *Lactobacillus acidophilus* ATCC 43121 on cholesterol metabolism in rats. *J Microbiol Biotechnol* 17(4):655–662
- Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S, Felin J, Perkins R, Borén J, Orešič M, Bäckhed F (2010) The gut microbiota modulates host energy and lipid metabolism in mice. *J Lipid Res* 51(5):1101–1112
- Usman HA (2000) Effect of administration of *Lactobacillus gasseri* on serum lipids and fecal steroids in hypercholesterolemic rats. *J Dairy Sci* 83:1705–1711
- Liong MT, Shah NP (2006) Effects of *Lactobacillus casei* symbiotic on serum lipoprotein, intestinal microflora and organic acids in rats. *J Dairy Sci* 89:1390–1399

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.