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ORIGINAL ARTICLE

Milk fermented with probiotic strains *Lactobacillus rhamnosus* MTCC: 5957 and *Lactobacillus rhamnosus* MTCC: 5897 ameliorates the diet-induced hypercholesterolemia in rats

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Abstract

The current study was intended to investigate the cholesterol-lowering potential of the two *Lactobacillus rhamnosus* probiotic strains, LR 5957 and LR 5897, isolated from 'dahi'. Cholesterol-lowering ability of both strains was determined in in vitro conditions. For in vivo investigations, the Wistar rats were randomly assigned into five groups and treated with different diets: standard diet (SD), high-cholesterol diet (HCD), HCD with Milk, HCD with LR 5957–fermented milk, and HCD with LR 5897–fermented milk. After 3 months of feeding, different parameters of hypercholesterolemia were measured in blood, feces, liver, and kidney. Both the strains, LR 5957 and LR 5897, showed the ability to grow in the presence of cholesterol and eliminate the cholesterol under in vitro conditions. In vivo results indicate that consumption of probiotic-fermented milk has significantly reduced the HCD-induced body weight, hyperlipidemia, and hepatic lipids (total cholesterol and triacylglycerol). Further, increased cholesterol excretion in feces was also observed in probiotic-fed groups. The studied fermented milk also helped to maintain healthy liver and kidney by increasing the antioxidant activities and decreasing the lipid peroxidation. Consumption of probiotic-fermented milk also found to decrease the mRNA expression of the inflammatory markers TNF-α and IL-6 in the liver. Overall, our results indicate that the *L. rhamnosus* strains, LR 5957 and LR 5897, are two potential probiotic strains that can ameliorate the diet-induced hypercholesterolemia.

Keywords Probiotics · Hypercholesterolemia · Probiotic-fermented milk · *Lactobacillus rhamnosus*

Introduction

Hypercholesterolemia is a common cause of concern for all humans since it constitutes a high risk factor for cardiovascular diseases (CVD) such as atherosclerosis, diabetes, and hypertension (Gielen and Landmesser 2014). Hypercholesterolemia constitutes abnormally high levels of total cholesterol (TC), low-density lipoprotein (LDL)-cholesterol, and triacylglycerol (TGs), and low levels of high-density lipoprotein (HDL)-cholesterol in the blood and blood vessels. Long-term cholesterol consumption causes dysregulated lipid metabolism and leads to hypercholesterolemia. Excess lipid accumulation in the body arouses the activity of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Coppack 2001) which plays a crucial role in the development of oxidative stress, which further triggers lipid peroxidation, tissue injury, and liver cirrhosis. In healthy subjects, enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione-related enzymes protect tissues from oxidative stress (Yoo et al. 2013). However, excessive consumption of cholesterol adversely affects above antioxidation mechanism and augments oxidative processes in the tissues causing DNA, protein, and lipid damage.

Excess dietary intake of cholesterol also leads to accumulation of cholesterol in blood vessels and causes atherosclerosis and associated diseases such as coronary heart disease and peripheral vascular disease. As treatment strategy, statins are widely used to normalize the elevated circulating cholesterol levels and can reduce CVD-related events. But, due to their potential association with adverse side effects such as liver damage and carcinogenicity, their safety has been questioned for a long time (Levine et al. 1995). Therefore, high mortality

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rates associated with CVD and increasing safety concerns associated with current treatment methods demand an alternative therapeutic strategy to combat CVD. Government policies favoring dairy industry in India besides growing awareness and rising preferences of consumers for probiotic usages have generated a great deal of attention towards probiotic foods. Many recent studies led to renewed interest in probiotic bacteria as a potential supplementation tool in combating CVD and associated complications. The cholesterol-lowering efficacy of probiotic bacteria is highly strain-specific. Probiotics were shown to attenuate hypercholesterolemia by rebalancing the blood lipid profile (Xiao et al. 2003), increasing the cholesterol excretion in feces (Salaj et al. 2013), promoting the activities of antioxidative defense system (Yoo et al. 2013), and decreasing the pro-inflammatory signaling cascade (Dai et al. 2012) in hypercholesterolemic subjects.

Lactobacillus rhamnosus MTCC: 5957 (LR 5957) and *Lactobacillus rhamnosus* MTCC: 5897 (LR 5897) were originally isolated in our lab from locally fermented milk product in India called “dahi” and characterized for their probiotic attributes in previous studies (Sharma et al. 2014; Kemgang et al. 2016). LR 5957 was studied for its ability to induce mucosal and systemic compartments of immune system (Kemgang et al. 2016). Whereas, LR 5897–fermented milk has been proved to resist immunosenescence during aging along with oxidative stress in the liver and red blood cells (Sharma et al. 2014). LR 5897 was also studied for its potential to alleviate allergic responses in newborn mice during weaning transition when fed as fermented milk to mothers and their babies (Saliganti et al. 2015). Considering the above probiotic attributes of these strains, in the present study, we aimed to explore the antihypercholesterolemic potential of LR 5957– and LR 5897–fermented milk in the Wistar rats.

Material and methods

Source and maintenance of probiotic cultures

The two indigenous probiotic strains, *Lactobacillus rhamnosus* MTCC: 5957 (LR 5957) and *Lactobacillus rhamnosus* MTCC: 5897 (LR 5897), used in present study were isolated from locally fermented milk product called “dahi” and characterized earlier for their probiotic attributes, immunomodulatory, and antioxidative properties (Sharma et al. 2014; Kemgang et al. 2016). Cultures were stored at − 80 °C in MRS Broth supplemented with 20% glycerol and propagated twice in MRS Broth prior to use. Before each experiment, purity and morphological identification of lactobacilli were confirmed microscopically by gram-positive and negative staining.

In vitro cholesterol removal activity

The effect of cholesterol on the growth of lactobacilli cultures was investigated according to Liong and Shah (2005). Briefly, probiotics were cultured in MRS Broth supplemented with cholesterol and 0.3% oxgal for 18 h in aerobic conditions. An aliquot of 2 mL of spent MRS media was taken from bacterial culture at every 2 h interval during culturing. Thereafter, bacterial growth was measured by spectrophotometer at 600 nm and the effect of cholesterol on bacterial growth was evaluated by plotting optical density and time interval on Y and X axis respectively. The cholesterol assimilating ability of growing, resting, and dead LR 5957 and LR 5897 bacterial cells was assessed by following the methods of Kimoto et al. (2002) and Liong and Shah (2005) with slight modifications. The cholesterol concentration in MRS was determined using commercial enzymatic kit, Span Diagnostics Pvt. Ltd., Surat, India, as per the standard protocol recommended by the company. The percentage of cholesterol reduction in broth was calculated comparing with control. Each result was the average of three independent assays.

$$\text{Cholesterol reduction \%} = [1-(\text{residual cholesterol in cell free broth})/(\text{cholesterol in control broth})] \times 100$$

Preparation of probiotic-fermented milk for treatment groups

Lactobacillus strains, LR 595 7 and LR 5897, were subcultured (1% *v*/*v*) in sterilized skim milk at 37 °C/18 h. Probiotic-fermented milk (PFM) was prepared by inoculating fresh milk (2.5% fat) with 1% activated LR 5957 and LR 5897 culture in skim milk followed by incubation at 37 °C/18 h. As a control, uninoculated sterilized milk was simultaneously incubated under similar conditions. The number of bacteria in the fermented milk was determined by plate counting on MRS agar plates after aerobic incubation at 37 °C/48 h. Milk was used as a vehicle because it is a convenient way of probiotic consumption in India.

Experimental design and feeding schedule

Six-week-old male Wistar rats ($n = 30$) approximately of similar body weight (BW), 155 g, were procured from the small animal house of ICAR-National Dairy Research Institute, Karnal, Haryana, India, to conduct the experiments. Rats were housed in polypropylene cages under controlled conditions of temperature (24 ± 1 °C), humidity (55 ± 5%), and light (12-h light/dark). After 2 weeks of adaptive period on standard diet,

30 rats were randomly assigned to five groups of six rats each and fed as follows:

1. SD group maintained on standard diet
2. HCD group maintained on high-cholesterol diet
3. HCD + milk group maintained on high-cholesterol diet supplemented with milk (2 mL/animal/day)
4. HCD + LR 5957group maintained on high-cholesterol diet supplemented with LR: 5957–fermented milk (2×10^9 cfu/animal/day in 2 mL PFM)
5. HCD + LR 5897 group maintained on high-cholesterol diet supplemented with LR: 5897–fermented milk (2×10^9 cfu/animal/day in 2 mL PFM).

The animals were maintained on 15 g standard diet (SD) or high-cholesterol diet (HCD) per day for 3 months. The components of SD and HCD diet are prepared and mixed according to AOAC (1990) as shown in Table 1. The fat source used for the preparation of the standard chew diet was refined soybean oil obtained from a commercial supplier (Fortune, Adani Wilmar Limited, India). A single lot of soybean oil was used for the whole experiment. The cellulose used in the diet was of analytical grade (Product code 025791) and obtained from Central Drug House Pvt. Ltd., India.Water was provided ad libitum and replaced daily throughout the 3 months of experimental feeding. The complete experimental design was shown in Fig. 1.

Effect of probiotic fermented milk on serum lipid profile

Blood samples were collected from the orbital venous plexus, using a capillary tube from overnight-fasted rats at regular intervals. At the end of the experiment, all fasting experimental rats were sacrificed and blood was collected from the heart by cardiac puncture. Serum was collected by centrifuging the blood samples at 4000×*g* for 10 min at 4 °C and was then stored at − 80 °C. Serum parameters including TC, TG, and HDL-C levels were determined using enzymatic colorimetric kits as per instructions of the manufacturer (Span Diagnostics Pvt. Ltd., Surat, India).

LDL-C and very low-density lipoprotein-cholesterol (VLDL-C) were calculated according to Friedewal's equation (Friedewald et al. 1972).

$$\text{LDL-C} = [\text{TC}- \text{HDL-C}—(\text{TGs}/5)]$$
$$\text{VLDL-C} = \text{TG}/5$$

Atherogenic index (AI) was calculated according to the method described by Liu et al. (1999) and expressed as:

$$\text{AI} = (\text{TC—HDL-C})/\text{HDL-C}$$

Coronary artery risk index (CRI) was calculated using the following formula (Boers et al. 2003).

$$\text{CRI} = \text{TC}/\text{HDL-C}$$

Effect of probiotic-fermented milk on feces

At 30 days of interval, feces were collected in a sterile tube gently stimulating the rectal part and were processed within 60 min of collection for enumeration of fecal bacteria and cholesterol concentration.

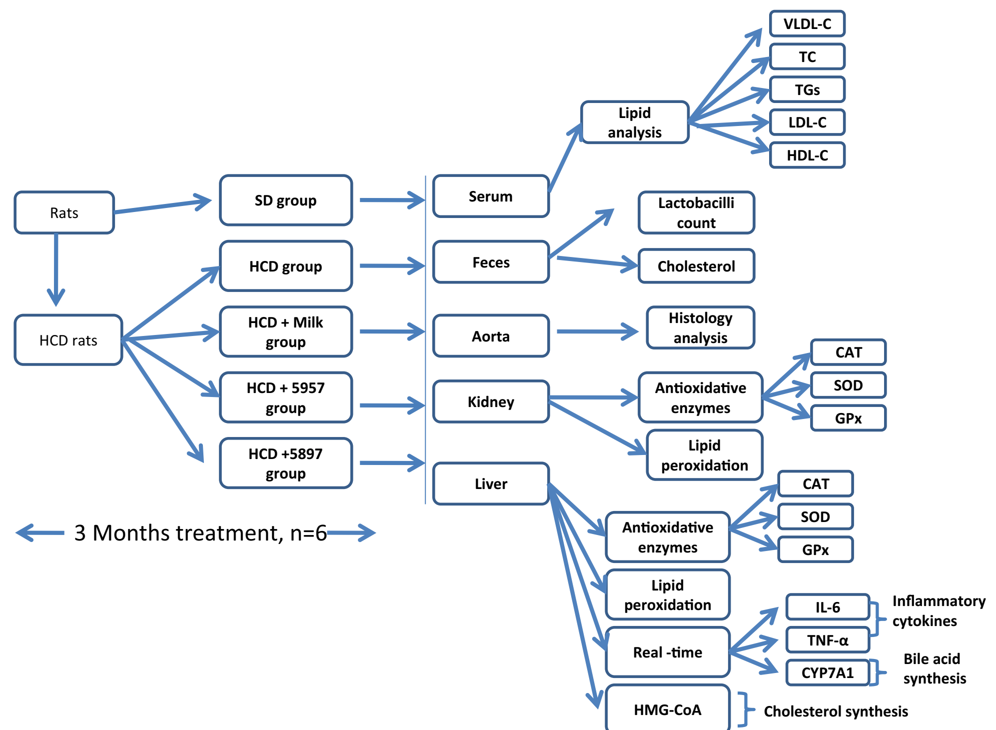
Feces were homogenized in sterile PBS (pH 7.2), serially diluted, followed by plating of appropriate dilutions (10^6–10^8) on MRS agar for fecal bacteria count on MRS. The plates were incubated at 37 °C for 24–48 h. The colonies were subjected to morphological and biochemical analysis.

The feces were homogenized in chloroform methanol (2:1) mixture in accordance with the method described by Folch et al. (1957). Amount of cholesterol excreted in feces was estimated by using enzymatic colorimetric kits as per instructions of the manufacturer (Span Diagnostics Pvt. Ltd., Surat, India). The kits were based on enzymatic colorimetric methods.

Table 1 Composition of standard diet and high-cholesterol diet (g/100 g ratio)

S. no.	Component	Standard diet (SD)	High-cholesterol diet (HCD)
1	Starch	53.200	51.325
2	Casein	20.000	20.000
3	Sucrose	10.000	10.000
4	Soybean oil	7.000	7.000
5	Cellulose	5.000	5.000
6	Vitamin mixture	1.000	1.000
7	Mineral mixture	3.500	3.500
8	Methionine	0.300	0.300
9	Cholesterol	–	1.500
10	Sodium cholate	–	0.375

Fig. 1 Experimental design



Effect of probiotic-fermented milk consumption on liver parameters

At the end of the experiment, liver tissues were carefully removed, washed with phosphate buffer saline, blotted dry, and processed for biochemical measurements. One gram of liver tissue was homogenized in 10 volume of ice-cold phosphate buffer (50 mM, pH − 7.4), using a homogenizer. The liver homogenate was centrifuged at 3000×*g* at 4 °C for 15 min, and the supernatant was analyzed for antioxidative enzyme activities, lipid peroxidation, and HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) activity. Total proteins in liver tissue were estimated by using the method described by Lowry et al. (1951). For estimation of hepatic TC and TGs, liver tissue was homogenized in 2 mL of chloroform:methanol (2:1) mixture in accordance with the method described by Folch et al. (1957). For gene expression, liver tissue was stored in RNA later (Sigma, USA) at − 80 °C until use.

The antioxidant activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were determined in the liver. CAT activity was measured spectrophotometrically by analyzing the rate of H_2O_2 decomposition at 240 nm (Aebi 1984). One unit of CAT corresponds to degradation of 1 μmol of H_2O_2 per min. SOD activity was measured according to the method of Marklund and Marklund (1974), and the unit activity was defined as the amount of enzyme that causes 50% inhibition of pyrogallol autoxidation under experimental conditions. GPx activity was assayed by measuring the rate of oxidation of NADPH, using cumene hydroperoxide as a substrate (Paglia and Valentine 1967). The enzyme activity was calculated using an extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}, and 1 unit was defined as 1 mmol of NADPH oxidized per min. Results are expressed as units/mg of total protein for liver enzymes.

Lipid peroxidation was assayed by monitoring thiobarbituric acid reactive substances (TBARS) level using previously described method (Kaushal and Kansal 2012).

HMGR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) activity in the liver was estimated by HMG-CoA Reductase Assay Kit (Sigma-Aldrich, USA).

Hepatic lipids including TC and TG levels were determined using enzymatic colorimetric kits as per instructions of the manufacturer (Span Diagnostics Pvt. Ltd., Surat, India).

The gene expression of CYP71A (cholesterol 7 alpha-hydroxylase) and pro-inflammatrory cytokines (TNF-α, IL-6) was measured in liver tissue.

Total RNA was isolated from liver tissue using TRIzol reagent (Sigma-Aldrich, USA). The quality of isolated RNA was analyzed by agarose gel electrophoresis (in 1.5% 80 V for 1 h). RNA was quantified by NanoQuant, Infinite M200Pro, Tecan. Purity of RNA was assessed based on readings at 260/280 nm, and the samples with acceptable purity (i.e., ratio 1.8–2.0) were quantified and used for reverse transcription. One microgram of total RNA was used to prepare cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). The prepared cDNA was stored at − 20 °C until further use. Quantitative real-time PCR (ABI PRISM 7700 Sequence

Detection System, Applied Biosystems) was used to assess the relative expression of CYP7A1, TNF-α, and IL-6 using the SYBR Green method. Primers used in the present study were as follows:

β-actin: Forward- CGTGGGCCGCCCTAGGCACCA, and Reverse- TTGGCCTTAGGGTTCAGGGGGG; (Ewaschuk et al. 2007)
TNF-α: Forward: ATGAGCACAGAAAGCATGATC, Reverse- TACAGGCTTGTCACTCGAATT; (Ewaschuk et al. 2007)
IL-6: Forward- CACAAAGCCAGAGTCCTTCAGAG and Reverse- CTAGGTTTGCCGAGTAGATCTC; (Ewaschuk et al. 2007)
CYP7A1: Forward- ATCTTGGCATGGCCCTGA and Reverse- GAGCATCTCCTGCCTCTC (Kumar et al. 2013). Amplification condition included initial denaturation at 95 °C for 10 min, followed by annealing temperature at 60 °C for 30 s. Amplification was carried out for 45 cycles. β-actin was used for normalization. The ΔΔCT method was used to calculate the relative mRNA expression of genes.

Effect of probiotic-fermented milk on oxidative status of kidney

Kidney tissues were carefully removed, washed with phosphate buffer saline, blotted dry, and processed for biochemical measurements. A total of 1 g tissue was homogenized in 10 volumes of ice-cold phosphate buffer (50 mM, pH − 7.4), using a homogenizer. Kidney homogenate was centrifuged at 3000×*g* at 4 °C for 15 min, and the supernatant was subjected to further analysis for the antioxidative enzyme activities and lipid peroxidation as described above for the liver. Total protein in kidney tissue was estimated by the method described by Lowry et al. (1951).

Statistical analysis All experimental data were presented as the mean ± SEM. ANOVA test was performed to determine the effect of significance followed by the Tukey test using GraphPad Prism5.0. Significance was acknowledged at a minimum $P < 0.05$.

Results

Probiotic growth and their cholesterol assimilation ability

The growth rates of LR 5957 and LR 5897 cultured in MRS supplemented with and without cholesterol at different time points were presented in Fig. 2a. The optical density of probiotic suspension grown on cholesterol at 600 nm after 18 h was 1.68 and 1.69 for LR 5957 and LR 5897 respectively, and the values get slightly decreased to 1.66 and 1.65 in the absence of cholesterol. But overall, both cultures have inherent mechanisms to grow on cholesterol without any significant effect on growth rate. Both the growing cultures were also found to be successful in their in vitro cholesterol-lowering ability (Fig. 2b). Specifically, after 18 h of incubation, LR 5957 and LR 5897 removed 62.2 ± 2.45% and 59.8 ± 2.31% of cholesterol respectively from MRS Broth by their cholesterol assimilating property. Resting cells of LR 5957 and LR 5897 have

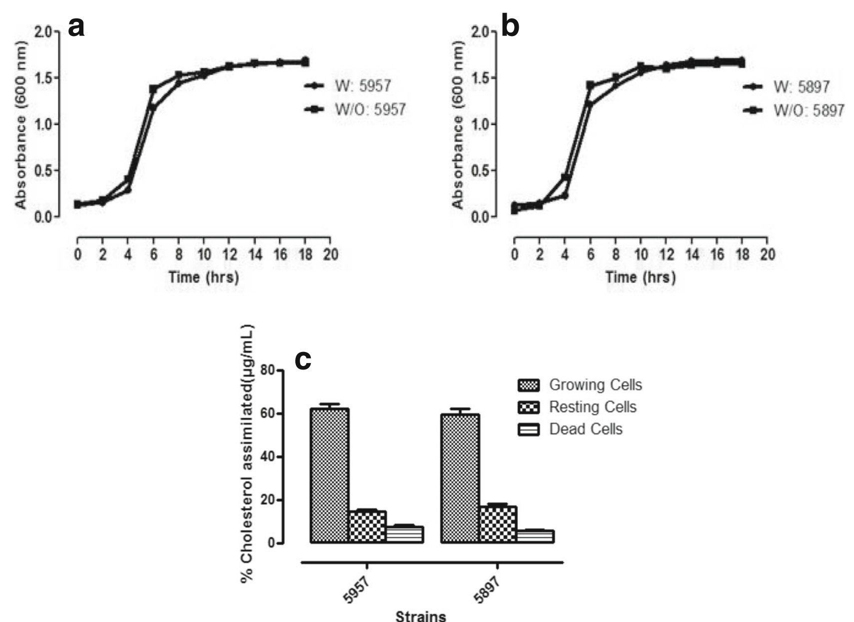


Fig. 2 Growth profiles of LR5957 (**a**) and LR5897 (**b**) in medium containing cholesterol (w), without cholesterol (W/O) and cholesterol removal by growing, resting, and dead LR5957 and LR5897 (**c**)

also found to remove the cholesterol, i.e., 14.7 ± 0.74% and 17.1 ± 1.03% respectively but much less than growing cells. Although, dead cells were also found to remove cholesterol from broth but the cholesterol removal ability was lowest among all studied growth phases, and it was found to be 7.5 ± 0.79% and 5.8 ± 0.29% for LR 5957 and LR 5897 respectively.

Effect of probiotic-fermented milk on body weight in hypercholesterolemic rats

Body weight of all the experimental rats was noted down 1 day before the start of experiment (Table 2), and the average body weight was 150 g. As the treatment period goes forward, body weight increased steadily in each group but the increase was more pronounced in HCD-fed groups. The body weight significantly increased in HCD-fed group compared with control group (41.1% increase vs control group) was observed by the end of 90th day, but it was significantly less in LR 5957 (21.2%) and LR 5897 (21.7%) PFM groups as compared to HCD treatment group whereas no obvious significant change was observed in group fed milk alone compared with HCD group.

Effect of probiotic-fermented milk on serum lipid profile, AI, and CRI in hypercholesterolemic rats

Serum lipid profile, AI, and CRI were estimated four times in the blood samples obtained from all the experimental rats in the total duration of the study with 30 days interval (Table 3). Serum TC, TG, HDL-C, LDL-C, and VLDL-C levels among different groups did not differ on 0 day, and estimated values were in the range of 66.15–89.93, 80.57–99.13, 29.26–34.56, 25.16–39.62, and 16.53–19.75 mg/dL respectively (Table 3). Rats fed on SD have showed no significant difference in serum lipid levels during the entire study period. But, in rats fed on HCD, the increase in serum lipid (TC, TGs, LDL-C and VLDL-C) levels were dramatic on the 30th day, which further increased on 60th and 90th day of experiment. On the 90th day, compared with the SD group, the HCD group showed 2.4-fold increase in TC, 1.3-fold increase in TGs, and nearly 6-fold increase in LDL-C. However, administration of PFM to HCD rats significantly attenuated the elevated levels of serum lipids as compared with HCD-fed group. On 90th day, serum TC, TGs, and LDL-C were significantly reduced in PFM-fed groups by 1.33-, 1.42-, and 1.43-fold in LR 5957 and 1.53-, 1.50-, and 1.8-fold in LR 5897, respectively as compared with HCD group. However, the group which was fed milk along with HCD showed a significant reduction only in serum TG levels (1.24-fold) compared to HCD group. These findings are very important, as LDL-C is the chief culprit in coronary heart disease. On the contrary, both the probiotics LR 5957 and LR 5897 successfully restored the HDL-C levels up to 1.23- and 1.31-fold respectively compared to HCD group. But, no such effects were seen in milk-fed group. Since serum VLDL-C values in all experimental groups were 1/5 part of TGs, therefore the trend in VLDL-C levels was similar to TGs level.

The effect of fermented milk on AI and CRI are shown in Table 3. There was a 6.4-fold increase in atherogenic index (AI) and 4.1-fold increase in coronary artery risk index (CRI) in HCD rats compared with SD rats. Milk and fermented milk with LR 5957 and LR 5897 significantly reduced AI and CRI values than those of HCD group. Administration of LR 5957–fermented milk significantly causes 1.7-fold and 1.6-fold reduction in the AI and CRI, whereas administration of LR 5897–fermented milk causes 1.6-fold and 1.7-fold significant reductions in the AI and CRI respectively. Milk administration also significantly decreased AI and CRI by 1.3-fold in HCD rats.

Effect of fermented milk on feces in hypercholesterolemic rats

The effects of probiotics on fecal bacteria count on MRS and cholesterol excretion in feces are shown in Table 4.

At 0 day, the colony-forming unit on MRS of all groups ranged from 8.5 to 8.9 log cfu/g. The colony-forming unit on MRS in SD group remained constant, while progressive decrease was seen in HCD-fed animals as the days progressed. On 90th day of experimental period, the colony-forming unit on MRS significantly decreased by 1.3-fold in HCD group compared with the SD group while the colony-forming unit on MRS was restored in different treatment groups by 1.35-fold in both PFM groups (LR 5957 and LR 5897) and by 1.2-fold in milk group as compared to HCD-fed group.

Table 2 Effect of PFM on body weight

BW(g)	SD	HCD	HCD + MILK	HCD + 5957	HCD + 5897
0 day	144.2 ± 3.67	156.7 ± 6.24	159.5 ± 6.05	153.3 ± 6.18	156.2 ± 5.02
30th day	181.0 ± 4.45	224.5 ± 10.58$^{\#}$	225.5 ± 6.09	216.0 ± 14.94	217.2 ± 4.55
60th day	243.5 ± 12.57	313.3 ± 9.23$^{\#\#\#}$	288.2 ± 12.05	270.8 ± 8.70*	269.0 ± 7.16*
90th day	272.2 ± 7.53	384.0 ± 7.64$^{\#\#\#}$	344.0 ± 13.37	302.5 ± 9.92**	300.5 ± 15.24***

Data expressed as mean ± SEM ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs HCD group

Table 3 Effect of PFM on serum lipid profile

Serum lipids (mg/dL)	Days	SD	HCD	HCD + MILK	HCD + 5957	HCD + 5897
TC	0	Initial range: 66.1–89.9 mg/dL				
	30	80.2 ± 1.76	162.1 ± 3.47$^{###}$	136.1 ± 7.00	122.1 ± 8.39**	110.1 ± 7.85***
	60	75.1 ± 4.29	173.8 ± 6.16$^{###}$	150.5 ± 12.81	134.1 ± 8.15**	115.0 ± 6.24***
	90	78.4 ± 3.66	188.2 ± 5.01$^{###}$	165.5 ± 9.76	141.1 ± 4.23***	122.5 ± 5.66***
HDL-C	0	Initial range: 29.2–34.5 mg/dL				
	30	30.7 ± 0.78	15.5 ± 0.88$^{###}$	17.4 ± 0.30	18.5 ± 0.40	18.8 ± 0.40*
	60	32.2 ± 0.67	18.8 ± 1.69$^{###}$	19.5 ± 0.70	21.1 ± 0.94	24.0 ± 1.39*
	90	32.6 ± 0.87	18.8 ± 0.84$^{###}$	21.4 ± 1.07	23.2 ± 2.01*	24.7 ± 1.34*
TGs	0	Initial range: 80.5–99.1 mg/dL				
	30	97.3 ± 4.25	128.9 ± 8.11$^{###}$	119.7 ± 3.31	110.8 ± 3.66	106.6 ± 4.21*
	60	105.8 ± 5.71	147.4 ± 6.90$^{###}$	123.2 ± 3.59*	112.3 ± 4.23***	104.5 ± 1.83***
	90	124.2 ± 7.88	168.8 ± 4.30$^{###}$	135.3 ± 3.59**	118.9 ± 5.29***	112.4 ± 3.91***
VLDL-C	0	Initial range: 16.5–19.7 mg/dL				
	30	19.4 ± 0.85	25.7 ± 1.62$^{###}$	23.9 ± 0.66	21.56 ± 0.94	22.17 ± 0.73*
	60	21.1 ± 1.14	29.4 ± 1.38$^{###}$	24.6 ± 0.71	23.48 ± 1.03***	22.46 ± 0.84***
	90	24.8 ± 1.57	33.6 ± 0.87$^{###}$	27.0 ± 0.72**	23.43 ± 1.01***	23.78 ± 1.05***
LDL-C	0	Initial range: 25.16–39.62 mg/dL				
	30	30.0 ± 1.92	120.8 ± 3.04$^{###}$	94.64 ± 7.11	81.4 ± 8.54**	70.0 ± 8.43***
	60	21.7 ± 3.53	125.5 ± 6.44$^{###}$	106.3 ± 12.90	90.4 ± 8.64*	70.0 ± 6.46**
	90	20.9 ± 4.71	135.7 ± 4.84$^{###}$	106.9 ± 8.39	94.3 ± 4.48***	75.2 ± 5.38***
AI	0	Initial range – 1.4 to 1.8				
	30	1.6 ± 0.05	9.5 ± 0.67$^{###}$	6.7 ± 0.35**	5.6 ± 0.52**	4.8 ± 0.44***
	60	1.3 ± 0.14	8.4 ± 0.67$^{###}$	6.7 ± 0.72	5.6 ± 0.52**	3.8 ± 0.39***
	90	1.4 ± 0.10	9.0 ± 0.41$^{###}$	6.7 ± 0.38**	5.3 ± 0.58***	3.9 ± 0.28***
CRI	0	Initial range − 2.4 to 2.8				
	30	2.6 ± 0.05	10.6 ± 0.67$^{###}$	7.7 ± 0.35**	6.6 ± 0.52***	5.8 ± 0.44***
	60	2.3 ± 0.14	9.4 ± 0.67$^{###}$	7.7 ± 0.72	6.4 ± 0.52**	0.9 ± 0.39***
	90	2.4 ± 0.10	10.0 ± 0.41$^{###}$	7.7 ± 0.38**	6.3 ± 0.586***	4.9 ± 0.28***

Data expressed as mean ± SEM ($n = 6$). $^{#}P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$ vs SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs HCD group

In addition, the feeding of cholesterol for 90 days to the experimental rats resulted in progressive increase in cholesterol excretion in feces (4-fold vs SD group). Cholesterol excretion increased significantly by 1.7-fold and 1.8-fold in fermented milk groups as compared to HCD group.

Table 4 Effect of PFM on fecal bacterial count on MRS and cholesterol

Fecal parameters	Days	SD	HCD	HCD + milk	HCD + 5957	HCD + 5897
Fecal bacterial count on MRS	0	Initial range: 8.5–8.9 log cfu/g				
	30	8.8 ± 0.03	7.8 ± 0.02$^{###}$	8.9 ± 0.02**	9.0 ± 0.03***	9.0 ± 0.00***
	60	8.8 ± 0.06	7.6 ± 0.07$^{###}$	8.5 ± 0.01***	9.1 ± 0.02***	9.1 ± 0.02***
	90	8.9 ± 0.03	6.7 ± 0.02$^{###}$	8.7 ± 0.02***	9.1 ± 0.04***	9.1 ± 0.02***
Fecal cholesterol	0	Initial range: 2.7–9.6 mg/dL				
	30	5.7 ± 0.96	13.2 ± 1.64$^{#}$	19.6 ± 2.04	21.2 ± 0.96*	23.3 ± 0.82**
	60	5.3 ± 1.21	18.1 ± 2.74$^{##}$	27.3 ± 1.93	29.5 ± 1.68*	29.6 ± 2.96*
	90	5.2 ± 0.71	21.5 ± 1.50$^{#}$	30.5 ± 3.06	38.3 ± 4.36*	38.9 ± 5.38*

Data expressed as mean ± SEM ($n = 6$). $^{#}P < 0.05$, $^{##}P < 0.01$, and $^{###}P < 0.001$ vs SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs HCD group

Effect of probiotic-fermented milk on different liver parameters

Effect of fermented milk on different liver parameters is shown in Fig. 3.

After 90 days of treatment, the activities of liver antioxidative enzymes (CAT, SOD, GPx) were performed and the results indicate the decrease in activities of CAT, SOD, and GPx by 2.3-, 2.7-, and 3.3-fold in animals of HCD group compared to SD group (Fig. 3). But, animal fed on HCD along with fermented milk with LR 5957 and LR 5897 showed a significant increase in CAT activity by 2- and 2.1-fold, SOD activity by 2- and 1.6-fold, and GPx activity by 2.2- and 2.6-fold respectively as compared to HCD group. No significant differences were observed in the case of the group fed with milk along with HCD.

In the case of lipid peroxidation (Fig. 3d), rats showed a significant reduction in levels of TBARS by 1.7-, 2.1-, and 1.2-fold in the groups fed with milk, PFM with LR 5957, and PFM with LR 5897, respectively.

TC and TG levels (Fig. 3e, f) were significantly elevated in the liver of HCD-fed experimental rats by 3.2- and 2.7-fold compared with SD group. This increase in TC was inhibited by supplementation of PFM with LR 5957 and PFM with LR 5897 TC by 2.0 and 1.9 respectively; TGs were decreased by 1.4-fold in both PFM with LR 5957 and PFM with LR 5897 compared with HCD-fed group.

The effect of HCD consumption on the activity key regulator of cholesterol biosynthesis, HMG-CoA, in the liver was determined in experimental rats and shown in Fig. 3g. The enzyme activity was significantly decreased in HCD group by 1.8-fold compared to SD group; however, 3-fold decrease in the activity was observed in probiotic treatment groups as compared to HCD group. Although the activity was found to decrease in milk group, the effect was not significant as compared with HCD group.

Estimation of hepatic mRNA expression of CYP7A1, the rate-limiting enzyme of bile acid synthesis expression (Fig. 3h) showed that CYP7A1 mRNA expression is significantly increased in HCD-fed group compared with SD group. PFM significantly ($P < 0.05$) decreases mRNA expression compared to HCD group.

The gene expression analysis of proinflammatory cytokines (TNF-α and IL-6) was estimated in the liver (Fig. 3i, j). TNF-α and IL-6 content was elevated significantly in HCD group by 5.7-fold and 4-fold respectively compared to SD

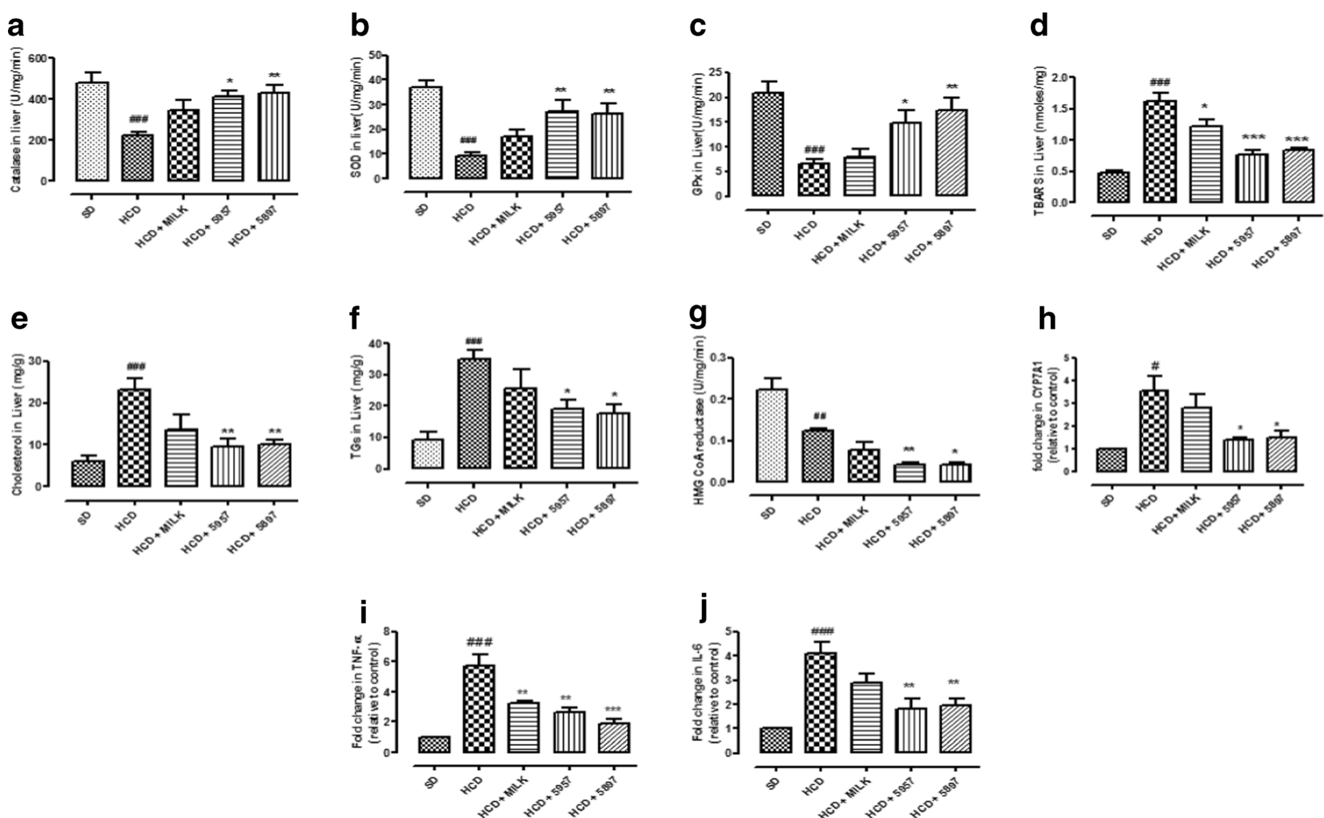


Fig. 3 Effect of milk and probiotic-fermented milk on different liver parameters, i.e., catalase (CAT) (**a**); superdisoxide dismutase (SOD) (**b**); glutathione peroxidase (GPx) (**c**); lipid peroxidation (**d**); hepatic lipids, i.e., total cholesterol and triacylglycerol (TGs) (**e** and **f**); HMG-CoA enzyme activity (**g**); mRNA expression of CYP7A1 (cholesterol 7 alpha-hydroxylase) (**h**); TNF-α (tumor necrosis factor alpha) (**i**); and IL-6 (Interleukin-6) (**j**) in rats. Data expressed as mean ± SEM ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs HCD group

group; whereas, milk and PFM significantly decreased TNF-α levels in the liver compared to HCD group. However, the significant decrease in IL-6 was observed only in probiotic-fermented milk. These results suggest that probiotic-fermented milk inhibit the production of inflammatory cytokines in the liver and thereby reduced inflammation.

Effect of fermented milk on antioxidative enzyme activities and lipid peroxidation in kidney of hypercholesterolemic rats

As shown in Fig. 4a–c, significant decrease in CAT, SOD, and GPx activities by 2.1-, 4-, and 3.1-fold respectively and increase in lipid peroxidation by 4-fold were observed in HCD treated rats as compared to SD rats. Both probiotic LR 5957 and LR 5897 supplementation showed a significant elevation in CAT activity (1.1-fold) compared to HCD group. However, in the case of SOD, only LR 5957 showed significant 1.3-fold increase in activity compared to HCD group. There was no statistically significant increase in GPx activity in milk and PFM treatment groups compared to HCD group. Further, significant decrease was observed in total lipid peroxidation in all supplementation groups, and the effects were more pronounced in the rats that received PMF and results are shown in Fig. 4d.

Discussion

Several epidemiological, clinical, genetic and animal studies showed that elevated level of cholesterol in blood is correlated with increased risk of cardiovascular diseases. Hypercholesterolemia is one of the major risk factors contributing to the severity of many cardiovascular diseases and remains a primary cause of death worldwide (Gielen and Landmesser 2014). The Wistar rats have been selected for the study, and hypercholesterolemia was created by feeding high-cholesterol diet containing 0.375% sodium cholate in addition to 1.5% cholesterol (Kalyan et al. 2018). As natural health remedy, probiotics have gained special attention to treat elevated cholesterol in body due to their cost effective and safety properties (Huang et al. 2013). Among probiotics, *Lactobacillus* and *Bifidobacterium* species have been well studied for their hypolipidemic effects in animal and human subjects. Choi and Chang (2015) showed that LAB strains can remove the cholesterol in vitro at different growth stages. We have observed similar results as both the lactobacilli strains, LR 5957 and LR 5897, successfully removed the cholesterol in vitro in different growth stages (growing, resting, dead). Increase in body weight is one of the major complications observed in hypercholesterolemia. Probiotics have shown to be effective in such conditions. A 4-week study showed that supplementation of VSL#3 reduced weight gain in healthy young men consuming a high-fat and high-energy diet (Osterberg et al. 2015). Similarly in another study, researchers have showed that *Lactobacillus rhamnosus,* CGMCC1 3724, helps obese women to achieve sustainable weight loss (Sanchez et al. 2014). In our study, we have observed similar kind of phenotype in rats fed with LR 5957– and LR 5897–fermented milk compared to HCD. This indicates the successful hypocholesterolemic effects of strains investigated.

To lower the incidences of CVD, it is important to reduce the serum/plasma cholesterol levels in hypercholesterolemic patients. In current study, the serum TC, TG, LDL and HDL concentrations were significantly reduced upon feeding fermented milk which was not observed in other feeding groups. Michael et al. (2017) showed that probiotics can lower cholesterol due to the presence of bile salt hydrolase activity. Zhang et al. (2017) showed that probiotic-fermented soymilk with mixture of *Bifidobacterium bifidum*, *Lactobacillus casei*, and *L. plantarum* remarkably reduced serum TC, TG, and LDL and increased HDL level in mice fed on HFD for 6 weeks. Al-Sheraji et al. (2012) found an increased HDL levels induced by consumption of yoghurt, which was fermented with B. *pseudocatenulatum* G4 and B. longum BB536. Oral administration of *L. fermentum* RS-2 increases intestinal lactobacilli count and reduces coliform count in streptozotocin-induced diabetic rats (Kumar et al. 2017). In current study, we have analyzed lactobacilli in rat feces. It demonstrated that the lactobacilli counts were decreased in

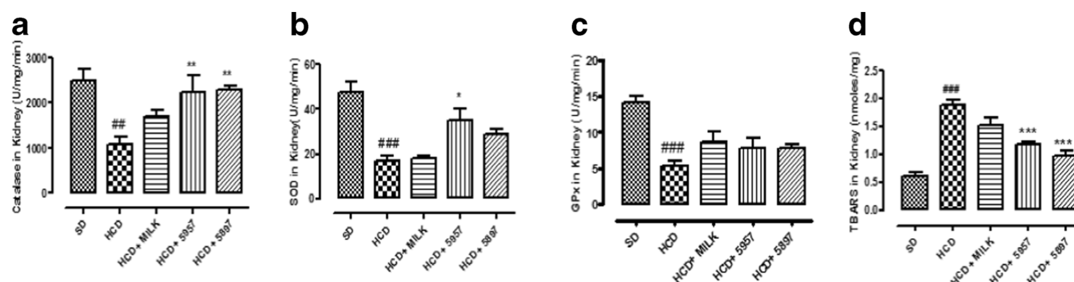


Fig. 4 Effect of milk and probiotic fermented milk on kidney parameters, i.e., catalase (CAT) (**a**), superdisoxide dismutase (SOD) (**b**), glutathione peroxidase (GPx) (**c**), and lipid peroxidation (**d**) in rats. Data expressed as mean ± SEM ($n = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs HCD group

HCD-fed group, implying that the high-cholesterol diet interferes with the intestinal microbiota. But feeding of fermented milk product has successfully ameliorated the number of lactic acid bacterial in the intestine and exerted beneficial effects. One plausible mechanism by which probiotic bacteria can influence plasma cholesterol levels is assimilation of cholesterol onto the plasma membrane of bacteria or cell walls, and then excreted via feces, which results in the inhibition of cholesterol resorption in intestine. Similar kind of observation was reported by Huang et al. (2013). They showed that *Lb. plantarum* Lp09 and Lp45 strains facilitate increasing amount of cholesterol excretion in feces and thus causes in lowering cholesterol level in blood. Various other studies also demonstrated that probiotic administration increases fecal lipid excretion in mice fed on high-fat diet (Yonejima et al. 2013). In current study, we also found that LR 5957 and LR 5897 supplementation increases significant excretion of cholesterol in feces in rats fed on cholesterol diet. Probiotics have also been shown to lower cholesterol levels by regulating its metabolism. Generally, cholesterol biosynthesis and bile acid synthesis are the two pathways that can describe the cholesterol metabolism in the liver. A well-known regulatory enzyme, HMG-CoA reductase, in the cholesterol synthesis pathway catalyzes the synthesis of mevalonate from HMG-CoA (Goldstein and Brown 1990) and is regulated at the posttranscriptional level. A hypercholesterolemic diet decreases cholesterol biosynthesis in the liver as high levels of circulatory cholesterol inhibit HMG-CoA reductase expression (Brown and Goldstein 1986). A similar kind of result was noticed in the present study that supports that the supplementation of the standard diets with cholesterol had an inhibitory effect on the HMG-CoA reductase activity. This negative effect of HCD was significantly ameliorated by probiotic supplementation. The other enzyme, cholesterol 7 α-hydroxylase, is the rate-limiting step in bile acid synthesis. Its transcription and activity are increased by endogenous and dietary cholesterols. Kumar et al. (2013) showed that dietary cholesterol supplementation upregulated CYP7A1 mRNA expression resulted in rats fed on HCD and which was found downregulated in *L. rhamnosus* GG plus aloe vera gel–treated group. This change could represent a compensatory mechanism used to maintain cellular cholesterol levels. As expected, ingestion of a HCD resulted in obvious elevations of hepatic cholesterol and TG contents in HCD group and eventually resulted in an unhealthy liver (Liu et al. 2017). The reduction in liver cholesterol and TG content in PFM groups proved that the cholesterol was reduced, not redistributed between blood and the liver. Supportingly, a recent study showed that LP96 supplementation reduces liver TC and TG in rats fed on high-cholesterol diet compared with the model group (Liu et al. 2017). These observations further indicated that serum lipid levels in probiotic-fed groups were actually reduced by decreasing the metabolism or absorption of intestinal lipids rather than just being redirected from blood to the liver. Our results further indicate that strong anti-inflammatory- and antioxidative-enhancing activity of the two probiotics could potentially ameliorate the tissue damage associated with hypocholesterolemia. The activities of antioxidative enzymes (CAT, SOD, and GPx) and levels of TBARS were imbalanced in rats fed only on HCD. A recent study by Kumar et al. (2017) reported the role of probiotics in restoring the activity of antioxidative enzyme which may protect against the oxidative stress developed in streptozotocin diabetic rats. TNF-α and IL-6 are potent pro-inflammatory cytokine produced by macrophages/monocytes. Previous studies have indicated that probiotics inhibited cell inflammatory signaling intestinal epithelium (Menard et al. 2004; Yan et al. 2007). Zhang et al. (2017) reported that soymilk fermented with probiotic mixture reduced expression of TNF-α and reduces hepatic inflammation. Hung et al. (2016) also reported that probiotic NTU101 decreases the mRNA expression of TNF-α and IL-6. The present study supports this conclusion.

Summary and conclusion

In current study, we analyzed the hypocholesterolemic effects of two *Lactobacillus rhamnosus* probiotic strains, LR 5957 and LR 5897. The initial in vitro assays preliminarily showed the cholesterol removal activity of LR 5957 and LR 5897 in MRS Broth under different growth stages. In in vivo investigations, we observed that consumption of LR 5957– and LR 5897–fermented milk significantly reduced different characteristics of hypercholesterolemia including HCD-induced body weight, hyperlipidemia, and hepatic lipids. Probiotics also increase cholesterol excretion in feces. The studied fermented milk helped to maintain healthy liver and kidney by modulating the oxidative and inflammatory responses. Overall, consumption of milk fermented with *Lactobacillus rhamnosus* strains, LR 5957 and LR 5897, may help to cure the diet-induced hypercholesterolemia.

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Compliance with ethical standards

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

Ethical statement The study was approved by the Institute Animal Ethical Committee (IAEC) (IAEC No. 101/16 dated 21.04.2016) of Indian Council of Agriculture Research-National Dairy Research Institute.

Informed consent was obtained from all individual participants included in the study.

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