Probiotics and Antimicrobial Proteins (2019) 11:509–518
https://doi.org/10.1007/s12602-018-9429-4

Consumption of Probiotic *Lactobacillus fermentum* MTCC: 5898-Fermented Milk Attenuates Dyslipidemia, Oxidative Stress, and Inflammation in Male Rats Fed on Cholesterol-Enriched Diet

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Published online: 13 May 2018
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Abstract

There is a growing and alarming prevalence that increased serum cholesterol is closely related to increased cardiovascular disease risk. Probiotic consumption could be a safe and natural strategy to combat. Therefore, we sought to examine the cholesterol-lowering potential of co-supplementation of probiotic bacteria *Lactobacillus fermentum* MTCC: 5898-fermented buffalo milk (2.5% fat) in rats fed cholesterol-enriched diet. Male Wistar rats were divided into three groups on the basis of feed, viz. group 1, fed standard diet (SD); group 2, fed cholesterol-enriched diet (CED); and group 3, fed cholesterol-enriched diet along with *L. fermentum* MTCC: 5898-fermented milk (CED+LF) for 90 days. At the endpoint, significantly higher levels of serum total cholesterol, low-density lipoprotein cholesterol, triacylglycerols, very low density lipoprotein cholesterol, atherogenic index, coronary artery risk index, hepatic lipids, lipid peroxidation, and mRNA expression of inflammatory cytokines (TNF-α and IL-6) in the liver while significantly lower levels of serum high-density lipoprotein cholesterol and anti-oxidative enzyme activities, catalase, superoxide dismutase, and glutathione peroxidase in the liver and kidney were observed in the CED group compared to the SD group. Compared to the CED group, these adverse physiological alterations were found significantly improved in the CED+LF group. Hence, this study proposes that *L. fermentum* MTCC: 5898 is a potential probiotic bacteria that can be consumed to tackle hypercholesterolemia.

Keywords Cardiovascular diseases · Probiotic-fermented milk · Cholesterol · Oxidative stress · Inflammation

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Introduction

Elevated level of body cholesterol is a devastating problem that can trigger a number of cardiovascular diseases (CVDs) and has been considered as the primary causes of death worldwide [1]. A recent report of the World Health Organization (WHO) [2] revealed that 17.5 million people have died from CVDs in 2012, representing 31% of all global deaths. The CVD-associated morbidity is expected to increase up to 23 million by 2030 in spite of the availability of various drug therapies. Moreover, various side effects have been reported due to drug therapies such as myalgia and muscle weakness, liver dysfunction, kidney failure, and risk of new onset of diseases [3, 4] which promoted the need of natural therapeutics like probiotics. Probiotics are "live microorganisms" that, when administered in adequate amounts, confer a health benefit on the host [5]. There has lately been a great interest in probiotics as food supplements. Various studies, examining the consumption of probiotic-fermented milk, have confirmed

different health benefits of probiotics such as decrease in lactose intolerance [6], decrease in the severity of diarrhea [7], anti-inflammatory [8], and ability to counteract alcohol-induced liver injury [9]. A number of studies have further identified different potential probiotics for minimizing the risk of CVDs and lowering the cholesterol level [10] and serum lipid levels in animal [11] and human [12].

Lactobacillus fermentum MTCC: 5898 (*L. fermentum* MTCC: 5898) used for this study was originally isolated from breast-fed human infant feces and characterized for its standard probiotic attributes such as acid resistance, bile tolerance, cell surface hydrophobicity, and adhesion to caco-2 cells [13]. *L. fermentum* MTCC: 5898 supplementation has been shown to augment healthy aging, alleviating immunosenescence and enhancing anti-oxidative enzyme activities in aging mice. Its supplementation showed enhanced neutrophil functions in aging mice. *L. fermentum* MTCC: 5898 consumption found to inhibit systemic infection by resisted invading pathogen in all organs and peritoneal fluid, in aging mice [13]. Hence, considering its widespread health benefits, we have used same probiotic strain to explore its hypocholesterolemic effects. At first, in vitro cholesterol lowering through bsh activity assay by using MRS media added sodium taurodeoxycholate (TDCA) and the cholesterol-lowering test by using MRS broth supplemented with cholesterol. Further hypocholesterolemic effect of *L. fermentum* MTCC: 5898-fermented milk was studied in vivo in rats fed cholesterol-enriched diet.

Material and Methods

In Vitro Bile Salt Hydrolyzing and Cholesterol Removal Activity Measurement

Firstly, the bsh (bile salt hydrolase gene) positive nature of probiotic *L. fermentum* MTCC: 5898 was analyzed using bacterial 9RNA, which is converted to cDNA and amplified using *L. fermentum* MTCC: 5898 by polymerase chain reaction. The primer sequences used were forward: CCGAGCAACACTTT GTCTTGT and reverse: AAAAGTCCGGGGTGAAGTCT.

After confirmation of expression of bsh gene, the ability of *L. fermentum* MTCC: 5898 to hydrolyze bile salt was assessed by qualitative direct plate assay as reported by Ahn et al. [14]. Next, the effect of cholesterol on the growth of *Lactobacillus* culture was investigated according to Liong and Shah [15]. Briefly, *L. fermentum* MTCC: 5898 was cultured in MRS supplemented with cholesterol (70 μg/mL) and 0.3 g/mL oxgal for 18 h. An aliquot of 2 mL of spent MRS media was taken from bacterial culture at every 2-h interval during culturing. Then, the bacterial growth was measured at 600 nm using spectrophotometer and the effect of cholesterol on bacterial growth was evaluated by plotting optical density and time interval on Y and X axis, respectively. Next, the cholesterol assimilating ability of growing, resting, and dead *L. fermentum* MTCC: 5898 was assessed by following the method of Liong and Shah [15] with slight modifications. The cholesterol concentration in MRS was determined using commercial enzymatic kits Span Diagnostics Pvt. Ltd., Surat, India, as per the standard protocol recommended by the company.

Preparation of Probiotic-Fermented Milk

Buffalo milk was collected from cattle yard of the ICAR-National Dairy Research Institute, Karnal, India, and fat percentage of milk was adjusted to 2.5%. The fat percentage was determined by Gerber method [16]. Buffalo milk was centrifuged at 2800×*g* for 30 min maintaining 4 °C to separate milk fat from whole milk. Further, fat in milk is adjusted to 2.5% by adding skim milk to the whole milk and this calculation is according to the Pearson's square method. Probiotic-fermented milk (PFM) was prepared by inoculating 1% of activated *L. fermentum* MTCC: 5898 (in skim milk) into the fresh milk (2.5% fat) followed by incubation at 37 °C for 18 h. The number of bacteria in the fermented milk was determined by plate counting on MRS agar plates after aerobic incubation at 37 °C for 48 h.

Animals and Experimental Design

Six-week-old male Wistar rats approximately of similar body weight (BW), 155 g, were procured from small animal house of ICAR-National Dairy Research Institute, Karnal, India, to conduct the experiments. The rats were housed in polypropylene cages (2 rats in each cage) 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, the rats were randomly divided into three groups of 6 rats each and fed as follows:

SD group maintained on standard diet; CED group maintained on cholesterol-enriched diet; and CED+LF group maintained on cholesterol-enriched diet supplemented with PFM prepared with *L. fermentum* MTCC: 5898. 2 mL PFM containing 2×10^9 cfu was offered to each rat per day at 9:00 AM every day and allowed the rats to consume it voluntarily. Later, after consumption of PFM, the animals were resumed on the experimental diet (CED). During the night, all groups of animals were provided water ad libitum, but it was removed at least 3 to 4 h before feeding their respective diets (SD and CED) in the morning. The food bowls were also removed from the cages each night. This ensured that animals felt an adequate urge for food and water in the morning, when experimental diets were supplied. Also, animals were trained to obtain food/fermented milk from feeding plates/bowls.

Diet Composition

The animals were maintained on 15 g of SD (Table 1) or CED (Table 1) for 3 months. Vitamin and mineral mixture were prepared and mixed according to AOAC [17]. Water was provided ad libitum and replaced daily throughout the three months of experimental feeding. Body weight was recorded at 30 days interval. On the 90th day, animals were sacrificed and organs (liver, spleen, adipose tissue, and kidney) were collected and weighed.

Assay for Serum Lipids

Blood samples were collected from the orbital venous plexus, using a capillary tube from overnight fasted rats at regular intervals of 30 days during 90 days of experimental period. At the end of the experimental period, 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 total cholesterol (TC), triglycerides (TGs), and high-density lipoprotein cholesterol (HDL-C) levels were determined using enzymatic colorimetric kits as per instructions of the manufacturer (Span Diagnostics Pvt. Ltd., Surat, India). Low-density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) in serum were calculated according to Friedewald's equation [18].

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

Atherogenic index (AI) in serum was calculated according to the method described by [19] and expressed as:

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

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

S. No.	Component	Standard diet	Cholesterol-enriched diet
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

* compostion of vitamin mixture as per AOAC, 1990 [17]

** compostion of mineral mixture as per AOAC, 1990 [17]

Coronary artery risk index (CRI) in serum was calculated using the following formula [20].

$$\mathrm{CRI} = \mathrm{TC}/\mathrm{HDL}$$

Assay for Hepatic Lipids (TC and TGs) and Fecal TC

After the sacrifice, the liver was removed, rinsed with physiological saline solution, blotted dried with filter paper, and weighed. The liver (TC and TGs) and fecal TC were extracted according to Folch method [21]. TC and TG levels in the liver and TC levels in feces were determined using enzymatic colorimetric kits as per instructions of the manufacturer (Span Diagnostics Pvt. Ltd., Surat, India).

Assay for Anti-oxidative Enzyme Activities in the Liver and Kidney

Catalase Assay

The method of Aebi [22] was followed to estimate the activity of catalase (CAT) enzyme. The enzyme activity in the sample was calculated using extinction coefficient of 0.0394 mM cm^{-1} and expressed as micromoles of H_2O_2 consumed per milligram per minute or Units per milligram per minute at absorbance 240 nm. Protein concentration was estimated by Lowry method [23].

Superoxide Dismutase Assay

The superoxide dismutase (SOD) activity was assayed by the method of Marklund and Marklund [24]. The inhibition of pyrogallol auto-oxidation by SOD at 420 nm is a measure of enzyme activity in this method. The amount of enzyme that inhibited the auto-oxidation of pyrogallol by 50% is defined as one unit of enzyme activity. Protein concentration was estimated by Lowry method [23].

Glutathione Peroxidase Assay

The glutathione peroxidase (GPx) was assayed spectrophotometrically by the method of Paglia and Valentine [25]. The method uses excess of glutathione reductase that couples the rate of oxidation of NADPH to reaction of the peroxidase with H_2O_2 and glutathione (reduced). The oxidation of NADPH was monitored by measuring the change in absorbance at 340 nm with time. The enzyme activity was calculated using extinction coefficient of 6.22 mM cm^{-1}, where one unit enzyme activity is 1 mmol of NADPH oxidized per minute or Units per milligram per minute. Protein concentration was estimated by Lowry method [23].

Lipid Peroxidation Analysis

Lipid peroxidation was assayed by monitoring the levels of thiobarbituric acid reactive substances (TBARS) as described by Kaushal and Kansal [26].

Gene Expression Analysis

The gene expression of proinflammatory 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 (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 analyze the mentioned gene expression using the SYBR Green method. Primers used in the present study were β-actin: forward: CAAGTTCAAGCTCAACAAGTCTG and reverse: GAAGTCCACCTCGTTGTCCT; TNF-α: forward: GGTACAAGTCCAAGTTTGCTG and reverse: TCCAGAGACTCGTTAGTCCC; and IL-6: forward: GCAGCCAACAAGAACAATGAC and reverse: TCCTCTAGCTCCCTCATCTG. The applied qPCR condition includes 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. The $2^{-\Delta\Delta C_T}$ method by Livak and Schmittgen [27] was used to calculate the relative mRNA expression of genes and represented as fold change by using the following equations.

$$\Delta C_{T\text{control group}} = C_{T\text{gene of interest}} - C_{T\text{housekeeping gene}}$$

$$\Delta C_{T\text{treatment group}} = C_{T\text{gene of interest}} - C_{T\text{ housekeeping gene}}$$

$$\Delta\Delta C_T = \Delta C_{T\text{gene of interest (treatment group)}} - \Delta C_{T\text{gene of interest (control group)}}$$

$$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

where

C_T Threshold cycle
ΔC_T Difference between threshold cycles
$2^{-\Delta\Delta C_T}$ Fold difference in mRNA abundance

Statistical Analysis

All experimental data were presented as the mean ± SEM. ANOVA test was performed to determine the effect of significance followed by Tukey test to find out the significance of each effect level using GraphPad Prism5.0. Values of $P < 0.05$ were considered statistically significant.

Results

Identification of Bile Salt Hydrolyzing Activity

The expression of bsh gene in *L. fermentum* MTCC: 5898 was analyzed by PCR amplification using species-specific primers. A clear single band with the molecular size of 171 bp in Fig. 1a indicated the presence of bsh gene in *L. fermentum* MTCC: 5898. After confirmation of bsh gene expression, agar plate assay was performed to qualitatively analyze the bsh activity. A clear precipitation zone was observed surrounding the bacterial colonies (Fig. 1b) in MRS agar supplemented with 0.5 g/mL concentration of sodium taurodeoxycholate (TDCA). It indicates that the *L. fermentum* MTCC: 5898 can deconjugate the TDCA. Further, probiotics showed the ability to grow and remove the cholesterol in cholesterol-supplemented MRS broth (Fig. 1c). Specifically, it has removed 71.5, 33.7, and 8.6% cholesterol from MRS broth in growing, resting, and dead cell stages, respectively (Fig. 1d).

Growth of Rats

The effect of different experimental diets is shown in Fig. 2(A). All the rats appeared healthy throughout the feeding period. Rats fed with the cholesterol-enriched diet exhibited significant gain ($P < 0.05$) in body weight compared to rats fed on SD. There was decrease in weight gain in rats fed fermented milk, but the decrease was not statistically significant in comparison to rats fed CED diet only.

Effect of PFM on Organ Weight in CED Rats

Weight of collected organs is shown in Fig. 2(B). The rats in the SD group were found to have the lowest liver, kidney, and epididymal fat levels whereas in the CED group, a significant increase in weight of the liver ($P < 0.001$), kidney ($P < 0.01$), and epididymal fat ($P < 0.05$) was seen compared to SD rats. The probiotic-fed group successfully maintained the epididymal fat levels unlike the CED group. The weight of cecum was increased significantly ($P < 0.01$) in the probiotic-fed group compared to the CED group. No change in spleen weight was observed in different experimental groups.

Effect of PFM on Lipid Profile in CED Rats

Estimated serum TC, HDL-C, LDL-C, TGs, and VLDL-C levels in the three groups are shown in Fig. 3a. Rats fed on CED showed dramatic increase in the levels of serum TC,

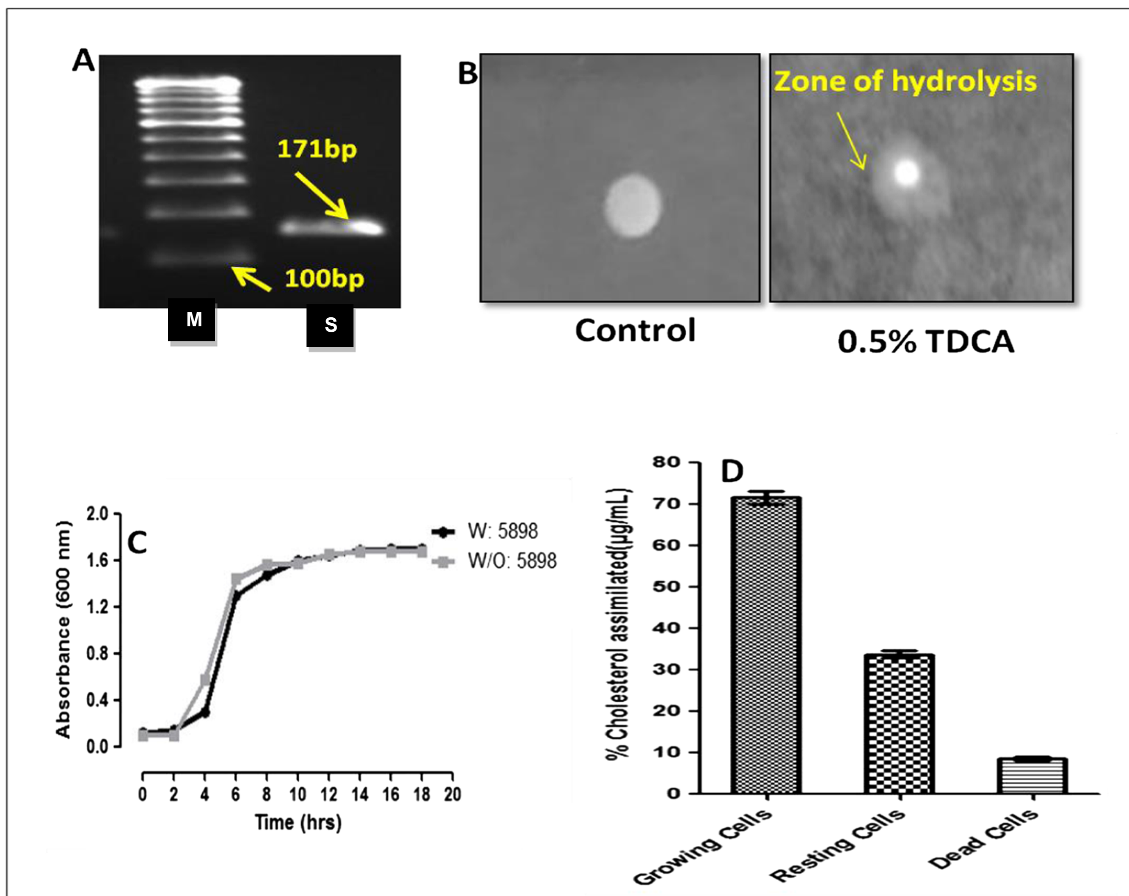

Fig. 1 bsh activity, growth curve, and cholesterol assimilation by *L. fermentum* MTCC: 5898. **a** Expression of bsh gene on agarose gel (M-DNA marker; S-sample). **b** De Man, Rogosa, and Sharpe (MRS) agar plates (left side) or MRS agar plates containing 0.5% sodium taurodeoxycholate (TDCA, right side) that were inoculated with *L. fermentum* 5898 for 72 h under anaerobic conditions. **c** Growth curve of *L. fermentum* MTCC: 5898 in MRS media supplemented with cholesterol 70 μg/mL (W:5898) and without cholesterol (W/O:5898). **d** % cholesterol assimilation by *L. fermentum* MTCC: 5898 in three stages: growing, resting, and dead cells in MRS broth containing 0.3% (*w*/*v*) oxgall and 70 μg/mL for 18 h under anaerobic conditions

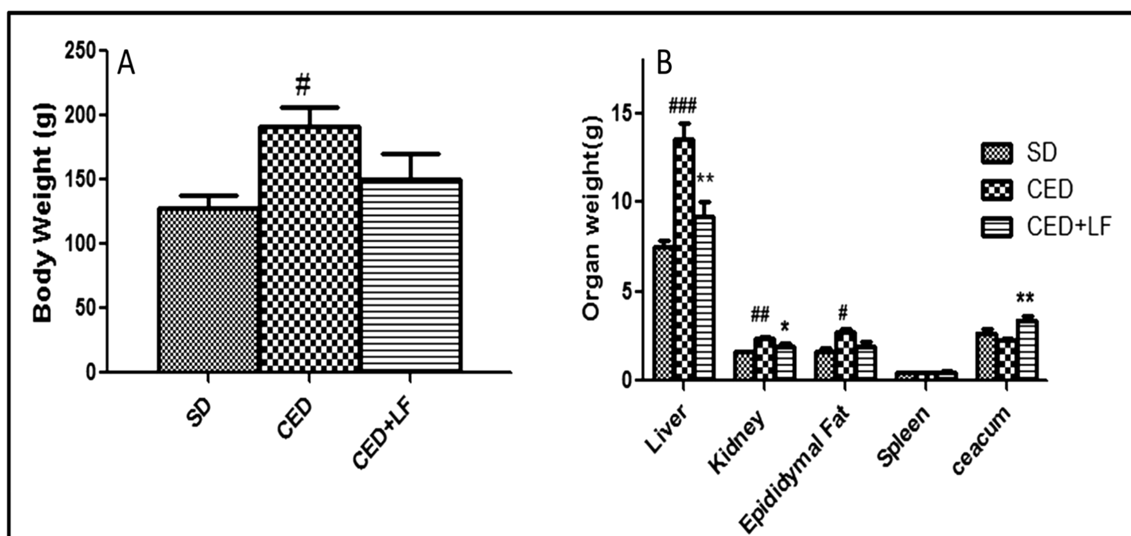

Fig. 2 Effect of probiotic-fermented milk on body weight gain (A) and weight of organs (liver, kidney, epididymal fat, spleen, and cecum) (B) in SD and CED rats. Data expressed as mean ± SEM ($n = 6$). Significance was measured by performing a one-way ANOVA followed by Tukey test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CED group

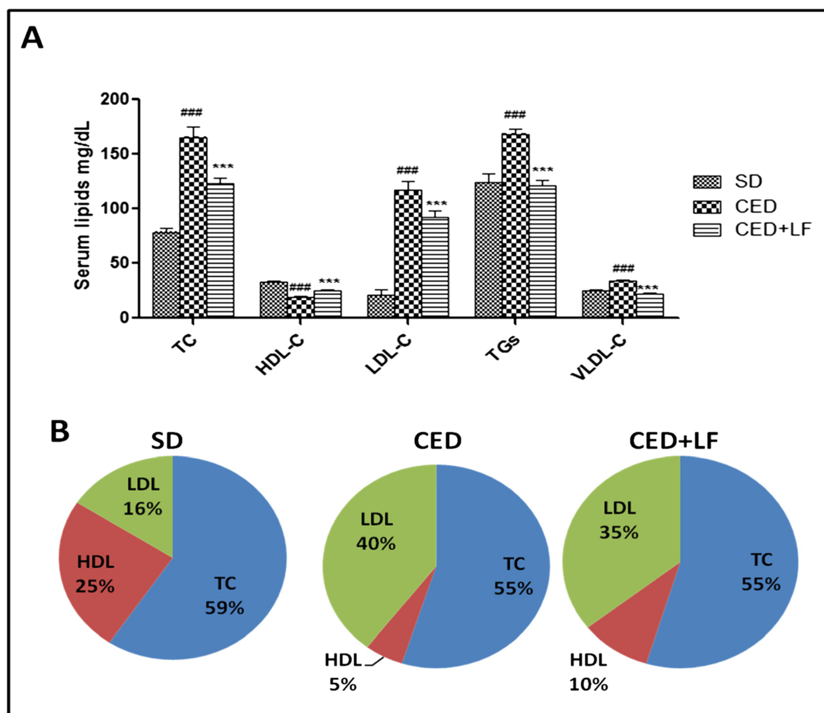

Fig. 3 Effect of probiotic-fermented milk on lipid profile (total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and very low-density lipoprotein cholesterol) (**a**) and lipid distribution pie charts showing high-density lipoprotein cholesterol and low-density lipoprotein cholesterol in different groups (**b**) in SD and CED rats. Data expressed as mean ± SEM ($n = 6$). Significance was measured by performing a one-way ANOVA followed by Tukey test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. SD. $^{*}P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CED group

LDL-C, TGs, and VLDL-C levels as compared to rats fed on SD. Specifically, 2.4-fold increase in TC (188.2 ± 5.0 vs. 78.4 ± 3.6 mg/dL, $P < 0.001$), 6.4-fold increase in LDL-C (135.7 ± 4.8 vs. 20.9 ± 4.7 mg/dL, $P < 0.001$), 1.4-fold increase in TGs (168.8 ± 4.3 vs. 124.2 ± 7.8 mg/dL, $P < 0.001$), and 1.4-fold increase in VLDL-C (33.6 ± 0.8 vs. 24.8 ± 1.5 mg/dL, $P < 0.001$) were observed in the group fed cholesterol-enriched diet compared with the SD group. The rats co-consumed PFM with CED were found to have reduced bad cholesterol levels compared with the CED group. Specifically, 1.3-fold reduction of TC (141.4 ± 6.3 vs. 188.2 ± 5.0 mg/dL, $P < 0.001$), 1.5-fold reduction in LDL-C (92.2 ± 5.7 vs. 135.7 ± 4.8 mg/dL, $P < 0.001$), 1.4-fold reduction in TGs (120.7 ± 4.9 vs. 168.8 ± 4.3 mg/dL, $P < 0.001$), and 1.5-fold reduction in VLDL-C (22.4 ± 0.7 vs. 33.6 ± 0.8 mg/dL, $P < 0.001$) were observed. Good cholesterol concentrations (HDL-C) were found to be highest in SD (32.6 ± 0.8 mg/dL, $P < 0.001$) > CED + PFM (25.1 ± 0.9, $P < 0.001$) > CED (18.8 ± 0.8) groups. As shown in Fig. 3b, the feeding of CED effects the HDL-C and LDL-C distribution by decreasing the HDL-C and increasing the LDL-C cholesterol. In the treatment group, a 5% increase in HDL-C was observed. It is reported that every 1% increase in HDL-C decreases the chances of CVD by 2–3%.

Effect of PFM on AI and CRI in CED Rats

The effect of PFM compared to SD and CED on AI and CRI is shown in Fig. 4. The SD group had the lowest AI and CRI among the three groups. The AI and CRI increased significantly ($P < 0.001$) in the CED group as compared to the SD group. Compared to the CED group, the AI and CRI were significantly lowered ($P < 0.001$) in the PFM-fed group.

Effect of PFM on Hepatic Lipids in CED Rats

Hepatic lipid concentrations are shown in Fig. 5(A). The SD group displayed the lowest levels of hepatic TC (5.9 ± 1.4 mg/g) and TGs (9.4 ± 2.4 mg/g), but their levels were found to be increased significantly nearly by 4-fold (23.0 ± 2.8 vs. 5.9 ± 1.4 and 35.0 ± 2.9 mg/g, $P < 0.001$), respectively, in the CED group compared to the SD group. The CED+LF group showed significant decrease in hepatic TC (12.3 ± 3.2 vs. 23.0 ± 2.8 mg/g, $P < 0.05$) and TGs (17.9 ± 2.6 vs. 35.0 ± 2.9 mg/g, $P < 0.01$) nearly 2-fold compared to the CED group.

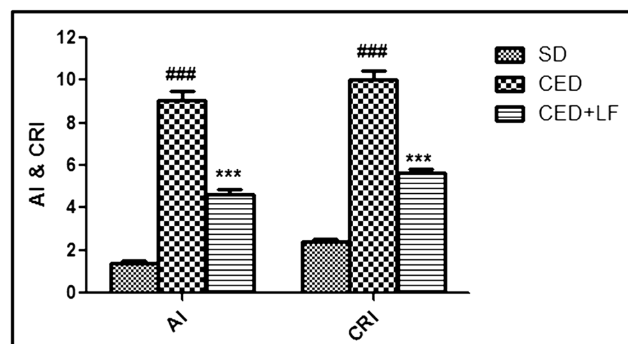

Fig. 4 Effect of probiotic-fermented milk on atherogenic index (AI) and coronary artery risk index (CRI) in SD and CED rats. Data expressed as mean± SEM ($n = 6$). Significance was measured by performing a one-way ANOVA followed by Tukey test. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.005$ vs. SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. CED group

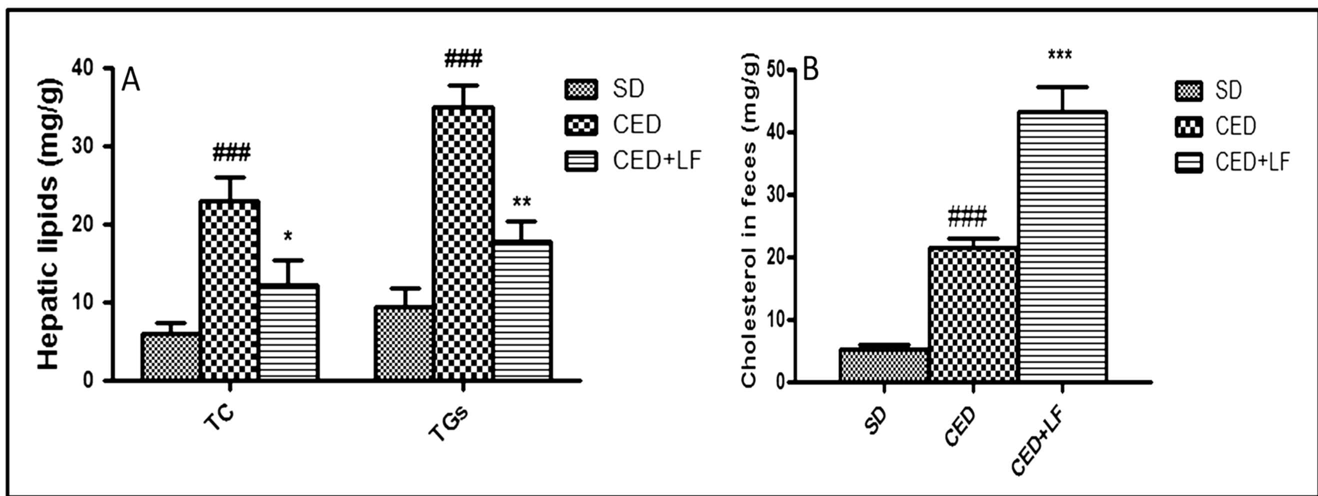

Fig. 5 Effect of probiotic-fermented milk on hepatic lipids (A) and cholesterol excretion in feces (B) in SD and CED rats. Data expressed as mean± SEM ($n = 6$). Significance was measured by performing a one-way ANOVA followed by Tukey test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CED group

Effect of PFM on Fecal Cholesterol in CED Rats

The cholesterol-enriched diet significantly increased (21.5 ± 1.5 mg/g) the fecal excretion of cholesterol by 4-fold compared to rats fed on SD (5.2 ± 0.7 mg/g). The cholesterol concentration in feces was further increased significantly (43.3 ± 3.9 vs. 21.5 ± 1.5 mg/g) by 2-fold in the CED+LF group compared with the CED group. Results are shown in Fig. 5(B).

Effect of PFM on Anti-oxidative Enzymes in CED Rats

The oxidative stress parameters in the liver and kidney samples collected from rats fed on different experimental diets are shown in Table 2. In both liver and kidney, activities of anti-oxidative enzymes, i.e., CAT, SOD, and GPx, were significantly decreased in the CED group compared to the SD group. In the liver, a significant increase ($P < 0.01$) was seen in the activity of CAT and SOD enzymes, whereas in the kidney, a significant increase ($P < 0.05$) was seen only in the activity of CAT enzyme, in the rats fed CED+LF group compared to the CED group. But no significant differences were observed in the activity of SOD enzyme in the kidney of probiotic-fed rats compared to the CED group. The activity of GPx enzyme in the liver and kidney did not show any significant difference compared to the CED group.

Effect of PFM on Lipid Peroxidation in CED Rats

TBARS levels indicate the oxidative damage in the tissues. Its levels were measured in the liver and kidney from all rats fed on different experimental diets at the end of the 90th day. The observed results are shown in Table 2. The levels of TBARS

Table 2 Effect of PFM on anti-oxidative enzyme activities and lipid peroxidation in the liver and kidney

	Liver			Kidney		
Enzyme activity (U/mg/min)	SD	CED	CED+LF	SD	CED	CED+LF
CAT	476.8 ± 52.8	226.2 ± 16.6$^{\#\#\#}$	413.9 ± 32.3**	2501.0 ± 238.1	1080.0 ± 174.7$^{\#\#}$	2285.0 ± 247.7*
SOD	36.9 ± 3.0	9.3 ± 1.3$^{\#\#\#}$	26.4 ± 3.4**	47.7 ± 4.3	17.0 ± 2.3$^{\#\#\#}$	25.4 ± 0.6
GPx	20.9 ± 2.2	6.6 ± 0.8$^{\#}$	12.9 ± 4.8	14.1 ± 0.8	5.3 ± 0.6$^{\#\#\#}$	8.1 ± 1.3
TBARS (nM/mg protein)	0.4 ± 0.0	1.6 ± 0.1$^{\#\#\#}$	0.7 ± 0.0***	0.6 ± 0.0	1.8 ± 0.0$^{\#\#\#}$	0.9 ± 0.0***

Data expressed as mean ± SEM (n=6). Significance was measured by performing a one- way ANOVA followed by tukey test

$^{\#}$ ($P < 0.005$)

$^{\#\#}$ ($P < 0.01$)

$^{\#\#\#}$ ($P < 0.005$) vs SD

* ($P < 0.05$)

** ($P < 0.01$)

*** ($P < 0.005$) vs CED group

in the liver and kidney drastically increased ($P < 0.001$) by 4-fold and 3-fold, respectively, in the CED group compared to the SD group. PFM was found to be effective in lowering TBARS levels significantly ($P < 0.001$) by 2.2-fold in the liver and by 2-fold in the kidney compared to the CED group.

Effect of PFM on Inflammatory Cytokines in CED Rats

Estimated levels of inflammatory cytokines, i.e., TNF-α and IL-6, in the liver are shown in Fig. 6. Cholesterol feeding (CED and CED+LF) increased the expression level of these cytokines in the liver compared to the SD group. But, TNF-α and IL-6 expression levels were found significantly ($P < 0.001$) higher in the CED group compared to the CED+LF group.

Discussion

Recently, probiotics have been considered as dietary adjuncts in novel functional foods especially to enhance the immune system and to maintain healthy life. It is well known that high blood cholesterol could be a predisposable factor associated with increased risk of CVD. Hence, therapeutics that prevents the increase in serum/plasma cholesterol is medically very important today. Probiotic dietary interventions could be the promising and cost-effective approach in this regard. Lactobacilli microorganisms constitute a part of intestinal microbial flora in human and other animals. *L. fermentum* MTCC: 5898 is a proved probiotic strain that has not been tested for its cholesterol-lowering abilities in animal models. Hence, the current study was designed to understand the anti-hypercholesterolic abilities of *L. fermentum* MTCC: 5898.

Initial in vitro experiments indicate that *L. fermentum* MTCC: 5898 could be a promising anti-hypercholesterolic agent as it successfully lowered the cholesterol concentrations in growth media at live, resting, and even dead cell stages as shown in Fig. 1. A similar kind of observations were reported by various studies [28, 29] with different probiotic strains. A close inspection of other reports indicates that the *L. fermentum* MTCC: 5898 is more effective in removing cholesterol (71.5%) in vitro in comparison with other strains *L. fermentum* SM-7 cells assimilated 61.5% cholesterol [30] and *L. fermentum* MTCC 8711 showed up to 50% reduction in cholesterol [31]. As evident from SEM results reported by Choi and Chang [28], removal of cholesterol by dead cells could be because of adsorption onto bacterial membrane. But in live cells, the cellular metabolism of cholesterol might be playing key role in cholesterol lowering [32]. Further experiments were carried out to understand the ability of *L. fermentum* MTCC: 5898 as cholesterol-reducing probiotic culture in the gastrointestinal system. Body weight has been considered as a primary parameter to visualize the effectiveness of PFM. As like Wang et al. [33], we have observed (Fig. 2) that feeding CED together with PFM did not induce body weight gain in rats. Cecum in rats is the major site of fermentation in rats; an increase in cecum weight in the probiotics group could indicate higher microbial content. Further, it is clearly observed that feeding PFM to rats successfully prevented the diet-induced increase in the concentrations of LDL-C, VLDL-C, TC, and TGs in serum compared to the CED-fed group (Fig. 3). Preventing the rise in serum bad cholesterol will prevent the animal from atherosclerosis and other CVDs. Similar kind of observation has been made in multiple studies [11, 19, 34–36]. Lactobacilli were reported to bind to cholesterol in the intestine and enhanced excretion of cholesterol in feces [37], which together resulting in decrease in total body cholesterol pool. Both AI and CRI ratio, which are indicators of the increased serum lipids associated with atherosclerosis and other CVDs, were also found to be decreased in the PFM group (Fig. 4), which is another important indicator showing the potential of *L. fermentum* MTCC: 5898. Diet supplemented with cholesterol could result in accumulation of cholesterol and triglycerides in the liver, leading to increased risk of CVD. Feeding PFM successfully prevented the accumulation of TC and TGs in the liver as PFM prevented accumulation of blood cholesterol (Fig. 5). These findings were in agreement with recent studies [11, 36]. Lactobacilli also enhance the production of short-chain fatty acids, which in turn reduce the synthesis of hepatic cholesterol. Higher excretion of TC through feces in rats fed CED indicated that changes had been brought about by ingestion of dietary cholesterol (Fig. 5). The results indicate that *L. fermentum* MTCC: 5898 can be effective in inhibiting the accumulation of cholesterol in the liver of the PFM group rats by further increasing the fecal TC content compared to CED rats. Lower level of hepatic TC will

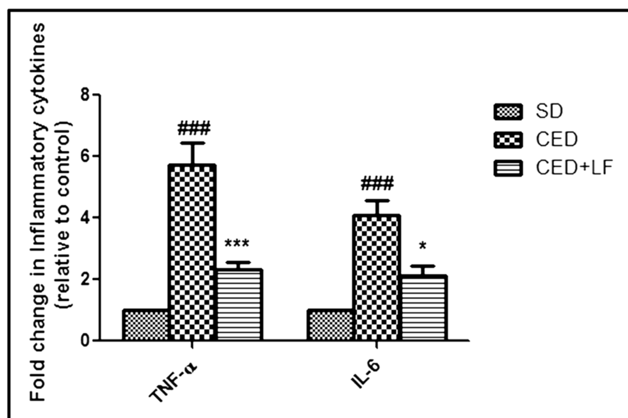

Fig. 6 Effect of probiotic-fermented milk on mRNA expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in SD and CED rats. Data expressed as mean ± SEM ($n = 6$). Significance was measured by performing a one-way ANOVA followed by Tukey test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CED group

promote influx of serum cholesterol to the liver, thus decrease the serum cholesterol. Also, oxidative stress is an important risk factor in the pathogenesis of hypercholesterolemia [38]. It has been suggested that cholesterol elevation and accumulation in the body cause high oxidative stress and might result in high susceptibility to lipid peroxidation. So the activity of anti-oxidative enzymes in the liver and kidney (CAT, SOD, and GPx) and measurement of lipid peroxidation based on determination of TBARS levels were carried out. In this study, it was observed that probiotic supplementation helped to repress the oxidative stress created by excess of cholesterol by increasing the anti-oxidative enzyme activities and by decreasing lipid peroxidation in the liver and kidney (Table 2). Similar to our findings, probiotic dahi was found to suppress streptozotocin-induced oxidative damage in diabetic rats by inhibiting the lipid peroxidation and preserving the activity of CAT, SOD, and GPx enzymes [39]. Also, a study [40] found that *L. casei* supplementation significantly increased the activity of anti-oxidative enzymes in serum and liver of hyperlipidemic rats. The role of inflammatory cytokines in hypercholesterolemia and its complications has been shown in some studies [41]. It was observed that PFM consumption prevented the increase of transcript abundance of TNF-α and IL-6 mRNAs in the liver of rats (Fig. 6). But, the increase in cholesterol in CED-fed rat resulted in increased synthesis of TNF-α and IL-6 cytokines which could be via activated immune cells due to increase in oxidative stress response in the body.

Conclusion

Feeding of *L. fermentum* MTCC: 5898-fermented milk to rats along with CED reduces the markers of hyperlipidemia, oxidative stress, and inflammatory responses. Therefore, it can be concluded that *L. fermentum* MTCC: 5898 is the new potential probiotic strain that can be consumed to prevent the diet-induced hypercholesterolemia.

Acknowledgements The authors are grateful to the Director of ICAR-National Dairy Research Institute, Karnal, for providing laboratory facilities to carry out this work.

Funding Information Funding is provided by ICAR-National Dairy Research Institute, Karnal.

Compliance with Ethical Standards

Ethical Approval The study was approved by the Institute's Animal Ethical Committee (IAEC) for Animal Experiments of National Dairy Research Institute (IAEC No. 101/16 dated 21 April 2016), Karnal, Haryana, India.

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

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