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Effect of *Lactobacillus fermentum* MCC2760-Based Probiotic Curd on Hypercholesterolemic C57BL6 Mice

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ABSTRACT: *Lactobacillus fermentum* MCC2760 is a probiotic strain proven earlier for cholesterol-reducing and anti-inflammatory properties *in vitro* and *in vivo*. This study investigates *L. fermentum* MCC2760-based probiotic curd in high-cholesterol diet (HCD)-fed C57BL6 mice. The mice were grouped into normal diet control, high-cholesterol diet control, normal diet with probiotic supplementation, and high-cholesterol diet with probiotic supplementation. Control groups and treatment groups were supplemented with market curd and probiotic curd, respectively, via oral gavage for eight weeks. The probiotic count was maintained at 10.95 log CFU/mL in the developed probiotic curd. The HCD group showed an increase in feed intake and body weight. Reduction in the levels of serum cholesterol, triglycerides, low-density lipoprotein cholesterol, glucose, aspartate aminotransferase, and alanine transaminase was observed in probiotic-supplemented groups. The probiotic-supplemented group resulted in an increase in *Lactobacillus* spp. count along with reduced pathogen count in the feces. Probiotic supplementation also showed a reduction in the bacterial translocation count in mesenteric adipose tissue. Expression of inflammatory markers by qPCR showed the decline in the fold change of TNF-α, IL-6, and IL-12 and elevation in the fold change of IL-10 in the adipose tissue of the probiotic-treated group. Probiotic supplementation also improved the expression of GLP-1, ZO-1, and CB2 in the intestine. They were thus possibly playing a role in the enhancement of barrier function. Histopathological sections showed improvement in the cellular infiltration and pathological indications due to the high-cholesterol diet intake. Our study also confirmed that probiotics could increase serum antioxidant enzymes in treated groups, showing their beneficial antioxidant activity. It suggests the anti-inflammatory, antioxidant effect, and gut barrier function of the given probiotic formulation, which ameliorate hypercholesterolemia.
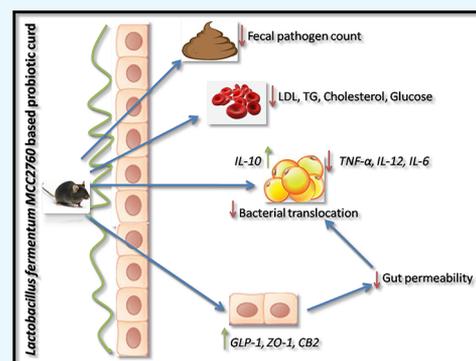
1. INTRODUCTION

Obesity and overweight are essential complications in both developed and developing countries. Obesity is the leading cause of weight gain; it is a disorder of physiology that is the source cause of numerous diseases, which compromises quality of life and poses an enhanced prospect of health troubles such as diabetes, hypertension, and cardiovascular disease (CVD).[1] Cholesterol is the principal lipid seen in bile, brain tissues, and blood, and it is expected to form cellular membranes and sterols. The higher serum cholesterol is a threat to CVD. The raised level of total cholesterol in the bloodstream causes hypercholesterolemia. The low high-density lipoprotein (HDL) cholesterol-to-low-density lipoprotein (LDL) cholesterol ratio is noted in hypercholesterolemia. LDL gives fatty sediments in the blood vessels. The collection of these fatty deposits blocks the blood supply to the organs by congesting blood vessels. Various laboratory, clinical, and epidemiological studies have established a link between hypercholesterolemia and enhanced CVD risk, a notable reason for death in Western countries.[1]

To manage hypercholesterolemia, potential food products and pharmaceuticals are continuously being developed.[2,3] The purpose of probiotic food products has got recognition from consumers and producers due to the evolution of a more health-conscious society.[4] In this link, probiotic lactic acid bacteria intake might be a natural route to lower serum cholesterol.[5] Preclinical and clinical studies have recommended that the use of fermented dairy products with probiotics (bifidobacteria and lactic acid bacteria) may lessen levels of serum lipids.[6−8] "Dahi" is a popular Indian fermented milk product made in most households, and it is available commercially as well. It is developed and consumed in many forms, such as sour curd, skim milk curd, conventional curd, and whole milk curd. Therefore, they can be utilized as an effective means for transporting probiotic microorganisms to consumers to confer health benefits.[9] *Lactobacillus fermentum*

MCC2760 is a novel probiotic strain that was studied for its *in vitro* probiotic attributes and anti-inflammatory and adhesion properties.[10,11] The anti-inflammatory potential of *L. fermentum* on carrageenan-induced paw edema in Wistar rats was also established.[12] The *L. fermentum* MCC2760 strain was conceived to have 62−65% *in vitro* cholesterol assimilation.[11] The effect of *L. fermentum* MCC2760 was also studied on high-fat diet-fed and diabetic Wistar rats where it proved anti-inflammatory and gut integrity functions.[13]

Thus, this study aimed to use *L. fermentum* MCC2760, a potential probiotic culture, to develop a fermented probiotic curd. The developed probiotic curd was tested on high-cholesterol diet-induced hypercholesterolemic C57BL6 mice and its influences on inflammatory markers, gut barrier function, antioxidant enzymes, and gut pathogen count.

2. RESULTS AND DISCUSSION

2.1. Changes in Feed Intake, Weight Gain, and Serum Biochemical Parameters during Probiotic Curd Intervention. Increased body weight was observed consistently for eight weeks in the high-cholesterol diet-fed group compared to all other groups. The groups treated with probiotic curd showed weight reduction (24.51 g) as opposed to the high-cholesterol diet control group (26.49 g). The significant weight difference between the HCD and other groups was noted from the fourth week to the eighth week (Figure 1).
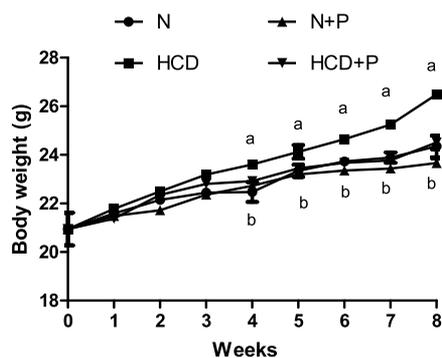

Figure 1. Effect of probiotic curd on body weight during different weeks in mice fed with normal and high-cholesterol diet. Data are presented as mean ± SD ($n = 5$); letters with different superscripts are significant at $P < 0.05$. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation; g, gram.

Similarly, the feed intake of groups fed with high-cholesterol diet increased significantly compared to the normal diet group. However, a slight decrease was seen in the probiotic-administered group (Figure 2).

Serum biochemical analysis showed an increase in the levels of cholesterol (243.27 mg/dL), LDL-C (183.41 mg/dL), triglycerides (234.14 mg/dL), and glucose (149.13 mg/dL) in the high-cholesterol diet control group as a result of high-cholesterol diet consumption. However, the levels were normal in the groups that received probiotic curd. There was no notable difference found in the total protein, uric acid, urea, creatinine, and albumin. Still, the rise in serum ALT and AST of the HCD group shows the damage in the liver function of the high-cholesterol diet-fed group (Table 1).

High-fat or high-cholesterol diet contributes to an increase in body weight. Few studies have reported the weight-lowering
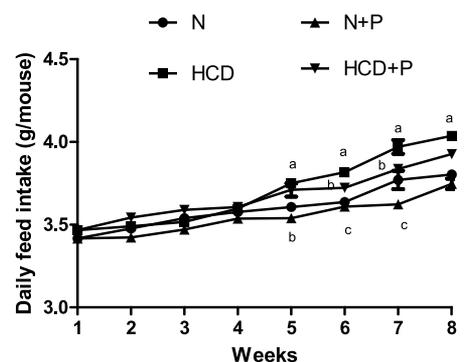

Figure 2. Effect of probiotic curd on feed intake during different weeks in mice fed with normal and high-cholesterol diet. Data are presented as mean ± SD ($n = 5$); letters with different superscripts are significant at $P < 0.05$. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation; g-, gram.

ability of LAB in such models. In our study, the groups supplemented with probiotic curd did show a significant weight reduction ($P < 0.05$). Certain strains of LAB have demonstrated the ability to prevent body weight gain.[14] It is said that the gut microbiota plays a role in digestion and absorption of fat in the small intestine. Increased excretion of bile acids in the feces may increase bile acid synthesis and contribute to weight loss, and the bile acid may induce energy expenditure.[15] Xie et al. demonstrated significant weight reduction upon treatment with *L. plantarum* 9-41-A in a high-cholesterol-fed rat model along with decreased liver and adipose tissue weight.[16] However, further studies are required to determine the exact mechanisms behind this effect. Some probiotic strains are known to reduce serum cholesterol levels by increasing the excretion of bile acids in feces and affecting cholesterol synthesis pathways.[17] Even though the usage of non-isocaloric diet, i.e., high-fat high-cholesterol diet when compared to AIN-93 diet was a limitation, it is inevitable to use high fat and high cholesterol for induction of hyper-cholesterolemia. Our results showed a significant reduction of glucose, LDL-C, cholesterol, and triglycerides in serum. Xie et al. reported reduced serum cholesterol levels in high-cholesterol diet-fed rats upon treatment with *L. plantarum* and suggested that the cholesterol-lowering effect could be due to deconjugation and precipitation of bile acids from the circulation and excretion through feces.[16] LAB may also assimilate cholesterol by incorporating them in their cell walls or cell membranes and then excreting through feces.[18] Therefore, our results correlate with several other studies that have also reported a reduction in LDL-C, triglycerides, and cholesterol content after probiotic intake.[19,20]

2.2. Effect of Probiotic Curd on Serum Antioxidant Enzymes. Several antioxidant enzymes, including glutathione transferase, glutathione peroxidase, catalase, and superoxide dismutase, are crucial in the cellular defense against ROS and free radicals. The serum antioxidant enzymes viz. glutathione transferase, glutathione peroxidase, catalase, and superoxide dismutase significantly ($p < 0.05$) increased in the probiotic curd-supplemented group as seen in Figure 3.

Similarly, Zommara et al. reported that the supplementation of *Bifidobacterium longum*, *L. acidophilus*, or *Streptococcus salivarius* subsp. *thermophilus* to rats increases the serum antioxidant enzymes such as glutathione peroxidase and superoxide dismutase.[21] In another study, when *L. plantarum*

Table 1. Serum Biochemical Changes in Normal and High-Cholesterol Diet-Fed Mice during Probiotic Curd Treatment[a]

groups	LDL-C (mg/dL)	HDL-C (mg/dL)	total cholesterol (mg/dL)	triglycerides (mg/dL)	total protein (g/dL)	uric acid (mg/dL)	urea (mg/dL)	creatinine (mg/dL)	glucose (mg/dL)	albumin (g/dL)	ALT (U/L)	AST (U/L)
N	96.87 ± 0.33[c]	58.29 ± 0.78[b]	177.73 ± 0.54[c]	115.18 ± 0.62[c]	6.71 ± 0.37[a]	3.12 ± 0.41[a]	8.84 ± 1.02[a]	1.66 ± 0.74[a]	119.31 ± 0.23[b]	3.14 ± 0.63[a]	37.61 ± 0.42[c]	40.12 ± 0.15[c]
HCD	183.41 ± 0.82[a]	37.90 ± 0.49[d]	243.27 ± 1.32[a]	234.14 ± 0.20[a]	6.62 ± 1.06[a]	3.63 ± 0.55[a]	8.98 ± 0.41[a]	1.70 ± 0.37[a]	149.13 ± 0.16[a]	2.17 ± 0.37[a]	63.15 ± 0.27[a]	59.29 ± 1.77[a]
N + P	94.12 ± 0.34[d]	60.07 ± 1.43[a]	160.20 ± 0.49[d]	108.25 ± 1.05[d]	6.75 ± 0.63[a]	3.03 ± 0.72[a]	8.77 ± 0.36[a]	1.73 ± 0.64[a]	116.07 ± 0.63[c]	2.86 ± 0.26[a]	34.11 ± 1.31[d]	38.17 ± 0.02[d]
HCD + P	129.20 ± 1.32[b]	49.34 ± 1.15[c]	202.56 ± 0.63[b]	167.17 ± 1.21[b]	6.70 ± 0.29[a]	3.52 ± 0.41[a]	8.90 ± 0.71[a]	1.65 ± 0.41[a]	117.12 ± 0.38[d]	2.67 ± 0.52[a]	39.26 ± 0.14[b]	43.12 ± 0.37[b]

[a]All of the values are mean ± SD (n = 5). Mean values with the same superscript letters are not significantly different, whereas those with different superscript letters are significantly (P < 0.05) different. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation.
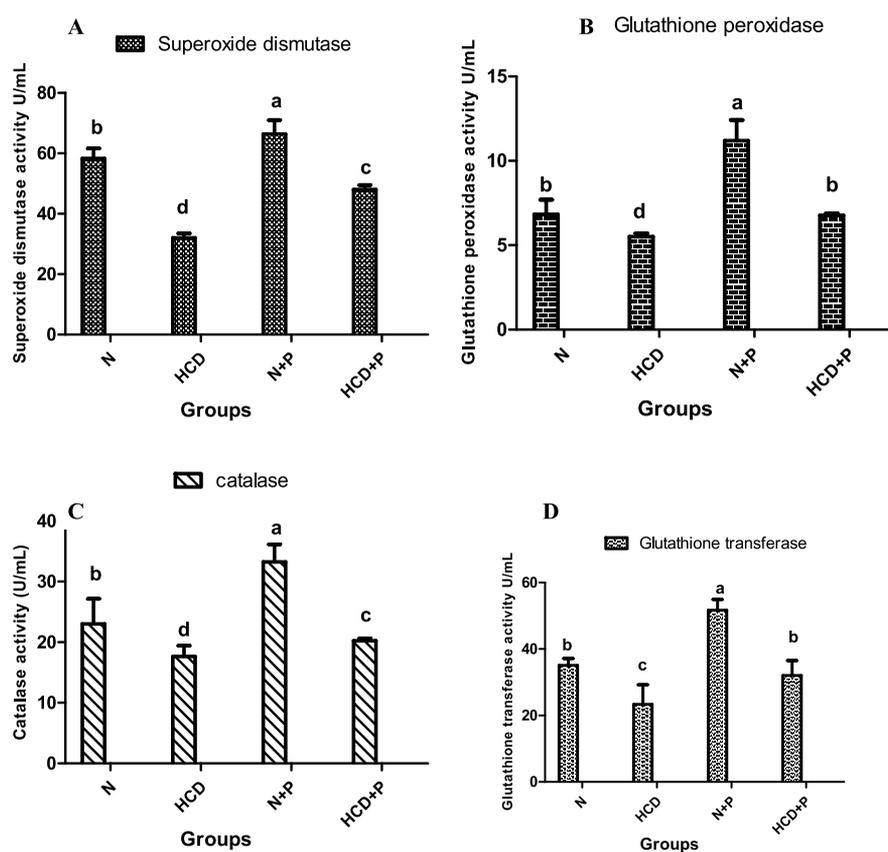

Figure 3. Effect of probiotic curd on serum antioxidant enzymes in mice fed with normal and high-cholesterol diet. (A) Superoxide dismutase. (B) Glutathione peroxidase. (C) Catalase. (D) Glutathione transferase. Data are presented as mean ± SD (n = 5); letters with different superscripts are significant at P < 0.05. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation

C88 was administered to senescent mice suffering oxidative stress induced by D-galactose, the serum superoxide dismutase activity, the glutathione peroxidase activity, and the total antioxidant capacity in liver increased significantly, while the level of malondialdehyde in liver decreased significantly.[22] Functional dietary antioxidant ingredients originating from fermented foods play an essential role in preventing the occurrence of diseases associated with oxidative stress by directly inhibiting lipid peroxidation, scavenging ROS, and regulating intestinal environment. Dietary antioxidants from fermented foods mainly include bioactive peptides, microbial enzymes, and metabolites. Hence, our test probiotic formulation increases the antioxidant activity and thus reduces the inflammation.

2.3. Effect on the Microbial Count in Feces and Bacterial Translocation to Mesenteric Adipose Tissue. The effect of the *L. fermentum* based probiotic curd in normalizing the disturbed gut microbiota during the high-cholesterol diet was evaluated by analyzing the microbial count of pathogenic and beneficial bacteria in the feces of mice. Evaluation of fecal microbial count revealed changes in the high-cholesterol diet-fed group compared to the normal control group. The count of pathogenic bacteria like *Staphylococcus* and *Campylobacter* increased significantly in the high-cholesterol diet control group (Figure 4).

However, the intervention of probiotic curd increased the LAB count. Consumption of a high-cholesterol diet did show elevation in the bacterial count in adipose tissue. However, the
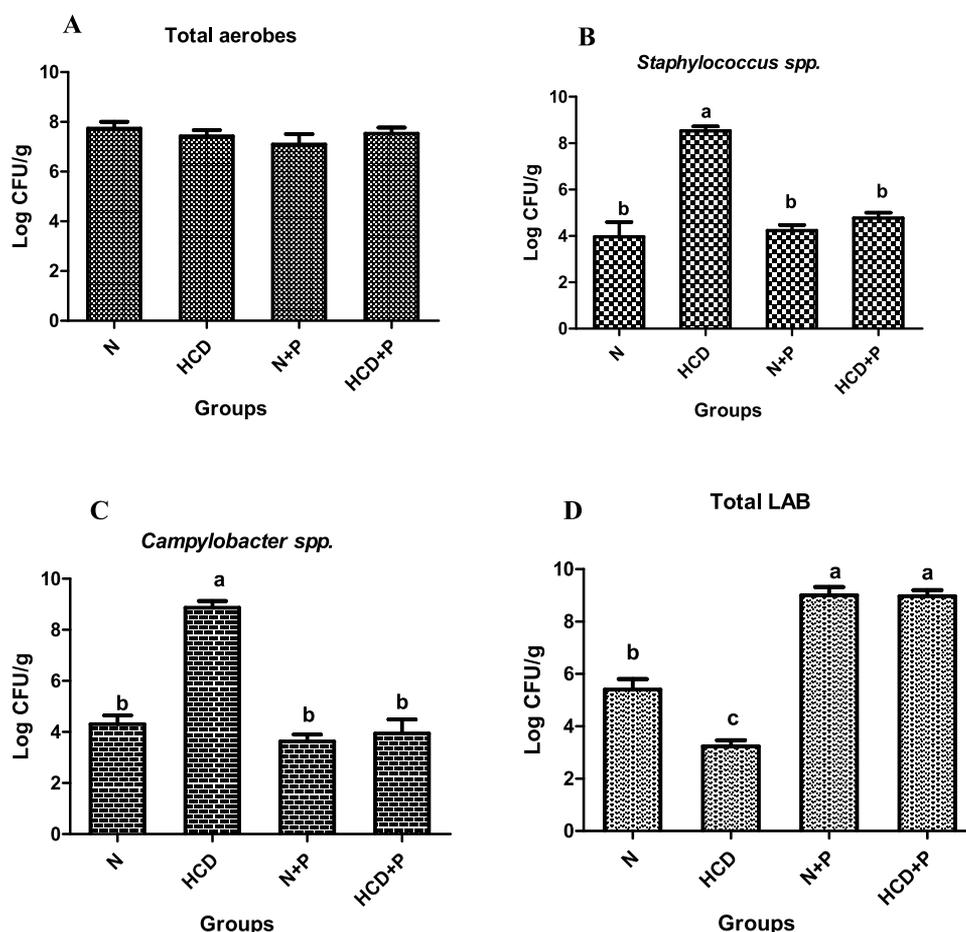
Figure 4. Fecal microbial count in normal and high-cholesterol diet-fed mice during probiotic curd intervention. (A) Total aerobes. (B) *Staphylococcus spp.* (C) *Campylobacter spp.* (D) Total LAB. Data are presented as mean ± SD ($n = 5$); letters with different superscripts are significant at $P < 0.05$. N, normal diet; HCD high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation.

bacterial translocation to adipose tissue count was significantly suppressed in groups that received probiotic curd (Figure 5).

Recent studies had reported that the gut microbiota plays a vital role in the development of metabolic diseases such as
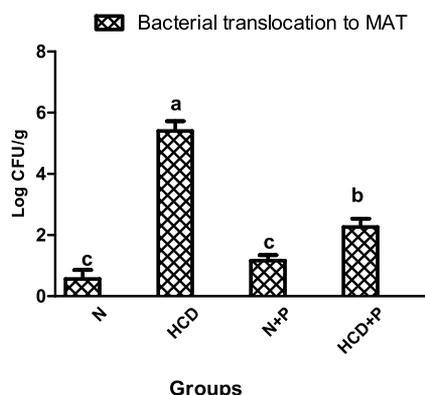

Figure 5. Bacterial translocation to MAT in normal and high-cholesterol diet-fed mice during probiotic curd intervention. Data are presented as mean ± SD ($n = 5$); letters with different superscripts are significant at $P < 0.05$. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation; MAT, mesenteric adipose tissue.

obesity and diabetes. Bäckhed et al. reported that microbiota from *ob/ob* mice increased the body weight of lean axenic mice upon colonization, suggesting that the obese microbiota caused increased energy harvest from non-digestible dietary polysaccharides, thus causing significant levels of serum glucose and insulin, further inducing lipogenesis.[23] In our study, high-cholesterol feeding seemed to increase the concentrations of pathogenic bacteria in the feces with decreased LAB count as previously reported by Cani et al. and Amar et al.[24,25] However, supplementation of probiotic *L. fermentum* resulted in the decrease in pathogen count and subsequent increase in LAB numbers. The ability of *L. fermentum* to combat pathogens could be attributed to their strong adhesion properties as previously mentioned, thus preventing the attachment of pathogenic bacteria. Additionally, the increase in LAB count suggests that *L. fermentum* facilitates the growth of other LAB and may have also improved the bifidobacterial count, which has the ability to inhibit pathogens by the production of several metabolites and other mechanisms. Xie et al. showed that supplementation of *L. plantarum* and *L. fermentum* strains reduced the count of *Escherichia coli* and increased the count of *Lactobacillus* and *Bifidobacteria* in rats fed with a high-cholesterol diet.[16] Consumption of *L. fermentum* by healthy overweight human subjects resulted in modulation of gut microbiota by reduction of pathogens and improvement of *Lactobacillus* count.[26] Our results are in
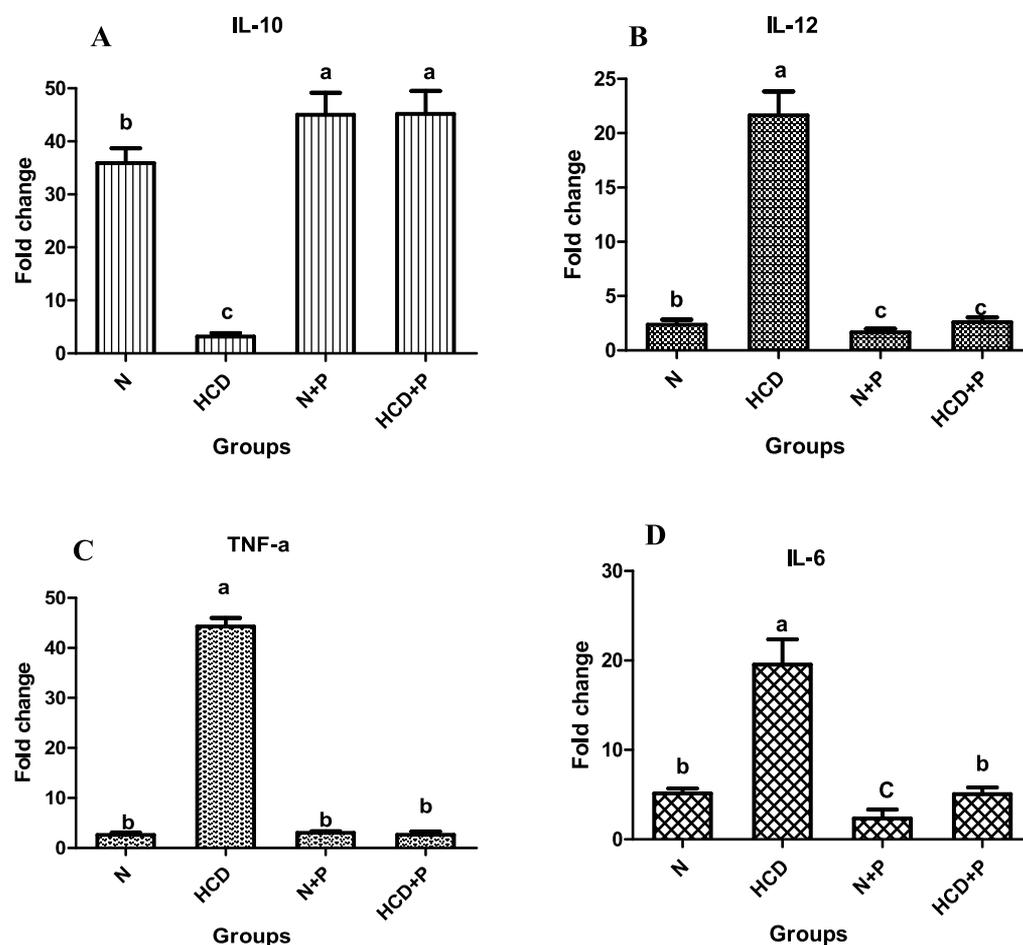
Figure 6. Expression changes in inflammatory markers in adipose tissue of normal and high-cholesterol diet-fed mice during probiotic curd intervention. (A) IL-10. (B) IL-12. (C) TNF-α. (D) IL-6. Data are presented as mean $\pm$ SD ($n = 5$); letters with different superscripts are significant at $P < 0.05$. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation.

accordance with the above reports and further confirm the gut microbiota-modulating effects of *L. fermentum*.

High-fat feeding by means of gut microbiota modulation and increased gut barrier permeability is said to facilitate higher endotoxemia.[27] Reduced levels of translocated bacteria were found in adipose tissue compared to the HCD group. This could be due to the gut microbial balance, i.e., reduction of pathogenic count and promotion of LAB counts.[16] *Bifidobacteria* have been found to lower bacterial translocation, thus reducing inflammatory reactions.[28] Additionally, probiotics are found to improve barrier function and decrease permeability, preventing bacterial translocation.[29] Thus, our results suggest the ability of *L. fermentum* MCC2760 to normalize the gut microbiota and prevent translocation in dysbiotic condition.

2.4. Effect on Inflammatory Markers in Adipose Tissue and Gut Barrier Markers. Adipose tissue showed an elevated expression level of TNF-α, IL-12, and IL-6 in a high-cholesterol diet-fed control group at the completion of the experimental period (Figure 6). However, the levels of the same were downregulated in the groups supplemented with probiotic curd. On the other hand, *L. fermentum* probiotic curd supplementation stimulated the increased expression of IL-10. It shows higher expression at a normal diet with a probiotic-supplemented group with a 51.65-fold change in adipose tissue (Figure 6).

Probiotic curd also stimulated the expression of GLP-1 (5.9-fold change) (Figure 7). It supports the mode of action of the given probiotics. The expression of the endocannabinoid (eCB) system, which plays a role in the functioning of gut permeability and plasma LPS levels, is thought to be modulated by the intestinal microbiota through the CB1 receptor.[30] The eCB system is also believed to modulate intestinal permeability by enhancing the distribution and localization of tight junction proteins.[31] It was shown that the stimulation of the CB2 receptor improves glucose tolerance in rats and is positively correlated with counts of *Lactobacillus* in the gut.[32] Probiotic *Lactobacillus* has been shown to upregulate the expression of the CB2 receptor in rats.[33] In our study, probiotic curd supplementation displayed the ability to influence the endocannabinoid (eCB) system by down-regulating the expression of CB1 and augmenting the expression of CB2, thereby reducing the gut LPS concentrations and improving barrier function. The enhanced barrier functionality can also be evident in the improved ZO-1 expression by probiotic treatment.

High-fat diet is said to be one of the major contributing factors for the development of metabolic disorders. In models of diet-induced obesity and diabetes, adipose tissue showed increased levels of the proinflammatory cytokine TNF-α.[34] Cytokines are thought to cause insulin resistance culminating into hyperinsulinemia and excessive lipid storage in adipose
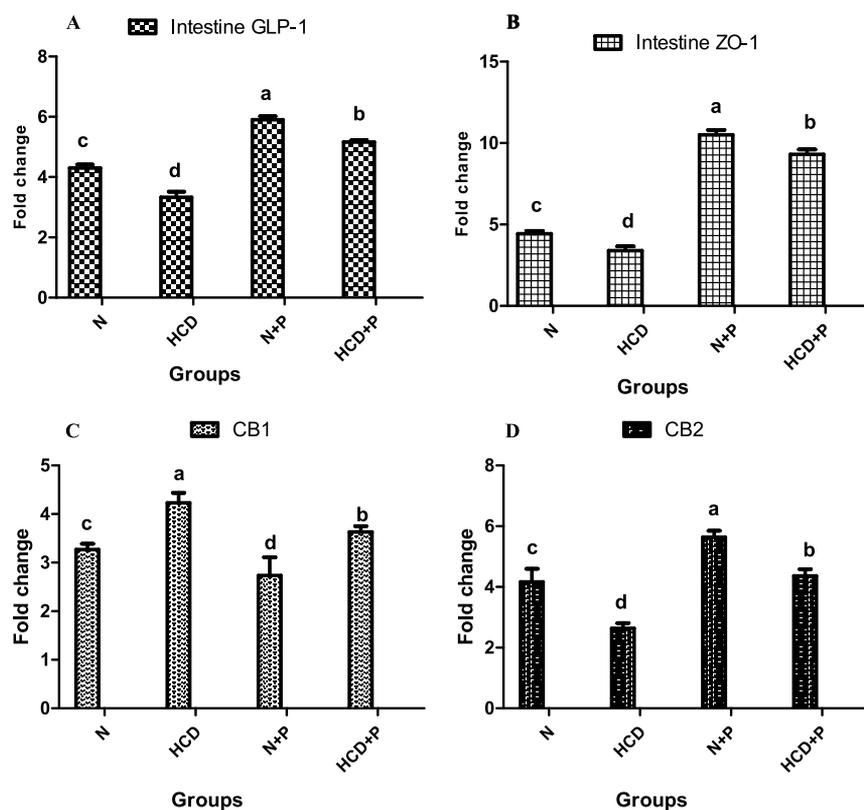
Figure 7. Expression changes in intestinal integrity markers in normal and high-cholesterol diet-fed mice during probiotic curd intervention. (A) GLP-1, glucagon-like peptide-1. (B) ZO, zonula occludens-1. (C) eCB-1, endocannabinoid system 1. (D) eCB-2, endocannabinoid system 2. Data are presented as mean ± SD ($n = 5$); letters with different superscripts are significant at $P < 0.05$. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation.

Figure 8. Effect of probiotic curd intervention on the liver, intestine, and adipose tissue histology of normal and high-cholesterol diet-fed mice. (A) Liver. (B) Intestine. (C) Adipose. N, normal diet; HCD, high-cholesterol diet; N + P, normal diet with probiotic curd supplementation; HCD + P, high-cholesterol diet with probiotic curd supplementation.
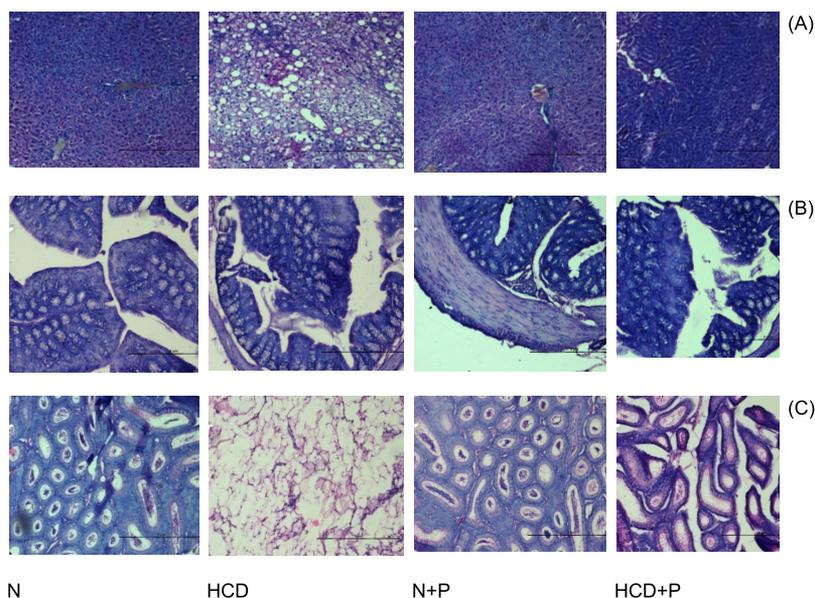
and hepatic tissues.[35] LPS is said to be the molecular link between high fat intake, microbiota, and inflammation and was found to trigger the onset of high-fat diet-induced obesity and type 2 diabetes.[24] Intestinal bacteria from the lumen were found to migrate to the mucosal layer where they get phagocytosed by dendritic cells and translocated to metabol-

ically active tissues.[25] Hence, high-fat diet increases the count of Gram-negative bacteria that produces LPS (antigenic and trigger inflammation), and translocation of these is a factor resulting in inflammation and insulin resistance. The inflammation is characterized by enhanced secretion of proinflammatory cytokines, which was observed in our study

upon feeding of high-cholesterol diet to mice for a period of 8 weeks. However, supplementation of probiotic curd showed downregulation of TNF-α in adipose tissue by stimulating the anti-inflammatory cytokine IL-10, suggesting its immunomodulatory potential. Probiotic bacteria like *L. fermentum* and probiotic mixture VSL#3 have shown similar immunomodulatory effects by stimulating anti-inflammatory IL-10 in the colitis model and high-fat diet model in rats, respectively.[36,37]

Supplementation of *L. fermentum* augmented the expression of GLP-1 in the intestine, which seems to play a role in improving inflammation and insulin resistance caused by high-fat diet intake.[38] GLP-1, an incretin secreted by L-cells of the intestine, stimulates glucose-dependent insulin secretion and inhibits the release of mass.[34] Probiotic consortium VSL#3 demonstrated gut microbiota modulation ability, which stimulated the production of SCFAs that in turn promotes secretion of GLP-1.[39] Our result suggests that *L. fermentum* via mechanisms involving modulation of gut microbiota and production of LAB count stimulates SCFA production, which acts upon GLP-1 secretion.

Thus, the results indicate that probiotic supplementation of *L. fermentum* collectively influences the effects of high-fat diet by modulating the immune response and anti-inflammatory effect and enhancing barrier function and other metabolic markers to reduce inflammation and prevent insulin resistance.

2.5. Histopathological Evidence. Hematoxylin and eosin (H&E) staining of liver sections of high-cholesterol diet (HCD) mice showed cellular infiltration of neutrophils, suggesting initial inflammatory response and formation of few vesicular structures, indicating the initiation of hepatic steatosis. However, both the inflammatory infiltration and steatosis formation were reversed in the groups treated with *L. fermentum* MCC 2760-based probiotic curd. Slight disruptions were observed in intestinal sections of high-cholesterol diet-fed mice, showing mucosal damage. The size and number of adipose tissues and neutrophil infiltrations were reduced in the probiotic intervened groups as compared to the controls (Figure 8).

Insulin resistance in the cells of different tissues is marked by the cellular infiltration of inflammatory molecules due to enhanced secretion of proinflammatory cytokines. Insulin resistance often combined with fat accumulation lead to the development of hepatic steatosis.[40] Infiltration of neutrophils indicates inflammation of the tissues as evidenced by qPCR results, which showed increased expression of proinflammatory cytokines. Reduction in the number of neutrophils upon probiotic treatment suggests the anti-inflammatory potential of *L. fermentum* MCC2760-based probiotic curd. Oral administration of *L. reuteri* improved hepatic steatosis and insulin resistance in liver tissues of high-fructose-fed rats.[41] These findings indicate inflammation and steatosis caused by consumption of high-cholesterol diet that may be ameliorated by the administration of *L. fermentum*. Consumption of high-cholesterol diet causes dysbiosis of the intestinal mucosal barrier.[42] However, probiotic administration can normalize the microbiota balance and enhance the barrier integrity by several mechanisms.

3. CONCLUSIONS

Consumers are converting to being more interested in including healthy foods into their diet. Dairy products such as yoghurt are an excellent vehicle to transport helpful microorganisms to consumers. In this study, the ability of *L.* *fermentum* MCC2760-based probiotic curd to ameliorate the implications of high-cholesterol diet-induced hypercholesterolemia *in vivo* by reducing the inflammation and gut microbial dysbiosis was studied. *L. fermentum* MCC2760 displayed a potent anti-inflammatory effect by stimulating the expression of IL-10, simultaneously downregulating the expression of proinflammatory cytokine TNF-α, IL-12, and IL-6 in adipose tissue. Probiotic curd supplementation also improved the expression of GLP-1, ZO-1, and CB-2 in the intestine. Thus playing a role in the improvement of barrier function and stimulating other gut functions. Reduction in levels of serum markers such as cholesterol, triglycerides, LDL-C and glucose and enhancement of the serum antioxidant enzymes are suggestive of its ability to ameliorate the cholesterol-lowering and pre-diabetic indications effects. *L. fermentum* MCC 2760 also showed the ability to modulate the fecal microbial count. Histopathological sections showed improvement in the cellular infiltration and pathological indications occurring due to the high cholesterol diet intake. From this study, it was demonstrated that the formulated *L. fermentum* MCC2760-based probiotic curd could alleviate the adverse effects of high-cholesterol diet by modulating the immune response from proinflammatory to anti-inflammatory, enhancing gut function by modulating the dysbiosis of gut microbiota. Since low-grade inflammation triggers inflammatory pathways causing insulin resistance, supplementation with *L. fermentum* MCC2760 could delay or prevent the onset of lifestyle-related or metabolic disorders. Hence, the formulated *L. fermentum* MCC2760-based probiotic curd is a potent probiotic formulation with the ability to modulate gut and immune function and influence the overall health of the host.

4. EXPERIMENTAL SECTION

4.1. Chemicals. Diet components and microbiological media such as Luria−Bertani agar, Brucella agar base with the supplement, and Mannitol agar were purchased from Himedia, Mumbai, India. Soybean oil and lard were purchased from the local market (Mysuru, Karnataka, India). Serum biochemical assay kits were procured from Agappe India Pvt. Ltd. (Cochin, Kerala). A transcriptor high-fidelity cDNA synthesis kit was purchased from Bionova-Roche chemicals (Bengaluru, India). Primers were synthesized from Eurofins Genomics Pvt. Ltd. (Bengaluru, India). Other chemicals were purchased from standard chemical manufacturers or suppliers.

4.2. Animal Maintenance and Diet. Male C57BL6 mice weighing 22 $\pm$ 2 g were kept at the animal house facility at CSIR-Central Food Technological Research Institute, Mysore, India. The experimental procedures and animals were approved by the Institutional Animal Ethics Committee of CSIR-CFTRI, Mysuru, India in line with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations. The approval number for the experimental procedure was CFT/IAEC/146/2019. The animals had access to food and water *ad libitum.* The animals were maintained at a 22 $\pm$ 2 °C temperature and humidity 50 $\pm$ 10% with a 12 h light and dark cycle in standard polycarbonate cages. The animals were grouped as follows, containing five mice in each group: (1) normal diet (N), (2) high-cholesterol diet (HCD), (3) normal diet with probiotic curd supplementation (N + P), and (4) high-cholesterol diet with probiotic curd supplementation (HCD + P). Oral administration of 0.2 mL of market curd and 0.2 mL of probiotic curd was done to the control and probiotic curd

group, respectively, daily for eight weeks. Then, 10.95 log CFU/mL of *L. fermentum* was maintained in the developed probiotic curd.

4.3. Diet and Curd Preparation. AIN-93 M diet was prepared and provided to animals in the normal diet group. High-cholesterol diet was provided for animals in the high-cholesterol diet group. The compositions of AIN-93 M diet and high-cholesterol diet are given (Table S1). The probiotic curd was prepared according to the Bureau of Indian Standards (BIS) − IS 9617 using *Lactococcus lactis* 11, a nisin producer as a starter culture and a potent probiotic *L. fermentum* MCC2760.

4.4. Feed Intake and Weight Gain. Daily feed intake and weekly body weight were recorded during the eight weeks of the experimental period.

4.5. Serum and Tissue Processing. Animals were euthanized under CO_2 anesthesia upon completion of the experiment. The cardiac puncture was done to collect blood, and it was allowed to clot at 4° C for 2 h followed by centrifugation at 2600 rpm for 20 min at 4° C, and serum was collected. The serum was stored at -20° C until further analysis. Tissues of the intestine and adipose tissues were collected and stored in RNAlater solution until further use.

4.6. Serum Biochemical Analysis. Levels of glucose, total cholesterol, high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), triglycerides, total protein, uric acid, urea, creatinine, albumin, AST, and ALT were analyzed in the serum using commercial analytical kits (Agappe, Diagnostics Ltd., Mumbai, India).

4.7. Analysis of Serum Antioxidant Enzymes. Serum antioxidant enzymes like catalase,[43] glutathione peroxide,[44] superoxide dismutase,[45] and glutathione transferase[46] were analyzed.

4.8. Evaluation of Marker Genes by Real-Time qPCR. Intestine and MAT tissues (1 g) were homogenized in a tissue homogenizer (Kinematica, Switzerland). The homogenate was subjected to RNA isolation using 1 mL of Trizol reagent (Sigma-Aldrich, Bengaluru, India) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a transcriptor high-fidelity cDNA synthesis kit (Bionova-Roche Chemicals, Bengaluru, India). Real-time qPCR (BioradCFX-96, Bengaluru, India) was carried out using diluted cDNA (1:25) as a template, SYBR Green Jumpstart Taq ReadyMix (Sigma-Aldrich, Bengaluru, India), and gene specific primers. The PCR program comprised an initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation (94 °C for 30 s), annealing (60 °C for 30 s), and elongation (72 °C for 30 s). Primer specificity and efficiency were evaluated from the melt curves. Data was obtained from triplicate sample runs along with the no-template control. By using the Primer3 software, oligonucleotide primers were designed (http://primer3.ut.ee/) by obtaining consensus sequences belonging to mice (*Mus musculus*) genes from NCBI and later synthesized from Eurofins Genomics India Pvt. Ltd. (Bengaluru, India). The primers related to inflammatory cytokine genes and other markers are listed in Table S2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control for normalization of gene expression. Gene expression results were expressed as a relative normalized expression (fold change) calculated using the $2^{-\Delta\Delta Ct}$ method.

4.9. Bacterial Translocation. Bacterial translocation was evaluated in adipose tissue post necropsy by plate count on LB agar. Adipose tissue (1 g) was washed with 1× sterile PBS, and it was homogenized with 1 mL of 1× PBS. Then, 100 μL of homogenate was plated on LB agar.

4.10. Fecal Microbiota Analysis by a Culture-Based Method. Feces of mice (150 mg) were collected and evaluated for the fecal microbial count. Feces was homogenized in saline (0.85%), serially diluted, and plated on their respective media. Total aerobes were obtained on Luria−Bertani (LB) agar, total LAB count on MRS agar, *Campylobacter* on basal media with supplement, and *Staphylococcus* spp. on Mannitol salt agar.

4.11. Histopathology. Tissues of the liver, intestine, and adipose tissue were sectioned and stained with hematoxylin and eosin (H&E) stain for histopathology evaluation. The stained tissue slides were observed under a bright-field microscope (Labomed, Burlington, NC, USA).

4.12. Statistical Analysis. Data were statistically investigated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). All data were represented as mean ± SD (n = 5). One-way ANOVA was used for the analysis of a single parameter, and two-way ANOVA analysis was used for comparison between groups. $P < 0.05$ was considered as significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00045.

> (Table S1) Composition of normal diet AIN-93 M and high-cholesterol diet (HCD) and (Table S2) list of primers used in this study (GAPDH, glyceraldehyde phosphate dehydrogenase; TNF, tumor necrosis factor; IL, interleukin; GLP-1, glucagon-like peptide-1; ZO, zonula occludens-1; eCB, endocannabinoid system; MOA, mode of action) (PDF)

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■ ABBREVIATIONS USED

CHD-coronary heart disease; MAT-mesenteric adipose tissue; g-gram; CFU-colony forming units; LAB-*Lactobacillus*; LDL-C-low-density lipoprotein cholesterol; HDL-C-high-density lipoprotein cholesterol; AST-aspartate aminotransferase; ALT-alanine transaminase; eCB-endocannabinoid system; GAPDH-glyceraldehyde phosphate dehydrogenase; TNF-tumor necrosis factor; IL-interleukin; GLP-1-glucagon-like peptide-1; ZO-zonula occludens-1

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