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RESEARCH

Protective Effects of *Limosilactobacillus reuteri* MSMC64 in Hyperlipidemia Rats Induced by a High-Cholesterol Diet

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Abstract

Hyperlipidemia, characterized by abnormally elevated levels of lipids such as cholesterol, is a significant risk factor for cardiovascular diseases (CVD), contributing to increased oxidative stress, inflammation, and disruption of gut immunity. Dysbiosis, or imbalance in the gut microbiome, plays a critical role in the pathogenesis of hyperlipidemia. Probiotics, as key components of the gut microbiome, have been shown to positively impact health. This study aimed to evaluate the effects of *Limosilactobacillus reuteri* MSMC64 on lipid profiles, blood glucose levels, hepatic steatosis, antioxidant capacity, inflammatory biomarkers, and colon barrier immunity in hyperlipidemic rats induced by a high-cholesterol diet. The results demonstrated that the administration of *L. reuteri* MSMC64 may improve lipid profiles and blood glucose levels, reduce hepatic steatosis and oxidative stress, and lower inflammatory biomarkers while maintaining colon barrier integrity. These findings suggest that *L. reuteri* MSMC64 has the potential to be developed as a probiotic supplement for mitigating risk factors associated with hyperlipidemia and CVD.

Keywords *Limosilactobacillus reuteri* · Hyperlipidemia · Metabolic disease · Cardiovascular disease

Introduction

One of the major risk factors for cardiovascular diseases (CVD), such as coronary artery disease, peripheral arterial disease, and stroke, is hyperlipidemia due to atherosclerosis [1]. Millions of CVD-related deaths worldwide are attributed to hyperlipidemia. Over the past few years, cholesterol levels have increased most in Asian countries such as Thailand, China, and Malaysia and have surpassed those in other Western countries [2]. Three types of hyperlipidemia, hypercholesterolemia, hypertriglyceridemia, and mixed hyperlipidemia, are distinguished by increased levels of total cholesterol, triglycerides, or both, respectively. There is a hereditary or acquired underlying reason for this disease [3]. The therapy of hyperlipidemia can be achieved using lipid-lowering agents; nevertheless, there have been reports of severe adverse effects, including hepatitis and rhabdomyolysis [4].

According to data from human trials and animal models, the development of hyperlipidemia is closely linked to gut microbiota dysfunction or gut dysbiosis [5]. It was discovered by Wang et al. that giving mice with high-fat diets (HFD) altered the balance of the gut microbiome and antioxidant capacity, which led to increased cholesterol levels [6]. Through host and gut microbiome interactions that support the gastrointestinal system's metabolic balance, probiotics can have various positive health impacts. According to the definition, probiotics are"live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [7]. It was discovered that administering some probiotic strains could help to improve lipid profiles [8]. Probiotics may compete with pathogens for adherence to epithelial cells, metabolic substrates, and immune system regulation [9].

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Limosilactobacillus reuteri MSMC64 produced reuterin and had antagonistic activity against enteric and other pathogens [10]. Gram-positive and gram-negative bacteria, as well as fungi and protozoa, are among the food-borne pathogens against which reuterin has demonstrated good broad-spectrum activity [11]. Moreover, *L. reuteri* MSMC64 demonstrated the capacity to endure in bile and acid environments, both of which are essential to its survival in the gastrointestinal system [10]. Therefore, in HFD-induced hyperlipidemia rats, the objective of this study was to examine the beneficial effects of *L. reuteri* MSMC64 on lipid profiles, blood glucose level, hepatic steatosis, antioxidant capability, anti-inflammation, and immune function within the colon barrier.

Materials and Methods

Probiotic Strains and Culture Condition

Limosilactobacillus reuteri MSMC64, which was isolated from healthy human infants and obtained from the Center of Excellence in Probiotics, Faculty of Medicine, Srinakharinwirot University, Thailand. *L. reuteri* MSMC64 was cultivated for 48 h at 37 ◦C in anaerobic conditions on de Man, Rogosa, and Sharpe (MRS) agar (Himedia, Mumbai, India). Probiotic was cultured in MRS broth using a single colony, which was then incubated under the same circumstances. The bacterial cells were then collected by centrifugation at 5000 × g, 4 ◦C for 10 min. Then, the cells were diluted to 10^9 colony-forming units (CFU)/mL in sterile phosphate-buffered saline (PBS, 0.1 M, pH 7.2).

Housing and Feeding of Animals

Fifteen male Sprague Dawley rats weighing between 120 and 140 g at eight weeks were acquired from Nomura Siam International, Bangkok, Thailand. The Ethics and Research Standardisation Section of Srinakharinwirot University's guidelines (approval number: COA/AE- 016–2564) were followed for all animal procedures. The rats were randomly assigned to three groups of five each, and they were kept in plastic cages with wire mesh walls in a room with a temperature of 22 ± 2◦C, a light–dark cycle of 12/12 h, and a relative humidity of 60 ± 5%. Before the experiments, the animals were acclimated for one week on a basal diet (082G/15, National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand) that contained 12.6% (w/w) moisture, 0.9% (w/w) phosphorus, 24% (w/w) protein, 4.5% (w/w) fat, 5% (w/w) fiber, and 52% (w/w) carbohydrate. After that, all rats were divided into three groups based on their average body weight at random. For twelve weeks, each group was given the following daily oral gavage of various supplements in addition to a basal diet and unlimited access to water: 1. (Normal control, NC): 2 mL of PBS was given; 2. (High fat-diet, HFD): 1 mL of HFD and 1 mL of PBS were given; 3. (*L. reuteri* MSMC64, MSMC64): 1 mL of HFD and 1 mL of 1×10^9 CFU/mL *L. reuteri* MSMC64 were given, which was the effective dose for high-cholesterol diet rats [12–14].

With a few minor adjustments, HFD was modified from Puttarat et al. with the main components comprising 5%(w/v) cholesterol, 0.5% (w/v) sodium cholate, 12.5% (w/v) sucrose, and 17.2% (w/v) fat [15]. Rats'body weight was assessed once a week, and the amount of food they ate each day was noted in the morning.

Sample Collection

All rats were forced to fast for ten hours before euthanasia after the experiment. They were sacrificed to draw blood by heart puncture. The liver, colon, and brain were blotted dry before extraction, cleaning, and weighing. Subsequently, 10% (v/v) formaldehyde was utilized to fix the liver and colon tissues. In addition, portions of liver tissue were preserved through freezing.

Biochemical Analysis

Plasma was obtained by centrifuging heparinized blood at 1,500 × g and 4 ◦C for 15 min. The plasma was tested for biochemical parameters by Professional Laboratory Management Corp, Co., Ltd., Bangkok, Thailand, which included total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting blood glucose (FBG), aspartate transaminase (AST), and alanine transaminase (ALT).

Histological Analysis of the Liver and Colon

A rat's liver was sectioned, and the right lobe was embedded in paraffin. Liver tissue sections measuring 5 µm in thickness were stained using Masson's trichrome, Hematoxylin and eosin (H&E), and Oil Red O. Liver tissue sections were deparaffinized and rehydrated with gradient alcohol for Masson's trichrome staining. After that, it was re-fixed in Bouin's solution for an hour at 56 °C, rinsed for five minutes under running tap water, and stained for five minutes with Weigert's iron hematoxylin. After another rinsing under running tap water, the sections were stained for 20 min in Biebrich's scarlet-acid fuchsin solution and then cleaned with distilled water. Following a 10-min differentiation in phosphotungstic-phosphomolybdic acid, the sections were moved to an aniline blue solution and left for eight minutes. They were then briefly rinsed in distilled water, dipped once in 1% acetic acid, and rinsed in distilled water before being

mounted on slides. Hematoxylin and eosin staining were applied to the slides. Morphology was examined using an Olympus BX53 light microscope (Olympus Corporation, Tokyo, Japan) and photos were taken. Frozen liver sections were fixed with sucrose and stained with Oil Red O (Sigma Aldrich Co., St. Louis, MO, USA) following the methods outlined by Guo D et al. [16]. Based on the NAFLD (Non-Alcoholic Fatty Liver Disease) activity score (NAS), obesity-induced liver damage scores were assessed. The total scores for lobular inflammation (0–3), hepatocyte ballooning (0–2), and steatosis (0–3) were computed to get the NAS, which has a range of 0 to 8 [17]. For the quantitative analysis of collagen density (% area) [18] and lipid accumulation (% area) [19], which were stained using Masson's trichrome and Oil Red O, respectively, ImageJ software was used for image analysis.

The 5 µm-thick paraffin-embedded colon was sectioned, and 10 randomly chosen locations were subjected to a semi-quantitative assessment using an inverted microscope (Olympus, UC50) at a 200 × magnification. We assessed ascending colon damage scores using the Geboes Score (GS). Examining erosions or ulcerations, infiltration of mononuclear cells, structural changes, and deterioration of crypts are all part of this extensive grading system. Scores for each condition ranged from 0 (no abnormality) to 5 (severe erosion or ulceration), 1–2 (immune cell infiltrate in the lamina propria), 3 (immune cell infiltrate in the epithelium), and 4 (crypt destruction). When evaluating the paracellular permeability of an ascending colon, zona occludens 1 (ZO- 1, Thermo Fisher Scientific) was utilized to gather data.

Measurement of Hepatic Cytochrome P450 7 A1 (CYP7 A1)

Using an Enzyme-Linked Immunosorbent Assay (ELISA) (Rat TNF-alpha DuoSet ELISA, R&D systems) for cholesterol 7-alpha-monooxygenase or cytochrome P450 7 A1 (CYP7 A1) detection, the levels of hepatic CYP7 A1 in homogenized liver tissues were determined following the manufacturer's instructions (My BioSource, USA).

Antioxidant Activities in Liver and Brain Tissues

Superoxide dismutase (SOD), and malondialdehyde (MDA) levels in homogenized liver and brain tissues were determined using colorimetric assay according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

Determination of Pro-inflammatory Cytokine in Liver and Brain Tissues

Liver and brain tissues were homogenized (10% w/v) with RIPA lysis buffer (Sigma-Aldrich, USA). Lysed tissue samples were sonicated on ice by ultrasonic homogenizers (Sonoplus, Bandelin, Germany). Then, samples were centrifuged at 300 × g, 10 min, 0 °C. Liver and brain supernatant were collected to quantify tumor necrosis factor-alpha (TNF-alpha) levels by ELISA.

Analysis of Colon Barrier Immunity (Toll-like Receptors (TLR))

Using quantitative reverse transcription polymerase chain reaction (qRT-PCR), interesting genes related to the inflammatory response were assessed. Thermo Fisher Scientific's TRIzol reagent was used to extract the colon's total ribonucleic acid (RNA), which was then transformed into complementary deoxyribonucleic acid (cDNA) using reverse transcriptase (Applied Biosystem, Warrington, UK). The resulting product was then run on an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System using SYBR® Green PCR master mix (Applied Biosystems). The comparative cycle threshold versus expression was utilised to normalize the transcription levels to target genes using beta-actin. Table 1 displays the primers for the PCR.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad software, version 8.0.2, San Diego, CA, USA). Comparisons of two groups were performed using unpaired, two-tailed Student's t-tests. Comparisons of three or more groups were performed using ANOVA with Dunnett's or Tukey's multiple comparison test. All experiments were performed three times independently. The results were expressed as means ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

Table 1 List of primers used in this study

Gene	Forward	Reverse
Toll-like receptor 2 (TLR- 2)	5′-CGCTTCCTGAACTTGTCC- 3′	5′-GGTTGTCACCTGCTTCCA- 3′
Toll-like receptor 4 (TLR- 4)	5′-GCATCATCTTCATTGTCCTTGAGA- 3′	5′-CTCCCACTCGAGGTAGGTGTTT- 3′
Beta-actin	5′-ACTGCCCTGGCTCCTAGCA- 3′	5′-GCCAGGATAGAGCCACCAATC- 3′

Results

Body Weight, Lipid Profile, Blood Glucose, and Liver Function Test

This study is the first study that investigated the effects of *Limosilactobacillus reuteri* MSMC64 in high-fat diet-induced hyperlipidemia rats. The body weight of rats in all groups increased with no statistically significant difference in body weight gain among *L. reuteri* MSMC64 (MSMC64), High fat-diet (HFD), and normal control (NC) groups during the study period (Fig. 1).

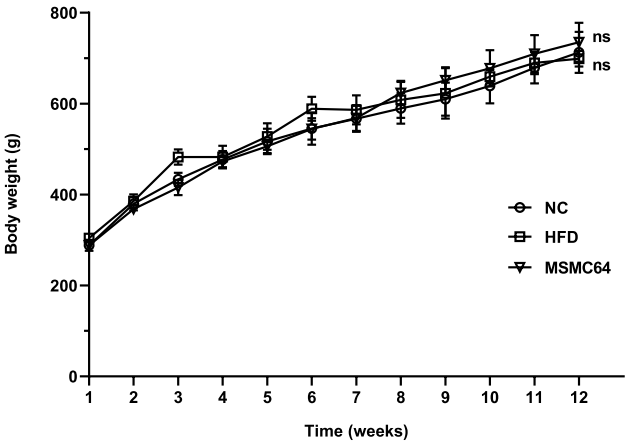

Fig. 1 Trend of body weight in rats (grams) NC, normal control group; HFD, high fat-diet group; MSMC64, *Limosilactobacillus reuteri* MSMC64 group

To evaluate the effect of *L. reuteri* MSMC64 in reducing total cholesterol, triglyceride, LDL-C, and FBG. Based on the result in Fig. 2, total cholesterol, triglyceride, and LDL-C levels of the HFD group increased when compared with the normal control group. Interestingly, administration of *L. reuteri* MSMC64 reduced total cholesterol, triglyceride, and LDL-C levels with statistical significance. *L. reuteri* MSMC64 increased HDL-C significantly. Moreover, *L. reuteri* MSMC64 reduces the FBG level statistically significant.

The main liver enzymes, such as ALT and AST, are important indicators of liver injury. In order to ascertain the lipid-lowering effect on HFD-induced hepatic steatosis, AST and ALT levels were determined in conjunction with the outcome of liver histology evaluation. The three groups had no significant changes in AST and ALT levels (Fig. 2).

Histological of the Liver and Colon

The liver histology depicted in Figs. 3A and C indicated that normal hepatocytes were present in the normal control and *L. reuteri* MSMC64 groups, respectively. H&E staining of the hepatic cells revealed distinct acidophilic and

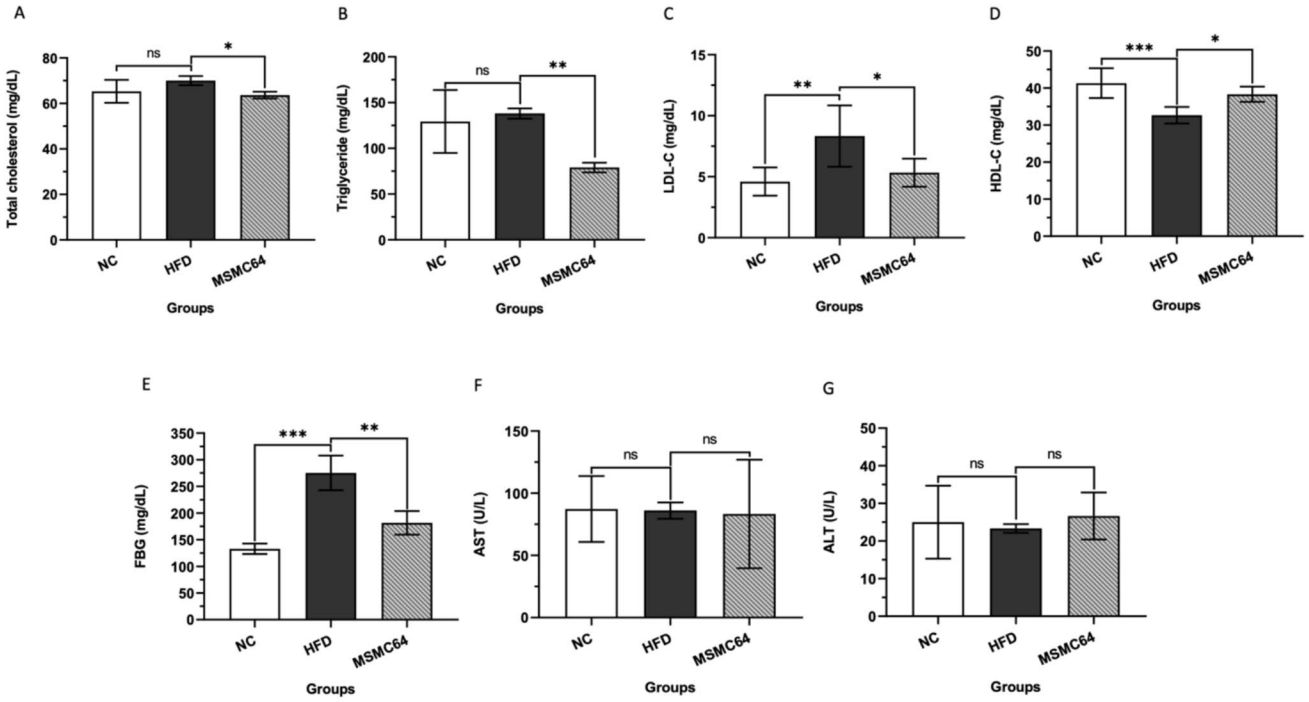

Fig. 2 Blood parameters: Total cholesterol (**A**), triglyceride (**B**), LDL-C (**C**), HDL-C (**D**), FBG (**E**), AST (**F**), and ALT (**G**). *, *p*-value <0.05; **, *p*-value <0.01; ***, *p*-value <0.001; ALT, alanine transaminase; AST, aspartate transaminase; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NC, normal control group; HFD, high fat-diet group; MSMC64, *Limosilactobacillus reuteri* MSMC64 group

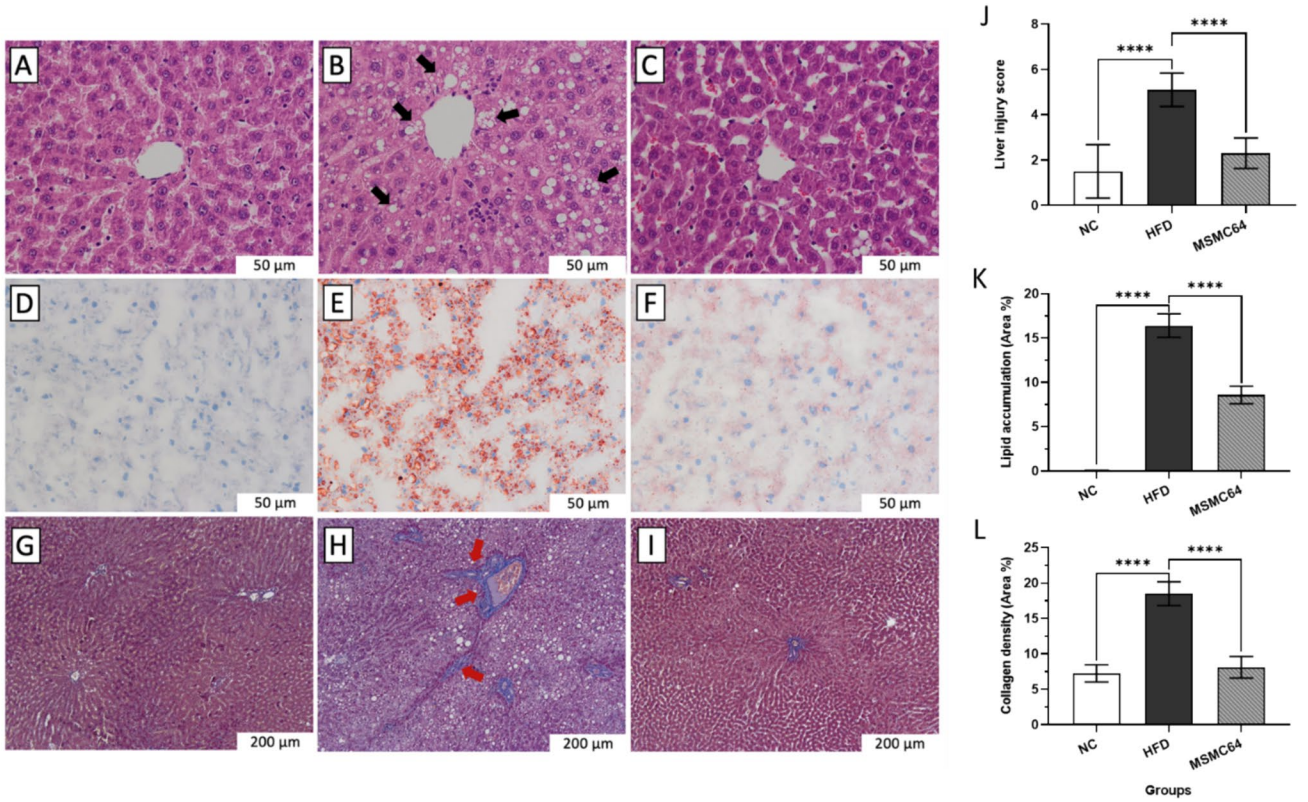

Fig. 3 The liver histology of normal control (NC), high-fat diet (HFD), and *L. reuteri* MSMC64 (MSMC64) groups from left to right in each row. Hematoxylin and eosin staining (**A**-**C**) (magnification of 400x), Oil Red O staining (**D**-**F**) (magnification of 400x), and Masson's trichrome staining (**G**-**I**) (magnification of 100x) of liver tissue sections of rats. The liver injury score (**J**), lipid accumulation (% area) (**K**), and collagen density (% area) (**L**) are shown. Error bars represent the mean ± SD.****, $p < 0.0001$

basophilic areas in the nucleus and cytoplasm, respectively. The nucleus was located in the middle of the cells, providing their microscopic structure a spherical appearance with distinct boundaries. Increasing lipid droplet vacuolization, on the other hand, widely distributed and accumulated in the hepatocytes of the HFD group (black arrows) (Fig. 3B). When compared to a normal control group, the HFD group had a statistically significant higher liver injury score. The liver injury score was considerably lower in the *L. reuteri* MSMC64 group than in the HFD group (Fig. 3J). Hepatic lipid accumulation in all groups was confirmed by Oil Red O staining (Figs. 3D-F), which showed that the *L. reuteri* MSMC64 groups significantly decreased lipid accumulation compared to the HFD group, as shown in Fig. 3K. The perisinusoidal space in the *L. reuteri* MSMC64 group was also comparable to that of normal hepatocytes. Masson's trichrome staining (Figs. 3G–I), is used to demonstrate collagen fibers that develop into fibrosis. The liver tissues of the normal control and *L. reuteri* MSMC64 groups lacked collagen fibers (Fig. 3G and I). Collagen fiber aggregation in the pericentral and periportal regions of the HFD group was the initial cause of fibrosis (red arrows) (Fig. 3H), which is consistent with the quantitative analysis of collagen density in Fig. 3L.

The colon histology is depicted in Fig. 4. The damage to the colon epithelium caused immune cells (blue arrows) to infiltrate the lamina propria and epithelium layer, resulting in crypt destruction in the HFD group (Fig. 4B). However, the normal control and MSMC64 groups exhibited normal morphology (Fig. 4A and C). Zona occludens 1 (ZO- 1) is a tight junction protein. The ZO- 1 stain was used to assess the paracellular permeability of colonocytes, as shown in Figs. 4D-F. The ZO- 1% in HFD was the lowest compared to the other groups with statistical significance. The effect of *L. reuteri* MSMC64 on ZO- 1 increased ZO- 1% relative to HFD with significance (Fig. 4G). Furthermore, HFD had the greatest colon injury score significantly when compared to the others. Colon injury score was dramatically reduced by *L. reuteri* MSMC64 compared to the HFD group as shown in Fig. 4H.

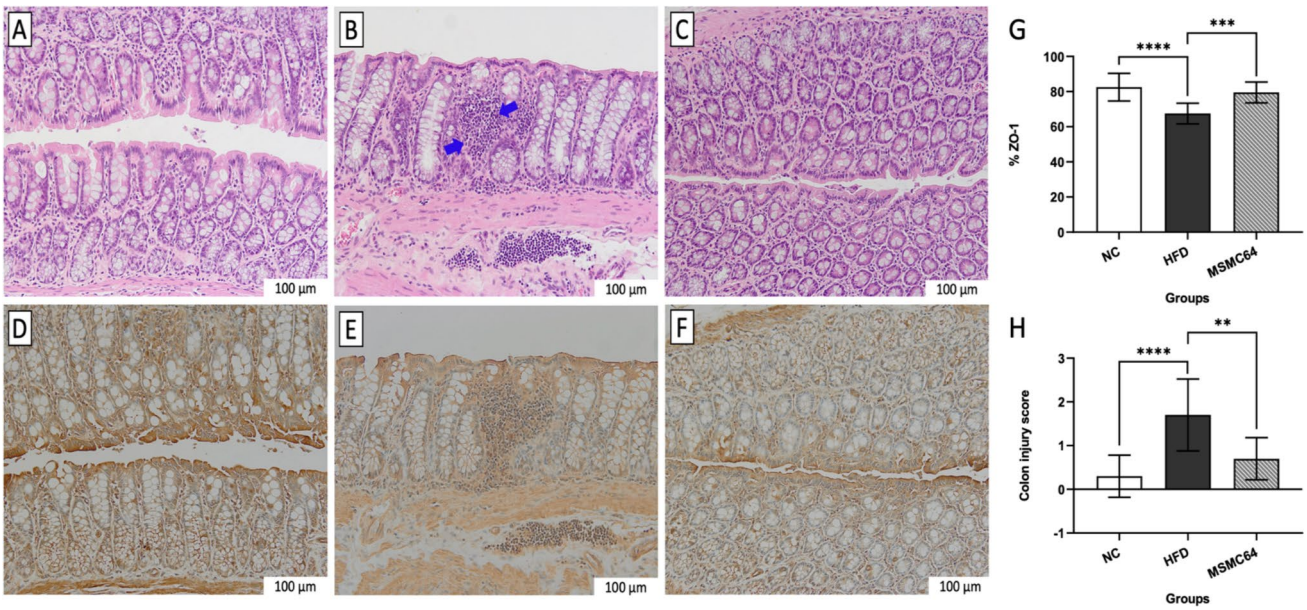

Fig. 4 The colon histology of normal control (NC), high-fat diet (HFD), and *L. reuteri* MSMC64 (MSMC64) groups from left to right in each row. Hematoxylin and eosin staining (**A**-**C**) and Zona occludens 1 staining (**D**-**F**) of colon tissue sections (magnification of 200x) of rats. The percentage of ZO- 1 is shown in Figure **G**. The colon injury score is illustrated in Figure **H**. Error bars show the mean ± SD. **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$

Hepatic Cytochrome P450 7 A1 (CYP7 A1) Level

The enzyme cholesterol 7-alpha-monooxygenase, or CYP7 A1 level, is encoded by the *CYP7 A1* gene and is essential for the metabolism of cholesterol and the generation of bile acid. Figure 5 demonstrates that the hepatic CYP7 A1 levels were lowest in the HFD group when compared to the other groups. When compared to HFD, *L. reuteri* MSMC64 significantly increased the CYP7 A1 level.

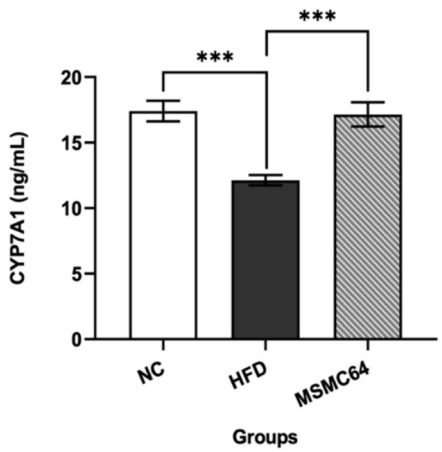

Fig. 5 Effect of *L. reuteri* MSMC64 administrations on levels of cholesterol 7-alpha-monooxygenase or cytochrome P450 7 A1 (CYP7 A1); NC, normal control group; HFD, high-fat diet group; MSMC64, *Limosilactobacillus reuteri* MSMC64 group. Error bars show the mean ± SD. ***, $p < 0.001$

Antioxidant Activities

One of the body's most important antioxidant defenses against oxidative stress is superoxide dismutase (SOD). This enzyme is a beneficial treatment for diseases caused by reactive oxygen species. When compared to the HFD group, *L. reuteri* MSMC64 significantly elevated the SOD levels in the brain and liver (Figs. 6A and B).

As a byproduct of free radicals oxidizing lipids, malondialdehyde (MDA) is a potent biological marker of systemic oxidative stress [20]. In the present study, the MDA level was the highest in the HFD group in both brain and liver tissues with statistical significance. *L. reuteri* MSMC64 decreased the brain and hepatic MDA levels compared to the HFD group significantly, as shown in Fig. 6C and D.

During acute inflammation, macrophages release a cytokine called tumor necrosis factor-alpha (TNF-alpha). TNF-alpha triggers various signaling processes in cells that might result in necrosis or death. In brain and liver tissues, TNF-alpha was markedly elevated in HFD compared to normal control. In comparison to the HFD group, *L. reuteri* MSMC64 significantly reduced the levels of TNF-alpha in the brain and liver (Fig. 6E and F).

Colon Barrier Immunity (Toll-like receptors (TLR))

TLR- 2 and *TLR- 4* gene expression was significantly increased in the HFD group compared to the normal control group (Fig. 7A and C). Lower *TLR- 2* and *TLR- 4* gene

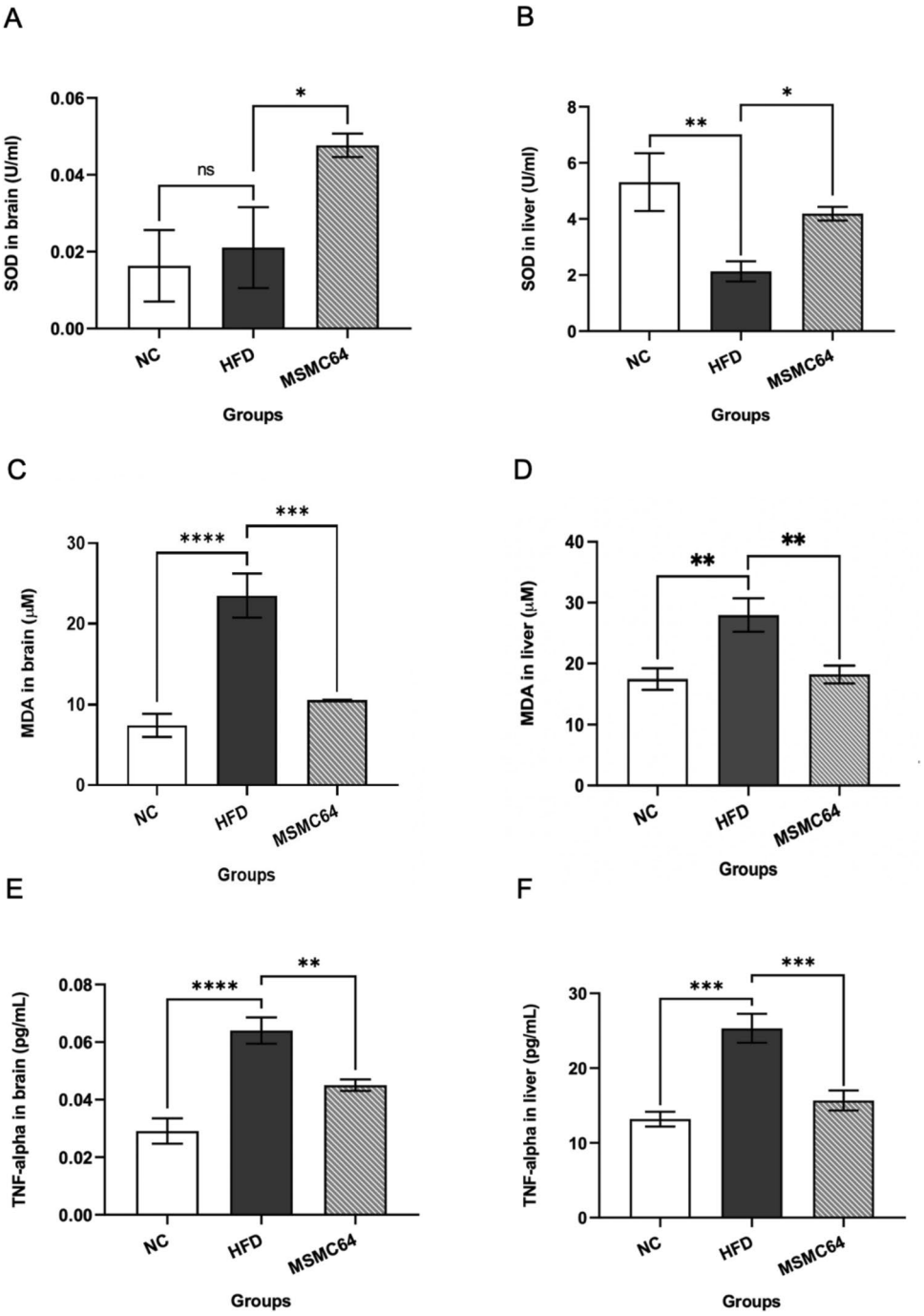

Fig. 6 Superoxide dismutase (SOD), Malondialdehyde (MDA), and tumor necrosis factor-alpha (TNF-alpha) levels in brain tissue (**A**, **C**, **D**) and liver tissue (**B**, **D**, **F**) in each group.; NC, normal control group; HFD, high-fat diet group; MSMC64, *Limosilactobacillus reuteri* MSMC64 group. Error bars show the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant

expression was shown by *L. reuteri* MSMC64, with statistical significance when compared with the HFD group (Fig. 7B and D).

Discussion

This investigation explores the effects of *Limosilactobacillus reuteri* MSMC64 on rats with high blood cholesterol levels due to a high-fat diet. Combination *L. reuteri* MSMC64 ameliorated steatosis, hepatic fibrosis, hepatic injury, colon injury, hyperlipidemia, and high fasting blood glucose levels in rat models, maybe due to reduced inflammation and oxidative stress. These data demonstrated the properties of *L. reuteri* MSMC64, even if the tests were limited to rats.

The findings of this study align with previous research demonstrating the cholesterol-lowering potential of *Lactobacillus reuteri* strains. A study by Kumar et al. investigated the hypocholesterolemic effects of *L. reuteri* LR6 in rats fed a high-cholesterol diet and found that supplementation

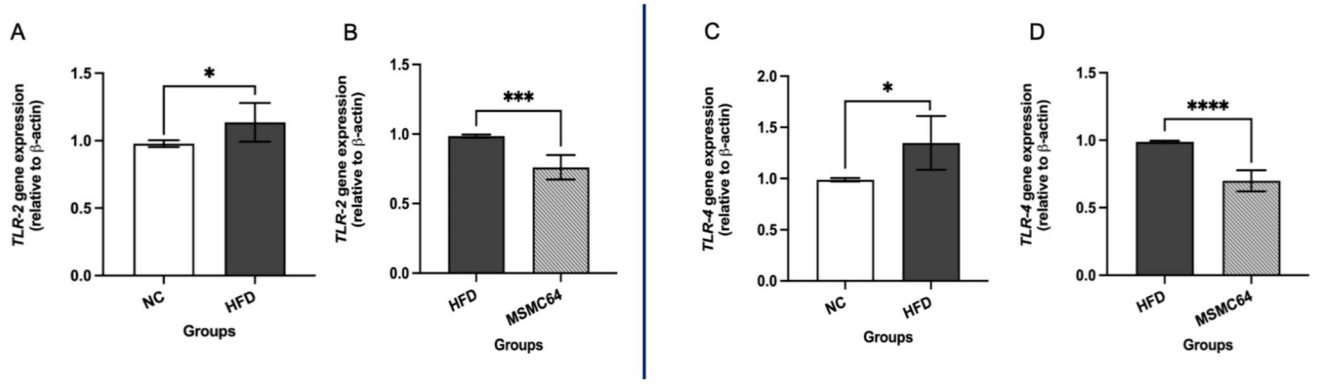

Fig. 7 Comparison of Toll-like receptor (TLR) gene expression; *TLR- 2* between NC and HFD (**A**), HFD and MSMC64 (**B**); TLR- 4 between NC and HFD (**C**), HFD and MSMC64 (**D**); NC, normal control group; HFD, high-fat diet group; MSMC64, *Limosilactobacillus reuteri* MSMC64 group; Error bars show the mean ± SD. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$

with *L. reuteri* LR6-fermented skimmed milk significantly reduced total cholesterol, triglyceride, and LDL-C levels compared to control groups. However, HDL-C levels did not show a significant increase. These findings indicate that the cholesterol-lowering effects of *L. reuteri* may be linked to its bile salt hydrolase (BSH) activity and bile tolerance. By hydrolyzing conjugated bile acids into their deconjugated forms, which are less soluble and more readily excreted, *L. reuteri* disrupts lipid emulsification in the intestine. This process reduces lipid absorption and, consequently, contributes to lower lipid levels [12, 21]. Consistent with the findings of the present study, a significant reduction in lipid profiles was also observed following *L. reuteri* MSMC64 supplementation, further reinforcing the role of specific *L. reuteri* strains in cholesterol modulation.

The cholesterol-lowering effects of *Lactobacillus* strains have been extensively studied, with evidence suggesting multiple mechanisms contributing to lipid metabolism regulation. A study by Zeng et al. evaluated the hypocholesterolemic properties of three *Lactobacillus* strains isolated from traditional koumiss and demonstrated significant reductions in total cholesterol, triglycerides, and LDL-C levels in rats fed a high-lipid diet, with concurrent increases in HDL-C levels. Additionally, probiotic supplementation led to reduced liver cholesterol content, increased fecal cholesterol excretion, and higher concentrations of total bile acids and short-chain fatty acids, particularly propionic acid and butyric acid, indicating potential interactions between gut microbiota and cholesterol metabolism, which resulted in a decreased cholesterol synthesis rate [22]. Similarly, the present study demonstrated a significant improvement in lipid profiles following *L. reuteri* MSMC64 supplementation, reinforcing the probiotic potential of specific strains in managing hyperlipidemia.

The accumulation of atheromatous plaque is finally facilitated by the transportation of excess cholesterol via LDL-C and its deposit in the wall of blood vessels. The risks of metabolic syndrome, stroke, high blood pressure, and CVD are increased by hypertriglyceridemia. Thus, CVD can be highly predicted by these lipid profile values [23]. By considerably lowering total cholesterol, LDL-C, and triglyceride and raising HDL-C, *L. reuteri* MSMC64 may be able to improve lipid profiles. This result is in agreement with several studies that demonstrated the benefits of *L. plantarum* and *L. reuteri* probiotics [24]. Furthermore, research involving HFD rats shown that certain *Lactobacillus* strains may improve lipid profiles [25, 26].

Hyperlipidemia can lead to several problems, one of which is nonalcoholic fatty liver disease (NAFLD). It is characterized by hepatocyte injury, resulting in high serum AST and ALT levels, as well as histopathological changes. To diagnose NAFLD, a histologic scoring system called the NAFLD Activity Score (NAS) is used. In this study, normal control, HFD, and MSMC64 rat groups had no significant difference in serum AST and ALT levels. It is possible since NAFLD can have normal or fluctuating AST and ALT values [18]. The liver histology stained with H&E and Oil Red O showed that HFD rats had increased lipid droplet vacuolization in the hepatocytes. Moreover, when stained with Masson's trichrome, collagen fiber aggregation in the pericentral and periportal regions was found, indicating the onset of hepatic fibrosis. MSMC64 rats had normal hepatocytes similar to the normal control group, regardless of H&E, Oil Red O, or Masson's trichrome staining. When evaluating the NAS, used to indicate liver injury score, a score equal to or greater than five has confirmed the diagnosis of nonalcoholic steatohepatitis (NASH) [27]. In the present study, a NAS score of five in the HFD group indicated the presence of NASH, while MSMC64 group had significantly lower NAS values. It indicated that *L. reuteri* MSMC64 can reduce fat and fibrosis accumulation in hepatocytes. This result may be explained by the increase of the enzyme CYP7 A1 level in

MSMC64 group which is similar to the control group and higher than in HFD rats. The enzyme CYP7 A1 is the first and rate-limiting enzyme in the bile acid synthesis pathway, which is responsible for the metabolism of cholesterol and the generation of bile acid, resulting in increased cholesterol utilization [28].

The enzyme CYP7 A1 is a key enzyme in the classical bile acid synthesis pathway and serves as the rate-limiting step in the conversion of cholesterol to bile acids, thereby facilitating cholesterol clearance and maintaining lipid homeostasis [28]. Its regulation is influenced by nuclear receptors, including the farnesoid X receptor (FXR), which suppresses CYP7 A1 transcription in response to bile acid accumulation [29]. The gut microbiota plays a crucial role in modulating bile acid metabolism by producing bile salt hydrolases, which deconjugate bile acids, altering their signaling properties and feedback inhibition on CYP7 A1 expression [30]. Dysbiosis, often seen in metabolic disorders, can disrupt this balance, leading to impaired cholesterol metabolism and increased circulating cholesterol levels. Probiotic interventions, such as *Limosilactobacillus reuteri* TF- 7, *Enterococcus faecium* TF- 18, and *Bifidobacterium animalis* TA- 1 have been shown to enhance bile acid deconjugation and upregulate CYP7 A1, promoting cholesterol catabolism and reducing hyperlipidemia [12]. These findings highlight the complex interplay between hepatic cholesterol metabolism and intestinal microbiota, suggesting that targeting CYP7 A1 through microbial and dietary interventions could offer novel therapeutic strategies for hyperlipidemia and related metabolic diseases.

Another mechanism of the lipid-lowering effect of *L. reuteri* is BSH [31]. BSH converts conjugated bile salts into deconjugated bile salts, resulting in decreased reabsorption in the intestines and increased excretion into feces. Consequently, the formation of new bile salts is stimulated. Therefore, more cholesterol is used, thus lowering serum cholesterol levels [32]. Moreover, probiotic cells can consolidate cholesterol into the plasma membrane, a mechanism called cholesterol assimilation [33]. Consequently, serum cholesterol level decreases, thus reducing the incidence of NAFLD. Furthermore, Riezu-Boj et al. found that *L. plantarum* DSM20174 reduced proinflammatory cytokine and M1-like/M2-like ratio of macrophages in white adipose tissue and liver in NAFLD rats. It also changed gut microbiota to have less Christensenellaceae and *Christensenella*. These two species are associated with increased body weight, triglyceride, and insulin resistance. Therefore, *L. plantarum* DSM20174 can reduce the progression of NAFLD [34]. In the previous study, Puttarat et al. studied both single and mixed strains of probiotics including *L. reuteri* TF- 7, *Enterococcus faecium* TF- 18, and *Bifidobacterium animalis* TA- 1 in hypercholesterolemic rats and found that single probiotic can reduce body weight gain, abdominal fat, total cholesterol, triglyceride, LDL-C, and hepatic steatosis. It can also improve the gastrointestinal microbiota. When combined, they further reduced total cholesterol and LDL-C levels close to those of the normal diet-fed group, indicating a synergistic effect. However, triglyceride, HDL-C, AST, and ALT levels were not different from a single probiotic [12]. In another previous study**,** mixed strains of probiotics including *B. longum, B. lactis, B. breve, L. reuteri*, and *L. plantarum* were used to evaluate the hypocholesterolemic effect in hypercholesterolemic rats and found that mixed strains of probiotics could reduce serum total cholesterol, triglyceride, and LDL-C levels with an increase in serum HDL-C level. It also reduces lipid accumulation in hepatic tissue through the mechanism of reducing sterol regulatory element-binding protein 1 (SREBP1), fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC) in the liver, which are hepatic cholesterol synthesis-related proteins [35]. As mentioned above, probiotics can alleviate metabolic syndrome which may be the future treatment in humans.

In the present study, in both the brain and the liver, *L. reuteri* MSMC64 reduced inflammation as demonstrated by elevated SOD levels, and decreased MDA and TNF-alpha. It was consistent with other studies that *B. animalis* MSMC83 was found to increase antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (GSH-Px) and decrease important biological marker of oxidative stress such as MDA. It also decreased pro-inflammatory cytokines such as TNF-alpha in rats with inflammatory liver [36]. This positive effect also happens in the brain. *B. longum* and *B. animalis* decreased TNF-alpha, IL- 1, and IL- 6 via inhibition of NF-KB and TLR4 signaling. They also decreased MDA activity and increased SOD, GSH-Px in the hippocampus of neuroinflammation-induced rats. This results in ameliorating age-related anxiety-like behavior, uncoordinated movement, and cognitive deficit [37]. According to this information, there is potential to use probiotics to treat diseases related to inflammation or aging such as dementia or Parkinson's disease.

The gastrointestinal tract is in charge of protecting the body from microorganisms in addition to absorbing essential nutrients from food. The intestinal barrier system consists of tight junctions, intestinal epithelial cells, immune cells, and gut microbiome [38]. When elements of this barrier system get compromised, intestinal permeability, a hallmark feature of dysbiosis, increases. Consuming a high-fat diet can increase intestinal permeability due to modulating the distribution of tight junctions and increasing barrier-disrupting gut microbiome [39]. The study on probiotics alters the gut microbiome of rats and reduces the expression of zonulin, a permeability biomarker, in the distal regions of the gut [40]. As anticipated by this study, immune cells to infiltrate the lamina propria and epithelium layer in the HFD rat's colon histology. Furthermore, in comparison to the HFD

group, *L. reuteri* MSMC64 had the impact of increasing zona occludens 1 (ZO- 1). This had been linked to lower colon injury scores.

In addition to facilitating communication between innate and adaptive immunity, toll-like receptors (TLR) can increase the expression of pro-inflammatory cytokines, proliferation, and survival mechanisms. Toll-like receptor 2 (TLR2) in the intestines directs immune responses to pathogens and controls the function of the epithelial barrier; mutations in TLR2 have been linked to the phenotype of inflammatory bowel disease [41].

Moreover, it has been shown that toll-like receptor 4 (TLR4) activation significantly affects the inflammatory signaling pathways in the gastrointestinal tract [42].

The gastrointestinal tract's inflammatory signaling pathways are significantly affected by TLR- 2 and TLR- 4. An overactivation of TLRs by a large number of invasive bacteria results in an overexpression of inflammatory cytokines, which damages epithelium and causes chronic inflammation [43]. As expected, the HFD group in this study exhibited elevated TLR- 2 and TLR- 4 gene expression and *L. reuteri* MSMC64 had lower expression of the TLR- 2 and TLR- 4 genes. This result was corroborated by the finding that in HFD rats, *L. plantarum* S9 lowered the expression levels of inflammatory markers, inhibited the activation of inflammatory signaling pathways, and decreased the expression level of TLR4 [25].

Several limitations must be acknowledged. First, the study was conducted in a rat model, which, while providing a controlled environment for assessing physiological effects, may not fully replicate the complexity of human lipid metabolism and gut microbiota interactions. Further clinical trials in human populations are necessary to confirm the probiotic's efficacy, safety, and long-term impact. Second, although this study explored key metabolic and inflammatory markers, it did not investigate the detailed molecular mechanisms underlying *L. reuteri* MSMC64's cholesterol-lowering effects, such as its influence on specific gut microbial compositions, bile acid metabolism, and host gene expression. Additionally, the study's duration was relatively short, limiting the ability to assess the long-term benefits or potential adverse effects of prolonged probiotic supplementation. Moreover, while CYP7 A1 is recognized as a key regulator of hepatic cholesterol metabolism, this study does not fully elucidate its interactions with bile acid metabolism, gut microbiota, and host regulatory pathways. Finally, a limitation of this study is the limited number of oxidative stress and inflammatory biomarkers assessed, which may not fully capture the complexity of these processes. Additional markers such as 8-isoprostane, high-sensitivity C-reactive protein (hs-CRP), or interleukins could provide a more comprehensive understanding of the underlying oxidative and inflammatory pathways. Expanding the panel of biomarkers would strengthen the analysis and allow for more nuanced conclusions. Future research should focus on extended longitudinal studies, dose optimization, and comparisons with existing lipid-lowering therapies to establish *L. reuteri* MSMC64 as a viable adjunct for managing hyperlipidemia and cardiovascular disease risk.

Conclusion

In conclusion, this investigation suggested that *Limosilactobacillus reuteri* MSMC64 may be a useful supplement for improving lipid profiles, blood glucose levels, oxidative stress, inflammatory biomarkers, and the tight junction protein and barrier immunity in the colon. Based on these findings, a probiotic supplement *containing L. reuteri* MSMC64 could potentially reduce the risk factors of hyperlipidemia associated with cardiovascular diseases.

Author Contribution Wongsakorn Luangphiphat contributed to the conceptualization, project administration, investigation, formal analysis, resources, methodology, data curation, writing of original draft, writing review and editing of the manuscript, validation, software, and supervision. Praewpannarai Jamjuree was involved in software development, validation, investigation, formal analysis, resource management, and methodology. Chantanapa Chantarangkul contributed to the review and editing process, validation, investigation, formal analysis, resources, and data curation. Onnicha Amornariyakool was responsible for reviewing and editing the manuscript. Malai Taweechotipatr contributed to the conceptualization, project administration, funding acquisition, writing, reviewing, and editing of the manuscript, supervision, software, validation, investigation, formal analysis, resources, methodology, and data curation.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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