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Influence of high-fat diet on host animal health via bile acid metabolism and benefits of oral-fed Streptococcus thermophilus

MN-ZLW-002

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Abstract

In this study, C57BL/6J male mice were fed normal chow (NC; control) or a high-fat diet (HFD) for 12 weeks, and HFD mice were supplemented with oral administration of *Streptococcus thermophilus* MN-ZLW-002 (HFD + MN002; n = 20/group. Body weight, visceral fat, blood glucose, blood lipids and liver lipid deposition increased in the HFD group, and the composition of gut microbiota, cecum short-chain fatty acids and fecal bile acids (BAs) also changed. Oral-fed MN-002 increased the relative abundances of *Ruminococcaceae*, *Lachnospiraceae* and *Streptococcaceae* and improved blood glucose, liver cholesterol deposition, and serum IL-10, CCL-3 and the fecal BAs composition.

Conclusion: The high-fat diet changed the composition of bile acids by shaping the gut microbiota into an obese type, leading to metabolic disturbances. *Streptococcus thermophilus* MN-ZLW-002 regulated gut microbiota by adjusting the composition of bile acids and improved the perturbation caused by high-fat diets. However, the effect of MN002 observed in animal experiments needs to be verified by long-term clinical trials.

Keywords: bile acids, glucose metabolism, gut microbiota, high-fat diets, *Streptococcus thermophilus*
Introduction

Since dietary fat consumption has sharply increased recently, the incidence of chronic metabolic diseases is increasing (such as obesity, non-alcoholic fatty liver and type 2 diabetes). High-fat diets (HFDs) have attracted great attention amongst disease-related risk factors for their induction of systemic chronic low-grade inflammation, which has been considered one of the characteristics of metabolic disorders [1, 2].

The gut microbiota acts as a huge metabolic ‘organ’ that regulates the energy, glucose and lipid metabolism of the host by changing energy absorption and participating in carbohydrate and bile acid (BA) metabolism [3–6]. Studies have confirmed that different diets can cause changes in the structure and metabolites of the gut microbiota, which contribute to metabolism differently [3]. For instance, Western diets, which are characterized by high-fat contents, have been proven to increase the Firmicutes/Bacteroidetes ratio with characteristic abundances of some specific bacteria. Thus, the metabolites of the gut microbiota changed as well [7–11]. Furthermore, studies have indicated that probiotics can reduce fasting blood glucose (FBG) and improve insulin resistance and liver function in patients with type 2 diabetes or non-alcoholic fatty liver disease [12]. Therefore, we believe that the gut microbiota could be an effective target for treating metabolic disorders and that supplementation with specific beneficial microbes (such as probiotics) might be a promising therapy.

Streptococcus thermophilus (S. thermophilus) has been widely used in fermented dairy products for its excellent fermentation characteristics and therefore has a long
consumption history [13, 14]. Emerging evidence has shown that fermented foods could increase food nutrition and have more health benefits for reducing the risk of metabolic syndrome, colorectal cancer, and obesity [15]. The metabolic benefits of \textit{S. thermophilus} have also been confirmed in several studies. For example, the \(\beta\)-galactosidase secreted by \textit{S. thermophilus} significantly inhibits colorectal tumorigenesis \textit{in vitro} and in a mouse model [16]. Furthermore, in the neonatal rat model, the probiotic mixture Golden Bifido (including active \textit{Lactobacillus bulgaricus}, \textit{Bifidobacterium}, and \textit{S. thermophilus}) upregulated the expression of mucin in the intestinal mucosa and reduced intestinal permeability, thereby improving neonatal bacteremia and meningitis caused by \textit{Escherichia coli} K1 [17]. Therefore, \textit{S. thermophilus} might be a promising probiotic strain [14, 18]. Our previous research results showed that inactivated MN002 has the ability to inhibit the differentiation of preadipocytes \textit{in vitro}, and in animal experiments, inactivated \textit{Streptococcus thermophilus} MN-ZLW-002 was observed to reduce the weight gain of mice induced by a high-fat diet on early stage [19]. However, the health benefits of live \textit{S. thermophilus} on HFD-induced metabolic disorders remain unclear.

Based on our previous study, the possible benefits of live \textit{S. thermophilus} MN-ZLW-002 on HFD-induced metabolic abnormalities and gut microbial dysbiosis were investigated in this study. C57BL/6J male mice were fed an HFD for 12 weeks and concurrently administered a new strain of \textit{S. thermophilus} MN-ZLW-002, isolated from traditional Chinese dairy products.
Materials and methods

**Mice.** Sixty 3-week-old specific-pathogen-free C57BL/6J male mice were purchased from Beijing Huafukang Bioscience Co., Inc. (Beijing, China). After one week of adaptive feeding/quarantine at the laboratory animal center of West China Second Hospital, Sichuan University (Sichuan, PR China), the mice were randomly divided into control, HFD and HFD + *S.thermophilus* MN002 (HFD+MN002) groups (five mice per cage). Mice were kept in an individual ventilated cage (IVC) system. Mice rearing cages, drinking bottles, bedding, feed, and sterile clothing for researchers were all sterilized by autoclaving (205.8kPa, 132°C, 10 minutes).

During the experiment, free access was provided to water and food, the breeding temperature was set to 25°C ± 2°C, and the humidity was at 50%–70% with a 12-h light/dark cycle. As bedding, spruce wood shavings (Chengdu Dossy Experimental Animals Co., Ltd.) were provided. This study was conducted according to the Guidelines for Animal Experiments at West China School of Public Health, Sichuan University (Sichuan, PR China). The protocol was approved by the West China Second University Hospital Ethics Committee of Sichuan University (Protocol Number 2019-032). All efforts were made to minimize suffering.

The body weight of the mice were measured weekly during the experiment. The fecal and cecum contents, epididymal fat, peri-bowel fat, perirenal fat, blood, and livers of mice were collected and weighed after sacrifice. Animals that unexpectedly died during the experiment were excluded from the data analysis.

**Blinding.** Because high-fat feed and normal chow (NC) have different colors and the...
characteristics of the MN002 solution and physiological saline were different, the experimenters could not be blinded.

**Diet and probiotic treatment.** As shown in Figure 1A, NC (270 kcal/100 g; 10% energy from fat, 20% from protein, and 70% from carbohydrates) was given to the control group. High-fat feed (D12492) was purchased from the Research Diets (New Brunswick, NJ, USA, 521 kcal/100 g; 60% of energy from fat, 20% from protein, and 20% from carbohydrates). Four-week-old mice in the HFD and HFD + MN002 groups were fed the HFD for 12 weeks.

MN-ZLW-002 was kindly supplied by Mengniu Dairy (Group) Co. Ltd. (Inner Mongolia of China). Freeze-dried live MN-ZLW-002 (2.0 × 10^{10} colony-forming units [CFU]/g) was dissolved in sterile saline to prepare bacterial suspensions of approximately 8.33 × 10^{9} CFU/ml. The daily intake of live MN-ZLW-002 was estimated to be 10^{9} CFU/mouse. The volume of suspension administered by gavage was 200 µl/mouse.

**Gavage procedure.**

1) Preparation of the solution: The mice in the Ctrl and HFD groups were treated with sterile normal saline (121°C, 20 minutes). For the mice in the MN002 group, sterile saline was used to dissolve the MN002 bacterial powder in an EP tube after high-temperature sterilization.

2) Disinfection: The prepared solution and the items used for gavage (such as gavage needles and sterile syringe) were disinfected by surface wiping (disinfectant solution containing 500mg/l of available chlorine) and ultraviolet irradiation and then entered
the IVCs system through the transfer window.

3) Gavage: Mice were held so that their head, neck, and body were in a straight line, and the gavage was performed. The sterile syringe used for gavage was changed daily. The gavage needles were sterilized at high temperature after use each day (121°C, 30 minutes).

Fasting blood glucose (FBG). During the experiment, the mice were fasted for 12 h every four weeks, and the blood glucose level of tail tip venous blood was measured using a blood glucose meter (Accu-Chek Performa, Roche Diagnostic, USA), which was used as the FBG level.

Increase in FBG = (last measured FBG level) – (initial FBG level).

ELISA and Luminex assays. Detection of the serum insulin content was performed according to the manufacturer’s instructions (EMD Millipore, Burlington, MA, USA), and the content was determined using a full-wavelength microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

A Luminex 200 was used to determine the serum contents of cytokines tumor necrosis factor-α (TNF-α), Interleukin-1 β (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12 (IL-12) and that of the chemokine CCL-3. All operations were performed according to the manufacturer’s instructions (R&D Systems China).

Measurement serum and liver lipids and liver function. Briefly, an automatic biochemical analyzer (Rayto Life and Analytical Sciences Co., Ltd., Guangdong, PR China) was used to detect high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), total cholesterol (TC), alanine
aminotransferase (ALT), and aspartate aminotransferase (AST). All operations were performed according to the manufacturer’s instructions (Changchun Huili Biotech Co., Ltd., Jilin, PR China). A bicinechninic acid assay was used according to the manufacturer’s instructions (Applygen Technologies Inc., Beijing, China) to quantify the TC and TG contents in the liver under a full-wavelength microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

**Histopathology.** Liver sections were fixed in 4% paraformaldehyde for 24 h and routinely stained with hematoxylin and eosin staining (H&E). After H&E staining, the liver pathological sections were observed under an optical microscope.

**Detection of the cecum short-chain fatty acid (SCFA) content.** Mouse cecum (100 mg) was collected into an EP tube, and then an internal standard (isohexanoic acid) solution and ether were added to fix the content. Gas chromatography was used to determine the acetic, propionic and butyric acid contents in the cecum.

**Detection of the fecal bile acid (BA) content.** Feces (10 mg) were pretreated with methanol, and the BA contents of fecal samples were determined using a liquid chromatograph-mass spectrometer method.

**DNA extraction of stool samples.** At the end of the experiment, stool samples were collected from the mice and frozen at −80°C. Total DNA was extracted using a TIANamp Stool DNA Kit (Tiangen Biotech Co. Ltd., Beijing, PR China) according to the manufacturer’s instructions.

**Amplification and sequencing of genes encoding 16S rRNA.** After extracting the stool sample DNA, PCR amplification was performed on the 16S rDNA V3–V4
region. Single-end sequencing was performed using a small fragment library and lon S5 XL sequencing platform. The sequences were clustered into operational taxonomic units (OTUs) with 97% identity, and then the OTU sequence and Silva132 database were annotated for species. An evolutionary tree was constructed by comparing representative sequences, and then QIIME scripts were used to calculate alpha-diversity indexes (Ace, Chao1, Shannon, Simpson, PD_whole_tree). QIIME 1.9.1 was used to calculate UniFrac distances to conduct principal coordinate analysis, and the multi-response permutation procedure (MRPP) was used for statistical analysis; linear discriminant analysis effect size (LEfSe) was used for differential species analysis.

**Statistical analysis.** Statistical analysis was performed using IBM SPSS Statistics v.20.0. All data are expressed as the mean ± standard deviation ( $\bar{x} \pm s$), median (M) or quartile (Q). For data with a normal distribution, one-way analysis of variance was used if variances are uniform, and comparison between groups was performed using the least significant difference. Data without a normal distribution were compared using the Kruskal–Wallis rank-sum test.

**Results**

**Body weight and visceral fat tissue of mice**

Since most studies have shown that high-fat diets lead to obesity, we first measured the body weights and visceral fat of the mice. There was no significant difference in initial body weights between groups. The body weights of the HFD and HFD +
MN002 groups were significantly higher than that of the control from 5 to 16 weeks of age ($P < 0.05$; Fig. 1B). There was no significant difference in body weight between the HFD and HFD + MN002 during the experiment.

The ratios of total visceral ($P < 0.001$), epididymal ($P < 0.001$), peri-bowel ($P < 0.01$) and perirenal ($P < 0.001$) fat to body weight were significantly increased in the HFD and HFD + MN002 groups compared with the control (Fig. 1C). There was no significant difference in body weight or visceral fat to body weight ratio between the HFD and HFD + MN002 mice.

**Glucose metabolism and blood lipids of mice**

Obesity is closely related to glucose and lipid metabolism. Since we found that a high-fat diet can lead to overweight in mice, we further measured the blood glucose of the mice. At the end of the experiment, the fasting blood glucose level of the HFD + MN002 group was significantly lower than those of the HFD and control groups (both $P < 0.05$; Fig. 2A). Although the initial fasting blood glucose level of the HFD-fed mice was significantly lower than that of the control, the growth of the fasting blood glucose in the HFD-fed mice was significantly higher than those of the control ($P < 0.01$) and HFD + MN002 mice ($P < 0.05$; Fig. 2B), the levels of which were similar.

Moreover, serum insulin was significantly increased in the HFD + MN002-fed mice compared with the control and HFD mice (both $P < 0.001$; Fig. 2C). The serum LDL-C, TG, and TC contents of the HFD group were significantly increased compared with the control ($P < 0.001$); conversely, HDL-C was significantly decreased ($P < 0.05$; Fig. 2D). However, *S. thermophilus* MN-ZLW-002
did not significantly improve blood lipids. The serum LDL-C, TC, and HDL-C contents in the HFD + MN002 group were significantly higher than those in the HFD group ($P < 0.05$, $P < 0.001$, and $P < 0.05$, respectively; Fig 2D).

**Lipid metabolism and liver function**

Dietary fat is metabolized in the liver, and we have found that a high-fat diet can cause dyslipidemia in mice. Therefore, we further determined the lipid metabolism capacity and function of the liver. In the control and HFD + MN002 groups, the structure of the hepatic lobule was clear. Hepatocytes were tightly and neatly arranged, and no lipid droplets or inflammatory changes were observed (Fig. 3A and Fig. 3C). In the HFD group, liver sinusoid and Disse’s space were unclear. Meanwhile, lipid droplets were observed in hepatocytes, and no collagen fiber deposition or necrosis was observed (Fig. 3B).

Moreover, there were significant increase in triglycerides and total cholesteryl ester of the liver in the HFD group compared with the control ($P < 0.001$ and $P < 0.05$, respectively; Fig. 3D and Fig. 3E), while mice in the HFD + MN002 group showed a significant decrease in the total cholesteryl ester of the liver compared with the HFD group ($P < 0.05$; Fig. 3E). There was no significant difference in the ALT/AST ratio between the groups (Fig. 3F).

**Serum inflammatory factors**

Obesity is also closely related to the inflammatory state of the body. Therefore, we measured serum inflammatory factors in the mice. No significant differences in serum inflammatory factors were found between the HFD and control groups. Nevertheless,
IL-10 and CCL-3 were significantly decreased and increased, respectively, in the HFD + MN002 group compared with the control and HFD groups ($P < 0.01$ and $P < 0.001$, respectively; Fig. 4).

**Cecum short-chain fatty acids**

As the research progressed, we began to explore the possible mechanisms behind the metabolic abnormalities induced by high-fat diets. Previous studies have found that a high-fat diet significantly alters the gut microbiota and its metabolites, such as short-chain fatty acids, so we measured the fecal short-chain fatty acid content. The results showed that acetic, propionic, and butyric acids were significantly decreased in the HFD and HFD + MN002 groups compared with the control ($P < 0.001$; Table 1.). However, *S. thermophilus* MN-ZLW-002 did not significantly improve the SCFA profile induced by the HFD.

**Fecal bile acid content of mice**

In recent years, emerging studies have begun to focus on the role of bile acids in regulating gut microbiota and host metabolism. Therefore, the fecal bile acid content was also measured in this study, and bile acids were divided into different types for analysis. Primary bile acids included: cholic acid (CA), chenodeoxycholic acid (CDCA), taurocholate acid (TCA), taurochenodeoxycholic acid (TCDCDA), glycocholic acid (GCA), and glycochenodeoxycholic acid (GCDCA). Secondary bile acids included: deoxycholic acid (DCA), lithocholic acid (LCA), tauroursodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), hyodeoxycholic acid (HDCA),
ursodioxycholic acid (UDCA), glycoursoxycholic acid (GUDCA), α-muricholic acid (α-MCA), and β-muricholic acid (β-MCA).

The results showed that the total bile acid contents of fecal samples were significantly increased in the HFD and HFD + MN002 groups compared with the control (P < 0.05; Fig. 5A). Moreover, the fecal secondary bile acid (SBA) contents were significantly higher in the HFD and HFD + MN002 groups than in the control (P < 0.05; Fig. 5B). However, there was no statistical difference in fecal primary bile acid (PBA) content between groups (Fig. 5B). Although the fecal SBA content was significantly higher in the HFD + MN002 group than in the HFD group, the PBA/SBA ratio was significantly decreased in the HFD + MN002 group compared with the control (P < 0.01; Fig. 5C).

Tauro-β-muricholic acid (T-β-MCA) and β-MCA were significantly increased in the HFD and HFD + MN002 groups compared with the control (P < 0.05; Fig. 5D). The T-β-MCA/β-MCA ratio was significantly decreased in the HFD and HFD + MN002 groups (P < 0.05 and P < 0.001, respectively). Furthermore, the T-β-MCA/β-MCA ratio of mice in the HFD + MN002 group was lower than that of mice in the HFD group, although there was no statistical difference (Fig. 5E).

CA, CDCA, DCA, LCA, TCA, TCDCA, TDCA, and TLCA act as farnesoid X receptor (FXR) agonists, while T-α-MCA, T-β-MCA, α-MCA and β-MCA act as FXR antagonists. DCA and LCA are considered cell surface-located G protein-coupled BA receptor (TGR5) agonists. The results showed that there were higher level of FXR and TGR5 agonists and FXR antagonists in the HFD group than
in the control \((P < 0.05)\). Interestingly, the mice in the HFD + MN002 group showed higher levels of FXR and TGR5 agonists and lower levels of FXR antagonists than the mice in the HFD group, although there was no statistical difference (Fig. 5F).

Fecal microbiota analysis by 16S rRNA sequencing

Finally, we analyzed the structure of the gut microbiota in mice by 16s rRNA to verify the role of gut microbiota in regulating the above indicators. The rarefaction curve reflects whether the sequencing depth is sufficient. When the number of sequences reaches about 10,000, the rarefaction curve tends to be flat, indicating that the amount of sequencing data is gradually becoming reasonable and that more data will only produce a small number of new species (OTUs; Fig. 6A). At the phylum level, *Firmicutes*, *Bacteroides*, and *Proteobacteria* were the three predominant fecal bacteria in all three groups (Fig. 6B). The relative abundances of *Firmicutes* and *Proteobacteria* were significantly increased in the HFD and HFD + MN002 groups compared with the control \((P < 0.05\) and \(P < 0.001\), respectively). In contrast, the abundances of *Bacteroidetes* were significantly decreased \((P < 0.01\) and \(P < 0.001\), respectively; Table 2.). The *Firmicutes/Bacteroidetes* ratio was significantly increased in the HFD and HFD + MN002 group compared with the control \((P < 0.05\) and \(P < 0.001\), respectively; Fig. 6C). However, there was no significant difference in the alpha-diversity indexes for the fecal microbiota among the groups (Table 3.). Analysis of the microbial beta-diversity with principal coordinate analysis based on weighted UniFrac distance clarified the differences in relative bacterial abundance and evolution between groups. The results showed that the fecal samples of the
HFD-treated mice clustered into an area, distinct from the control group, despite whether they were treated with MN002 or not (Fig. 6D). Therefore, the HFD might explain 77.86% of the variability on axis PC1, which emphasizes the influence of a HFD on bacterial community composition. Furthermore, the MRPP analysis showed that the bacterial community composition was significantly different in the HFD and HFD + MN002 groups than in the control; however, a significant difference was also detected between the HFD and HFD + MN002 groups ($P < 0.01$; Table 4.).

Linear discriminant analysis effect size could detect statistically different biomarkers among the groups. It showed that the biomarkers in the intestinal bacterial among the control group were *Muribaculaceae*, *Lactobacillaceae*, and *Rikenellaceae* at the family level; *Lactobacillus* and *Alistipes* at the genus level; and *Lactobacillus reuteri* at the species level. In the HFD group, the biomarkers among the intestinal bacteria were *Bacteroidaceae* and unidentified *Clostridiales* at the family level; *Bacteroides*, *Intestinimonas*, *Blautia*, unidentified *Clostridiales*, and *Tyzzerella* at genus level; and *Bacteroides sartorii* and *Desulfovibrio* sp. ABHU2SB at the species level. Meanwhile, the biomarkers among the intestinal bacterial in the HFD + MN002 group were *Ruminococcaceae*, *Lachnospiraceae*, and *Streptococcaceae* at the family level and *Ruminiclostridium* and unidentified *Lachnospiraceae* at the genus level (Fig. 6E).

**Discussion**

High-fat diets have recently attracted great research interest, and more studies have begun to focus on the health hazards of HFDs. These diets can provide excessive energy, thereby causing obesity. Changes in the structure of gut microbiota might be
one of the possible underlying mechanisms for this [3]. Studies have shown that HFDs increase body weight, visceral fat [17], hyperglycemia [18], dyslipidemia [20], and abnormal liver lipid metabolism [21, 22]. Moreover, HFDs can significantly change the structure of gut microbiota [23, 24], modifying the content of gut microbiota metabolites, such as short-chain fatty acids and bile acids [25, 26]. Short-chain fatty acids improve insulin response [27] and have anti-inflammatory effects [28, 29]. However, HFDs can lead to lower short-chain fatty acid contents [25]. Furthermore, they can also induce intestinal and systemic inflammation [30–32], thereby promoting the occurrence and distant metastasis of colorectal cancer [33, 34]. In this study, body weight, visceral fat, and growth of fasting blood glucose and blood lipids increased significantly in the HFD group. The pathological sections of the liver and measurements of liver lipid content showed that lipid deposits in the liver increased in the HFD mice. Simultaneously, changes were found in the structure and function of fecal microbiota. Moreover, the acetic, propionic, and butyric acid contents of the cecum decreased, while the fecal bile acid content was significantly altered. The above results of this study agreed well with those of the previous studies [1, 2, 6–11, 17–26], which stated that HFDs contributed to obesity and that glucose and lipid metabolic perturbations were related to gut microbiota dysbiosis. These results further verify the health hazards of HFDs. Unfortunately, although this study observed that MN002 could reduce the liver total cholesterol content in mice fed a high-fat diet, MN002 was not effective in reducing the liver triglyceride content. A recent meta-analysis showed that probiotic products had a significant effect on serum
total cholesterol and LDL/HDL cholesterol concentrations, but not triglyceride concentrations, in patients with hyperlipidemia [35]. Another study also showed that probiotics had no significant effect on improving triglyceride levels in overweight/obese people [36]. Overall, we believe that different probiotics have different effects on triglyceride/total cholesterol content and that not all strains have the capacity to reduce triglyceride and total cholesterol at the same time.

In other similar studies, diet-induced obesity was found to be accompanied by a significant increase in serum inflammatory factors in the tested host animals, suggesting that obesity could stimulate inflammation in the body [37-39]. The body weights of the tested HFD mice in this study increased by less than 1.1 times that of the control group, which was much less than the 1.5 times observed in these previous studies [37-39]. Therefore, the HFD-induced obesity in this study was not serious enough to induce inflammation in the tested HFD mice. However, unlike the previous studies, this study did not find that the HFD caused significant changes in the content of inflammatory factors. Based on the current research, we believe that MN002 may not be effective to improve inflammation caused by obesity.

As mentioned earlier, a HFD can change the structure of the gut microbiota and its metabolite content (such as SCFAs). Kurdi et al. [40] found that bile acids caused mucosal damage, leading to the inhibition of growth of lactobacilli and bifidobacteria, and thus, changing the gut microbiota structure. Masamichi et al. [41] showed that the Firmicutes/Bacteroidetes ratio increased after the administration of cholic acid to rats on a normal diet. Similar changes occurred after administering a HFD, which verified
the possibility of bile acids directly regulating the gut microbiota structure [41].

Subsequent studies by Atsushi et al. [42] emphasized that elevated levels of BAs in HFDs play a decisive role in the structural changes of gut microbiota. Therefore, more studies have begun paying attention to the role of bile acids in regulating gut microbiota and host metabolism.

Emerging evidence shows that bile acids act as steroid molecules to promote lipid absorption and as signal molecules to regulate metabolism by binding to their receptors. These receptors include the nuclear receptors FXR, pregnane X receptor, TGR5, and vitamin D receptor. Related studies have highlighted that the binding of bile acids binding to their receptors plays an important role in regulating host glucose and lipid metabolism, hepatic metabolism, and energy expenditure [43-51], especially FXR and TGR5.

In another study, the activation of intestinal FXR reduced liver bile acids synthesis through the fibroblast growth factor 15 (FGF15) - fibroblast growth factor receptor 4 (FGFR4) axis, consequently reducing the utilization of liver cholesterol [43]. In this study, MN002 further increased the content of FXR agonists and reduced the antagonist content. However, it was observed that HFD + MN002 decreased the liver cholesterol content. These results might be related to the complex interactions of FXR agonist/antagonist-related bile acids in the liver but not limited to the regulation of the FGF15-FGFR4 axis. Alternatively, the activation of TGR5 in intestinal L-cells might increase glucagon-like peptide-1 (GLP-1) expression and improves insulin sensitivity, which would eventually ameliorates glucose metabolism [51, 52]. Studies have shown
that LCA and DCA have a strong TGR5 agonistic effects, thus acting as TGR5 agonists [43, 53]. Chaudhari et al. [54] found that, in diet-induced obese mice, cholic acid-7-sulphate (CA7S) can TGR5-dependently reduce blood glucose in mice by upregulating GLP-1 and insulin level, which accompanied by an increase in the Tgr5 gene expression level in the colon. In this study, MN002 improved the impairment of fasting blood glucose caused by the HFD and increased the serum insulin content. In contrast, the Tgr5, LCA, and DCA contents also increased. Therefore, the present and previous studies suggest that S. thermophilus MN-ZLW-002 could activate intestinal TGR5 by upregulating bile acids, consequently inducing GLP-1 release and increasing the serum insulin content, thereby decreasing FBG. However, further studies are needed to detect the alterations in bile acids receptors and GLP-1 secretion to clarify the underlying mechanisms of the modulations caused by MN002.

In this study, the total fecal BAs content increased after MN002 administration, which indicates that the body excreted more bile acids. This might relieve the toxic effects of bile acids on the gut microbiota, providing it an opportunity to rebuild its structure. Furthermore, there is a crosstalk between bile acids and gut microbiota. Although bile acids regulate gut microbiota, they are also regulated by it. Studies have demonstrated that BAs that are not reabsorbed enter the colon and are metabolized by gut microbiota [43]. There is also evidence supporting the suggestion that conjugated BAs are converted into deconjugated BAs and SBAs in the colon by gut microbiota. Primary bile acids undergo 7α-dehydroxylation under the action of gut
microbiota to generate SBAs. Intestinal inflammation is associated with significant depletion of SBAs [55]. Connors et al. [56] found that patients with Crohn’s disease, who had been treated with exclusive enteral nutrition (EEN) but did not sustain remission and experienced more severe intestinal inflammation, had a PBA-dominant profile with significantly reduced SBA percentage. A study by Duboc et al. [57] on patients with irritable bowel syndrome (IBS) drew a similar conclusion, which was that fecal PBAs were significantly higher in subjects with IBS than in healthy subjects.

Moreover, Sinha et al. [58] confirmed that the family *Ruminococcaceae* is positively correlated with the SBA concentration and that a reduced relative abundance of *Ruminococcaceae* results in SBA deficiency. In this study, we observed that the SBAs in the HFD + MN002 group were the highest and that the PBA/SBA ratio was the lowest. Meanwhile, the relative abundance of *Ruminococcaceae*, which was capable of transforming PBAs in SBAs, as demonstrated in a study, was the highest. Furthermore, serum IL-10 and CCL-3 were significantly altered by MN002. Studies have shown that alterations in bile acid profiles can alter the number and composition of intestinal immune cells, thereby regulating the immune response of the host [59]. Therefore, we speculated that MN002 could increase the SBAs level by increasing the relative abundance of SBAs-producing bacteria, such as *Ruminococcaceae*, which might have beneficial effects in activating the immune response in the presence of intestinal inflammation caused by a HFD. However, the detection of intestinal inflammation is required to verify the improvement effect of MN002 on HFD-induced
inflammation.

Bile salt hydrolase (BSH), produced by specific gut (intestinal) microbes, has proven to have a crucial role in catalyzing the hydrolysis of conjugated bile acids to deconjugated bile acids. Studies have shown that Bacteroides, Eubacterium, Clostridium, Lactobacillus, Listeria, Bifidobacterium, Mycobacterium, Parabacteroides, Bacillus, Staphylococcus, Enterococcus, Blautia, Peptoclostridium, Streptococcus, Fusobacterium, Rhodopseudomonas, Yersinia, and Vibrio exert BSH activity [60–66]. T-β-MCA is produced by CYP2C70 in mice [62] and deconjugated into β-MCA by BSH [56]. Therefore, the T-β-MCA/β-MCA ratio could be used to predict the BSH activity. In this study, HFD-fed mice showed a lower T-β-MCA/β-MCA ratio than the control, indicating that the HFD increased BSH activity. The underlying mechanism for this might be a significant HFD-induced increase in the relative abundance of bacterial strains with BSH activity, such as Bacteroides, Clostridium, and Blautia. Meanwhile, Song et al. [59] found that Firmicutes rather than Bacteroides had the most bacterial strains with BSH activity. In this study, we confirmed that the relative abundance of Firmicutes in the HFD + MN002 group was even higher than that in the HFD group, which might have been due to the relatively higher BSH activity in the HFD + MN002 group than in the HFD group. Furthermore, Chen et al. [67] confirmed that increased BSH activity relieves liver steatosis and aortic arch damage, and this was consistent with the results indicating that liver lipid deposition was reduced by MN002 in the present. Several lines of evidence have confirmed that S. thermophilus could
strain-dependently regulate the immunity of the host [41, 68] and glucose metabolism [69]. *Streptococcus thermophilus* MN-ZLW-002 was newly isolated from traditional Chinese dairy products [16]. Our study found that heat-inactivated MN002 strain-dependently activated the immune response of mouse macrophages, which significantly inhibit the differentiation of preadipocytes *in vitro* [19]. In summary, MN002 improves the composition of bile acids and the structure of the gut microbiota under an HFD, thereby improving fasting blood glucose and liver cholesterol metabolism. Regrettably, the oral administration of MN002 could not protect host animals from increases in body weight, visceral fat, and blood lipids or decreases in short-chain fatty acids production caused by the HFD. Our previous study found that heat-inactivated *Lactobacillus gasseri* TMC0356 significantly improved blood lipids and inflammation in rats with metabolic syndrome induced by high-fat and high-salt diets [70]. This may be related to the strain specificity of the regulatory effects of probiotics on blood lipids and inflammation. Nevertheless, in this study, glucose and cholesterol metabolism of the liver in MN002-fed mice were improved. Interestingly, the gut microbiota and bile acids were also been significantly altered by MN002 in the HFD-fed mice. This suggests that MN002 could be considered as a probiotic candidate to strain-dependently improve HFD-induced metabolism in host animals. However, the regulatory effect of MN002 on metabolism observed in animal experiments needs to be verified by the results of long-term clinical trials.
Conclusion

HFDs alter the composition of bile acids, thereby converting the gut microbiota into an obese type, leading to metabolic disturbances. The present study suggests that *S. thermophilus* MN-ZLW-002 can regulate the gut microbiota by adjusting the composition and content of bile acids to improve, at least partially, the perturbation caused by HFDs. However, the regulatory effect on metabolism observed in animal experiments needs to be verified by the results of long-term clinical trials.

Data Availability Statement

All data included in this study are available in Supporting Information.

Declaration of interests

The authors have declared that no competing interests exist.

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Author contributions

F.H., R.Y.C., X.S., and Y.T.L. designed the study. J.N.W., Q.Q.Z., Z.H.M. and H.J.L. helped with the animal experiment. Y.T. L. and R.Y.C. wrote the manuscript. F.H. and R.Y.C. revised the manuscript. All authors reviewed the manuscript.

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**Figure Legends**

Figure 1. (A) Experiment design. (B) Changes in mouse body weight during the experiment. (C) Visceral fat tissue/body weight of mice.

Figure 2. (A) Changes of fasting blood glucose in mice during the experiment. (B) Growth of fasting blood glucose. (C) Content of blood insulin. (D) Blood contents of serum high-density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), triglyceride (TG) and total cholesterol (TC).

Figure 3. (A) H&E histological cross sections of liver in the control group. (B) H&E histological cross sections of liver in the HFD group. (C) H&E histological cross sections of liver in the HFD+MN002 group. (D) Triglyceride in the liver. (E) Total cholesteryl ester in the liver. (F) Alamine aminotransferase (ALT)/aspartate transaminase (AST) ratio of mice.

Figure 4. Content of serum inflammatory factors.

Figure 5. (A) Total fecal bile acids concentrations of mice. (B) Fecal primary and secondary bile acids concentrations of mice. (C) Fecal primary bile acid/secondary bile acid ratios of mice. (D) Fecal T-β-MCA and β-MCA concentrations of mice. (E) Fecal T-β-MCA/β-MCA ratios of mice. (F) Fecal FXR agonist, FXR antagonist, and TGR5 agonist concentrations of mice.

Figure 6. (A) Rarefaction curve for fecal samples. (B) Top 10 species according to relative abundance at the phylum levels among the three sets of stool samples. (C) The lg10 values for the *Firmicutes/Bacteroidetes* ratios. (D) Analysis of microbial beta diversity with principal
coordinates analysis (PCoA) based on weighted UniFrac distance. (E) Biomarkers of each group detected by linear discriminant analysis effect size (LDA score).
Fig 1. (A) Experiment design. There were 20 mice per group. Control group was fed on normal chow and supplemented with oral administration of normal saline for 12 weeks. HFD group was fed on high-fat diet with oral administration of normal saline for 12 weeks. HFD+MN002 group was fed on high-fat diet with oral administration of *Streptococcus thermophiles-MN-ZLW-002* for 12 weeks. (B) Changes in mouse body weight during the experiment (n=20/group). (C) Visceral fat tissue/body weight of mice (n=20/group).

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, compared with control group. Two ends of the horizontal line represent of the duration at which a statistical difference occurs or the comparison of groups.
Fig 2. (A) Changes of fasting blood glucose in mice during the experiment (n=20/group). (B) The Growth of fasting blood glucose. Growth of fasting blood glucose = (Last measured fasting blood glucose value) - (initial fasting blood glucose value). (C) The content of blood insulin (n=20/group). (D) Blood contents of serum lipoprotein-Cholesterol (HDL-C), low density lipoprotein-Cholesterol (LDL-C), Triglyceride (TG) and Total Cholesterol (TC) (n=20/group).

*: P < 0.05, **: P < 0.01, ***: P < 0.001, compared with control group. #: P < 0.05, compared with HFD group. Two ends of the horizontal line represent the comparison of groups.
Figure 3

(A) The H&E histological cross-sections of liver in control group. (B) The H&E histological cross-sections of liver in HFD group. (C) The H&E histological cross-sections of liver in HFD+MN002 group. (D) Triglyceride of liver (mmol/g, n=20/group). (E) Total cholesteryl ester of liver (mmol/g, n=20/group). (F) Alamine aminotransferase (ALT)/aspartate transaminase (AST) ratio of mice. (n=20).

*: P < 0.05, ***: P < 0.001, compared with control group. #: P < 0.05, ##: P < 0.01, compared with HFD group. Two ends of the horizontal line represent of the comparison of groups.
Figure 4

![Graphs showing inflammatory factors](image)

Fig 4. The content of serum inflammatory factors (pg/mL, n=20/group).
*: $P < 0.05$, **: $P < 0.01$, compared with control group. ###: $P < 0.001$, compared with HFD group. Two ends of the horizontal line represent the comparison of groups.
Figure 5

(A) Total fecal bile acids concentration of mice (µg/g, n=6/group). (B) Fecal primary and secondary bile acids concentration of mice (µg/g, n=6/group). (C) Fecal primary bile acids/secondary bile acids ratio of mice (n=6/group). (D) Fecal T-β-MCA and β-MCA concentration of mice (n=6/group). (E) Fecal T-β-MCA / β-MCA ratio of mice (n=6/group). (F) Fecal FXR agonist, FXR antagonist and TGR5 agonist concentration of mice (n=6/group).

Primary bile acids: CA, CDCA, TCA, TCDCA, GCA and GCDCA.
Secondary bile acids: DCA, LCA, TDCA, TLCA, GDCA, GLCA, HDCA, UDCA, GUDCA, α-MCA and β-MCA.

FXR agonist: CA, CDCA, DCA, LCA, TCA, TCDCA, TDCA and TLCA.
FXR antagonist: T-α-MCA, T-β-MCA, α-MCA and β-MCA.

TGR5 agonist: DCA and LCA.

*: P < 0.05, **: P < 0.01, ***: P < 0.001, compared with control group. Two ends of the horizontal line represent the comparison of groups.
Figure 6

A

B

C

D

E

Figure 6

A

B

C

D

E
### Supplementary Table 1. Cecum short-chain fatty acids (μg/g, n=6) (X ± S)

<table>
<thead>
<tr>
<th>Group</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>547.02 ± 99.55</td>
<td>149.49 ± 34.00</td>
<td>218.30 ± 60.56</td>
</tr>
<tr>
<td>HFD</td>
<td>235.41 ± 110.02***</td>
<td>48.82 ± 23.07***</td>
<td>85.56 ± 46.83***</td>
</tr>
<tr>
<td>HFD+MN002</td>
<td>122.03 ± 38.02***</td>
<td>29.38 ± 7.49***</td>
<td>49.37 ± 20.83***</td>
</tr>
</tbody>
</table>

***: P < 0.001, compared with the control group.

### Supplementary Table 2. Top 3 relative abundance of microbiota at phylum level (X ± S, n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Firmicutes</th>
<th>Bacteroidetes</th>
<th>Proteobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>0.40 ± 0.09</td>
<td>0.55 ± 0.12</td>
<td>0.020 ± 0.003</td>
</tr>
<tr>
<td>HFD</td>
<td>0.61 ± 0.13*</td>
<td>0.31 ± 0.16**</td>
<td>0.039 ± 0.015***</td>
</tr>
<tr>
<td>HFD+MN002</td>
<td>0.72 ± 0.03***</td>
<td>0.21 ± 0.04***</td>
<td>0.037 ± 0.010**</td>
</tr>
</tbody>
</table>

*: P < 0.05, compared with the control group. **: P < 0.01, compared with the control group. ***: P < 0.001, compared with the control group.

### Supplementary Table 3. Alpha diversity indexes (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>shannon</th>
<th>simpson</th>
<th>chao1</th>
<th>ACE</th>
<th>PD_whole_tree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>6.54</td>
<td>0.98</td>
<td>450.81</td>
<td>451.07</td>
<td>23.05</td>
</tr>
<tr>
<td>HFD</td>
<td>6.63</td>
<td>0.97</td>
<td>463.12</td>
<td>461.83</td>
<td>22.88</td>
</tr>
<tr>
<td>HFD+MN002</td>
<td>6.62</td>
<td>0.98</td>
<td>455.88</td>
<td>457.91</td>
<td>22.49</td>
</tr>
</tbody>
</table>

### Supplementary Table 4. MRPP analysis between groups (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>observed-delta</th>
<th>expected-delta</th>
<th>A</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl v.s HFD</td>
<td>0.38</td>
<td>0.60</td>
<td>0.36</td>
<td>0.005**</td>
</tr>
<tr>
<td>Ctrl v.s HFD+MN002</td>
<td>0.34</td>
<td>0.58</td>
<td>0.42</td>
<td>0.005**</td>
</tr>
<tr>
<td>HFD v.s HFD+MN002</td>
<td>0.37</td>
<td>0.43</td>
<td>0.13</td>
<td>0.003##</td>
</tr>
</tbody>
</table>

**: P < 0.01, compared with the control group. ##: P < 0.01, compared with the HFD group.