

Article

The Effect of Fermented *Momordica charantia* with *Leuconostoc mesenteroides* MKSR on Metabolic Complications Induced by High-Fat High-Cholesterol Diet in C57BL/6 Mice

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Abstract: This study aimed to investigate the beneficial effects of *Momordica charantia* (MC) extract and MC fermented with *Leuconostoc mesenteroides* MKSR (FMC) on high-fat and high-cholesterol diet-induced metabolic complications. Male C57BL/6 mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD with 1% MC extract (HFCD + 1M), HFCD with 4% MC extract (HFCD + 4M), HFCD with 1% fermented MC (HFCD + 1F), and HFCD with 4% fermented MC (HFCD + 4F). After 12 weeks of dietary intervention, the consumption of MC fermented with *L. mesenteroides* MKSR resulted in significant decreases in white adipose tissue weights (epididymal adipose tissue and retroperitoneal adipose tissue), serum alanine aminotransferase activity, and hepatic triglyceride levels. FMC also lowered total hepatic cholesterol content, improved glucose clearance during the oral glucose tolerance and insulin tolerance tests, and increased fecal cholesterol efflux from the enterohepatic circulation. Furthermore, the FMC notably increased hepatic mRNA expressions, which may indicate a compensatory mechanism against induced cholesterol efflux. Moreover, FMC induced both adipogenic (sterol regulatory element-binding protein 1c) and lipolytic (lipoprotein lipase, peroxisome proliferator-activated receptor alpha, and *adiponectin*) mRNA expressions. These findings suggest that fermentation with the probiotic *L. mesenteroides* MKSR enhances the beneficial effects of MC, preventing metabolic complications associated with a high-fat diet.

Keywords: *Momordica charantia*; high-fat high-cholesterol diet; metabolic syndrome; *Leuconostoc*



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1. Introduction

Metabolic syndrome (MetS) is a combination of multiple risk factors that significantly contribute to the development of metabolic complications, including excessive fat accumulation [1], type 2 diabetes mellitus [2], atherosclerotic cardiovascular disease [3], and hypertension [4,5]. The onset and development of MetS are closely intertwined with various factors, such as dietary choices [6], energy expenditure throughout life events [7], and genetic backgrounds [8]. Individuals living a contemporary lifestyle are increasingly busy and lead sedentary lives, resulting in less time for meal preparation and physical activity [9]. Consequently, such people tend to rely on convenient and energy-dense foods that are quick to consume but often lack nutritional density [10]. Fatty acids play a significant role in increasing the energy density of foods, given their high energy content (9 kcal/g)

compared to that of other macronutrients like carbohydrates and proteins, which provide 4 kcal/g [11]. Ideally, transitioning away from a high-fat diet is the recommended approach to prevent metabolic complications associated with high fat intake, which include insulin resistance and excess white adipose tissue (WAT) accumulation [12]. However, if adopting an alternative diet is not feasible or preventive, individuals at risk for MetS may consider incorporating functional nutrients to help modulate energy metabolism [13].

MC, commonly known as bitter melon, is a tropical and subtropical edible plant widely used in traditional medicine to modulate metabolic complications, including diabetes, obesity, and dyslipidemia [14–16]. Charantin has been widely accepted as a major functional compound in MC that improves insulin sensitivity by increasing cellular glucose uptake and promoting hepatic and muscular glycogen synthesis [17]. Additionally, charantin exhibits antioxidant [18] and anti-inflammatory properties [19], which may contribute to other potential health benefits in preventing complications associated with metabolic disorders. MetS, including obesity, insulin resistance, and dyslipidemia, is often associated with low-grade chronic inflammation, which can be influenced by several factors and contribute to the development of chronic inflammation and pathological processes [20]. Therefore, charantin supplementation to mitigate MetS-induced adipocyte and systemic inflammation could potentially prevent prolonged inflammation-mediated oxidative and endoplasmic reticulum stress, which can exacerbate the development of MetS-related complications [21].

However, there are disadvantages to isolating charantin from MC. Given the hydrophobic nature of charantin, organic solvents need to be used during the extraction process [22]. Unfortunately, the use of organic solvents to extract compounds from edible plants has negative implications for product development. One crucial issue related to organic solvent use is safety, particularly concerning the presence of residual solvents in the final product [23]. Even after completing the extraction process, traces of organic solvents may remain in the extracted compound, including charantin. These residual solvents are undesirable, especially when the compounds are intended for consumption and their biological functionality is of importance. Therefore, ensuring that all purification steps are thoroughly performed is important to remove any residual solvents from the product. Additionally, the organic solvents typically used for extraction, such as methanol, ethanol, or acetone, can negatively impact the environment [24]. Improper disposal of organic solvents and spills can contribute to environmental contamination, potentially harming ecosystems and human health [25]. As such, implementing proper waste management practices is crucial to minimizing the environmental impact associated with the use of organic solvents during extraction processes.

An alternative and sustainable method using hot water extraction was employed to overcome the safety, technical, and environmental disadvantages of using organic solvents for MC extracts. Therefore, the MC extract used in this study may possess different physicochemical and biological functional properties compared to the conventional charantin-containing MC extract obtained using organic solvents. Additionally, to enhance the biological functionalities of the hot water-extracted MC, the extract was fermented with probiotics, specifically *Leuconostoc mesenteroides* MKSR. Recent research has shown that *L. mesenteroides* MKSR could potentially modulate glucose metabolism by inhibiting α -glucosidase activity and cholesterol metabolism through bile salt hydrolase activity in test tubes [26]. Furthermore, hot water MC extracts fermented with *L. mesenteroides* MKSR have remarkably increased antioxidative activity, as evidenced by their increased ferric-reducing antioxidant power, DPPH radical scavenging, and superoxide dismutase-like activity. Hot water MC extracts fermented with *L. mesenteroides* MKSR may generate functional metabolites from either MC or *L. mesenteroides* MKSR [27].

We had previously reported on the potential beneficial functionalities of hot water MC extracts fermented with *L. mesenteroides* MKSR in test tubes [27]. In this study, we aimed to investigate whether dietary intervention with either hot water MC extract or hot water MC extract fermented with *L. mesenteroides* MKSR could prevent high-fat, high-

cholesterol-induced MetS-related disorders, such as weight gain, insulin resistance, and dyslipidemia, in experimental mice.

2. Materials and Methods

2.1. Mice Experiments and Diets

A total of 60 male C57BL/6 mice aged 4 weeks were purchased from DooYeol Biotech (Seoul, Republic of Korea) and housed in shoebox cages under controlled husbandry conditions (temperature 22–24 °C, relative humidity 55–65%, 12 h light/dark cycle). Experimental mice were acclimatized for a week and then randomly assigned into 6 different dietary intervention groups ($n = 10$ per group): normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). Given that both MC and FMC are rich dietary sources of fiber, we replaced the fiber portion of either the ND or HFCD with MC and FMC (Table 1). The fermented MC was prepared according to the method reported by Yoon and Kim [28]. Briefly, a probiotic strain, *Leuconostoc mesenteroides* MKSR KCTC 15665P ($6.7 \log \text{CFU} \cdot \text{mL}^{-1}$), was inoculated into the sterilized MC extract in the fermentation medium containing 394 mmol of sucrose, 143 mmol of maltose, $5 \text{ g} \cdot \text{L}^{-1}$ of yeast extract, $5 \text{ g} \cdot \text{L}^{-1}$ of peptone, $20 \text{ g} \cdot \text{L}^{-1}$ of K_2HPO_4 , $0.2 \text{ g} \cdot \text{L}^{-1}$ of MgSO_4 , $0.13 \text{ g} \cdot \text{L}^{-1}$ of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $0.01 \text{ g} \cdot \text{L}^{-1}$ of $\text{FeH}_{14}\text{O}_{11}\text{S}$, $0.01 \text{ g} \cdot \text{L}^{-1}$ of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, and $0.1 \text{ g} \cdot \text{L}^{-1}$ of NaCl. The mixture was left to ferment at 30 °C for 24 h. Experimental mice had access to the designated diet and water ad libitum for 12 weeks, and their body weight was measured every week. Experimental mice fasted for 12 h before sacrifice via thoracotomy after CO_2 narcosis. Whole blood was collected via cardiac puncture, allowed to clot at room temperature for 30 min, and then centrifuged (LaboGene, Lyngby, Denmark) at $1000 \times g$ at 4 °C for 15 min to separate the serum [29]. Liver and WATs, including epididymal adipose tissue (EAT), retroperitoneal adipose tissue (RAT), mesenteric adipose tissue (MAT), and perirenal adipose tissue (PAT), were collected and weighed [30]. Feces were collected from the cecum and transferred to 1.5-mL centrifuge tubes (SPL Life Sciences, Pocheon, Republic of Korea). These tissue, serum, and feces samples were stored at -80°C until further analysis [31].

Table 1. Composition of experimental diet.

Diet Composition (g)	ND	HFCD	HFCD + 1M	HFCD + 4M	HFCD + 1F	HFCD + 4F
Casein	200	200	200	200	200	200
L-cystine	3	3	3	3	3	3
Sucrose	100	90	90	90	89.76	89.04
Corn starch	397.486	259.49	259.49	259.49	259.49	259.49
Dextrose	132	125	125	125	124.12	121.49
Lard		145	145	145	145	145
Soybean oil	70	70	70	70	70	70
Cholesterol	0	10	10	10	10	10
Cellulose	50	50	40	10	41.12	14.46
Mineral mix	35	35	35	35	35	35
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin mix	10	10	10	10	10	10
t-Butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014
MC			10	40		
FMC					10	40
Total	1000	1000	1000	1000	1000	1000

2.2. Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)

After 9 weeks of dietary intervention, an OGTT was performed on the experimental mice following a 12 h fast. Mice were orally administered a glucose solution (Sigma, St. Louis, MO, USA) at a dose of 2 g/kg body weight (1 g/kg body weight as previously

described) [32]. Blood glucose levels were then measured from the tail vein 0, 15, 30, 60, and 120 min after the oral glucose challenge using a blood glucose meter (Accu-Chek, Seoul, Republic of Korea). After 12 weeks of dietary intervention, an ITT was performed using the prescribed method [33] on the experimental animals following a 12 h fast. Mice were intraperitoneally injected with insulin (2 U/kg body weight; Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, blood glucose levels were measured from the tail vein 0, 30, 60, and 90 min after the insulin injection using a blood glucose meter.

2.3. Biochemical Analysis in Serum, Liver and Feces

Lipid levels such as triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) in serum, liver, and feces were measured using commercial kits purchased from (Asanphram Co., Seoul, Republic of Korea) according to the manufacturer's instructions. Briefly, the sample was mixed with enzyme kits and incubated for 5–10 min at 37 °C water bath. Then, absorbance of the reaction products was measured at 500 nm for TC and HDL-C and 550 nm for TG using a spectrophotometer. Lipid components of the liver and feces were analyzed after extraction using a chloroform/methanol solution (1:2, v/v) and centrifugation at $1800 \times g$ for 5 min [34].

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were determined according to the manufacturer's instructions using commercial kits purchased from (Asanphram Co., Seoul, Republic of Korea). Briefly, substrate solution was mixed with a serum sample, incubated at 37 °C for 30 min for the ALT reaction or 60 min for AST, and mixed with color reagent. The reaction was terminated by adding 0.4N NaOH, and absorbance of the reaction product was measured at 505 nm using a spectrophotometer.

2.4. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted using the NucleoZOL reagent (Macherey-Nagel, GmbH & Co. KG, Düren, Germany). Total RNA (1 µg) was reverse transcribed to cDNA using a reverse transcription kit (Toyobo: FSQ-201, Osaka, Japan) following manufacturer's instructions. The resulting cDNA was amplified with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) [35]. The relative gene expression was normalized to that of *Gapdh*, which did not significantly vary with dietary intervention. The average fold changes in mRNA expression were calculated using the $2^{-\Delta\Delta C_t}$ analytical method [36]. The forward and reverse PCR primer sequences used in this study are available in Table 2 [37].

Table 2. qRT-PCR primer sequences (5' to 3').

Transcript	Forward	Reverse
<i>Hmgcr</i>	CTTGTGGAATGCCTTGATG	AGCCGAAGCAGCACATGAT
<i>Ldlr</i>	ACTCATGCAGCAGGAACG	GTCATTTTCACAGTCTAC
<i>Srebp1c</i>	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG
<i>Acc</i>	TGGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
<i>Srebp2</i>	GTGGGTGACCGCCGTTCTG	CTCGGCAGGCCTGAGCACAC
<i>Pparg</i>	TCATGACCAGGGAGTTCCTC	CAGCAGGTTGTCTTGGATGT
<i>Ppara</i>	AGAGCCCCATCTGTCTCTC	ACTGGTAGTCTGCAAAACCAAA
<i>Lpl</i>	GTGGCCGACGACGACGAGGAAGA	ATGCGAGCACTTCACCAGCTGGTC
<i>Adiponectin</i>	CTGGCTTTCTTCTTCCATGATAC	GTGTCGACGTTCCATGATTCTC
<i>Gapdh</i>	CATCGCCTTCCGTGTTCTA	GCGGCACGTCAGATCCA

2.5. Statistical Analysis

All experimental data are expressed as means $\pm$ standard deviations. Statistical significance was analyzed using one-way analysis of variance, followed by Duncan's post-hoc test. Statistical significance was set at $p < 0.05$, and means without a common letter were considered significantly different. All statistical analyses were performed using XLSTAT

2012 (Addinsoft Inc., Paris, France), and figures were created using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of the Dietary *Momordica charantia* on Body and Organ Weight

Dietary intervention with MC prevented high fat-induced body weight and WAT gain. Consumption of a high-fat diet significantly increased the final body weight by 31.9% after a 12-week intervention compared to the consumption of an ND (Figure 1a). However, MC decreased the final body weight by 7.12%, 8.81%, 1.17%, and 6.73% in the HFCD + 1M, HFCD + 4M, HFCD + 1F, and HFCD + 4F groups, respectively, compared to the HFCD group (Figure 1b). Among the dietary interventions with MC, only HFCD + 4M promoted a significant decrease in final body weight, whereas other interventions did not (Figure 1c,d). Regardless of dietary intervention with MC, a high-fat diet triggered hepatomegaly, unlike the HFCD. However, MC treatment did not prevent ectopic fat accumulation in the liver induced by the consumption of a high-fat diet (Figure 2a). Intriguingly, dietary consumption of 4% MC significantly prevented high fat-induced WAT expansion in the experimental mice (Figure 2b). A 12-week dietary intervention with a high-fat diet increased WAT by 46.2% compared to that with an ND. Neither 1% MC nor FMC prevented high fat-induced WAT expansion. Interestingly, however, 4% FMC significantly prevented high fat-induced WAT mass gain by 25.7%. Regardless of the anatomical location of the WAT, consumption of a high-fat diet significantly increased WAT mass compared to that of an ND. High-fat diet consumption elevated EAT, RAT, MAT, and PAT 3.8, 3.5, 2.3, and 2.6-fold, respectively, compared to ND consumption (Figure 2c–f). Interestingly, the HFCD + 4F group had a 29.3% and 28.6% lower EAT and RAT mass than did the ND group, respectively. In the case of MAT, the HFCD + 4M significantly attenuated WAT mass by 33.3% compared to the HFCD.

3.2. Effect of Dietary MC on Oral Glucose Tolerance and ITT Results

During the 9th and 11th weeks of the dietary intervention with MC, OGTT and ITT were performed to assess whether experimental mice consuming MC or FMC showed improved cellular glucose utilization, resulting in decreased body and WAT weight (Figures 1a and 2b). In the ND and HFCD groups, blood glucose levels peaked 15 min after the glucose challenge. However, the groups receiving MC intervention showed a delay in reaching peak blood glucose levels (i.e., peak levels were reached 30 min after OGTT) (Figure 3a). Interestingly, the HFCD + 1M, HFCD + 1F, and HFCD + 4F groups exhibited significantly lower blood glucose levels after the OGTT than did the HFCD group. The calculated area under the curve (AUC) values after the OGTT were overall higher in the HFCD groups than in the ND group; however, the AUC value in the HFCD + 4F group was lower, similar to that in the ND group (Figure 3b).

Given that the dietary intervention with MC remarkably attenuated body weight gain and improved insulin sensitivity as seen in the OGTT, we also conducted an ITT after the 11th week of dietary intervention (Figure 3c). Regardless of the dietary interventions, blood glucose levels peaked 30 min after the insulin treatment. Notably, the HFCD + 1M, HFCD + 1F, and HFCD + 4F groups showed a significant decrease in blood glucose levels after the ITT compared to the HFCD group, similar to the findings from the OGTT. However, no significant differences in the calculated AUC values were observed after the ITT (Figure 3d).

3.3. Effect of Dietary MC on Hepatic Functional Indices

Enzymatic activities of ALT and AST in the serum are commonly used indicators to assess normal liver function [32]. Compared to the ND group, the HFCD group exhibited a significant increase in serum ALT and AST activities by approximately 2.33- and 1.25-fold, respectively (Figure 4). Treatment with all doses of MC (HFCD + 1M, HFCD + 4M, HFCD + 1F, and HFCD + 4F) promoted a decrease in serum ALT activities by 11.6%, 26.6%,

31.0%, and 44.2%, respectively. Notably, fermented MC resulted in lower serum ALT activities than did non-fermented MC, and significant reductions were observed only in the HFCD + 4F group. Similarly, all MC-treated groups tended to have reduced serum AST activities, ranging from 3.8% to 12.5%. However, no significant differences were observed.

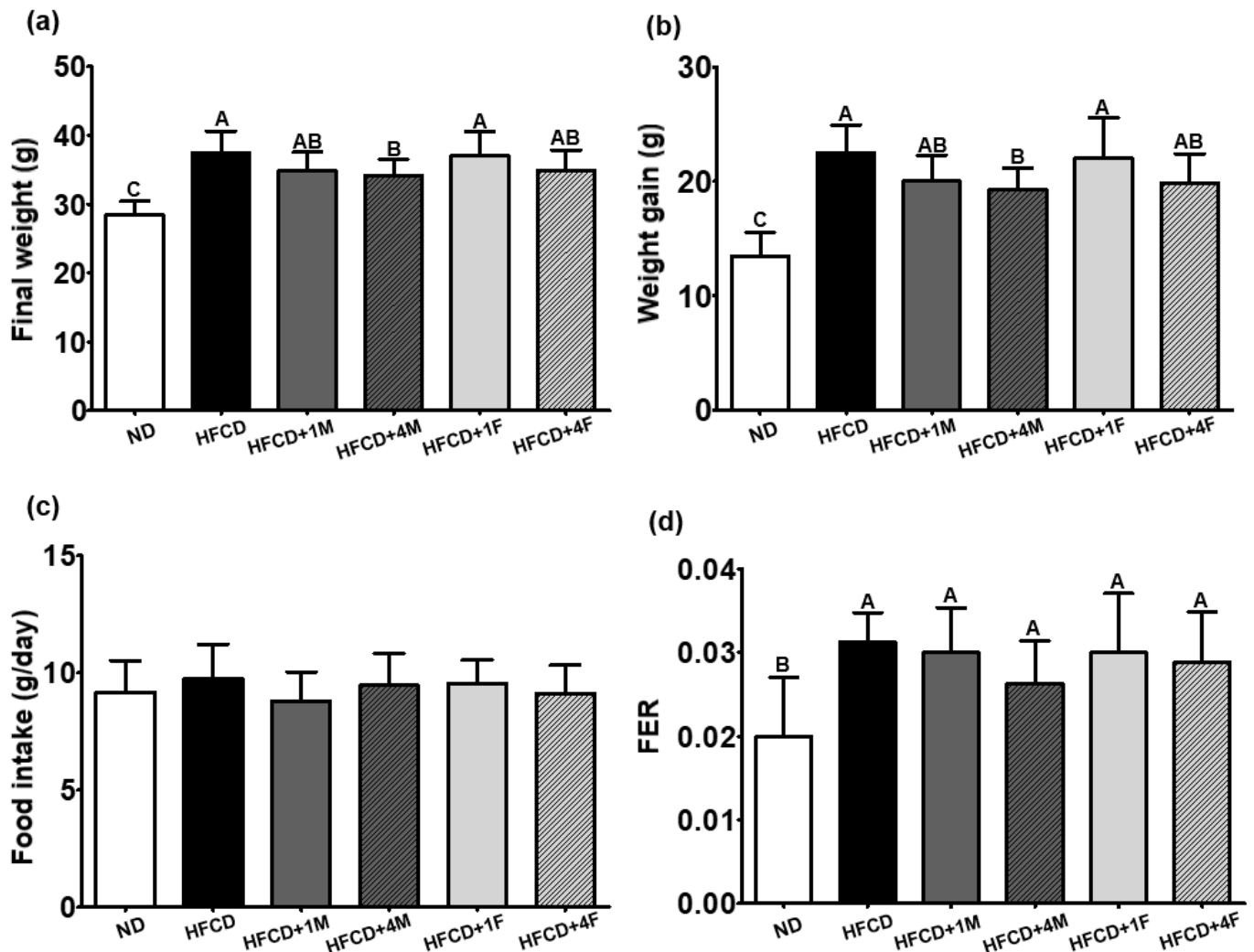


Figure 1. Effect of the dietary *Momordica charantia* on body weight, food intake, and FER. Mice were divided into six groups ($n = 10$): normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Final body weight; (b) weight gain (the final body weight—the initial body weight); (c) food intake during experimental period; (d) food efficiency ratio (FER = [weight gain (g/day)]/[food intake (g/day)]). Different capital letters of the labels on the bar are significantly different among the groups ($p < 0.05$).

3.4. Effect of the Dietary MC on Serum Lipid Profiles and Cardiovascular Parameters

To investigate the lipid-lowering effects of MC consumption in mice fed a high-fat diet, we assessed the serum lipid profiles associated with the risk of cardiovascular disease. As hypothesized, the overall HFCD + 1M, HFCD + 4M, HFCD + 1F, and HFCD + 4F groups showed a 24.1%, 15.5%, 12.3%, and 23.2% lower serum TG level than did the HFCD group, respectively (Figure 5a), showing a similar pattern to that for the reduction in WAT (Figure 2b). Among the four MC-treated groups, only the HFCD + 1M and HFCD + 4F groups showed a significant decrease in serum TG levels. However, regardless of type, such as HDL-C, and LDL-C, the MC-treated groups showed no difference in blood cholesterol levels when compared to the HFCD group (Figure 5c,d).

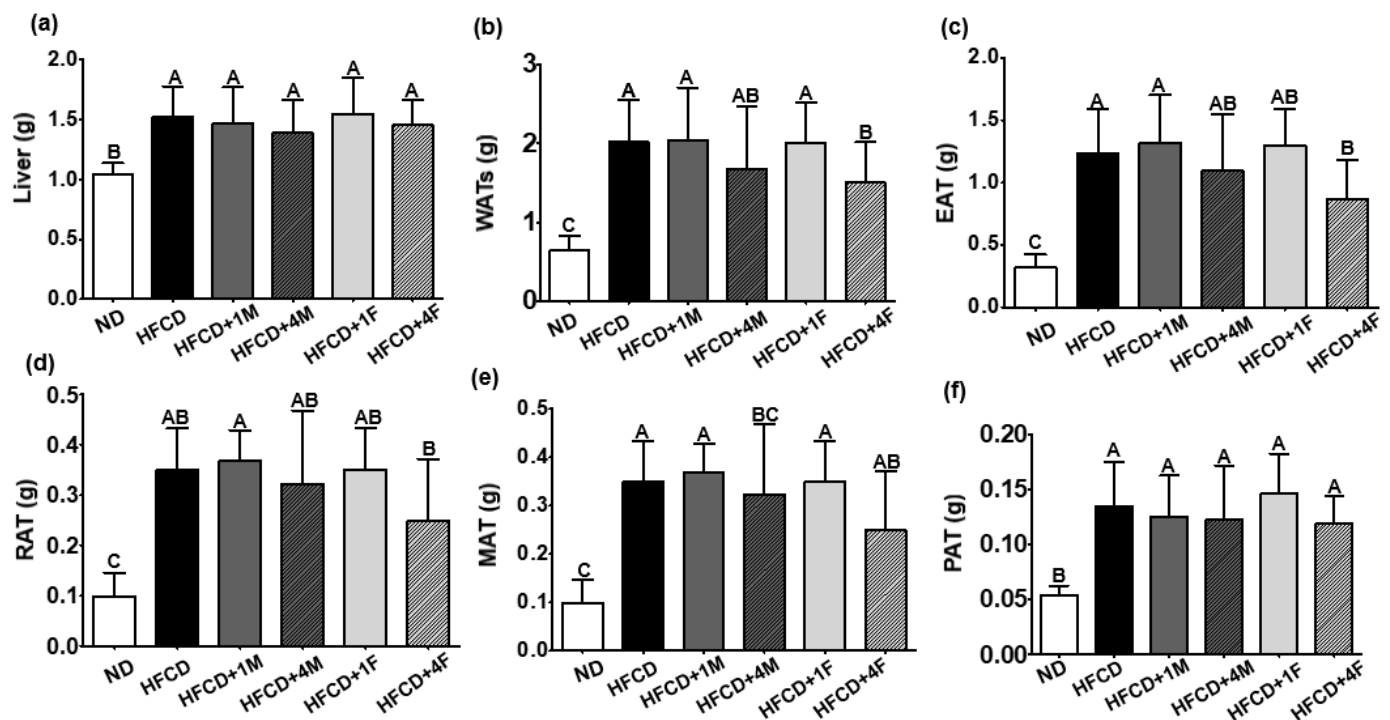


Figure 2. Effect of the dietary MC on organ weight. Mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Liver weight; (b) white adipose tissues (WATs) weight; (c) epididymal adipose tissue (EAT); (d) retroperitoneal adipose tissue (RAT); (e) mesenteric adipose tissue (MAT); (f) perirenal adipose tissue (PAT). Values are means $\pm$ standard deviation, ($n = 10$). Different capital letters of the labels on the bar are significantly different among the groups ($p < 0.05$).

3.5. Effect of the Dietary MC on Hepatic and Fecal Lipid Profiles

The enterohepatic circulation of serum TG and cholesterol is a complex process in which cholesterol is absorbed in the small intestine, transported to the liver, and subsequently reabsorbed back into the intestine, influencing blood lipid levels. As shown in Figure 5, MC significantly prevented the increase in serum TG levels but did not significantly affect serum cholesterol levels. This prompted us to question whether MC influences the enterohepatic circulation of lipids. To address this added research question, we measured hepatic and fecal lipid levels. Accordingly, we found that all groups consuming a high-fat diet, regardless of MC consumption, showed elevated hepatic TG accumulation (Figure 6a). Interestingly, however, the HFCD + 4F group showed significant alterations in both hepatic and fecal TC levels (Figure 6b). Specifically, compared to the HFCD group, the HFCD + 4M and HFCD + 4F groups exhibited a 26.4% and 21.5% decrease in hepatic TC accumulation, respectively. Additionally, fecal cholesterol efflux significantly increased, reaching levels approximately 2.7-fold higher than those in the ND group. Notably, all groups receiving fermented MC (HFCD + 1F and HFCD + 4F) demonstrated a 1.54- and 2.51-fold increase in fecal cholesterol efflux, respectively, compared to the HFCD group (Figure 6c). Furthermore, the HFCD + 4F group exhibited a greater increase in fecal cholesterol efflux than did the other groups, indicating the potential role of MC consumption in preventing enterohepatic cholesterol circulation.

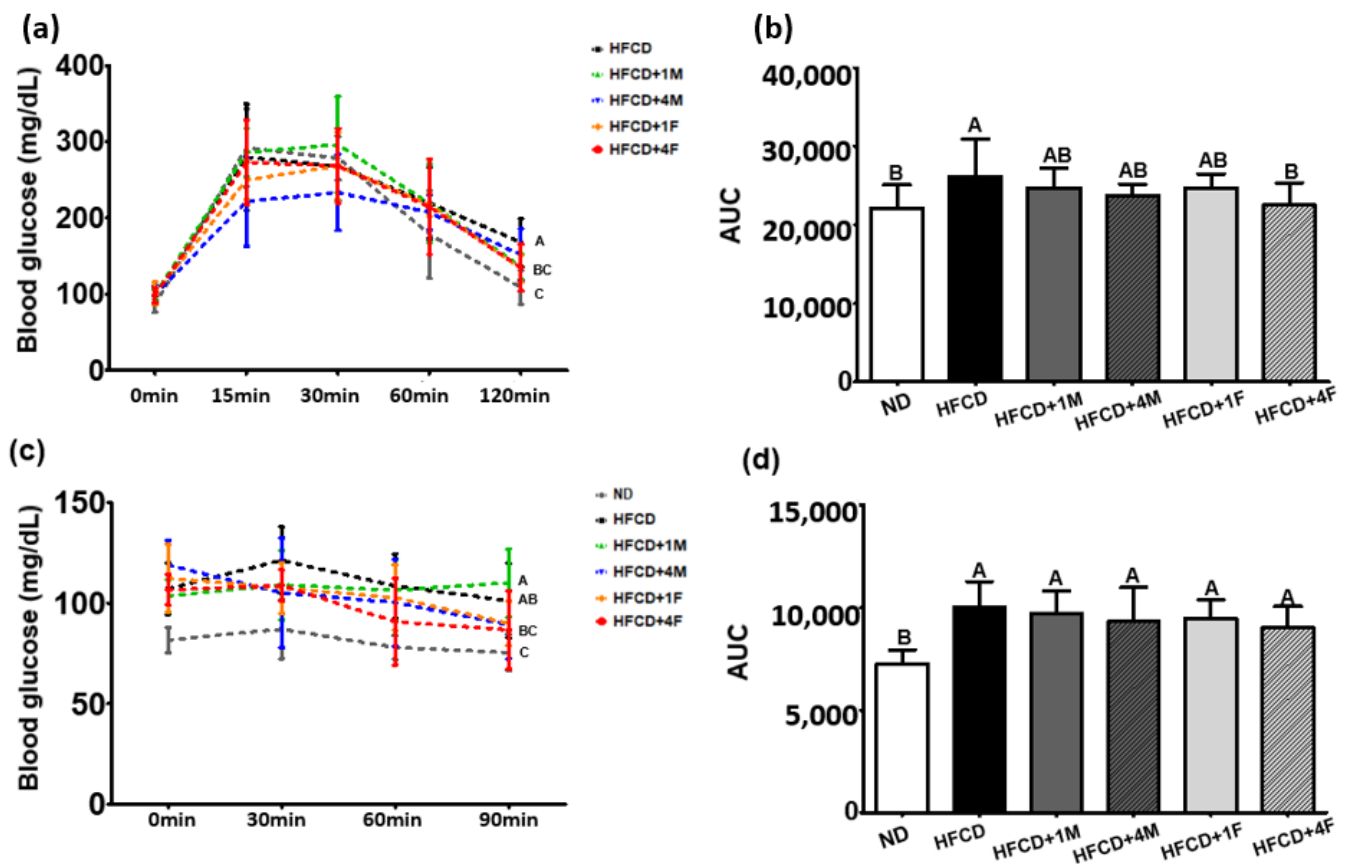


Figure 3. Effect of dietary MC on the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). Mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Glucose curve after OGTT; (b) the area under the curve (AUC) after OGTT; (c) blood glucose curve after ITT; (d) the area under the curve (AUC) after ITT. Values are means $\pm$ standard deviation, ($n = 10$). Different capital letters of the labels next to the dot at 120 min or 90 min and on the bar are significantly different among the groups ($p < 0.05$).

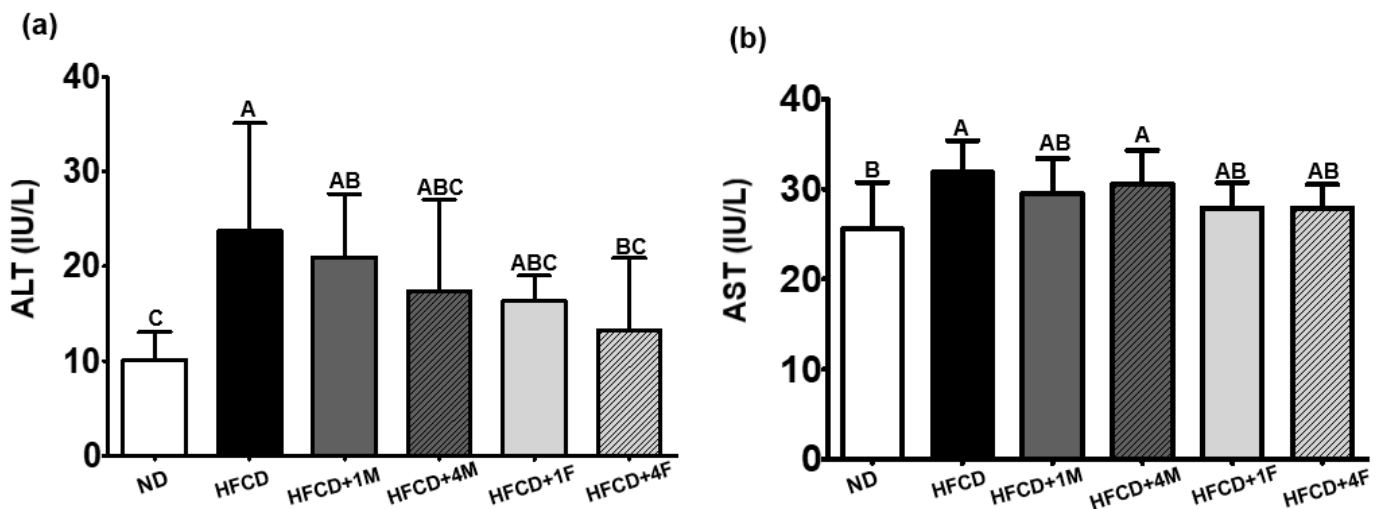


Figure 4. Effect of dietary MC on hepatic functional indices. Mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M),

HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Alanine aminotransferase (ALT); (b) aspartate aminotransferase (AST). Values are means $\pm$ standard deviation ($n = 10$). Different capital letters of the labels on the bar are significantly different among the groups ($p < 0.05$).

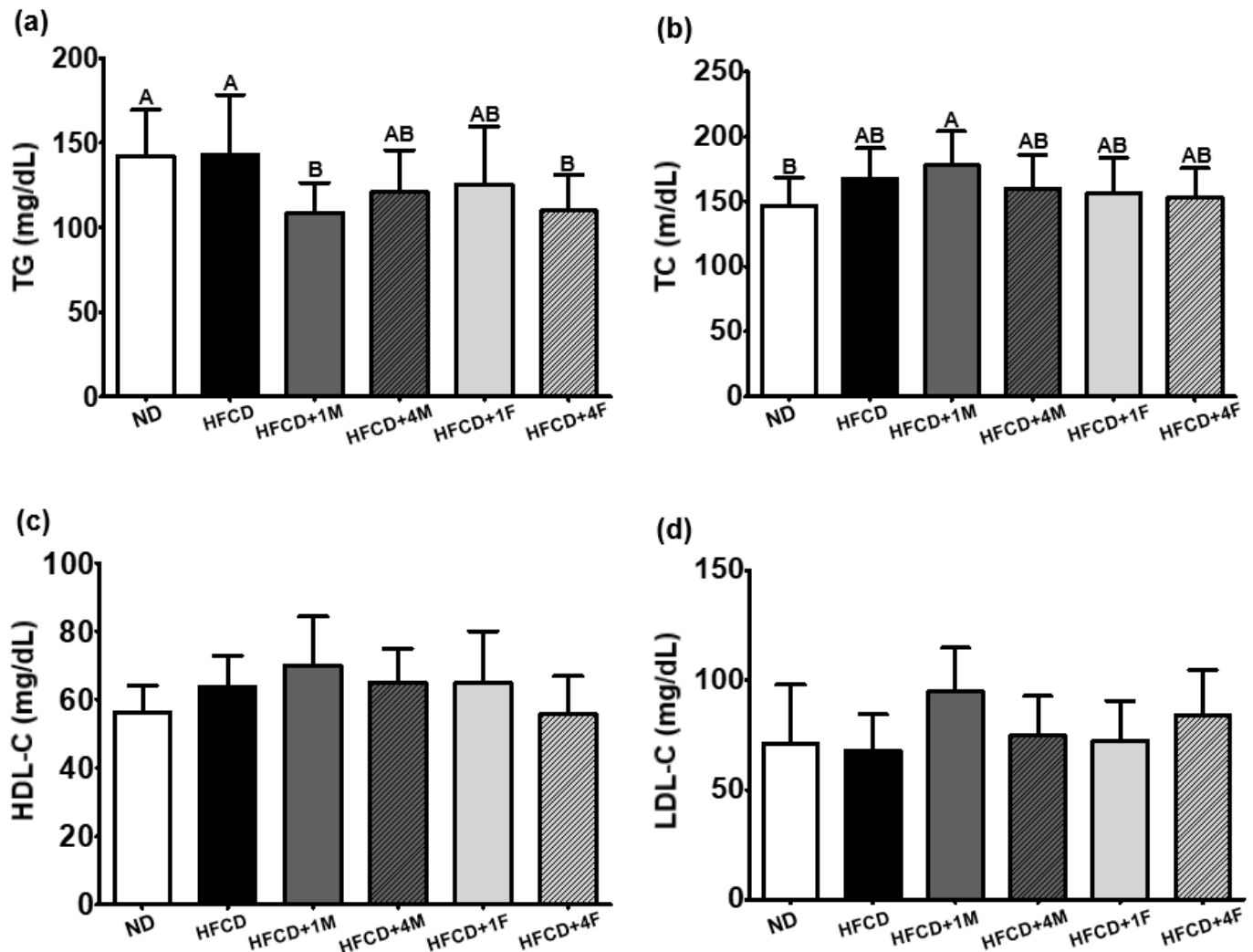


Figure 5. Effect of dietary MC on serum lipid profiles. Mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Triglyceride (TG); (b) total cholesterol (TC); (c) high-density lipoprotein cholesterol (HDL-C); (d) low-density lipoprotein cholesterol (LDL-C = [total cholesterol-HDL-C-(triglyceride/5)]). Values are means $\pm$ standard deviation ($n = 10$). Different capital letters of the labels on the bar are significantly different among the groups ($p < 0.05$).

3.6. Effect of the Dietary MC on Metabolic mRNA Expressions in the Liver

To explain the possible working molecular mechanism by which MC ameliorates blood TG and hepatic TC, we measured the cholesterol metabolism and anabolic, catabolic, and inflammatory mRNA expression levels in the liver. The 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*) is a rate-limiting enzyme involved in the mevalonate pathway by converting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid, which is responsible for cholesterol synthesis [38,39]. Therefore, *Hmgcr* inhibition is the specific target of statins, which are widely prescribed to lower cholesterol levels in patients with hypercholesterolemia [40,41]. In our experimental setting, HFCD inherently attenuated hepatic *Hmgcr* mRNA expression due to excess dietary fat consumption; how-

ever, HFCD + 4F elevated hepatic *Hmgcr* mRNA expression by approximately 3.90-fold compared to the HFCD (Figure 7a). A significant elevation in hepatic *Hmgcr* mRNA expression may reflect high fecal cholesterol efflux (Figure 6c). Another important molecular regulator of hepatic cholesterol metabolism is the low-density lipoprotein receptor (*Ldlr*). The main function of the hepatic *Ldlr* is binding and internalizing LDL particles from the bloodstream to the liver [42]. Hepatic *Ldlr* mRNA expression had a tendency to increase in the overall HFCD-fed groups (Figure 7b). Interestingly, groups treated with 4% MC (HFCD + 4M and HFCD + 4F) exhibited a 2.30-fold greater elevation in hepatic *Ldlr* mRNA expression than did the HFCD group; however, no significant differences were observed.

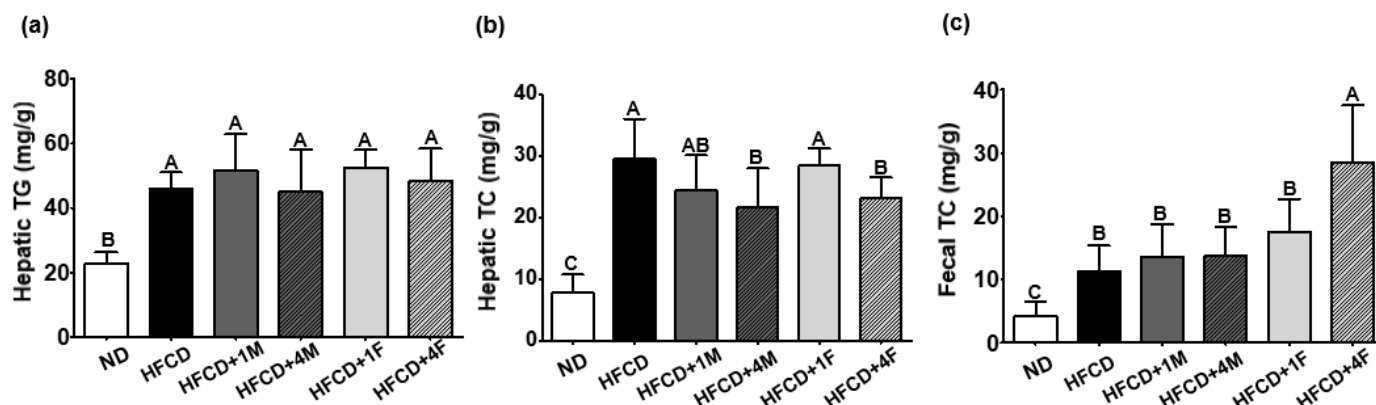


Figure 6. Effect of the dietary MC on hepatic and fecal lipid profiles. Mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Hepatic triglyceride (TG); (b) hepatic total cholesterol (TC); (c) fecal total cholesterol (TC). Values are means $\pm$ standard deviation ($n = 10$). Different capital letters of the labels on the bar are significantly different among the groups ($p < 0.05$).

Our experimental protocol observed increased cholesterol efflux and decreased hepatic TC content in the HFCD + 4F group (Figure 6). To investigate the underlying molecular mechanisms responsible for these changes, we examined the expression of hepatic mRNA involved in anabolic and catabolic processes. Despite the significant reduction in hepatic TC content in the HFCD + 4F group, we found an increase in adipogenic mRNA expression in the liver. Specifically, the expression of sterol regulatory element-binding protein (*Srebp1c*), a key factor in hepatic lipogenesis [43], and acetyl-CoA carboxylase (*Acc*), an enzyme involved in the production of malonyl-CoA from acetyl-CoA [44], were remarkably increased in the HFCD + 4F group compared to the control group, with fold increases of approximately 2.00- and 1.87-fold, respectively (Figure 7c,d). Additionally, other adipogenic transcription factors in the liver, namely *Srebp2* and peroxisome proliferator-activated receptor gamma (*Pparg*), were increased by approximately 2.32- and 1.61-fold in the HFCD + 4F group, respectively, although this increase was not statistically significant (Figure 7e,f).

Despite the reduced hepatic TG accumulation, the HFCD + 4F group showed increased expression of de novo adipogenic transcription factors compared to the control group. Therefore, we logically hypothesized that HFCD + 4F may induce beta-oxidation in the liver. *Ppara*, a transcription factor that stimulates hepatic beta-oxidation and promotes the uptake, utilization, and catabolism of free fatty acids [45,46], was 58% lower in the control group than in the normal diet group (Figure 7g). However, the HFCD + 4F group exhibited a significant 2.90-fold increase in hepatic *Ppara* mRNA expression compared to the control group. Alongside the induction of *Ppara*, *Lpl*, an enzyme involved in TG hydrolysis and receptor-mediated lipoprotein uptake [47,48], was also 2.94-fold higher in the HFCD + 4F group than in the control group (Figure 7g,h).

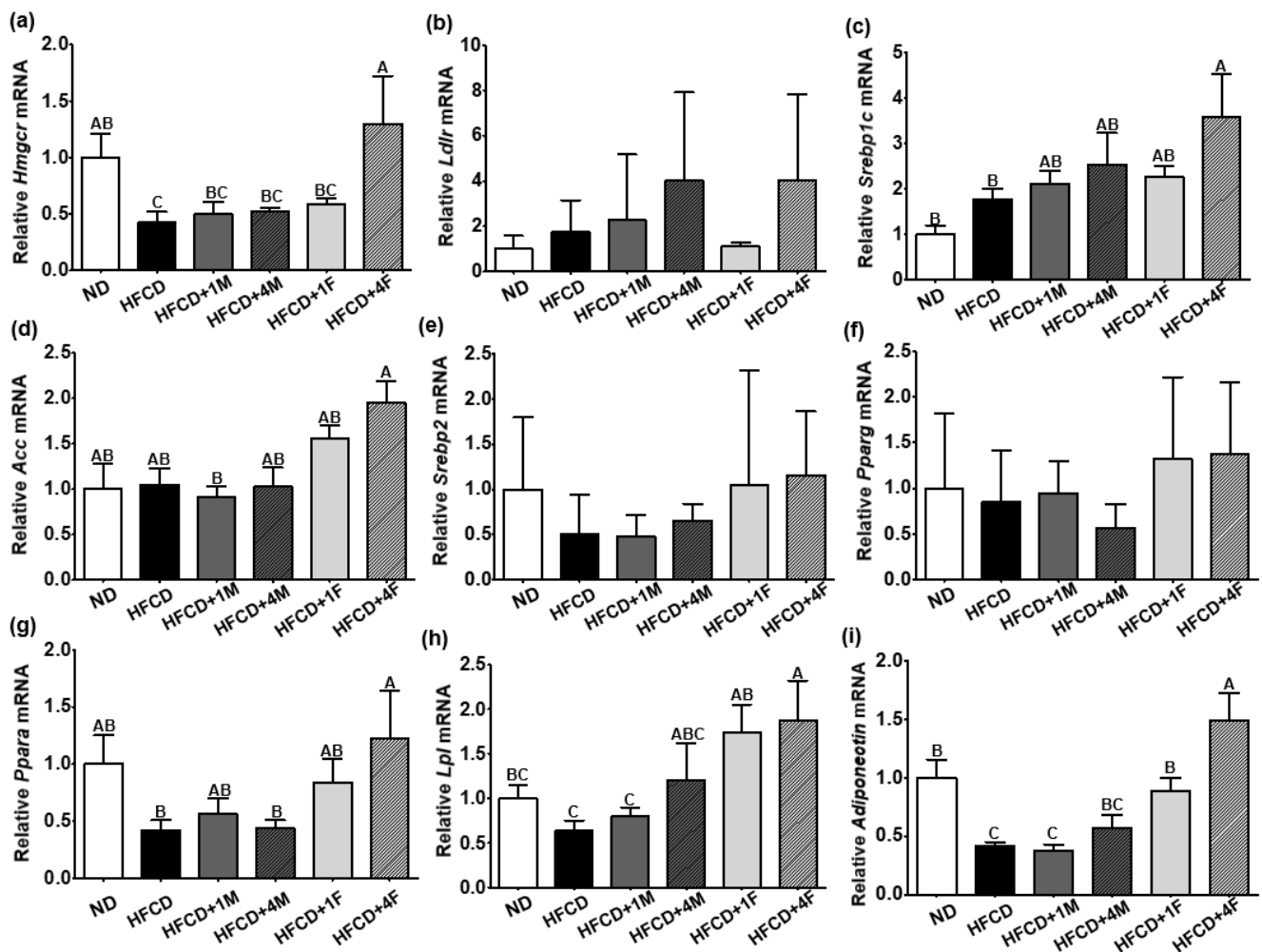


Figure 7. Effect of dietary MC on metabolic mRNA expressions in the liver. Mice were divided into six groups: normal diet (ND), high-fat and high-cholesterol diet (HFCD), HFCD mixed with 1% MC (HFCD + 1M), HFCD mixed with 4% MC (HFCD + 4M), HFCD mixed with 1% fermented MC (HFCD + 1F), and HFCD mixed with 4% fermented MC (HFCD + 4F). (a) Relative 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*Hmgcr*) mRNA expression; (b) relative low-density lipoprotein receptor (*Ldlr*) mRNA expression; (c) relative sterol regulatory element-binding protein (*Srebp1c*) mRNA expression; (d) relative acetyl-CoA carboxylase (*Acc*) mRNA expression; (e) relative *Srebp2* mRNA expression; (f) relative peroxisome proliferator-activated receptor gamma (*Pparg*) mRNA expression; (g) relative *Ppara* mRNA expression; (h) relative lipoprotein lipase (*Lpl*) mRNA expression; (i) relative *adiponectin* mRNA expression. Values are means $\pm$ standard deviation ($n = 10$). Different capital letters of the labels on the bar are significantly different among the groups ($p < 0.05$).

Adiponectin is an adipokine that regulates glucose metabolism and β -oxidation in adipocytes. Lower levels of adiponectin have been strongly associated with obesity [49] and diabetes [50] given its regulatory role in metabolism. However, higher levels of adiponectin may attenuate low-grade chronic inflammatory responses induced by MetS [51]. As mentioned earlier, the HFCD + 4F significantly altered the expression of various metabolic mRNAs in the liver. Therefore, we logically hypothesized that HFCD + 4F would also affect hepatic *adiponectin* mRNA expression. As expected, HFCD significantly reduced hepatic *adiponectin* mRNA expression by 59%, and MC did not prevent this reduction (Figure 7i). However, FMC remarkably prevented the high-fat diet-induced reduction in hepatic *adiponectin* mRNA expression. Consequently, the HFCD + 1F and HFCD + 4F promoted significantly higher hepatic *adiponectin* mRNA expression, with 2.2- and 3.6-fold increases, respectively, than did the HFCD. The increase in the expression of hepatic

adiponectin has been negatively associated with pro-inflammatory responses [52], given that MetS is considered a manifestation of low-grade inflammation [51]. Therefore, we further analyzed the expressions of hepatic inflammatory mRNA, such as *Tnfa* and *Il-6*; however, we did not observe any significant differences among the dietary interventions.

4. Discussion

High dietary fat intake promotes excessive calorie consumption [53], causing WAT or ectopic fat accumulation in the liver and muscles [54,55], ultimately increasing the risk of obesity and metabolic complications [56]. Obesity has been strongly associated with various metabolic complications, including diabetes [57], heart disease [58], hypertension [59], and dyslipidemia [60]. Reducing dietary fat is an important dietary intervention considering that total dietary fat intake has been considered a significant predictor of obesity due to its high energy content [61,62]. Current research focuses on not only cutting back on high-calorie fat consumption, but also consuming functional nutraceutical components to prevent metabolic complications induced by high-fat diets [63,64]. The current study investigated the potential of MC as a nutraceutical candidate to modulate high-fat diet-induced metabolic complications in experimental animals [16,65]. MC was extracted using hot water to avoid the use of organic solvents, after which the extracted MC was treated with probiotics, specifically *L. mesenteroides* MKSR. This probiotic strain has been reported for its various biological functionalities, including α -glucosidase inhibitory, bile salt hydrolase, and radical scavenging activities [26].

In the present study, the experimental animals were assigned into six different dietary intervention groups: ND, HFCD, HFCD + 1M, HFCD + 4M, HFCD + 1F, and HFCD + 4F. Except for the ND group, all groups were fed a high-fat diet in which a certain percentage of the energy was derived from fat. To examine the specific biological effects of MC and FMC, we incorporated MC and FMC into the diet by substituting them for the fiber portion as cellulose rather than providing them as separate supplements, as commonly practiced in animal experiments [66–68]. After 12 weeks of dietary intervention, the HFCD + 4M group showed a decrease in final body weight, delta body weight (excluding WATs), and hepatic TC levels compared to the HFCD group. Interestingly, consumption of MC fermented with *L. mesenteroides* MKSR significantly reduced WAT weights (EAT and RAT), serum ALT activity, and TG levels. It also lowered hepatic total cholesterol content, improved glucose clearance during OGTT and ITT, and increased fecal cholesterol efflux. Furthermore, the fermented MC groups showed significantly higher hepatic *Hmgcr*, *Srebp1c*, *Lpl*, *Ppara*, and *adiponectin* mRNA expression than did the HFCD group. These findings indicate that fermentation with *L. mesenteroides* MKSR enhances the biological activities that prevent metabolic complications induced by a high-fat diet.

Charantin, which gives MC its bitter taste, is widely recognized as one of the key bioactive compounds responsible for the potential beneficial health effects of MC against metabolic complications [22,69]. Charantin is a mixture of two steroidal glycosides that may enhance insulin sensitivity by increasing insulin secretion from the pancreatic beta cells [22,70], as well as sensitizing gastric glucose absorption [71] and peripheral glucose uptake [72]. However, extracting charantin from MC requires the use of organic solvents, such as methanol, ethanol, and/or chloroform, considering the lipophilic nature of charantin [65,73]. The potential use of organic solvents has raised concerns among potential customers seeking natural ingredients to prevent or mitigate metabolic complications through the supplementation of nutraceuticals [23–25]. An alternative extraction method that avoids the use of organic solvents is water extraction; unfortunately, water extraction may not be a viable method for obtaining charantin from MC. However, it should be noted that charantin in MC may not be the sole factor responsible for mitigating metabolic complications by enhancing insulin sensitivity. Other water extracts of MC, which likely do not contain charantin, have shown decreased insulin resistance in animal experiments (refer to Table 3). Notably, hot water-extracted MC has been found to increase insulin sensitivity in experimental models of both type 1 and type 2 diabetes. Moreover, other

studies have shown that water-extracted MC had the ability to attenuate hyperglycemia in animal models of type 1 diabetes induced by streptozotocin or alloxan, which destroy pancreatic beta cells [74,75]. Additionally, MC partially prevented metabolic complications in animal models of type 2 diabetes induced by high-fat diet consumption [66,76].

Regardless of the type of diabetes, MC extract has shown the ability to mitigate metabolic complications by preventing dyslipidemia [77] and WAT weight gain [72], as well as altering the microbiome caused by hyperglycemia [66]. Furthermore, the aforementioned peer-reviewed animal study used water-extracted MC, suggesting that the mitigating effects of MC supplementation on metabolic complications may be entirely independent of charantin. Unless we consider the beneficial biological effects of charantin, fermented fiber may potentially explain the beneficial effects of MC in our animal experiment. Fiber is categorized as either water-soluble or insoluble [78]. Water-soluble fibers, such as pectins, gums, and mucilages, dissolve in water and form a gel-like substance, which can help lower blood glucose and cholesterol levels [79,80]. In contrast, cellulose, hemicellulose, and lignin are major examples of insoluble fiber found in MC, which can enhance insulin sensitivity and promote bowel movement [81,82]. Considering that MC is a rich source of insoluble fiber, its ability to enhance insulin sensitivity, lower lipids, and increase cholesterol efflux in our experiments may partly be attributed to its fiber content. However, the amount of dietary fiber had been made consistent across all groups, and the amount of food consumed did not vary between different interventions. Therefore, the amount of fiber in the HFCD + 4F may not sufficiently explain its ability to mitigate the high-fat diet-induced metabolic complications better than the other interventions. We may also attribute the beneficial effects of MC to its large amount of fiber.

Given that MC contains other bioactive compounds, the results obtained from studying the efficacy of charantin by extracting only charantin may be insufficient. Hence, it is not enough to characterize charantin as the representative physiologically active substance of MC. Wang et al. [17], who studied mice with type 1 and type 2 diabetes, showed that an extract from the Taiwanese *M. charantia*, which is rich in charantin, increased insulin sensitivity instead of providing protection against β -cell dysfunction. As shown in Table 3, previous research conducted animal experiments by inducing both type 1 and 2 diabetes through the injection of STZ or alloxan. Our experimental model was intended to examine type 2 diabetes by inducing MetS with a high-fat diet. Notably, we found that the consumption of MC in the absence of charantin improved insulin sensitivity. Fermentation may increase potency to increase insulin sensitivity exclusively from charantin from MC. In our previous test tube experiment, fermentation with *L. mesenteroides* MKSR significantly inhibited α -amylase activity [27]. Additionally, fermentation with *Lactobacillus plantarum* in MC (medium of cultivation) reduced α -amylase activity [83]. The suppression of α -amylase activity by MC fermentation with microorganisms occurred independently of the biological effects of charantin resulting from the use of hot water extraction. Therefore, the improvement in insulin sensitivity observed in this experiment with MC fermentation using *L. mesenteroides* MKSR may be attributed to the independent biological effects of charantin.

After eliminating common denominators in both MC and FMC to determine their beneficial effects against high-fat diet-induced metabolic complications, the one remaining variable was probiotics—*L. mesenteroides* MKSR—which we used to ferment MC. In our previous study, wherein MC was fermented with MKSR and compared with MC, we confirmed that considerable amounts of lactic acid, mannitol, dextran, and oligosaccharides were present in MC fermented with MKSR [27]. Our experiment showed that these produced compounds may trigger superior effects in mitigating metabolic complications than would other dietary interventions. In particular, highly water-soluble dextran and oligosaccharides in MKSR-MC may have acted as prebiotics and promoted the production of short-chain fatty acids (SCFAs), such as butyrate, acetate, and propionate [84]. SCFAs provide energy to the cells of the colon, promote a healthy gut environment, and have various positive effects on metabolism and overall health [85]. As suggested by our findings, extra consumption of either dextran or oligosaccharides lowered blood cholesterol

levels and alteration of colonic microbiome [86,87]. Furthermore, the consumption of oligosaccharides has demonstrated cholesterol-lowering effects in high-fat-inducible animal experimental settings [88,89].

To explain how HFCD + 4F facilitates the efflux of cholesterol from the enterohepatic circulation, we examined the mRNA expressions relevant to cholesterol and lipid metabolism using qRT-PCR. Surprisingly, we observed a significant increase in *Hmgcr*, a key rate-limiting enzyme that converts HMG-CoA to mevalonic acid. In the pharmacological aspect, targeting HMG-CoA reductase (e.g., with statins) is a crucial approach in mitigating dyslipidemia and preventing cholesterol synthesis [38,39]. However, increasing cholesterol efflux to fecal matter through the consumption of natural compounds tends to upregulate HMG-CoA reductase expression as a compensatory mechanism against decreasing cholesterol levels [90]. Additionally, HFCD + 4F increased both anabolic (*Srebp1c*) and catabolic (*Lpl*, *Ppara*, and *adiponectin*) mRNA expressions related to lipid metabolism in the liver. This elevation of catabolic reactions in hepatic ectopic fat induced by a high-fat diet likely occurs through increased β -oxidation. Meanwhile, HFCD + 4F increased de novo mRNA expression of the adipogenic *Srebp1c*, which may indicate an increase in hepatic TG synthesis from catabolized free fatty acids originating from WATs (EAT and RAT; Figure 2). Adiponectin plays an anti-inflammatory role in WATs; thus, we anticipated that HFCD + 4F would attenuate hepatic pro-inflammatory responses. However, throughout our experimentation, we did not observe any significant differences in the expression of inflammatory mRNA.

The current study showed that FMC treatment significantly prevented high-fat diet-induced MetS. Our study is a novel endeavor that investigates the effects of dietary intervention with fermented MC in a murine model while precisely controlling the variables and matching dietary fiber contents across all dietary groups. To date, multiple studies have indicated the beneficial effects of MC against MetS [14–19]. However, limited information has been available on which fermented MCs are suitable for potential clinical trials. Therefore, our experimental results strongly suggest that fermented MC may have the potential to modulate lipid and cholesterol metabolism. Although our animal experimental protocol lasted for 12 weeks, we may consider extending the duration of dietary intervention to better observe the effects of fermented MC. In the liver, both de novo adipogenesis and β -oxidation were elevated; therefore, long-term observations may provide a vivid understanding on how fermented MC prevents high-fat diet-induced MetS. In potential future studies, we aim to determine the factor that primarily contributes to the beneficial effects of FMC given that the exact working mechanism is currently unknown. We also plan to explore the potential chemical compounds generated from fermented MC. Additionally, we will investigate the potential probiotic effects of fermented MC by analyzing the microbiome.

Table 3. Effects of MC in animal models with metabolic disease.

Strain	Inducer	Treatment	Biological Markers	Ref.
Wistar Rats	STZ (30 mg/kg) after HFD for 8 weeks	MC (water extract, fermented by <i>Lactobacillus plantarum</i>), 10 mL/kg of BW for 4 weeks	BW, FBG, serum insulin ↓ HOMA-IR ↓ Serum TC, TG, LDL-C levels ↓ Serum HDL-C ↑ Enhance oxidative stress (SOD ↑, CAT ↑, MDA ↓) Improve gut microbiome	[75]
C57BL/6J	HFD	MC (methanol extract), 0.2–1.0 g/kg BW for 4 weeks	BW ↓ <i>Lpl</i> mRNA expression in adipose tissue ↑ Blood glucose level ↓ Serum TG, TC, insulin levels ↓ Epididymal WAT <i>Pparg</i> mRNA expression ↑ Hepatic <i>Ppara</i> mRNA expression ↑	[91]

Table 3. Cont.

Strain	Inducer	Treatment	Biological Markers	Ref.
Sparague-Dawley	HFD	MC powder, 300 mg/kg BW for 8 weeks	Serum TG, TC ↓ HOMA-IR ↓ TNF- α , IL-6, MCP-1 IL-10 ↑ Improve gut microbiome	[66]
Sparague-Dawley	STZ (50 mg/kg)	MC (water extract), 1.5 g/kg BW for 28 days	Serum TC, TG, LDL ↓ Serum HDL ↑ MDA ↓, NO ↑ e NOS expression ↑	[68]
Albino rats	STZ (45 mg/kg)	MC (diluted with distilled water), 10 mL/kg BW for 21 days	Serum glucose, TG, TC ↓ Serum HDL, insulin ↑ Serum TAOC ↑ Pancreatic GSH ↑, MDA ↓	[67]

↑, increased; ↓, decreased; HFD, high-fat diet; STZ, streptozotocin; BW, body weight; MC, *Momordica charantia*; FBG, fasting blood glucose; HOMA-IR, homeostatic model assessment of insulin resistance; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; *Lpl*, lipoprotein lipase; WAT, white adipose tissue; *Pparg*, peroxisome proliferator-activated receptor gamma; *Ppara*, peroxisome proliferator-activated receptor alpha; *TNF- α* , tumor necrosis factor- α ; *IL-6*, interleukin-6; MCP-1, monocyte chemoattractant protein-1; *IL-10*, interleukin-10; NO, nitric oxide; e NOS, endothelial nitric oxide synthase; TAOC, total antioxidant capacity; GSH, reduced glutathione.

5. Conclusions

The current study investigated the potential of non- and fermented MC as a nutraceutical candidate to modulate high-fat diet-induced metabolic complications in C57BL/6 mice. The findings suggest that *L. mesenteroides* MKSR-fermented MC has beneficial effects in mitigating metabolic complications associated with a high-fat diet. The study demonstrated that consumption of fermented MC reduced WAT weights, serum ALT activity, and TG levels. It also lowered hepatic total cholesterol content, improved glucose clearance, and increased fecal cholesterol efflux. Additionally, the fermented MC groups showed increased mRNA expression of key genes involved in cholesterol and lipid metabolism. Although charantin has been recognized as a key bioactive compound in MC, our result suggests that the beneficial effects of MC may not solely rely on charantin. Other water extracts of MC have shown decreased insulin resistance, and the fermentation process itself may enhance the physiological activities of MC. The findings of this study support the potential use of fermented MC as a dietary intervention to prevent and mitigate metabolic complications associated with high-fat diets. Further research is needed to better understand the specific mechanisms of action and to explore the potential chemical compounds and probiotic effects of fermented MC. Longer-term studies are also warranted to evaluate the sustained effects of fermented MC and its potential for clinical applications.

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