pISSN 1225-4339 eISSN 2287-4992
Korean J. Food Nutr. Vol. 35. No. 5, 332~342 (2022)
https://doi.org/10.9799/ksfan.2022.35.5.332

THE KOREAN JOURNAL OF
한국식품영양학회지
FOOD AND NUTRITION

Hepatoprotective Effects of *Streptococcus thermophilus* LM1012 in the Methionine-Choline Deficient (MCD) Diet Induced Nonalcoholic Steatohepatitis Mice Model

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is recognized one of the leading metabolic diseases globally, and the younger age population with the disease is rapidly growing, especially in developed countries. Since there has been no approved medicine, losing weight is known to be the only best remedy to control or reverse the disease. Recently, the field of microbiome has attracted much attention to offer more practical choices for patients. Here, we provide experimental evidence that *Streptococcus thermophilus* LM1012 (LM1012), a safe probiotic strain, is effective for improving NAFLD indexes. In the methionine-choline deficient (MCD) diet induced C57BL/6 mouse model, administration of LM1012 promoted marked reductions of aspartate transaminase (23.8%), total bilirubin (27.8%), hydroxycholesterol (64.2%), triglyceride (29.7%) and IL-1β (68.3%) compared to the MCD diet alone group. Also, the histopathological data imply that LM1012 inhibited fat accumulation and inflammation in the liver, which are the key biomarkers for progression of the disease. Together, these findings suggest that human consumption of LM1012 as a healthy nutritional supplement, may be helpful in reducing the risk of liver damages in NAFLD patients.

Key words: probiotics, nonalcoholic fatty liver disease, health supplement, microbiome

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common metabolic dysfunction and is characterized by high fat content in the liver. Most patients with NAFLD in the early stages of NAFLD do not suffer from life-threatening health issues, except for the development of a fatty liver. However, ~20% of the patients develop non-alcoholic steatohepatitis (NASH), which causes chronic inflammation and fibrosis of the liver, and a significantly higher risk of death is reported in such population. Obesity is considered the primary cause of NAFLD, which has no available treatment yet; therefore, maintaining a healthy weight by lifestyle modification has been the first-line treatment recommended by medical professionals. Patients with NAFLD lack secondary causes of hepatic fat accumulation, such as excessive alcohol consumption, chronic viral hepatitis, autoimmune hepatitis, congenital hepatic disorders, or long-term use of steatosis-inducing medications (Park et al. 2020). Generally, NAFLD has no symptoms and is diagnosed by elevated liver enzyme levels, including alanine transaminase (ALT) and aspartate transaminase (AST) in the serum (Kwong & Puri 2021). Approximately 34% of adults in the United States reportedly have NAFLD, and the incidence of NAFLD is increasing especially among the young population. As NAFLD is a collective term for liver-related diseases with various features, including NASH, fibrosis, severe cirrhosis, and hepatocellular carcinoma (Jang et al. 2014), its pathogenesis is quite complex and remains unclear in many aspects. Hence, many studies have been conducted to elucidate this disease and allow more treatment options for patients (Xiao et al. 2014; Friedman et

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al. 2018).

Consumption of potential health supplements is a good alternative, since changing lifestyles for weight loss can be difficult to maintain on a daily basis. Silymarin, an extract from milk thistle, is the most cited food supplement for improving liver health. Several clinical trials of silymarin have been conducted to confirm its effectiveness in NAFLD, and some benefits and doubts in clinical outcomes have been reported (Cacciapuoti et al. 2013; Navarro et al. 2019; Gillessen & Schmidt 2020). Among other food supplements, vitamin E, vitamin D, polyunsaturated fatty acids, and astaxanthin have been reported to have some degree of potential for liver health (Cicero et al. 2018). In Korea, the extract from *Acer tegmentosum* Maxim (Seo et al. 2012), mulberry leaf tea fermented by *Monascus pilosus* (Lee et al. 2013), and *Curcuma longa* L. extract (Lee et al. 2020) have been examined as potential health supplements for NAFLD.

Recent studies on gut microbiota have provided new perspectives on human diseases in various ways. Interestingly, several non-clinical and clinical reports have indicated that the changes in intestinal microbiota affect liver status through the "gut-liver axis". Moreover, evidence of the correlation between the disease and microbiome is rapidly accumulating, and it is now considered one of the vital contributors among the numerous pathophysiological mechanisms leading to NAFLD (Gkolfakis et al. 2015; Machado & Cortez-Pinto 2016; Xie & Halegoua-DeMarzio 2019; Kwong & Puri 2021). The normal intestinal mucosal barrier prevents endotoxins from being absorbed by bacteria in the intestine and passing into the bloodstream. Patients with NAFLD exhibit not only increased intestinal permeability but also high concentrations of endotoxins (*e.g.*, lipopolysaccharide) in the blood. Therefore, harmful toxins in the bloodstream may reach the liver, thus triggering inflammation and accelerating fat accumulation in the liver (Darnaud et al. 2013). Additionally, a close relationship between gut microbiota and liver function was revealed. For example, in children with NAFLD, intestinal *Bifidobacterium* was reduced; however, *Lactobacillus* was increased in contrast to that in healthy children (Nobili et al. 2018). Therefore, consuming probiotics to maintain a healthy balance of gut microbiota may restore healthy microbiota and improve clinical outcomes by stabilizing and strengthening the intestinal mucosal barrier. Accordingly, several non-clinical and small-scale human clinical trials have been conducted over the past few decades to investigate the therapeutic potential of probiotics in patients with NAFLD, and most trials have confirmed that probiotics improve the clinical progress, development, and prognosis of patients with NAFLD (Xie & Halegoua-DeMarzio 2019).

Previously, we demonstrated that *Streptococcus thermophilus* LM1012 is a safe strain without hemolysis, antibiotic resistance, or cytotoxicity in HepG2 cells. We also confirmed that LM1012 has potent hepatoprotective effects in diesel exhaust particulate matter (DEPM)-treated HepG2 and Institute of Cancer Research mice (Jeon et al. 2020). In this study, we assessed the hepatoprotective effects of LM1012 using a methionine–choline-deficient (MCD) diet-induced C57BL/6 mouse model, a commonly accepted *in vivo* model with good reproducibility for testing oxidative stress-mediated steatohepatitis. The pathological phenotypes were induced after the administration of the MCD diet for 8 weeks, and the effect of LM1012 on protecting the liver was assessed by feeding LM1012 with an MCD diet. For comparison, silymarin, the most widely used liver supplement, was administered to mice. Here, we provide experimental evidence of the hepatoprotective efficiency of LM1012 in an MCD diet-induced NAFLD mouse model, suggesting that LM1012 could be utilized as a probiotic supplement for liver health.

Materials and Methods

1. Animals and diets

Animal experiments were conducted at the nonclinical Contract Research Organization institution (KNOTUS, Incheon, Korea). Five-week-old male specific pathogen-free C57BL/6NHsd mice were obtained from Koatech (Gyeonggi-do, Korea). All the animals were housed under a 12-hour light/12-hour dark cycle at 23±3℃, humidity 55±5%, and illuminance 150~300 lx. After 7 days, the mice were divided into four groups (n=10 per group) as follows: control group (mice that were fed a regular diet); MCD diet group (mice that were fed an MCD diet); silymarin group (silymarin 100 mg/head; oral administration once a day during the entire experimental duration); and LM1012 group (LM1012 10^9 CFU/head; oral administration once a day during the entire experimental duration). The experimental duration was 8 weeks. The body weights of the mice were measured on the day of administration initiation and at three times a week during the experimental period. The mice were sacrificed at the end of the experiment. After overnight fasting, the mice were anesthetized, and blood was collected in a vacutainer tube with a clot activator.

After 15 min at room temperature, the tube was centrifuged at 1,500 ×*g* for 10 min to separate the serum. Serum samples were stored at −70℃ in a deep freezer until use. All animal care and experimental procedures were approved by the Animal Care and Use Committee (IACUC) of KNOTUS (IACUC # KNOTUS IACUC 20-KE-433).

2. Biochemical analyses

The ALT, AST, glucose, gamma-glutamyl peptidase (GGT), total cholesterol, total protein, triglyceride (TG), high-density lipoprotein, low-density lipoprotein, and total bilirubin (TBIL) levels in the plasma were measured using a biochemistry automatic analyzer (7180 clinical analyzer, Hitachi High-Technologies, Tokyo, Japan).

The levels of hepatic biomarkers were quantified using enzyme-linked immunosorbent assay (ELISA) analysis kits: hydroxy cholesterol (24(S)-hydroxycholesterol ELISA Kit (cat. no. ab204530); Abcam, Cambridge, UK) and malondialdehyde (MDA) assay kit (competitive ELISA) (cat. no. ab238537), Abcam, Cambridge, UK). The amount of hydroxyproline in the liver tissues was determined using a hydroxyproline assay kit (Biovision, California, USA). Hepatic TGs were measured using a TG assay kit (cat. no. ab65336, Abcam, Cambridge, UK).

3. Real-time quantitative polymerase chain reaction (real-time qPCR) of cytokines

To measure the expression levels of pro-inflammatory cytokines in the liver, total RNAs was extracted from the liver lobes of the sacrificed mice using TRIzol reagent (Invitrogen), and 1 μg of total RNAs was used for cDNA synthesis. Real-time qPCR was performed on CFX96 Touch (Bio-Rad, California, USA) using the primer sets listed in Table 1, and the mRNA levels of tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interleukin (IL)-6, and IL-1β were presented as fold changes relative to the glyceraldehyde-3-phosphate dehydrogenase control.

4. Histopathological examination

After the mice were sacrificed, their livers were harvested, and the tissues were fixed immediately with 10% neutral buffered formalin. The fixed tissues underwent general tissue processing procedures (trimming, dehydration, and paraffin embedding), and the paraffin-embedded blocks were cut into thin (5-μm thick) slide samples for histopathological examination (Sadeghipour & Babaheidarian 2019). Subsequently, hematoxylineosin (H-E) staining was performed. Histopathological changes were observed using an optical microscope (BX53; Olympus, Tokyo, Japan).

Table 1. Primer sequence information used in real-time qPCR

Gene	Primer	Sequence (5′ to 3′)
TNF-α	Forward	ATGAGCACAGAAAGCATGATC
	Reverse	TACAGGCTTGTCACTCGAATT
TGF-β	Forward	TTGCTTCAGCTCCACAGAGA
	Reverse	TGGTTGTAGAGGGCAAGGAC
IL-6	Forward	AGTTGCCTTCTTGGGACTGA
	Reverse	CAGAATTGCCATTGCACAAC
IL-1β	Forward	GGGCCTCAAAGGAAAGAATC
	Reverse	TACCAGTTGGGGAACTCTGC
GAPDH	Forward	AATTCCATCGGCACCGTCAAG
	Reverse	ATCGCCCCACTTGATTTTGG

TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

5. Statistical analysis

The results of all the experiments were analyzed using parametric or non-parametric multiple comparison procedures, assuming the normality of the data. When the results of the one-way analysis of variance were significant, a post-test was performed using Dunnett's multiple comparison test. When the results of the non-parametric Kruskal-Wallis H-test were significant, a post-test was performed using Dunn's multiple composite tests. Statistical analysis was conducted using Prism 7.04 (GraphPad Software Inc., San Diego, CA, USA), and significance was set at $p<0.05$.

Results

1. LM1012 on body and liver weights

Feeding an MCD diet to mice is known to damage the liver by oxidative stress from reactive oxygen species generation and decrease β-oxidation levels, ultimately leading to hepatic fibrosis and steatosis (Anstee & Goldin 2006; Rinella et al. 2008; Ramadori et al. 2015). Therefore, we assessed the role of LM1012 in protecting the liver from damage caused by an MCD diet in the presence or absence of either LM1012 or silymarin for 8 weeks. First, the body and liver weights of mice were

compared. Loss of body weight with the MCD diet was observed in all the MCD diet groups, including silymarin and LM1012, compared to the normal diet group (Fig. 1A). Weight loss (body weight) is a well-known characteristic of the MCD diet model, which exhibits some degree of clinical differences compared to patients with NAFLD. Initially, we anticipated that the LM1012-treated group would demonstrate significantly less loss of body and liver weights; however, the data indicated that LM1012 did not prevent the damage from the toxicity of the MCD diet (Fig. 1B). Nonetheless, considering that no improvement was observed in the positive control group (silymarin), we postulated that the weight loss alone did not represent the actual physiological condition of the mice. Therefore, other physiological measurements involved in the liver conditions were also analyzed.

2. Liver protective effects of LM1012 on hepatic enzymes and TBIL

To evaluate the physiologically relevant biomarkers involved in liver health, the ALT, AST, GGT, and TBIL levels were measured using serum samples from mice. As presented in Fig. 2, all the ALT, AST, GGT, and TBIL levels deteriorated in the MCD diet alone group, which implies that the MCD diet induces liver damage, as expected. Moreover, the degree of deterioration was significantly weakened in the MCD-fed mice treated with LM1012 or silymarin; in particular, both the AST and TBIL levels were significantly improved. For LM1012, the administration promoted a significant reduction in both AST and TBIL levels (23.8% and 64.2%, respectively) compared to that in the MCD diet alone group. Considering that the MCD-fed mice treated with silymarin demonstrated such improvement only in ALT levels, these data suggest that feeding LM1012 protects the liver from MCD diet-induced damage. Although up to 50% of patients with NAFLD demonstrate normal ranges of both AST and ALT enzymes, the progression of the disease is known to be correlated with the elevation of both. Therefore, the relevance of measuring liver enzymes remains clinically important (Neuschwander-Tetri & Caldwell 2003).

3. Inhibition of lipid-related biomarkers by LM1012

Hydroxycholesterols are oxygenated cholesterols, which are

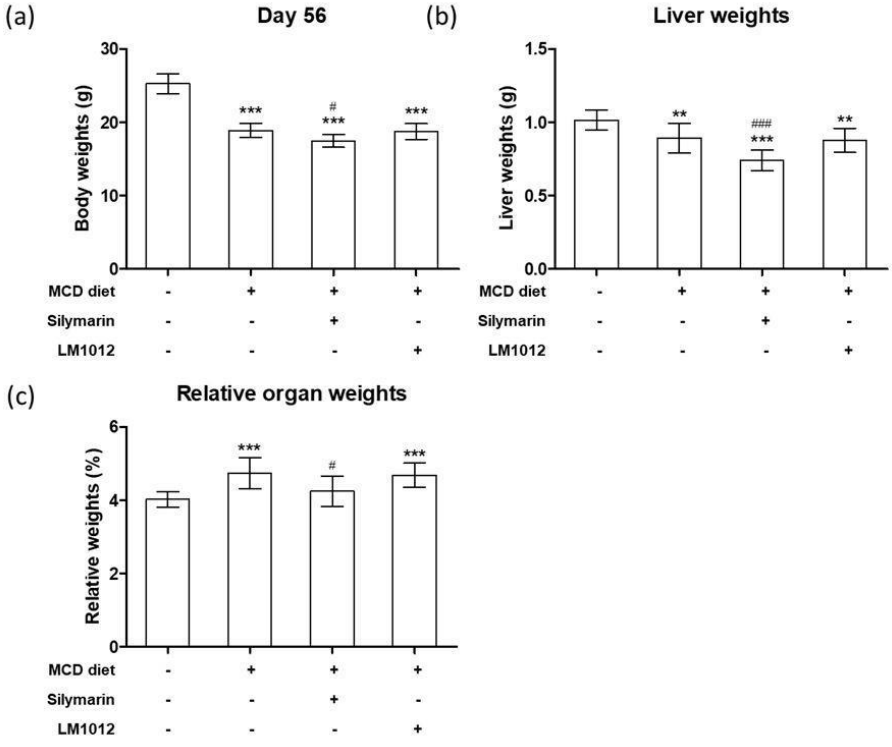

Fig. 1. Body and liver weight changes during experimental periods. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ against the normal diet group (control). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ against the methionine-choline-deficient (MCD) diet-alone group.

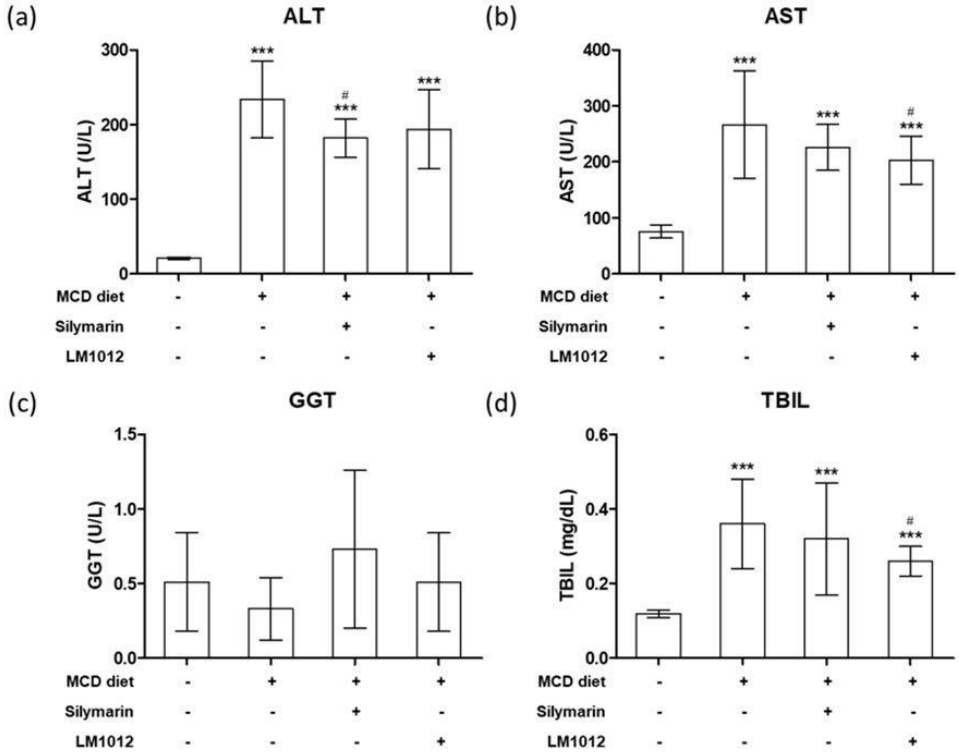

Fig. 2. Various liver associated biomarkers level in the serum. (a) alanine transaminase (ALT), (b) aspartate transaminase (AST), (c) gamma-glutamyl peptidase (GGT) and (d) total bilirubin (TBIL) levels. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ against the normal diet group (control). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ against the methionine-choline-deficient (MCD) diet alone group.

precursors of bile acids and steroid hormones, and one of these roles is involved in maintaining cholesterol homeostasis in the body (Horn et al. 2022). High levels of hydroxycholesterols have been reported in human and mouse serum in NAFLD and NASH conditions (Ikegami et al. 2012; Raselli et al. 2019). Hydroxycholesterols are known to activate fatty acid synthesis in the liver by binding to the liver X receptor α; therefore, elevated levels of hydroxycholesterols in blood circulation cause fat accumulation in the liver (Horn et al. 2022). Administration of the MCD diet to the mice may increase the levels of hydroxycholesterol in the serum, although the increase was not significant compared to that of the normal diet group. Nevertheless, the addition of either LM1012 or silymarin in the same MCD diet significantly reduced the levels of hydroxycholesterol (Fig. 3A). Hence, lowering hydroxycholesterol by either LM1012 or silymarin may protect the liver by inhibiting fat synthesis or lipid export in the liver. Moreover, MDA, a biomarker for lipid peroxidation (Marin et al. 2017), was compared in serum samples as accumulated evidence has indicated that oxidative stress is a key contributor to inflammation (Ucar et al. 2013). The data for MDA again strengthened the disease model that we applied for NAFLD, as the administration of the MCD diet boosted MDA levels compared to providing a normal diet; however, silymarin treatment may be significantly effective in MCD diet-fed mice (Fig. 3B). TGs are well-known indicators of NAFLD, and elevated levels of TG in the bloodstream result in fat accumulation in the liver (Kawano & Cohen 2013; Alves- Bezerra & Cohen 2018). As expected, the MCD diet increased TG serum levels, and the MCD diet-mediated increase was surprisingly reversed when the mice were treated with LM1012 or silymarin (Fig. 3C). Hydroxyproline is a marker for the diagnosis or measurement of hepatic fibrosis (Gabr et al. 2017). The level of hydroxyproline in the MCD diet group was remarkably higher than that in the normal diet group with no significant differences observed in the LM1012- or silymarin- treated MCD diet group compared to the MCD diet only group (Fig. 3D).

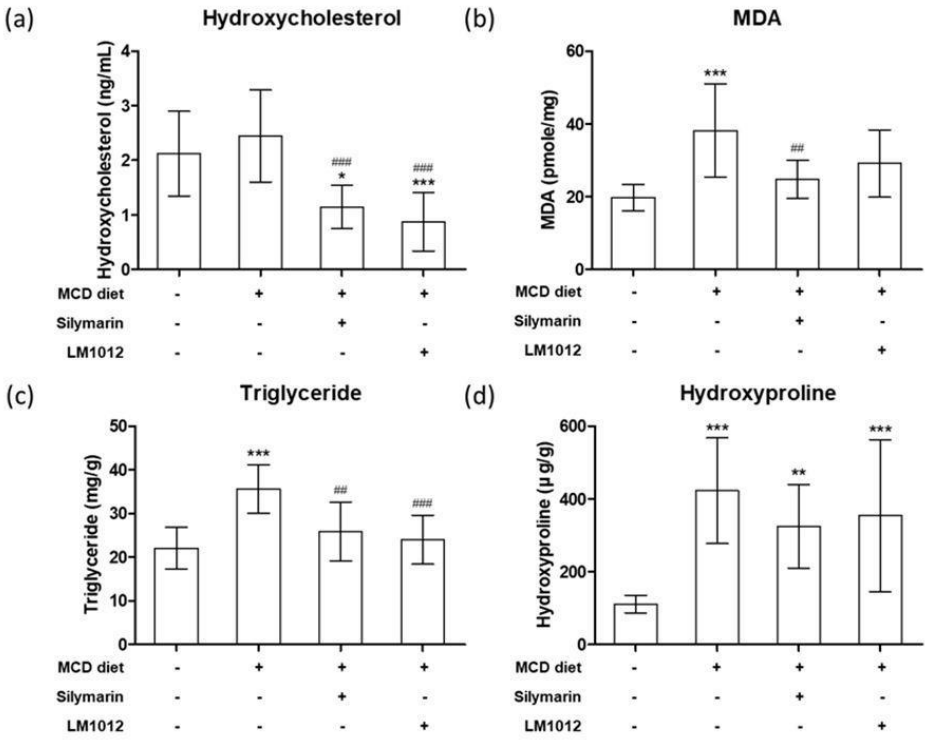

Fig. 3. Changes in metabolite levels in the liver tissues. (a) Hydroxycholesterol, (b) malondialdehyde (MDA), (c) triglyceride, and (d) hydroxyproline. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ against the normal diet group (control). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ against the methionine-choline-deficient (MCD) diet-alone group.

4. Inhibition of pro-inflammation cytokines by LM1012

Liver inflammation is triggered in part by fat accumulation in the liver of patients with NAFLD (Tilg & Moschen 2010). To evaluate the anti-inflammatory effects of LM1012 in the MCD diet model, all liver samples were collected at the end of the experiment, and pro-inflammatory cytokines were examined by real-time qPCR. As presented in Fig. 4, pro-inflammatory cytokines including TNF-α, TGF-β, IL-6, and IL-1β were increased in the MCD diet-fed mice compared to those in the normal diet-fed mice, demonstrating that the MCD diet induced liver inflammation properly. Compared to the MCD diet alone-fed mice, LM1012 co-treated MCD diet-fed mice exhibited some degree of reduction in pro-inflammatory cytokines, although no other significant levels except IL-1β levels were noted. In the silymarin group, all pro-inflammatory cytokines were significantly reduced compared to those in the MCD diet-alone group. Altogether, these findings suggest that LM1012 and silymarin may inhibit inflammatory pathways in the liver and relieve the damage caused by the MCD diet.

5. Reduced fat accumulation and inflammation by LM1012

Histopathological changes were measured by H-E staining, and the validity of LM1012 in this model was tested. Steatosis was monitored by measuring accumulated lipid vacuoles in the tissues. As presented in Fig. 5B, feeding the MCD diet caused many fat vacuoles in the liver lobules, and several inflammatory foci were also formed by infiltrating immune cells. Inflammation in hepatocytes eventually heals, although it could result in wounds, which may ultimately lead to liver fibrosis (Koyama & Brenner 2017). By contrast, inflammatory foci were barely observed in either silymarin or LM1012-treated MCD diet-fed mice. Moreover, fat vacuole formation was significantly less than that in the MCD diet-alone group (Figs. 5C and 5D).

Discussion

To evaluate hepatic protection by administering LM1012,

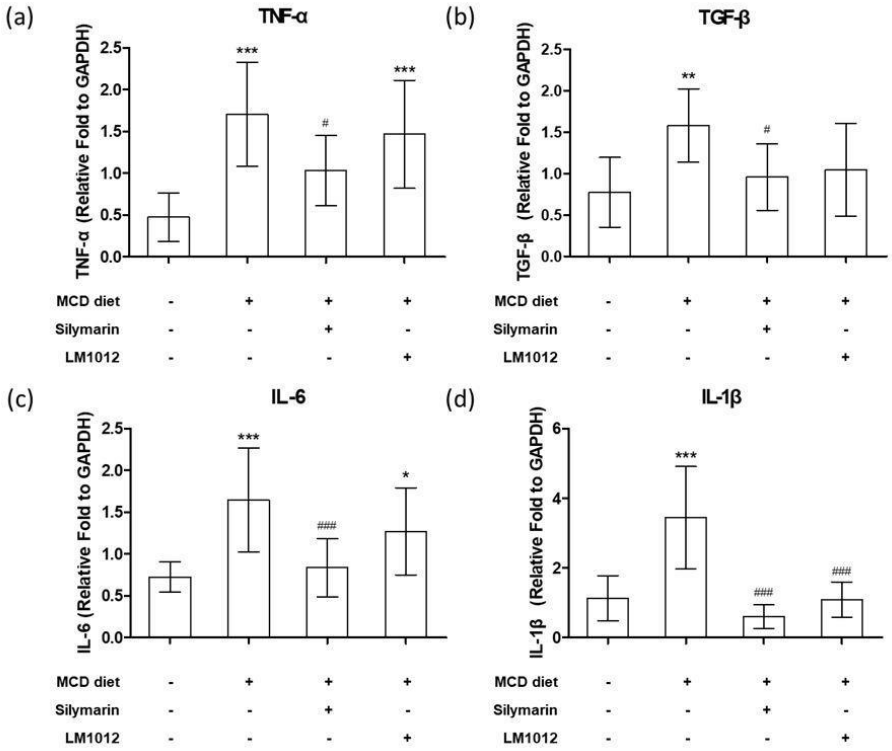

Fig. 4. Cytokine mRNA expression level of liver lobes. (a) tumor necrosis factor-α (TNF-α), (b) transforming growth factor-β (TGF-β), (c) interleukin (IL)-6, and (d) IL-1β. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ against the normal diet group (control). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ against the methionine-choline-deficient (MCD) diet alone group.

changes in body and liver weights, hepatic and lipid biomarkers, and histopathology were examined in a mouse model of MCD diet-induced NAFLD for 8 weeks. Body weight was significantly reduced in the group induced with NAFLD, which was consistent with previous reports, and this was due to the unique feature of the MCD diet-induced NAFLD mouse model (Lee et al. 2019; Mahzari et al. 2019). As described earlier, we anticipated that LM1012 would prevent MCD diet-induced weight loss; however, no significant improvement was observed. The same weight loss was not avoided when silymarin, a scientifically proven food supplement for liver health, was administered to MCD diet-fed mice. Hence, changes in body and liver weights may be the ultimate consequences of many physiological events related to hepatological factors, including lipid synthesis and transport, oxidative stress, fat accumulation, and inflammation, leading to liver damage. Moreover, the weight loss caused by the MCD diet is a feature of the animal model; therefore, it is natural to recognize it as a limitation of the MCD diet-induced NAFLD mouse model.

Other liver-related biomarkers were improved by the administration of LM1012, as demonstrated in the results. First, blood biochemical tests revealed that LM1012 clearly improved MCD diet-induced elevation of both AST and TBIL levels, which are the major indices for liver health. Compared to silymarin, which may be effective only in ALT levels, LM1012 demonstrated a protective effect in the NAFLD mouse model. Additionally, LM1012 treatment decreased the levels of both hydroxycholesterol and TG, which mediate lipid accumulation in the liver. The reduced degree of fat accumulation and inflammation were also confirmed by histopathological studies. Considering that IL-1β, a pro-inflammatory cytokine, was also markedly reduced by LM1012, inhibition of lipid pathways by LM1012 may be the main mechanism leading to less fat and inflammation in the liver of MCD diet-fed mice.

In a previous report, treatment with LM1012 inhibited oxidative stress and inflammatory cytokines by DEPM, a major component of air pollutants, in HepG2 cells derived from human hepatoma, and the administration of LM1012 ameliorated

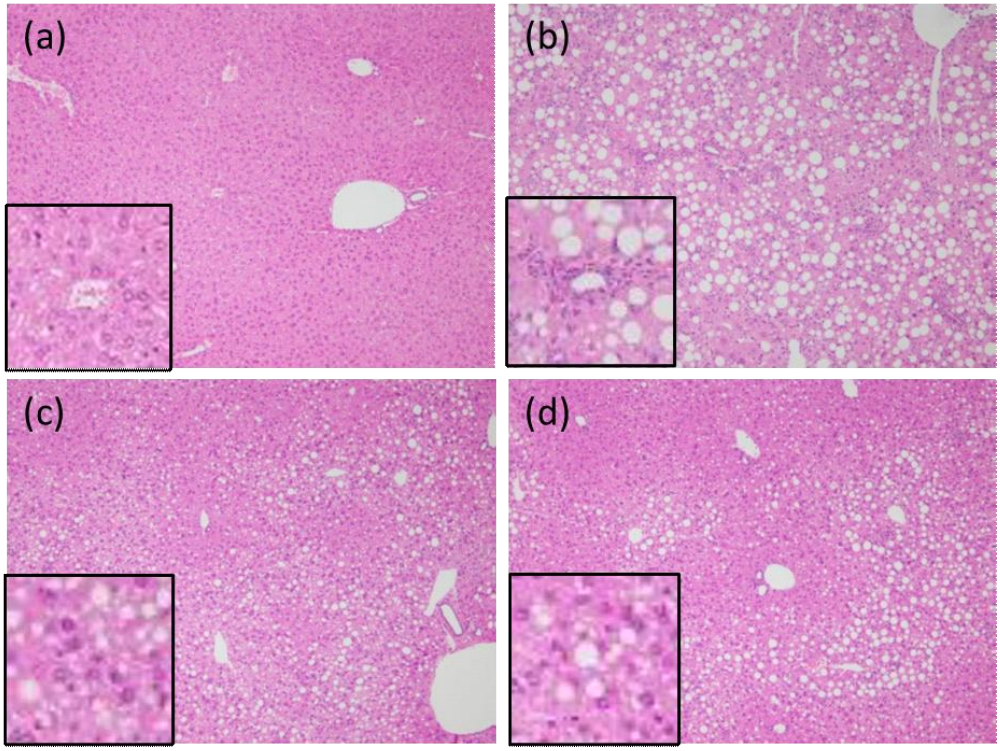

Fig. 5. Histological images (×100) of the liver tissue from each group by hematoxylin-eosin staining. (a) normal diet group, (b) methionine-choline-deficient (MCD) diet group, (c) silymarin group, and (d) LM1012 group. The squares with black lines represent the magnified areas from the respective images.

DEPM-induced elevation of liver indices, including AST and ALT levels in mice (Jeon et al. 2020). In addition to LM1012, many reports have demonstrated the beneficial effects of probiotic strains in NAFLD. Probiotic strains, including *Lactobacilli*, *Bifidobacterium*, *Streptococcus*, and *Saccharomyces*, have been tested for their effectiveness in NAFLD, and some degree of success has been observed in animal experiments and human clinical trials (Meroni et al. 2019; Yao et al. 2021). For example, VSL#3, a probiotic mixture of eight species, has been reported to be effective against liver inflammation in animal experiments (Li et al. 2003; Esposito et al. 2009). Interestingly, a human clinical trial has demonstrated that the combination of both *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (the species of LM1012) was effective in reducing ALT and AST levels compared to placebo (Aller et al. 2011).

The beneficial effects of probiotics on liver health are understood as follows. First, harmful microbes are inhibited by competitive growth in the gastrointestinal tract; therefore, the liver is exposed to less toxic microbial metabolites. Experimental evidence supports the idea that some microbes related to hepatopathology overgrow in the small intestine and translocate to the mesenteric lymph nodes of rats with cirrhosis (Runyon et al. 1994; Garcia-Tsao et al. 1995). Second, anti-obesity effects have been observed for some probiotic strains. *Lactobacillus fermentum* CQPC07 slows weight gain and decreases hyperlipidemia by affecting lipid metabolism (Wu et al. 2021). In addition, *Lacticaseibacillus paracasei* K56 demonstrated similar improvements in body weight, fat accumulation, and insulin resistance (Miao et al. 2022). Considering that obesity is considered a primary cause of NAFLD, the anti-obesity effects of probiotics may benefit liver health. In this study, we did not observe any anti-obesity effects of LM1012, and one of the causes may be related to the MCD diet model, which is known to promote weight loss due to nutrient deficiency.

As a probiotic strain, LM1012, when orally administered, reaches the gastrointestinal tract. We postulate that the metabolites released and the cellular debris derived from LM1012, which are absorbed through the intestine, are the main active ingredients that benefit the liver. Other investigators have suggested that the metabolites secreted from microbes and digested components of

microbial cells are more easily transferred from the gut to the systemic bloodstream (Rodríguez- Romero et al. 2022). Moreover, the living LM1012 itself might translocate from the gut to the liver, and exert liver protective effects. For LM1012, no evidence for translocation has been recorded; however, recent experimental reports have demonstrated that microbial action between the gut and liver exists and functions, which is now understood as a part of the mechanisms of the gut-liver axis (Barrow et al. 2021; Song & Zhang 2022). Altogether, the LM1012 may benefit the liver through the gut-liver axis. Further investigation is necessary to elucidate the precise mechanism by which LM1012 affects liver health.

In conclusion, we have demonstrated that the administration of LM1012 to NAFLD-induced mice is effective in protecting the liver from MCD diet-induced damage. Additionally, as a probiotic strain, LM1012 has been proven safe for human consumption. Therefore, it is a good candidate for human clinical trials to evaluate its potential in liver health.

Funding Information

This research was financially supported by Ministry of SMEs and Startups of Korea under the regional specialized industry development program (S2906501).

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Received 22 July, 2022
Revised 02 September, 2022
Accepted 02 October, 2022