2,3,5,4'-tetrahydroxy-stilbene-2-*O*-*β*-*D*-glucoside attenuates methionine and choline-deficient diet-induced non-alcoholic fatty liver disease

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Abstract. Previous studies have suggested that 2,3,5,4'-tetr ahydroxy-stilbene-2-*O*-*β*-*D*-glucoside (TSG) prevents progression of non-alcoholic fatty liver disease (NAFLD) induced by high-fat diet. The present study aimed to evaluate whether TSG could reverse NAFLD induced by a methionine and choline-deficient (MCD) diet and identify the possible mechanism of action. C57BL6/J mice were fed a MCD diet and were treated with TSG, fenofibrate, and resveratrol for 9 weeks. Regulatory effects of several cytokines and enzymes, including Nod-like receptor protein 3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), caspase-1, interleukin (IL)-18, IL-1β, and gut microbiota balance were investigated. TSG significantly reduced NAFLD biochemical indexes, including total cholesterol, triglyceride, low density lipoprotein cholesterol, very low density lipoprotein cholesterol, aspartate aminotransferase and free fatty acid. Middle dosage (TSG.M, 35 mg/kg) of TSG reduced the expression of ASC and caspase-1. Furthermore, TSG displayed gut microbiota regulatory effects on MCD-induced NAFLD mice. The results of the present study suggested that TSG prevented the occurrence and development of MCD diet-induced NAFLD. The data further indicated that TSG may serve as a promising lead compound that may aid with intervention in NAFLD therapy.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive fat deposited in hepatocytes (1). NAFLD has a high prevalence both in both developed and developing countries (2). In a recent publication, National Health and Nutrition Examination Survey (NHANES) announced a NAFLD prevalence of 30% in the United States (3). In a large population prospective cross-sectional study including 2,493 volunteers recruited from the general population and the Red Cross Transfusion Center in Hong Kong, the prevalence of NAFLD in the general Chinese population was 42% (4). In patients with metabolic syndrome (MetS), the NAFLD prevalence is particularly high, and approximately 45% of patients with MetS have fatty liver disease (5). In total, 1.2 and 0.002% of NAFLD patients progress to liver fibrosis and cirrhosis, respectively (4).

Traditionally, the development of NAFLD has been associated with genetics, systolic blood pressure, serum cholesterol, fasting glucose, gender, age, and waist circumference (6). In recent years, it was gradually realized that gut microbiota and related inflammatory process are, to a great extent, also involved in the development of NAFLD (7). Nod-like receptor protein 3 (NLRP3) inflammasome, which has the ability to sense intracellular danger signals, is highly linked to alterations in the gastrointestinal microflora (7). NLRP3 recognizes gut microbial, stress and damage signals, resulting in direct activation of caspase-1, thereby leading to the secretion of potent pro-inflammatory cytokines and pyroptosis (8). Thus, NLRP3 inflammasome partly aggravates the progression of NAFLD/non-alcoholic steatohepatitis (NASH), and causes MetS via a cell-extrinsic effect of modulation of gut microbiota through activation of the effector protein IL-1β (9). In mice, NLRP3 inflammasome gain of function leads to early and severe onset of methionine and choline-deficient (MCD) diet-induced steatohepatitis (10-12). Furthermore, NLRP3 inflammasome activation is required in the development of fibrosis in NAFLD (13).

In our previous *in vivo* and *in vitro* studies (14-18), 2,3,5,4'-tetrahydroxy-stilbene-2-*O*-*β*-*D*-glucoside (TSG), a naturally occurring stilbenoid, showed beneficial lipid accumulation regulation effects in hepatocytes. The data showed that TSG could partly cut off free fatty acid (FFA) supply, regulated the balance of gut microbiota, improved intestinal mucosal barrier function, and reduced the content of serum lipopolysaccharide (LPS). Prevention of high fat diet (HFD)-induced NAFLD as induced by TSG was mediated by modulation of the gut microbiota via gut-liver axis. Furthermore, TSG suppressed the activation of Toll-like

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receptor 4/nuclear factor-κB (TLR4/NF-κB) signaling pathway, which may alleviate chronic low grade inflammation by reducing exogenous antigen load on the host.

However, activities and capabilities of TSG on NAFLD induced by factors other than HFD are presently unknown. Whether TSG affects the activation of NLRPs, related effector proteins, activation of caspase-1 and further pyroptosis remains to be elucidated. Therefore, we established an MCD diet-induced NAFLD/NASH model to investigate the anti-NAFLD activity of TSG.

In this study, the effects of TSG and resveratrol on regulating NAFLD were compared. Resveratrol, which has a similar backbone as TSG, has been widely recognized for its great bioactivity. Previous studies demonstrated that resveratrol-mediated improvements in glycemic control and NAFLD were associated with alterations in hepatic metaflammation that was related to the NLRP3 inflammasome (19). Thus, this study may provide more powerful evidence for anti-NAFLD activities of TSG compared to resveratrol.

Materials and methods

Chemicals. TSG was purchased from Nanjing Jingzhu Bio-technology Co., Ltd., (Nanjing, China). TSG was purified using a high performance liquid chromatography-diode array detector and the purity was over 98%. Fenofibrate (Laboratoires Fournier S.A., Dijon, France) was used as positive control for serum and hepatic lipid regulation.

In all liver homogenates samples levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), very low-density lipoprotein cholesterol (VLDL-C), and FFA were measured by assay kits purchased from Cusabio Biotech Co., Ltd. (Wuhan, China) and Nanjing Jiancheng Bioengineering Institute, Co., Ltd. (Nanjing, China). Antibodies directed against NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and caspase-1 were purchased from Proteintech Group, Inc. (Chicago, IL, USA). IL-18 and IL-1β were detected by ELISA kits purchased from Cusabio Biotech Co., Ltd.

MCD diet and its control, methionine and choline supplemented (MCS) diet, were purchased from Trophic Animal Feed High-tech Co., Ltd. (Nantong, China).

Animals and treatments. Seventy C57BL/6 male mice (20±2 g, approximately 6-week-old) were provided by Beijing HFK Bioscience Co., Ltd. (Beijing, China). Mice (n=10 per cage) were housed in stainless steel cages, containing sterile paddy husk bedding in ventilated animal rooms (temperature 22±1˚C; 60±10% humidity; and a 12/12 h light/dark cycle), and had free access to water. All animal experiments were performed in compliance with Institutional Ethical Committee on Animal Care and Experimentations of Yunnan University of Traditional Chinese Medicine (Kunming, China; R-0620150019). The ethics committee provided ethical approval for the animal experiments performed in this study. All reasonable efforts were made to minimize the animals' suffering.

After adaptive feeding for three days, mice were randomly assigned to 7 groups (n=10 per group): A, normal control group; B, MCD diet-induced NAFLD model group; C, low dosage of TSG (TSG.L, 17.5 mg/kg); D, middle dosage of TSG (TSG.M, 35 mg/kg); E, high dosage of TSG (TSG.H, 70 mg/kg); F, fenofibrate (26 mg/kg) served as a positive control; G, resveratrol (35 mg/kg) (Table I). Mice in groups B to G were fed with MCD diet for 0-7 weeks and MCS diet for 8-9 weeks.

Mice were fasted for 2 h every day before administration of TSG, fenofibrate and resveratrol. Then, all TSG, fenofibrate and resveratrol treatment was administered (0.2 ml/20 g) by gavage.

Morphology observations. At termination of the experiment, mice were sacrificed by cervical dislocation. For light microscopic observations, liver samples were fixed in formalin fixative and embedded in paraffin following a routine procedure. Next, 5 μm-thick sections were cut, stained with hematoxylin and eosin (H&E), and examined using a light microscope.

Evaluation of liver TC, TG, lipoprotein, IL-1β, and IL-18 levels. Mice were sacrificed by cervical dislocation and liver tissue samples were harvested and immediately processed for biochemical analysis. A total of 100 mg of liver sample from mice in each group were cut into pieces and homogenized on ice using 1 ml of physiological saline. Homogenates were centrifuged at 9,600 x g for 10 min at 4˚C. The supernatants were stored at -80˚C until further analysis. Levels of AST, ALT, TG, TC, LDL-C, HDL-C, VLDL-C, and FFA were determined in liver homogenates by enzymatic colorimetric method using commercial standard enzymatic assay kits. Moreover, levels of IL-1β and IL-18 in the supernatant were measured by commercial Elisa kits (Cusabio Biotech Co., Ltd.).

Western blot analysis. Liver tissue was minced and homogenized in a glass homogenizer on ice. Homogenates were centrifuged at 9,600 x g for 10 min at 4˚C. The supernatants were stored at -80˚C until further evaluated by Western blot analysis. Protein levels in the supernatant were measured by the bicinchoninic acid (BCA) method. In brief, 10-50 mg of total protein per sample was separated on a 10-15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinilide fluoride (PVDF) membranes. Membranes were blocked by incubation with 5% skim milk in TBS at room temperature for 1 h, followed by incubating with primary antibodies directed against NLRP3, ASC, caspase-1, IL-18, and IL-1β for overnight at 4˚C, respectively. Next, membranes were washed three times with TBS/Tween-20 (TBST) at room temperature and incubated with secondary antibodies (Proteintech Group, Inc.). Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system and quantified with ImageJ software.

Overall structural changes of gut microbiota. Feces were collected at the last day of the experiment and stored in sterilized centrifuge tubes at -80˚C. All feces samples of mice in the same group were mixed, transferred into a mortar and grinded to a fine powder. DNA was extracted according to the instruction of test kits (Omega Bio-Tek, Inc., Norcross, GA, USA).

Table I. Animal groupings and treatments.

Groups	Diet		Treatment	
	0-7 weeks	8-9 weeks	0-9 weeks	Dosage (mg/kg)
A	MCS	MCS	-	-
B	MCD	MCS	-	-
C	MCD	MCS	TSG	17.5
D	MCD	MCS	TSG	35.0
E	MCD	MCS	TSG	70.0
F	MCD	MCS	Fenofibrate	26.0
G	MCD	MCS	Resveratrol	35.0

MCD, methionine and choline-deficient; MCS, methionine and choline supplemented; TSG, 2,3,5,4'-tetrahydroxy-stilbene-2-*O*-*β*-*D*-glucoside.

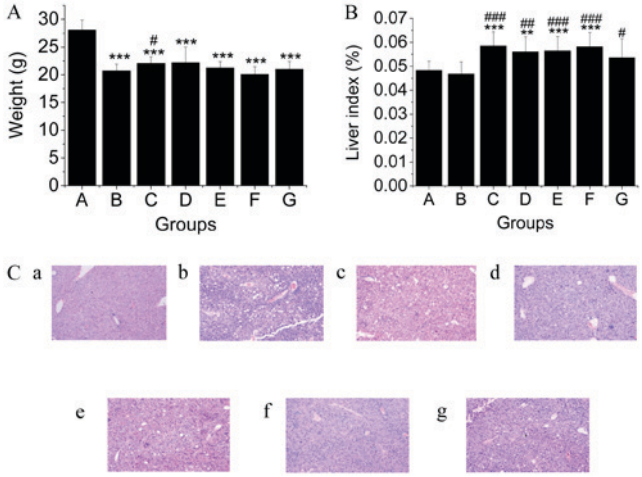

Figure 1. Shown are (A) body weight, (B) liver index, and (C) microscopic morphology of liver of mice in all groups. In this study, the changes of (A) body weight, (B) liver index and (C) microscopic morphology of liver in groups were evaluated, 10 times of magnification. (C-a) Normal control group (C-b) MCD diet group, (C-c) low dosage of TSG (17.5 mg/kg) group, (C-d) middle dosage of TSG (35 mg/kg) group, (C-e) high dosage of TSG (70 mg/kg) group, (C-f) fenofibrate (26 mg/kg) group, (C-g) resveratrol (35 mg/kg) group. Liver atrophy and fat drop accumulation were apparent in mice in the MCD group compared with the normal group. TSG (TSG.L, 17.5 mg/kg; TSG.M, 35 mg/kg; and TSG.H, 70 mg/kg) significantly reduced the degree of fatty liver degeneration. Fenofibrate also effectively decreased the fatty degeneration of liver cells. $^{**}P<0.01$, $^{***}P<0.001$ (*, compared with the control group). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ ($^{\#}$, compared with the model group). MCD, methionine and choline-deficient; TSG, 2,3,5,4'-tetrahydroxy-stilbene-2-*O*-*β*-*D*-glucoside.

To determine the diversity and composition of the bacterial species in the feces samples from each group, we used the protocol as described by Caporaso *et al* (20). PCR amplifications were conducted with the 515f/806r primer set, which allowed for the V4 region of the 16S rDNA gene to be amplified. The reverse primer contained a 6-bp error-correcting barcode that was unique for each sample. DNA was amplified following a protocol described previously (21). Sequencing was conducted on an Illumina MiSeq platform (NoVogene, Beijing, China).

Statistical analysis. Data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was employed to analyze the data when multiple group comparisons were performed. Graphics were created using Origin 6.1 software (MicroCal Software, Northampton, MA, USA). Operational taxonomic units (OTUs) clustering was analyzed by the software system of Mev Development Team (TMEV) Clustering.

Results

Effects of TSG on the general physiological features of NAFLD mice. At the end of the experiment, the body weights of mice in the MCD diet groups were significantly lower compared to that of mice in the regular feed group. However, treatment with TSG.L alleviated this weight reduction (Fig. 1A). Entirely different from HFD-induced NAFLD, fatty liver induced by MCD diet possessed a similar liver index when compared to normal mice (Fig. 1B), indicating that liver atrophy was not significantly different in mice in the MCD group. Treatment with TSG, fenofibrate, and resveratrol effectually attenuated this liver atrophy (Fig. 1B). Morphology observations indicated that in our study, an MCD diet-induced NAFLD model was successfully established. TSG effectively relieved lipid accumulation in the liver of mice (Fig. 1C). Resveratrol also decreased lipid accumulation induced by MCD, however, lipid drops in hepatic cells could also be observed in resveratrol group (Fig. 1C).

Subsequently, we determined the levels of AST, ALT, TG, TC, LDL-C, VLDL-C, and FFA in liver tissue (Fig. 2). Levels of TC and TG in mic in the NAFLD group were higher compared to that of mice in the normal group. The levels of TC and TG were significantly decreased in TSG treatment groups ($P<0.05$). TSG treatment significantly reduced TC and TG accumulation in the liver in a dose dependent manner. Moreover, TSG treatment demonstrated a more attractive hepatic lipid control effect compared to fenofibrate and resveratrol. LDL-C contents were increased in MCD diet-induced NAFLD mice. This increase was inhibited by treatment with TSG.M, TSG.H, and fenofibrate. The mechanism for stetosis in mice on a MCD diet appeared to be related with impaired VLDL-C secretion due to the lack of phosphatidyl choline synthesis (22). Our results confirmed that VLDL-C content of mice in the MCD diet-induced NAFLD group was reduced by about 50%. Unfortunately, none of these treatments could reverse this VLDL-C reduction. In NAFLD mice, hepatic FFA levels dramatically increased about 243.5%. This increase was reduced after treatment of TSG.M, TSG.H, fenofibrate, and resveratrol. Thus, these results confirmed that TSG could partly reduced FFA supply as presented in our previous study (14).

To evaluate the possible effect of MCD diet and treatments on mouse hepatic function, hepatic AST and ALT levels were measured. AST levels were slightly elevated after mice were fed an MCD diet, however, the difference was not significant. Interestingly, the increase in AST levels was inhibited by treatment with TSG and resveratrol. In contrast, hepatic ALT levels remained at relative stable levels and were unrelated to the MCD diet. Treatment with TSG, fenofibrate, and resveratrol did not influence hepatic ALT levels.

Effects of TSG treatment on regulation of NLRP3 inflammasome and hepatic metaflammation. The regulatory effects of

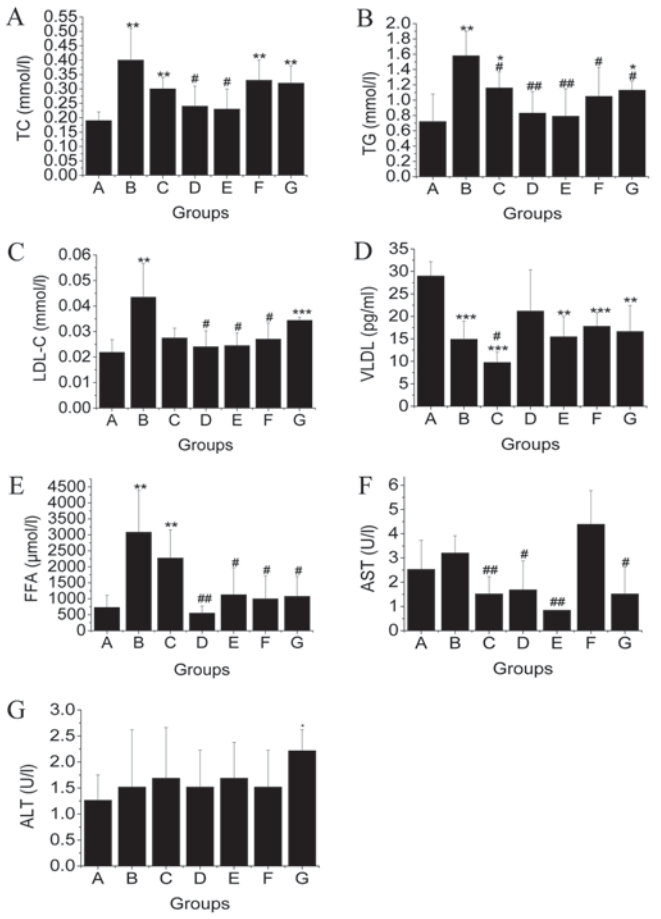

Figure 2. Levels of (A) TC, (B) TG, (C) LDL-C, (D) VLDL-C, (E) FFA, (F) AST, and (G) ALT in liver tissue of mice in the different groups (mean ± SD, *n*=10). In this study, levels of TC, TG, LDL-C, VLDL-C, FFA, AST, and ALT were measured. The contents of TG, TC, LDL-C, AST, and FFA were significantly increased in mice that were fed an MCD diet. Moreover, TSG treatment effectively reduced the contents of TG, TC, LDL-C, AST, and FFA in the liver. $^{\#}$P<0.05; $^{\#\#}$P<0.01 ($^{\#}$, compared with MCD diet-induced NAFLD model group). *P<0.05; **P<0.01; ***P<0.001 (*, compared with the control group). TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; FFA, free fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

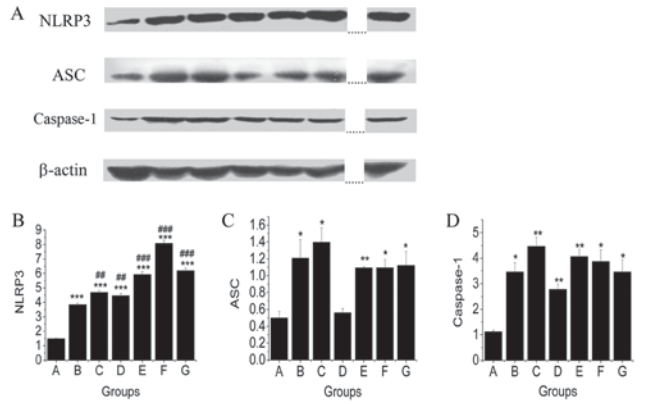

Figure 3. The effects of TSG on protein expression of NLRP3, ASC and caspase-1 in livers of MCD diet fed mice. Protein imprinting of NLRP3, ASC, and caspase-1. (A) As a matter of fact, nine samples were included in the original gel/experiment. The antepenultimate and penultimate pair were omitted from the figure (as denoted by the dotted lines) since they were not relevant for a discussion of the experiment at hand. Densitometric analysis of (B) NLRP3, (C) ASC, (D) caspase-1. Mice fed an MCD diet showed increased levels of NLRP3, ASC and caspase-1 protein expressions compared with mice in the control diet-fed group. The results showed that the middle dosage of TSG downregulated ASC and caspase-1 levels. $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 ($^{\#}$, compared with MCD diet-induced NAFLD model group). *P<0.05; **P<0.01; ***P<0.001 (*, compared with the control group). TSG, 2,3,5,4'-tetrahydroxy-stilbene-2-*O*-*β*-*D*-glucoside; NLRP3, Nod-like receptor protein 3; ASC, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain; MCD, methionine and choline-deficient; NAFLD, Non-alcoholic fatty liver disease.

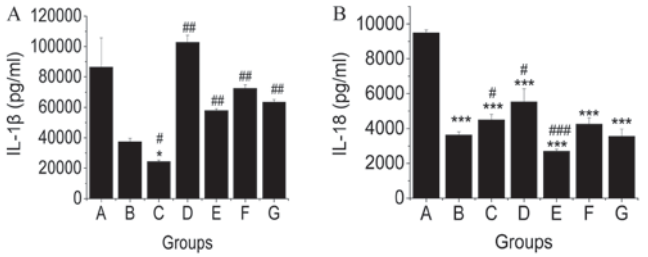

Figure 4. Effects of TSG on the protein expression of (A) IL-1β and (B) IL-18 in livers of MCD diet-fed mice. At the end of the experiment, the levels of IL-1β and IL-18 in liver tissue were evaluated. Low dosage of TSG reduced the levels of IL-1β, and high dose of TSG showed that IL-18 levels were reduced compared with the model group. Data are shown as the mean ± SD ($^{\#}$, compared with the MCD diet-induced NAFLD model group). $^{\#}$P<0.05; $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001. *P<0.05; ***P<0.001 (*, compared with the control group). TSG, 2,3,5,4'-tetrahydroxy-stilbene-2-*O*-*β*-*D*-glucoside; IL, interleukin; MCD, methionine and choline-deficient; NAFLD, non-alcoholic fatty liver disease.

TSG treatment on levels of NLRP3, ASC, and caspase-1 in liver were investigated by Western blot analysis. As shown in Fig. 3, MCD diet fed mice showed an increase in protein expression levels of NLRP3, ASC, and caspase-1 compared with mice in the control group. TSG treatment had no significant effect on the level of NLRP3 (Fig. 3B). However, TSG.M treatment reduced the level of ASC to a similar level of mice in the control group (Fig. 3C). The level of caspase-1 was also reduced by TSG.M treatment. These results showed that TSG.M, TSG.H, fenofibrate, and resveratrol down-regulated the ASC content. Compared to model group, treatment with TSG.M and resveratrol resulted in caspase-1 lowering activities (Fig. 3D).

In general, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by the inflammasome, thereby activating the cysteine protease caspase-1 that, in turn, will result in the maturation of the proinflammatory cytokines interleukin (IL)-1β and IL-18 (23). In this study, IL-1β and IL-18 levels were evaluated in liver tissue of mice. We found that levels of IL-1β and IL-18 of mice in the NAFLD group were not significantly different compared to that in normal mice. TSG.L treatment reduced the level of IL-1β (Fig. 4A) compared to that in NAFLD mice, whereas TSG.H treatment reduced IL-18 content (Fig. 4B).

Effects of TSG treatment on regulating relative proportions of intestinal microbial. Numerous studies have suggested that disruption of the relative proportions of gut microbial populations may contribute to the progress of NAFLD. Therefore, increased attention has been paid to the status and therapy of the intestinal microbial balance in NAFLD.

An MCD diet may modify the gut microbiota and gut permeability (24-26). Several findings suggested that

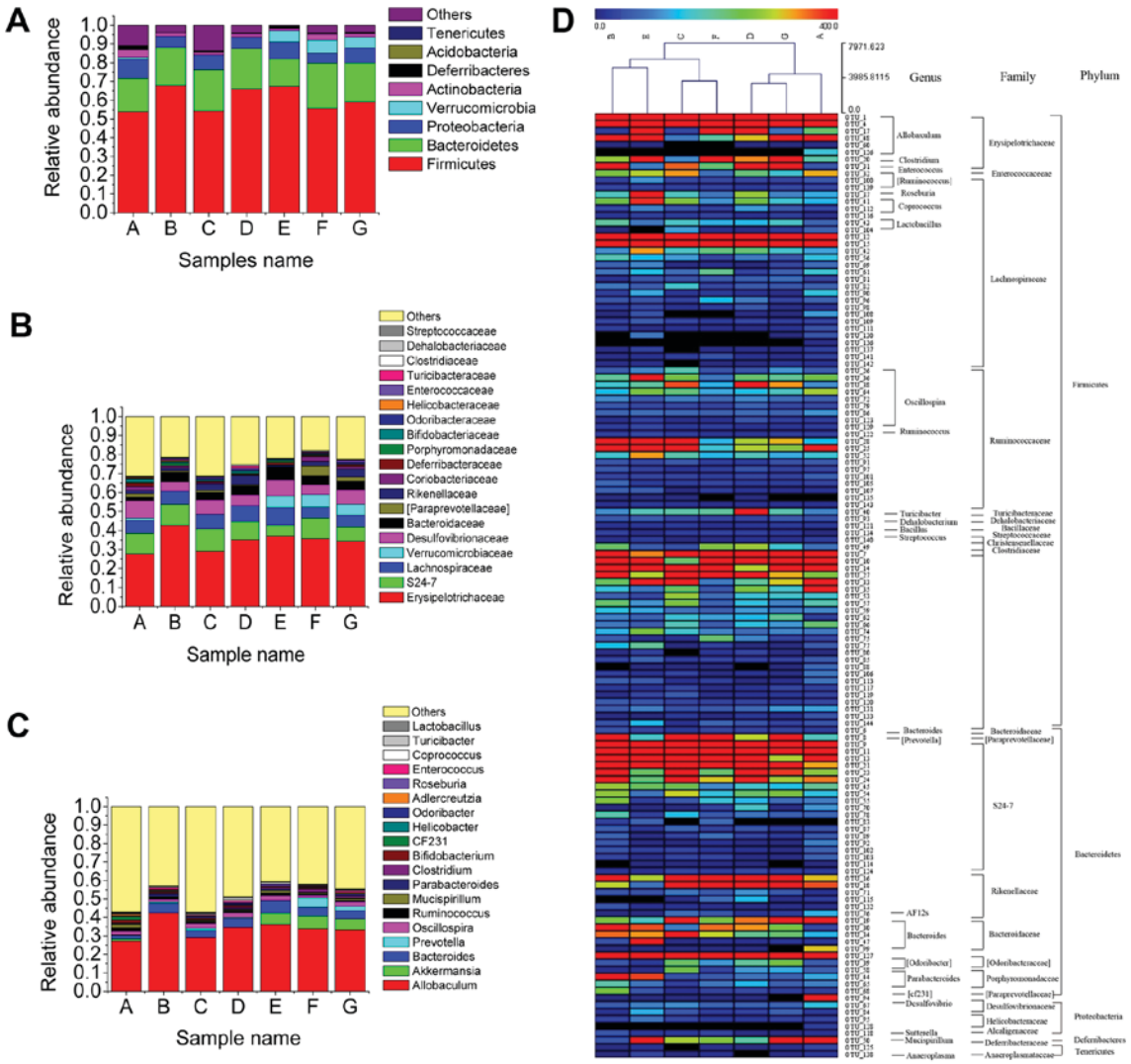

Figure 5. The microbial structure of mice in different experiment groups were analyzed by different species classification level of gut microbiota. The relative abundance of microbial species in (A) Phylum, (B) Family, (C) Genus level in different groups and (D) heat map of top 134 OTU are shown.

circulating LPS levels are elevated in rodent MCD diet-induced NAFLD (24-26).

The relative abundance of microbial species in phylum revealed that the presence of Firmicutesin MCD diet fed mice (67.7%) was high compared with that in normal mice (53.8%). An increase in the relative abundance of Firmicutes was also observed in HFD-induced and genetically engineered obese mice as well as in obese humans when compared with lean control subjects (27). Mice in Group C, D, E, F, and G showed a reduction in relative abundance of Firmicutes (54.2, 66.1, 67.3, 55.68, and 59.10%, respectively) (Fig. 5A).

Erysipelotrichaceae abundance (42.85%) in mice in Group B was significantly higher compared to that of mice in the control group (27.65%) (Fig. 5B). Several species of Erysipelotrichaceae were identified as potential candidates to play a causal role in the pathogenesis of parenteral nutrition-associated liver injury (PNALI) (28). Moreover, the abundance of Erysipelotrichaceae seemed was affected after treatment with TSG.L, TSG.M, TSG.H (29.20, 35.18, 37.15%), fenofibrate (35.70%), and resveratrol (34.43%). It was also worth mentioning that an MCD diet increased the relative abundance of Helicobacteraceae (0.557%) (Fig. 5B), which was usually considered as pathogenic species (29). Moreover, *Helicobacter pylori* may activate the inflammasome and caspase-1 in antigen-presenting and other cells, resulting in processing and release of caspase-1-dependent cytokines (30). Mice in groups C, D, E, F and G showed a reduction in the relative abundance of Helicobacteraceae (0.071, 0.065, 0.15, 0.043, and 0.10%, respectively), which suggested that TSG treatment effectively controlled Helicobacteraceae abundance. Mice in groups D, E, F, G, H and I showed increased abundance of Verrucomicrobiaceae (Fig. 5B). It was previously reported that decreased Verrucomicrobia in prediabetes was consistent with the findings by Barlow *et al* that *Akkermansia* may play a substantial role in regulating host adiposity and weight loss (31).

Furthermore, MCD diet intake resulted in an increase of *Allobaculum* and *Parabacteroides* (Fig. 5C). Abundances of *Allobaculum* and *Parabacteroides* showed a significant increase in NAFLD/NASH patients compared with controls (29). The relative abundances of *Allobaculum* and *Parabacteroides* in the gut negatively correlated with the expression levels of tight junction protein (ZO-1 and occludin) and anti-inflammatory genes, such as IL-10 and Foxp3 (27).

Treatment with TSG effectively inhibited their abundances to a normal level (Fig. 5C).

Given that *Allobaculum* genera belong to the Erysipelotrichaceae family, which belong to Firmicutesphylum, we proposed that TSG treatment reduced the abundance of Firmicutesphylum by affecting Erysipelotrichaceae family and *Allobaculum* genera.

On the contrary, an MCD diet (0.038%) decreased the relative abundance of *Akkermansia* (10.93%). Several reports demonstrated that *Akkermansia muciniphila* treatment reversed high-fat diet-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance (32). TSG.M, TSG.H, fenofribrate, and resveratrol increased the relative abundance of *Akkermansia*.

Effects of TSG treatment on regulating intestinal microbial balance. In this study, we identified 134 key variables, which were significantly altered after treatment with TSG and resveratrol. C clustering analysis of 134 OTUs (Fig. 5D) showed that mice in the MCD diet group were significantly different from the control group. TSG.M treatment shortened its distance compared to the control group. Thus, these results indicated that TSG treatment partially recovered the gut microbiota equilibrium.

Comparison of TSG and resveratrol in the treatment of NAFLD. In this study, we compared the effects of TSG and resveratrol treatment on NAFLD regulation. Our data indicated that TSG treatment had a better alleviating effect on liver atrophy compared to resveratrol. The levels of TC, LDL-C, and ALT in mice in the TSG-treated Group were lower compared to that of mice in Group G. TG levels of mice in Group C and D were lower than those of mice in Group G. Considering VLDL-C and FFA regulation, TSG treatment demonstrated a better activity compared to resveratrol. Moreover, when compared to resveratrol, treatment with TSG.L and TSG.M displayed better NLRP3-lowering activities. Levels of ASC and caspase-1 were significantly decreased in TSG.M-treated mice compared to mice treated with resveratrol.

Discussion

Previous studies have suggested that TSG, a promising anti-NAFLD candidate, prevented HFD-induced NAFLD. In this study, we tried to investigate whether TSG could reverse NAFLD induced by an MCD diet and if this effect was related to gut microbiota and the NLRP3 inflammasome.

It had been reported that mice fed an MCD diet might loss >40% of body weight and >60% of liver weight after 30 weeks. In addition, 2 weeks after switching back to the control diet, both body and liver weights significantly recovered (33). We found that an MCD diet impaired mitochondrial β-oxidation, which led to increased production of reactive oxygen species (ROS), mitochondrial DNA damage, and apoptotic cell death, in addition to hepatic stellate cell (HSC) activation and extracellular matrix deposition (34). Furthermore, oxidative stress had been implicated as an etiological factor in various acute and chronic liver diseases, including NAFLD and NASH (35).

It has been shown that inflammasome-mediated dysbiosis regulated the progression of NAFLD and obesity (6). In mice, NLRP3 inflammasome activation resulted in hepatocyte pyroptosis, liver inflammation, and fibrosis (36). Previous studies indicated that resveratrol may ameliorate hepatic metaflammation and inhibit NLRP3 inflammasome activation in HFD-induced obesity mice (19). Therefore, in this study, components of the NLRP3 inflammasome complex and proinflammatory markers were analyzed to test whether NLRP3 inflammasome and related hepatic metaflammation were involved in TSG-mediated improvement of MCD diet-induced hepatic stetosis.

Gut microbiota and related inflammatory process played to a great extent a role in the development of MCD diet-induced NAFLD. Moreover, gut microbiota and bacterial endotoxin were involved in several underlying mechanisms of NAFLD as well as in its progression to NASH. Management of gut microbiota dysbiosis may become a cornerstone for future treatment of liver diseases (37). Alterations in the gut microbiota population and/or changes in gut permeability promote microbial translocated into the portal circulation, thus directly to the liver, via the NLRP3 inflammasome (38). Several studies have demonstrated that activation of the NLRP3 inflammasome resulted in severe liver inflammation and fibrosis, while hepatocyte pyroptotic cell death was identified as a novel mechanism of NLRP3-mediated liver damage (36).

In our study, we demonstrated that the levels of TG, TC, LDL-C, AST, and FFA in the liver were significantly increased in C57BL6/ mice fed an MCD diet. This increase could be effectively reduced by treatment with TSG. On the other hand, TSG reduced the accumulation of TG in the liver by lowering the expression of LDL-C. Moreover, MCD-diet elevated levels of AST and ALT were also alleviated after treatment with TSG. The protein expression levels of NLRP3, ASC, and caspase-1 were increased by MCD diet, and TSG.M treatment reduced the levels of ASC and caspase-1.

Gut microbiota of MCD diet-induced NAFLD mice were significantly altered by treatment with TSG. TSG decreased relative abundances of Firmicutes phylum, Erysipelotrichaceae, and Helicobacteraceae in MCD diet-induced NAFLD mice. However, levels of Verrucomicrobiaceae were increased after TSG treatment. Moreover, abundances of *Allobaculum* and *Parabacteroides,* which may affect the incidence and progress of NAFLD, were also raised after TSG treatment. The clustering analysis of 134 key OTUs showed that TSG partly recovered gut microbiota alteration induced in MCD diet-induced NAFLD mice. However, some inadequacies also existed in our research, due to the small sample size, the fecal samples from mice in each experimental group were mixed (instead of analyzed in each individual mouse) and used to determine the effect of TSG and resveratrol on the gut microbiome.

Finally, we found that TSG possessed better effects on MCD diet-induced NAFLD when compared to resveratrol. Although TSG and resveratrol are both natural stilbenoids, they may regulate NF-κB differently. Resveratrol was reported to primarily decrease the production of pro-inflammatory factors by impairing phosphorylation and nuclear translocation of NF-κB (39,40). However, TSG may protect brain tissue from ischemia by impairing the DNA binding activity of NF-κB (41).

In addition, several *in vivo* studies in both animals and humans indicated a very low intestinal uptake of resveratrol, leading to trace amounts in the bloodstream based on extensive metabolism in the gut and liver (42). An effective approach to stabilize resveratrol derivatives was accomplished by methylation (43) or glycosidation (44) of resveratrol to form methylated or glycosylated resveratrol (45). TSG possessed better stability and water solubility compared to resveratrol. This may contribute to its increased effect on MCD diet-induced NAFLD compared to resveratrol.

In summary, our findings demonstrated that TSG reversed the occurrence and development of NAFLD by affecting the intestinal microbial content and by partly regulating the subsequent inherent immune system. Thus, TSG may be a promising compound for NAFLD therapy.

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