RESEARCH ARTICLE

Molecular Nutrition & Food Research
www.mnf-journal.com

Soyasaponin A_2 Alleviates Steatohepatitis Possibly through Regulating Bile Acids and Gut Microbiota in the Methionine and Choline-Deficient (MCD) Diet-induced Nonalcoholic Steatohepatitis (NASH) Mice

*Fei Xiong, Zhongdaixi Zheng, Lingyu Xiao, Chuhong Su, Junbin Chen, Xiangfu Gu, Jiaqi Tang, Yue Zhao, Huiyu Luo, and Longying Zha**

Scope: Nonalcoholic steatohepatitis (NASH) is a chronic progressive disease with complex pathogenesis of which the bile acids (BAs) and gut microbiota are involved. Soyasaponins (SS) exhibits many health-promoting effects including hepatoprotection, but its prevention against NASH is unclear. This study aims to investigate the preventive bioactivities of SS monomer (SS-A_2) against NASH and further clarify its mechanism by targeting the BAs and gut microbiota.

Methods and Results: The methionine and choline deficient (MCD) diet-fed male C57BL/6 mice were intervened with obeticholic acid or SS-A_2 for 16 weeks. Hepatic pathology is assessed by hematoxylin-eosin and Masson's trichrome staining. BAs in serum, liver, and colon are measured by ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC-TQMS). Gut microbiota in caecum are determined by 16S rDNA amplicon sequencing. In the MCD diet-induced NASH mice, SS-A_2 significantly reduces hepatic steatosis, lobular inflammation, ballooning, nonalcoholic fatty liver disease activity score (NAS) scores, and fibrosis, decreases *Erysipelotrichaceae (Faecalibaculum)* and *Lactobacillaceae (Lactobacillus)* and increases *Desulfovibrionaceae (Desulfovibrio)*. Moreover, SS-A_2 reduces serum BAs accumulation and promotes fecal BAs excretion. SS-A_2 changes the BAs profiles in both liver and serum and specifically increases the taurohyodeoxycholic acid (THDCA) level. *Faecalibaculum* is negatively correlated with serum THDCA.

Conclusion: SS-A_2 alleviates steatohepatitis possibly through regulating BAs and gut microbiota in the MCD diet-induced NASH mice.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as the accumulation of fat in the liver in patients who do not consume excessive alcohol.[1] NAFLD has emerged as a major cause of chronic liver disease. It is growing in prevalence worldwide due to the increase in obesity and metabolic syndrome. The estimated prevalence of NAFLD in general population is 27–34% in United States of America (USA) and 20–40% in western countries.[2] NAFLD represents a series of liver abnormalities ranging from simple steatosis, nonalcoholic steatohepatitis (NASH), and fibrosis. About 30% of patients with NAFLD progress into NASH, which is a more severe form of liver disease characterized by steatosis, hepatocellular ballooning, and lobular inflammation with varying degrees of fibrosis.[3] Furthermore, a subset of NASH patient can progress to fibrosis, which ultimately leads to cirrhosis and hepatocellular carcinoma (HCC).[4] The pathogenesis of NAFLD and its progression is a complex process and is still not fully understood. There are several hypothesized mechanisms on the pathogenesis of NAFLD, among which the "multiple-hit" theory relatively obtains a common recognition, although it still cannot give the complete explanation.[5,6] The latest studies show that the bile acid-gut microbiota crosstalk plays an important role in the pathogenesis and progression of NAFLD/NASH.[7,8]

Bile acids (BAs) are a component of bile and well known for their role in aiding fat absorption. BAs are synthesized from cholesterol via the classical pathway or the alternative pathway.[7] Cholic acid (CA) is produced via the classical pathway in which the rate-limiting step is the catalysis of cholesterol into 7α-hydroxycholesterol by the enzyme cholesterol 7α-hydroxylase (CYP7A1). Chenodeoxycholic acid (CDCA) is synthesized via the alternative pathway, which is initiated by the

Dr. F. Xiong, Dr. Z. Zheng, L. Xiao, Dr. C. Su, Dr. J. Chen, Dr. X. Gu, J. Tang, Y. Zhao, H. Luo, Prof. L. Zha
Department of Nutrition and Food Hygiene
Guangdong Provincial Key Laboratory of Tropical Disease Research
School of Public Health
Southern Medical University
Guangzhou, Guangdong 510515, P. R. China
E-mail: lyzha@smu.edu.cn

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202100067

DOI: 10.1002/mnfr.202100067

enzyme sterol 27-hydroxylase (CYP27A1)-catalyzed synthesis of 25-hydroxycholesterol and 27-hydroxycholesterol.[8] Both CA and CDCA are called primary Bas, which are usually conjugated with either glycine or taurine in humans or taurine in rodents.[9] Following synthesis, BAs are exported via the bile salt export pump (BSEP) into the gallbladder to form bile. BAs and other biliary components enter the small intestine, where they function in the emulsification and absorption of dietary fat, cholesterol, and fat-soluble vitamins.[10] In the intestine, primary conjugated BAs are deconjugated via bile salt hydrolases (BSH)-active bacteria and 7α-dehydroxylated to secondary BAs, such as deoxycholic acid (DCA) and lithocholic acid (LCA). At the terminal ileum, the majority (95%) of BAs are reabsorbed via the apical sodium-dependent bile acid transporter (ASBT) into enterocytes and are secreted into the portal circulation.[11,12] BAs are then taken up by sodium/taurocholate cotransporting polypeptide (NTCP) and organic anion-transporting polypeptide 1 (OATP1) into hepatocytes. The cyclic process by which bile acids are secreted from the liver into the intestine, reabsorbed in the ileum and returned to the liver via the portal vein is termed enterohepatic circulation.[8,9,13] Under normal conditions, the pool size of BAs remains in a steady state and regulated by negative feedback inhibition through the nuclear receptor farnesoid X receptor (FXR), while limiting the accumulation of BAs in the liver.[13,14] FXR is highly expressed in both the liver and ileum.[15,16] In the liver, BA-activated FXR induces the expression of small heterodimer partner (SHP), which binds to liver receptor homolog-1 (LRH-1) and inhibits the expression of CYP7A1 and BAs synthesis.[15,16] In the distal ileum, FXR is activated by BAs and induces the expression of fibroblast growth factor (FGF) 15 (FGF19 in humans).[10] FGF15/19 reaches the liver via portal vein blood and binds to the FGF receptor 4 (FGFR4)/b-klotho heterodimer complex, triggering the JNK1/2 and ERK1/2 signaling cascades that also inhibits the expression of CYP7A1.[17] Moreover, ileum FXR activation can also inhibit the expression of ASBT and increase excretion of BAs.[8]

Gut microbiota is believed to play a role in the development of NAFLD/NASH through regulating the pool size and composition of BAs and modifying the chemical and signaling properties of BAs.[8,9] Gut microbiota can deconjugate and subsequently metabolize the primary BAs into secondary BAs in the gut and thus alter the activation and signaling of FXR.[10] The homeostasis of BAs is found to be disrupted in experimental NASH mouse models and in clinical human NASH patients.[11] Moreover, the content and composition of BAs and gut microbiota were both altered in patients and mice with NASH.[6,12–14] Therefore, pathways linked to the BAs and gut microbiota crosstalk have been implicated as novel targets for the prevention and treatment of NAFLD and NASH.[11,18–23]

In recent years, phytochemicals, a large category of secondary metabolites from fruits and vegetables, whole grains and other plant foods have attracted much attention because of their potential roles in the prevention and treatment of chronic diseases including NAFLD/NASH.[24,25] Soyasaponins (SS) are a family of phytochemicals from soybean and its products with an average content ranging from 0.17% to 6.16 %.[26] SS are amphiphilic oleanane triterpenoid glycosides with polar sugar chains conjugated to a nonpolar pentacyclic ring. SS have been classified into group A, B, E, and DDMP (2, 3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) based on different soyasapogenols and aganlycone structure.[27] Group A and B SS are relatively richer in soybean and its related products.[28] The group A SS has soyasapogenol A as its core structural aglycone and includes members of the monomers (A_1, A_2, A_3, A_4, A_5, A_6, A_a or acetyl A_4, A_b or acetyl A_1, A_c, A_d, A_e or acetyl A_5, A_f or acetyl A_2, A_g or acetyl A_6, A_h or acetyl A_3 and A_X). The group B SS uses soyasapogenol B as its core structural aglycone and contains monomer members of B_a (V), B_b (I), B_c (II), $B_{b'}$(III), $B_{c'}$(B_X) and IV.

A number of studies have shown that SS have hepatoprotective bioactivities. Kuzuhara et al. reported that soyasapogenol A reduced the number of infiltrating inflammatory cells in the liver and significantly lowered the elevated plasma level of tumor necrosis factor (TNF-α) and alanine transaminase (ALT) in the concanavalin A (Con A)-induced hepatitis model in mice, suggesting its preventive bioactivity of liver damage.[29] Similarly, soyasapogenol B also lowered the elevated plasma ALT level in the Con A-induced hepatitis model in mice, with two hydroxyl groups on the A ring of soyasapogenol B required for the amelioration.[30] Yang et al. found that supplement of soyasaponins-rich extract (SRE) prevented alcohol-induced hepatic steatosis necrosis, inflammation, and swelling by decreasing the levels of aspartate transaminase (AST), ALT, alkaline phosphatase, lactate dehydrogenase as well as hepatic triglyceride, total cholesterol, and malondialdehyde (MDA) levels in the Institute of Cancer Research (ICR) mice.[31] Both soyasapogenol B and soyasaponin III had weak protective effects against the cytotoxicity of tert-butyl hydroperoxide to human liver-derived cells.[32] Moreover, soyasapogenol A and soyasapogenol B could protect against aflatoxin B_1-induced cytotoxicity to liver cells.[33] Hong et al. found that soybean embryo powder containing bioactive substances such as isoflavones and soyasaponins exhibited hepatoprotective effects by enhancing adiponectin-mediated AMP-activated protein kinase (AMPK) α pathway in high-fat and high-cholesterol diet-induced NAFLD mice and suggested that the regular supplementation of this powder might be a useful treatment for preventing NAFLD and associated complications.[34] Our recent study found that SS (A_1, A_2, and I) alleviated the hepatic steatosis and inflammation in high-fat diet (HFD)-induced C57BL/6J obese mice.[35] In addition to the hepatoprotective effects, SS also have antioxidant, anti-inflammatory, hypoglycemic, and hypocholesterolemic activities.[26–28,36–38] Results from these studies together suggest that SS may have the potentials to prevent NAFLD/NASH since hepatic injury, oxidation, inflammation hyperglycemia, hypercholesterolemia all are risk factors linked to the occurrence and progression of NAFLD/NASH. However, the preventive effects of SS (especially purified monomers) against NAFLD/NASH are still unclear. Therefore, the first objective of this study is to investigate the preventive effect of soyasaponin A_2 (SS-A_2) against NASH by using a methionine and choline deficient (MCD) diet-induced NASH mice model. We used SS-A_2 as study object because we have previously found that it exhibited relatively stronger alleviative activities against hepatic steatosis and inflammation in HFD-fed obese mice.[35]

As described above, the crosstalk between BAs and gut microbiota play an important role in the development and progression of NASH. Several lines of existing evidence indicate that SS may

possibly be involved in the regulation of BAs and gut microbiota. It has been shown that SS can combine with cholesterol to form insoluble complexes and inhibit the absorption of endogenous and exogenous cholesterol in the intestine.[38] Thus, the inhibition of cholesterol absorption by SS affect the process of BAs synthesis by cholesterol as a precursor in the liver. In addition, SS can also affect the hepatoenteral circulation of BAs through forming a mixed micelle with BAs and effectively blocking the reabsorption of BAs at the end of the ileum.[27,39] Furthermore, SS are found to be degraded into soyasapogenol by gut microbiota in chick, rat and mice.[27] Hu et al. found that the gut microbiota also metabolized SS into soyasapogenol in healthy women.[40] We previously found that SS-A_2 alleviate hepatic steatosis and improved the serum lipid profiles in HFD-fed obese mice at least partly by enhancing fecal excretion of cholesterol, BAs and triglyceride (TG).[35] This suggested that SS-A_2 might regulate BAs in HFD-fed obese mice. Therefore, the second objective of the present study is to test the hypothesis that SS-A_2 might prevent NASH through regulating the BAs and gut microbiota.

2. Results

2.1. Animal Growth and Feed Consumption

As shown in Figure S1, mice in the MCD group, obeticholic acid (OCA) group and all three SS-A_2 intervention groups (LSS, MSS, and HSS) had significantly lower ($p < 0.05$) body weight (BW) (Figure S1A), body length (Figure S1B), feed consumption (Figure S1C), and Lee's index (Figure S1D) as compared to those in the methionine and choline supplemented (MCS) group. Mice in the OCA group had decreased ($p < 0.05$) Lee's index (Figure S1D) but had no change ($p > 0.05$) in the BW (Figure S1A) and food consumption (Figure S1C) when compared to those in the MCD group. The BW (Figure S1A), body length (Figure S1B), food consumption (Figure S1C), and Lee index (Figure S1D) of mice in the SS-A_2 intervention groups (LSS, MSS, and HSS) were not significantly different ($p > 0.05$) from that of mice in the MCD group. No significant changes of the live index (Figure S1E) was found among all groups ($p > 0.05$). These results showed that MCD diet caused growth retardation and feed consumption reduction in mice. However, SS-A_2 intervention cannot improve the MCD diet-caused growth retardation and feed consumption reduction. Interestingly, OCA intervention resulted in a decrease of body length in MCD diet-fed mice.

2.2. SS-A_2 Alleviates Steatohepatitis in the MCD Diet-induced NASH Mice

The MCD diet-induced steatohepatitis mice model is a well-characterized model for studying NASH.[41] In this study, the male C57BL/6J mice were fed with MCD diet for 16 weeks to establish the NASH model and then were given preventive intervention with OCA or graded doses of SS-A_2 (1, 50, and 100 mg kg^{-1} BW). As evidenced in **Figure 1**, the MCD diet, as compared to the MCS diet, induced significant hepatic steatosis, lobular inflammation, and ballooning inflammation as determined by HE staining (Figure 1A) as well as obvious fibrosis as assessed by Masson's trichrome staining (Figure 1B). The results from the NAS semi-quantitatively scoring system (Figure 1C) showed that mice in the MCD group had significantly higher ($p < 0.05$) scores for all categories (hepatic steatosis, lobular inflammation, ballooning, NAS, and fibrosis) than those in the MCS group. Mice in the OCA and SS-A_2 intervention groups (LSS, MSS, or HSS) had markedly decreased ($p < 0.05$) scores for hepatic steatosis, lobular inflammation, ballooning and NAS as compared to those in the MCD group. Similarly, mice in the OCA and SS-A_2 intervention groups (MSS or HSS) had significantly reduced ($p < 0.05$) scores for fibrosis when compared to those in the MCD group.

Furthermore, MCD diet-fed mice had significantly elevated ($p < 0.05$) levels of inflammatory cytokines (tumor necrosis α, TNF-α and interleukin 6, IL-6) in serum (**Figure 2**A) and liver tissues (Figure 2S), and liver function index (alanine transaminase, ALT and aspartate aminotransferase, AST) (Figure 2B) in serum than the MCS diet-fed mice. Mice fed with all three dosages of SS-A_2 (LSS, MSS, or HSS) or OCA had significantly decreased ($p < 0.05$) levels of ALT in serum and mRNA expression of cytokines (TNF-α, IL-6, and interleukin β, IL-β) in liver tissues (Figure 2S) as compared to the MCD diet-fed mice (Figure 2B). Meanwhile, mice fed with medium (MSS) and high dosage (HSS) of SS-A_2 had also reduced ($p < 0.05$) level of TNF-α in serum when compared to those fed with the MCD diet (Figure 2A).

Together, these results indicated that male C57BL/6J mice fed with the MCD diet for 16 weeks developed typical characteristics of NASH. Both SS-A_2 and OCA exhibited preventive bioactivities against steatohepatitis in MCD diet-induced NASH mice.

2.3. Effects of SS-A_2 on the Levels of Lipids in Serum, Liver, and Feces in the MCD Diet-induced NASH Mice

As shown in Figure 2C, mice in the MCD group had significantly lower ($p < 0.05$) levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) in serum than those in MCS group. Mice in the OCA, LSS, and MSS groups had no significant change ($p > 0.05$) on the levels of TG, TC, LDL-C, and HDL-C in serum as compared to those in the MCD group. However, mice in the HSS group had increased ($p < 0.05$) levels of TG, TC, and HDL-C in the serum than those in the MCD group.

As seen in Figure 2D, mice in the MCD group had significantly elevated ($p < 0.05$) levels of cholesterol and TG in the liver as compared to those in the MCS group. Mice in the OCA, LSS, MSS, and HSS groups had reduced ($p < 0.05$) levels of TG in the liver than those in the MCD group.

As indicated in Figure 2E, mice in the MCD, OCA, LSS, MSS, and HSS groups had significantly lower ($p < 0.05$) levels of cholesterol and TG in the feces when compared to those in the MCS group. However, mice in the OCA, LSS, MSS, and HSS groups had no statistically different ($p > 0.05$) levels of cholesterol and TG in the feces as compared to those in the MCD group.

Together, these results showed that feeding mice with MCD diet resulted in elevated levels of cholesterol and TG in the liver, and decreased levels of TG, TC, LDL-C, and HDL-C in the serum,

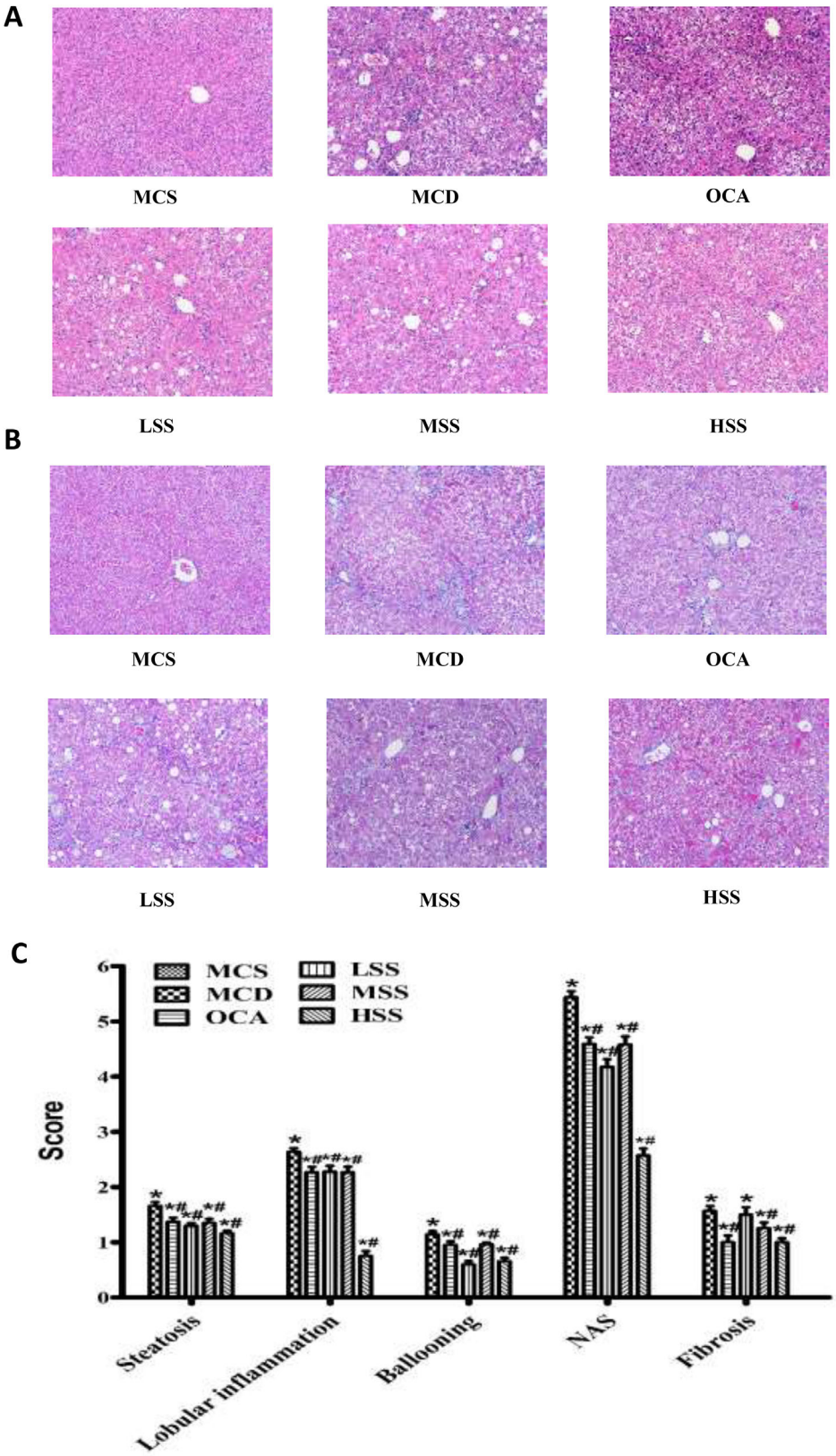

Figure 1. Effects of soyasaponin A_2 on hepatic steatosis, lobular inflammation, ballooning, and fibrosis in the methionine and choline deficient (MCD) diet-induced nonalcoholic steatohepatitis (NASH) Mice. The hepatic tissues were stained by (A) hematoxylin-eosin (HE) staining and (B) Masson's trichrome staining. The representative pictures (200× magnification) were presented. (C) The scores of hepatic steatosis, lobular inflammation, balloon-

and reduced levels of cholesterol and TG in the feces. OCA and SS-A_2 (all three dosages) can reduce the TG level in the liver in MCD diet-induced NASH mice. Meanwhile, high dosage of SS-A_2 (HSS) can increase the levels of TG, TC, and HDL-C in the serum in MCD diet-induced NASH mice.

2.4. SS-A_2 Alters the Composition of Gut Microbiota in the MCD Diet-induced NASH Mice

To obtain further insights into the alleviative effect of SS-A_2 on NASH, we investigated the composition of gut microbiota by using 16S rDNA amplicon sequencing. A total of 2 457 612 sequencing clean reads were obtained from a total of 36 cecal contents DNA samples ($n = 6$ in each group).

The indexes of Chao1, ACE, Shannon, and Simpson are usually used to predict the α-diversity value of gut microbiota. As shown in Table S1, mice in the MCD group had significantly lower ($p < 0.05$) indexes of Chao1, ACE, Shannon and Simpson than those in the MCS group, suggesting MCD diet could cause the decrease in α-diversity of gut microbiota. Mice in the LSS and HSS groups had higher ($p < 0.05$) Chao1 index than those in the MCD group. Meanwhile, mice in the LSS and MSS groups had higher ($p < 0.05$) ACE index than those in the MCD group. These results showed that SS-A_2 might increase the α-diversity of gut microbiota in MCD diet-induced NASH mice.

The unweighted UniFrac principal coordinate analysis (PCoA), which evaluates phylogenetic similarities between microbial communities, is often used to calculate the β-diversity values. As indicated in **Figure 3**A, all groups (MCS, MCD, OCA, LSS, MSS, and HSS) were clearly separated into different clusters (Anosim, $p < 0.05$, R>0) following PCoA analysis. The top 10 most abundant taxa at the phylum (Figure 3B), family (Figure 3C), and genus (Figure 3D) levels were further presented.

The LEfSe analysis at different phylogenetic levels (from phylum to genus level) was performed to further identify the distinguishing key phylotypes in the gut microbiota of the different groups (Figure 3E). The key gut microbiota with significant change as identified by LEfSe analysis (LDA score (log10) > 4.0) were selected for further analysis on the following levels.

At the phylum level (Figure 3F), the relative abundance of OTUs (%) of *Firmicutes* in the MCD group was significantly increased ($p < 0.05$) as compared to that in the MCS group. The relative abundance of OTUs (%) of *Bacteroides* and *Protebacteria* in the MCD group was significantly decreased ($p < 0.05$) as compared to that in the MCS group. The relative abundance of OTUs (%) of *Actinobacteria* in the MCD group was not statistically different ($p>0.05$) from that in the MCS group. The relative abundance of OTUs (%) of *Firmicutes* in the LSS group was significantly decreased ($p < 0.05$) as compared to that in the MCD group. However, the relative abundance of OTUs (%) of *Bacteroides* in the LSS group was significantly increased ($p < 0.05$) as compared to that in the MCD group. The relative abundance of OTUs (%) of *Actinobacteria* in the OCA, MSS and HSS groups was significantly reduced ($p<0.05$) as compared to that in the MCD group. These results suggested that SS-A_2 could reduce the relative abundance of *Firmicutes* and *Actinobacteria* but increase that of *Bacteroides* in the MCD diet-induced NASH mice. Interestingly, OCA can increase the relative abundance of *Proteobacteria* but reduce that of *Actinobacteria* in the MCD diet-induced NASH mice.

At the family (Figure 3G) and genus (Figure 3H) levels, mice in the MCD group had significantly increased ($p<0.05$) relative abundance of *Lactobacillaceae* (and *Lactobacillus)* and *Erysipelotrichaceae* (and *Faecalibaculum*), but decreased ($p<0.05$) relative abundance of *Desulfovibrionaceae* (and *Desulfovibrio*) as compared to those in the MCS group. The relative abundance of *Lactobacillaceae* (and *Lactobacillus*) of mice in the LSS group was significantly reduced ($p<0.05$) as compared to that of mice in the MCD group. The relative abundance of *Unidentified_Clostridiales* (both at family and genus levels) of mice in the OCA and HSS groups was significantly elevated ($p<0.05$) as compared to that of mice in the MCD group. The relative abundance of *Erysipelotrichaceae* (and *Faecalibaculum*) of mice in the OCA, LSS, MSS and HSS groups was significantly decreased ($p<0.05$) as compared to that of mice in the MCD group. The relative abundance of *Desulfovibrionaceae* (and *Desulfovibrio*) in the OCA and MSS groups was significantly increased ($p<0.05$) as compared to that of mice in the MCD group. These results indicated that SS-A_2 might decrease the relative abundance of *Lactobacillaceae* (and *Lactobacillus*) and *Erysipelotrichaceae* (and *Faecalibaculum*) and increase that of *Desulfovibrionaceae* (and *Desulfovibrio*) and *Unidentified_Clostridiales* in the MCD diet-induced NASH mice.

2.5. SS-A_2 Promotes the Colonic Excretion of BAs and Reduces the Serum Accumulation of BAs in the MCD Diet-induced NASH Mice

The homeostatic disorder of BAs is involved in the pathogenesis of NAFLD.[42] Thus, we further measured the contents of BAs in liver tissues, serum and colonic contents. The content of BAs in liver tissues was shown in Table S2. Mice in the MCD group had significantly elevated ($p < 0.05$) levels of TβMCA and THCA, and reduced ($p < 0.05$) levels of THDCA, 6_ketoLCA, muroCA, HDCA, and TDCA in liver tissues as compared to those in the MCS group. Mice in OCA group had increased ($p < 0.05$) hepatic levels of THDCA and decreased ($p < 0.05$) levels of UDCA, CA, and THCA as compared to those in the MCD group. When compared to those in the MCD group, mice in the LSS group had decreased ($p < 0.05$) levels of TβMCA and THCA, and mice in the HSS group had elevated ($p < 0.05$) levels of THDCA, TLCA, and TDCA, and reduced ($p < 0.05$) level of THCA.

The content of BAs in serum was shown in Table S3. Mice in the MCD group had significantly higher ($p < 0.05$) levels of TUDCA, TαMCA, TβMCA, TCA, NorDCA, 12_ketoLCA, UDCA, 7_DHCA, ωMCA, αMCA, βMCA, ACA, CA, and TDCA, and lower ($p < 0.05$) level of THDCA in serum as compared to

ing, and fibrosis were quantified by using the nonalcoholic fatty disease activity score (NAS) semi-quantitatively scoring system independently by two technicians. Data were statistically analyzed by using independent-samples *t* test of SPSS 20.0 software. Results presented are means ± SEM of samples from six to seven ($n = 6$~7) mice in each group. *: $p < 0.05$ versus methionine and choline supplemented (MCS) group. #: $p < 0.05$ versus MCD group.

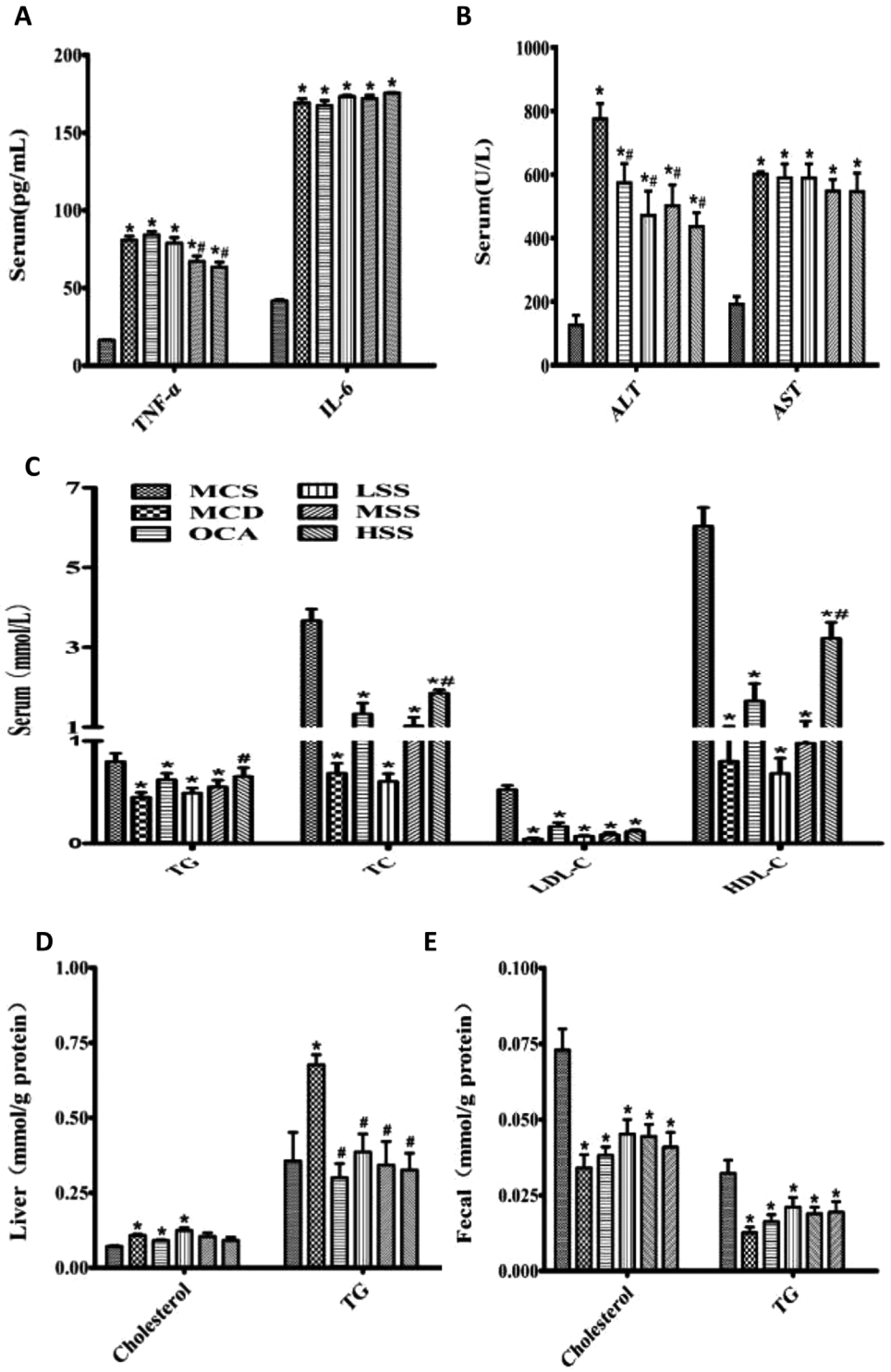

Figure 2. Effects of soyasaponin A_2 on the inflammatory cytokines and liver function index in serum and the lipids in serum, liver, and feces in the MCD diet-induced NASH mice. The levels of inflammatory cytokines (TNF-α and IL-6) (A) and the liver function index (ALT and AST) in serum (B). The lipid profile (TG, TC, LDL-C, and HDL-C) in serum (C), and the levels of cholesterol and TG in liver (D) and feces (E). Data were statistically analyzed by using one-way ANOVA of SPSS 20.0 software. Results presented are means ± SEM of samples from six to seven (n = 6~7) mice in each group. *: $p < 0.05$ versus MCS group. #: $p < 0.05$ versus MCD group.

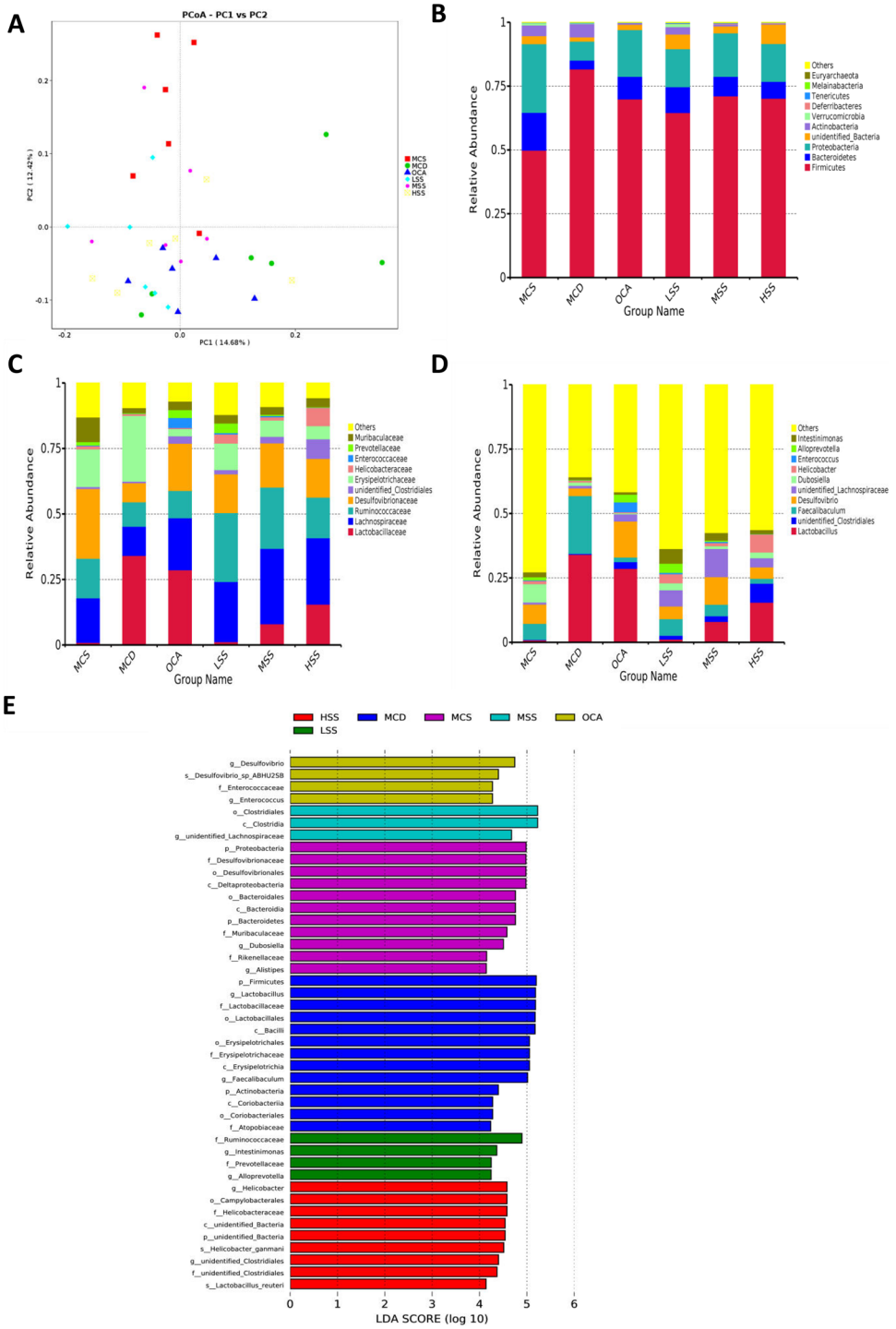

Figure 3. Effects of soyasaponin A_2 on the composition of gut microbiota in the MCD diet-induced NASH mice. The gut microbiota was determined by using 16S rDNA amplicon sequencing. The composition of gut microbiota in mice from different experimental groups was analyzed by different species classification level of gut microbiota. (A) Principal coordinate analysis (PCoA) of the gut microbiota was made based on unweighted UniFrac metric. Top 10 most abundant taxa were presented at the level of phylum (B), family (C), and genus (D). LDA effect size (LEfSe) analysis was applied to find the biomarkers with statistical differences between groups. LEfSe bar plot (E) listed the significantly differential taxa based on effect size (LDA score (log10) > 4) and represented taxonomic levels from phylum to genus levels. The biomarkers of gut microbiota presented relative abundance of OTUs (%) in the cecal contents at the phylum (F), family (G), and genus (H) level. Data were statistically analyzed by using the LDA Effect Size (LEfSe) analysis. Results presented are means ± SEM of samples from six ($n = 6$) mice in each group. *: $p < 0.05$ versus MCS group. #: $p < 0.05$ versus MCD group.

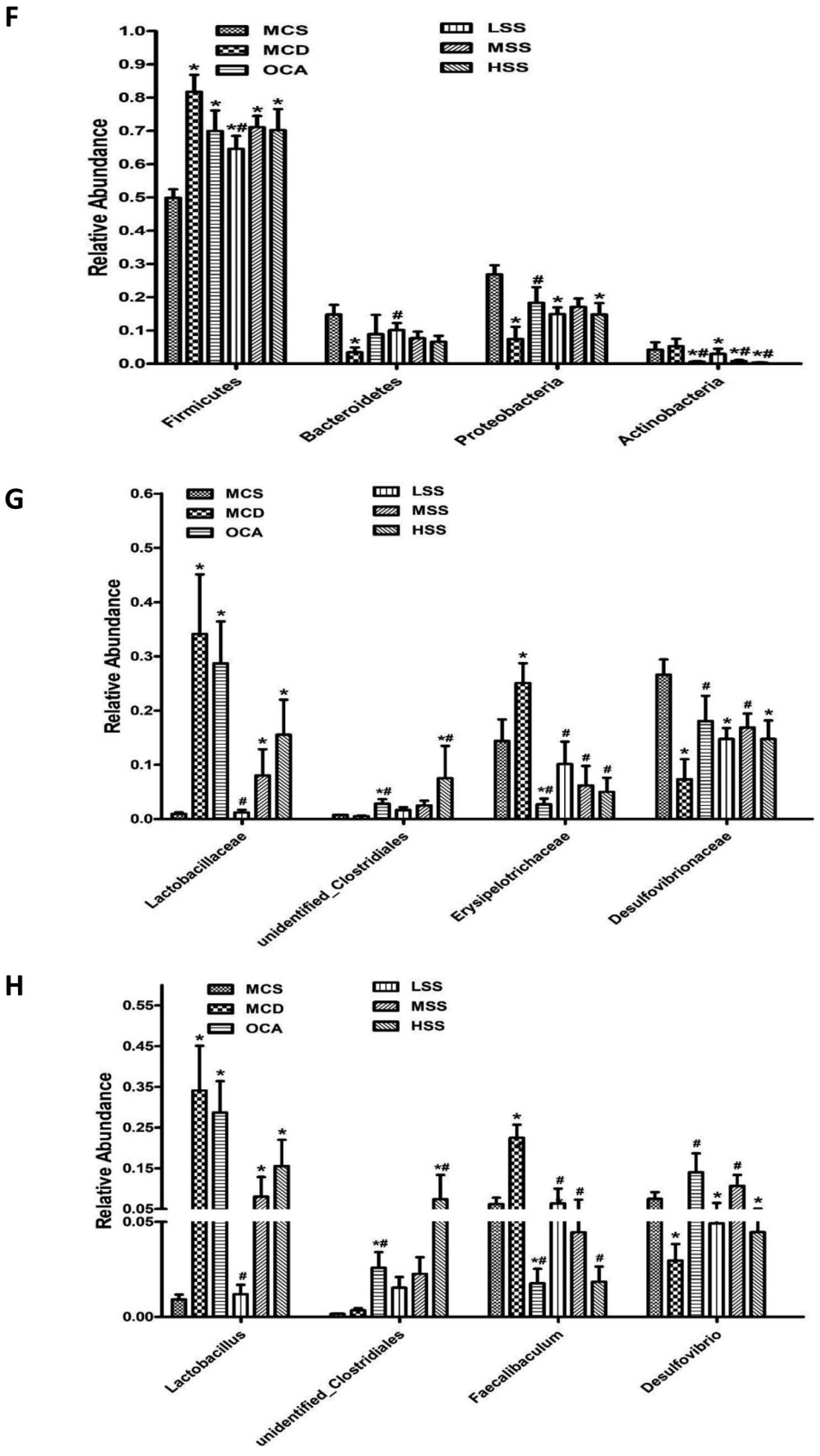

Figure 3. *Continued*

those in the MCS group. Mice in the OCA group had increased ($p < 0.05$) levels of THDCA and decreased ($p < 0.05$) levels of NorDCA, UDCA, 7_DHCA, ωMCA, αMCA, βMCA, ACA, and CA when compared to those in the MCD group. When compared to those in the MCD group, mice in the LSS group had decreased ($p < 0.05$) serum levels of 7_DHCA, βMCA, and CA, and mice in the MSS group had decreased ($p < 0.05$) serum level of TβMCA, NorDCA, 7_DHCA, and CA, and mice in the HSS groups had decreased ($p < 0.05$) serum levels of NorDCA, 7_DHCA, βMCA, and CA, and increased ($p < 0.05$) serum levels of THDCA and TDCA. Furthermore, mice in the MCD group had higher ($p < 0.05$) levels of total BAs, primary BAs, secondary BAs, conjugated BAs and unconjugated BAs in serum than those in the MCS group. Mice in OCA, LSS, and HSS groups had decreased ($p < 0.05$) serum levels of total BAs, primary BAs, secondary BAs, and unconjugated BAs as compared to those in the MCD group. When compared to those in the MCD group, mice in the MSS group had reduced ($p < 0.05$) serum levels of total BAs, primary BAs, conjugated BAs, and unconjugated BAs.

The content of BAs in colonic contents was shown in Table S4. Mice in the MCD group had reduced ($p < 0.05$) levels of THDCA, TωMCA, TCA, CDCA, DCA, TLCA, 6_ketoLCA, muroCA, UDCA, HDCA, ωMCA, αMCA, and TDCA as compared to those in the MCS group. Mice in the OCA group had increased ($p < 0.05$) levels of THDCA, TCA, CDCA, muroCA, UDCA, HDCA, ωMCA, αMCA, βMCA, and ACA as compared to those in the MCD group. When compared to those in the MCD group, mice in the MSS group had increased ($p < 0.05$) levels of CDCA and UDCA, and mice in the HSS group had increased ($p < 0.05$) levels of THDCA, TCA, CDCA, muroCA, UDCA, HDCA, αMCA, βMCA, ACA, TDCA, and GCA. Furthermore, mice in the MCD group had reduced ($p < 0.05$) levels of total BAs, secondary BAs, conjugated BAs, and unconjugated BAs in colonic contents than those in the MCS group. When compared to those in the MCD group, mice in the OCA and HSS groups had elevated ($p < 0.05$) levels of total BAs, primary BAs, secondary BAs, conjugated BAs, and unconjugated BAs.

Taken together, these results showed that the MCD diet-induced NASH mice had increased accumulation of BAs in serum and decreased colonic excretion of BAs. Both OCA and **SS-A$_2$** could promote the colonic excretion of BAs and reduce the serum accumulation of BAs in the MCD diet-induced NASH mice. One interesting point was that the MCD diet-induced NASH mice had decreased level of THDCA in liver, serum, and colonic contents, which could be elevated by the preventive intervention of both OCA and **SS-A$_2$**.

To explore the correlation between gut microbiota and the contents of BAs in liver, serum, and colon, the Spearman correlation analysis was further performed. As shown in **Figure 4**A, *Desulfovibrio* was negatively correlated ($p < 0.05$) with the contents of CDCA, UDCA and muroCA in liver tissues. *Lactobacillus* was positively correlated ($p < 0.05$) with the hepatic levels of TCA, THCA, TωMCA, and was negatively correlated ($p < 0.05$) with the hepatic level of UDCA. *Unidentified_Clostridiales* was positively correlated ($p < 0.05$) with the hepatic levels of TωMCA and GCA, and was negatively correlated ($p < 0.05$) with the hepatic levels of UDCA and muroCA. *Faecalibaculum* was positively correlated ($p < 0.05$) with the hepatic level of THCA.

As indicated in Figure 4B, *Faecalibaculum* was positively correlated ($p < 0.05$) with the levels of CA, ACA, 7_DHCA, CDCA, UDCA in serum and was negatively correlated ($p < 0.05$) with the serum level of THDCA. *Lactobacillus* was positively correlated ($p < 0.05$) with the level of TCA in serum.

As seen in Figure 4C, *Desulfovibrio* were positively correlated ($p < 0.05$) with the level of NorDCA in colonic contents. *Unidentified_Clostridiales* was positively correlated ($p < 0.05$) with the colonic levels of NorDCA and GCA. *Faecalibaculum* was negatively correlated ($p < 0.05$) with the colonic levels of TCA, muroCA, TβMCA, and GCA.

2.6. SS-A$_2$ Increases the Expressions of FXR and FGF-15 in Ileum of the MCD Diet-induced NASH Mice

It is known that the bile acid transport receptors are vital in maintaining the homeostasis of BAs.[7,8] Thus, we further investigated the effects of SS-A$_2$ on the bile acid transport receptors in liver (CYP7A1, FXR, NTCP, and BSEP) and ileum (FXR and FGF-15). The MCD diet-fed mice had significantly reduced ($p < 0.05$) levels of CYP7A1 (**Figure 5**A) and NTCP (Figure 5C) in liver as compared to those fed with the MCS diet. However, the expressions of FXR (Figure 5B) and BSEP (Figure 5D) in the liver of the MCD diet-fed mice were not statistically different ($p > 0.05$) from that of the MCS diet-fed mice. The expressions of CYP7A1, FXR, NTCP, and BSEP in the liver of mice in the OCA or soyasaponins (LSS, MSS, HSS) groups were not statistically different ($p > 0.05$) from that of mice in the MCD group (Figures 5A–5D).

The expressions of FXR (Figure 5E) and FGF-15 (Figure 5F) in ileum of mice in the MCD group were significantly lower ($p < 0.05$) than that of mice in the MCS group. The expressions of FXR (Figure 5E) and FGF-15 (Figure 5F) in ileum of mice in the LSS and HSS groups were significantly higher ($p < 0.05$) than that of mice in the MCD group.

Collectively, these results indicated that SS-A$_2$ could increase the expressions of FXR and FGF-15 in ileum but not affect the expressions of CYP7A1, FXR, NTCP, and BSEP in liver in the MCD diet-induced NASH mice.

3. Discussion

In this study, we aimed to first investigate the preventive bioactivities of SS-A$_2$, a monomer member from group A soyasaponin, against steatohepatitis in the MCD diet-induced NASH mice and further explore its underlying mechanisms by focusing on the BAs and gut microbiota. We made several novel findings here.

First, SS-A$_2$ alleviates steatohepatitis in the MCD diet-induced NASH mice. It has been shown that mice fed with the MCD diet develop the typical characteristics of NASH similar to NASH in human including hepatic steatosis, lobular inflammation and pericelluar fibrosis in liver.[41,43,44] In this study, the male C57BL/6J mice fed with the MCD diet for 16 weeks produced significant hepatic steatosis, lobular inflammation, ballooning, and obvious fibrosis, indicating the successful induction of typical characteristics of NASH. We administrated the preventive intervention in the MCD diet-fed mice by using OCA and graded

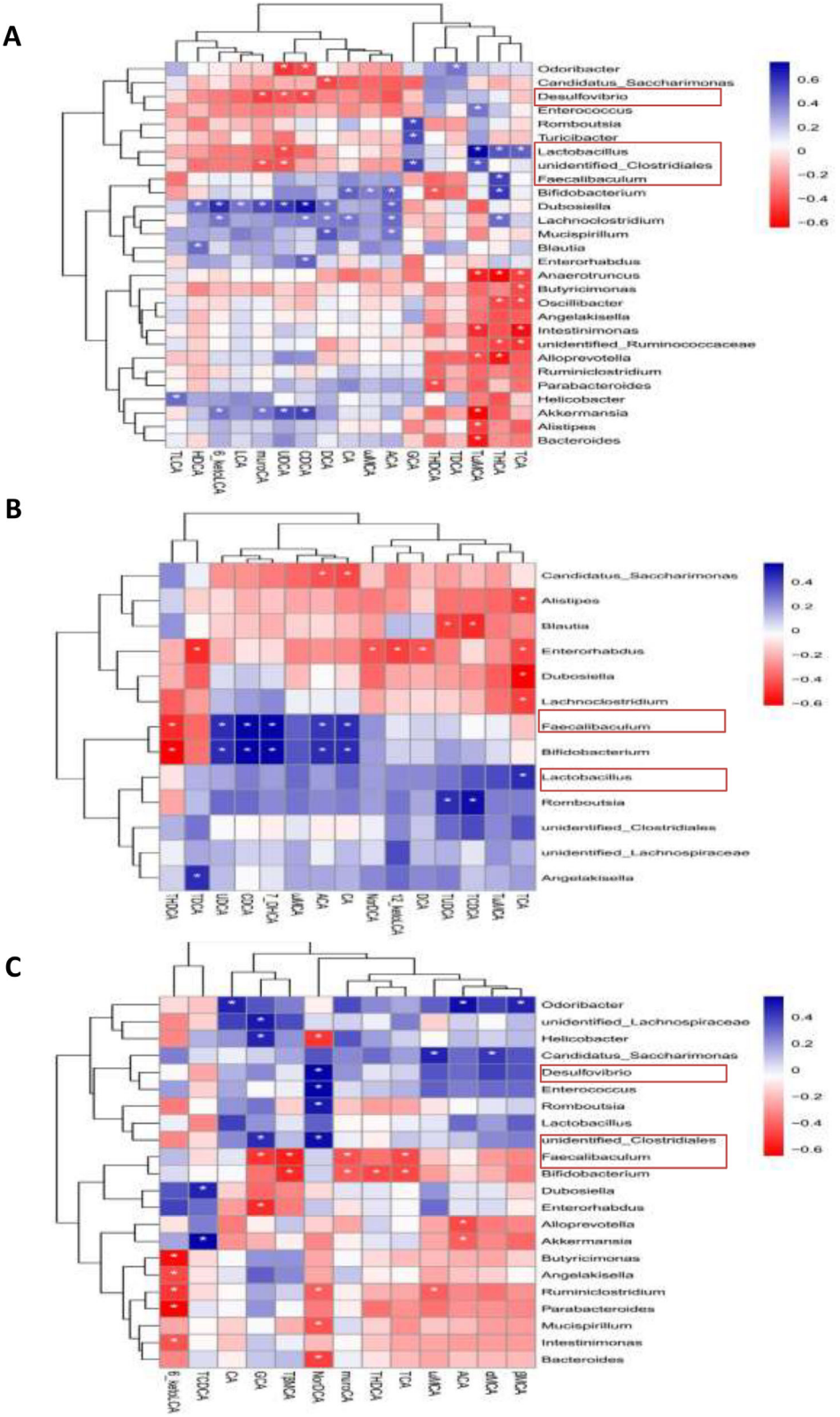

Figure 4. The correlations between gut microbiota and bile acids in the MCD diet-induced NASH mice. The correlation between the relative abundance of gut microbiota and the concentrations of bile acids in liver (A), serum (B), and colon contents (C) was analyzed by using the Spearman correlation analysis. The *r* values were represented by gradient colors, where blue and red cells indicate positive and negative correlations, respectively. *: means significant correlations ($p < 0.05$) between the gut microbiota and the bile acids.

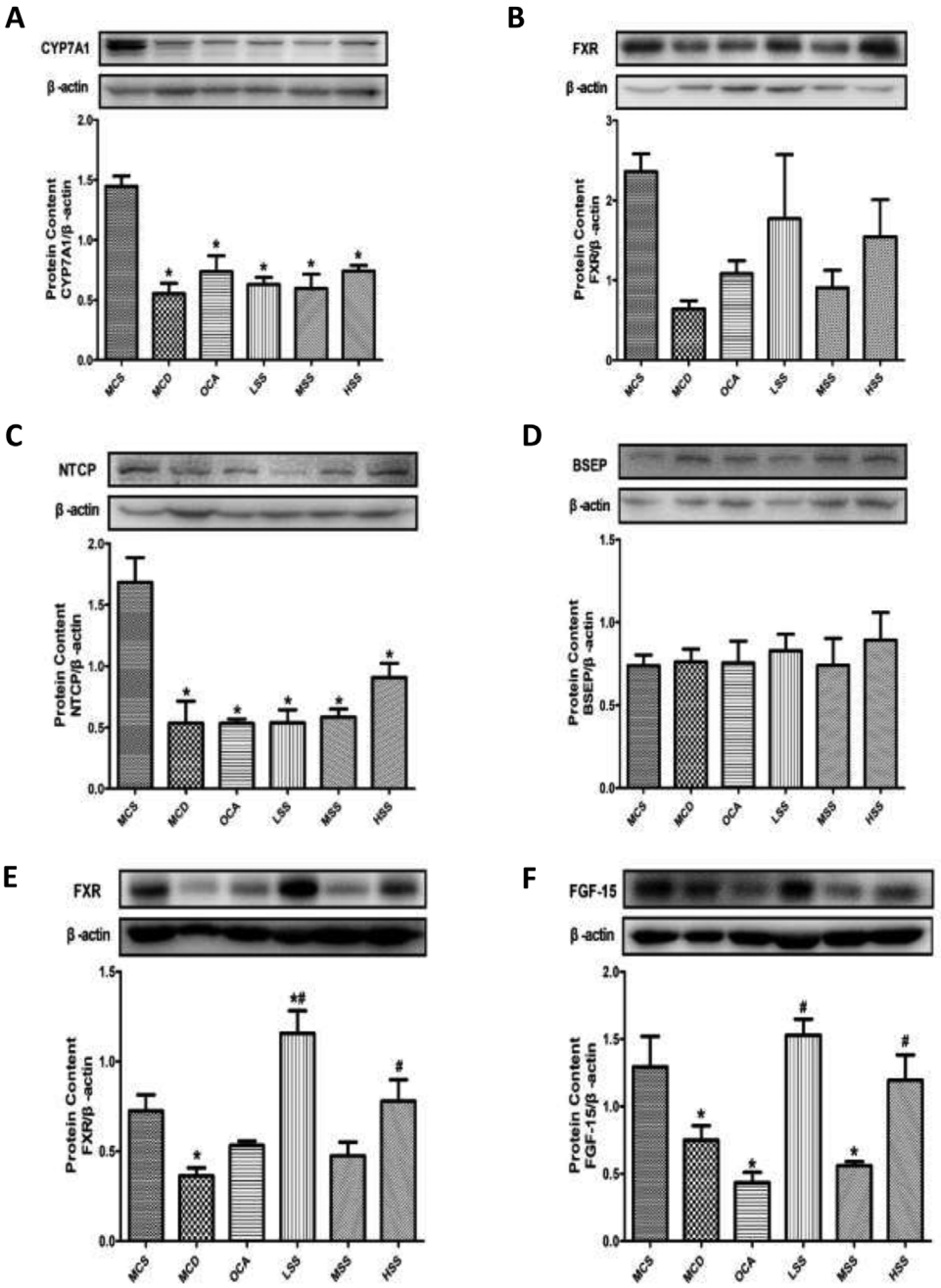

Figure 5. Effects of soyasaponin A_2 on the expression of bile acid transport receptors in liver and ileum in the MCD diet-induced NASH mice. The expression of bile acid transport receptors CYP7A1 (A), FXR (B), NTCP (C) and BSEP (D) in liver and FXR (E) and FGF-15 (F) in terminal ileum were determined by western blotting. Data were statistically analyzed by using one-way ANOVA of SPSS 20.0 software. Results presented are means ± SEM of tissue samples from six to seven ($n = 6\sim7$) mice in each group. *: $p < 0.05$ versus MCS group. #: $p < 0.05$ versus MCD group.

dosages of SS-A_2. We found that both OCA and all three dosages (1, 50, 100 mg kg^{-1}•BW) of SS-A_2 alleviated hepatic steatosis, lobular inflammation, ballooning and NAS scores as assessed by the well-defined NAS semi-quantitatively scoring system. Moreover, OCA and medium (50 mg kg^{-1}•BW) and high (100 mg kg^{-1}•BW) dosages of SS-A_2 reduced the fibrosis score. OCA is a potent FXR agonist.[19–21] Our result of OCA here is consistent with the previous reports by others. Neuschwander-Tetri et al. reported that OCA treatment improved the histological features (steatosis, lobular inflammation, ballooning, and fibrosis) of NASH in patients in a multicenter, randomized, placebo-controlled clinical trial.[20] Goto et al. found that OCA protected against hepatocyte death and liver fibrosis in a murine model of NASH.[45] Evidences from both in vitro experiments[32,33,46,47] and in vivo animal studies[29–31] supported that soyasaponins have hepatoprotective bioactivities. Furthermore, different structures

of soyasaponins vary in their hepatoprotective bioactivities.[46,47] We recently found that three types of soyasaponin monomers (A_1, A_2, and I) alleviated liver inflammation and steatosis in high-fat diet (HFD)-induced C57BL/6J obese mice.[35] However, the histological characteristics of NASH were not assessed in that study. Furthermore, the results obtained from studies using HFD-induced NAFLD/NASH model are usually inconsistent although HFD are the commonly used diet for establishing the model of NAFLD/NASH in mice or rat. For example, HFD could induce hepatic steatosis of varying degrees and patterns in some studies, while it did not lead to the development of hepatic fat accumulation in other experiments.[43] Therefore, till now no direct evidence is available regarding the preventive bioactivities of soyasaponin monomers against NASH. In the present study, we used the MCD diet-fed mice as the model for studying NASH. The MCD diet-induced NASH model is one of the best defined mice model of NASH and is widely used to study NASH although it has drawbacks like loss of body weight and lack of insulin resistance. The MCD diet induces reproducible histological changes of hepatic steatosis, steatohepatitis or fibrosing steatohepatitis that simulates the histological picture of NASH.[43] Results of SS-A_2 here thus, for the first time, provided direct evidence that SS-A_2 can alleviate steatohepatitis in the MCD diet-induced NASH mice.

Feeding mice with the MCD diet results in rapid accumulation of TG in the liver with the subsequent development of steatohepatitis and fibrosis. This is because that methionine and choline are essential for hepatic β-oxidation and production of very low-density lipoprotein (VLDL) and the absence of both methionine and choline substantially hampers the export of TG via VLDL packaging from hepatocytes.[41,43] It is reported that the MCD diet-induced NASH mice had increased levels of TG and TC in liver, and also decreased levels of TG, TC, LDL-C, and HDL-C in serum.[43,44] In this study, mice fed with the MCD diet had significantly elevated levels of cholesterol and TG in the liver and decreased levels of TG, TC, LDL-C, and HDL-C in serum than those fed with the MCS diet. Our results are thus consistent with the reports from others. This further supported that the MCD diet-induced NASH mice model has good repeatability. Our present results showed that 100 mg kg^{-1}•BW of SS-A_2 significantly increased the levels of TG, TC, and HDL-C in the serum of the MCD diet-induced NASH mice. All three dosages (1, 50, and 100 mg kg^{-1}•BW) of SS-A_2 reduced the TG level in the liver but had no effects on the fecal levels of cholesterol and TG in the MCD diet-induced NASH mice. In HFD-fed mice, total soyasaponins decreased the levels of TC, TG and LDL-C in serum, increased the HDL-C level in serum, and reduced the levels of TC and TG in liver tissues.[48] In HFD-fed hamsters, group B soyasaponins significantly lowered plasma TC, non-HDL-C, and TG and increased the excretion of fecal BAs and neutral sterols.[49] In HFD-fed obese mice, soyasaponins A_1 and A_2 decreased TC, TG, and LDL-C in serum, and soyasaponin I reduced TG and LDL-C in serum, and all three soyasaponins (A_1, A_2, and I) increased HDL-C in serum.[35] Meanwhile, soyasaponins (A_1, A_2, and I) lowered the levels of cholesterol and TG in liver and increased the fecal excretion of cholesterol and BAs.[35] In HFD-fed ApoE$^{-/-}$ mice, soyasaponins A_1 reduced TC, TG, and LDL-C and increased HDL-C in serum. **SS-A_2** decreased TC, TG, and LDL-C but did not affect HDL-C in serum. Meanwhile, soyasaponins A_1 increased TG, **SS-A_2** increased TC, and both of them increased BAs in the feces.[50] It is obvious to find that the effects of soyasaponin on the serum lipid profiles in the MCD diet-induced NASH mice is different from that observed in the HFD-fed mice. It is traditionally considered that soyasaponins improve the serum lipid profile through promoting the fecal excretion of cholesterol in HFD-fed animals. However, in the MCD diet-fed mice, high dosage (100 mg kg^{-1}•BW) of SS-A_2 increased the levels of TG, TC, and HDL-C in the serum. Meanwhile, SS-A_2 did not affect the levels of lipids in feces. This may be because of the metabolic difference of lipids in HFD and MCD diet-fed mice. Anyhow, SS-A_2 decreased the accumulation of TG in liver in both HFD and MCD diet-fed mice suggesting the preventive effect of SS-A_2 on steatosis.

Moreover, the MCD diet-induced NAFLD/NASH model reduce weight and liver/body ratio, and does not develop insulin resistance.[41] Weight loss associated with the MCD diet is thought to be due to hypermetabolism[51] caused by increased sympathetic nervous system outflow to adipose tissue, leading to increased mitochondrial uncoupling and reduced efficiency in extracting energy from nutrients.[52] In this study, the MCD diet-fed NASH mice had decreased body weight, Lee's index, and feed intake, which were not reversed by preventive intervention of OCA and SS-A_2. Furthermore, the MCD diet-fed mice did not develop insulin resistance which was either not affected by the preventive intervention of OCA and SS-A_2 (data not shown).

It is known that the liver injury induced by the MCD diet is accompanied by elevated serum levels of ALT, AST, and pro-inflammatory cytokines (such as IL-1β, IL-6, and TNF-α).[41,43] In this study, the MCD diet-fed mice had significantly elevated levels of inflammatory cytokines (TNF-α and IL-6) and liver function index (ALT and AST) in serum than the MCS diet-fed mice, suggesting the induction of liver injury and inflammation in the model. Both SS-A_2 and OCA significantly decreased the serum ALT level and liver cytokines expression, and medium and high dosage of SS-A_2 also reduced the serum TNF-α level in the MCD diet-fed mice. These results are consistent with our previous studies supporting that SS-A_2 had hepatoprotective[35] and anti-inflammatory[35,37,53,50,54] bioactivities. Other types of soyasaponins were also shown to have hepatoprotective and anti-inflammatory bioactivities. Soyasaponins-rich crude extracts exhibited protection against the acute alcohol-induced liver damage by decreasing serum levels of AST and ALT, and inflammation.[31] Soyasapogenol A was also reported to reduce the liver macrophage infiltration, plasma TNF-α, and ALT levels in hepatocytes in a concanavalin A-induced hepatitis model.[29]

Second, SS-A_2 can modulate the composition of gut microbiota in the MCD diet-induced NASH mice. Diet or bioactive food component can modulate gut microbiota, which has been shown to contribute to the host health.[55] It is known that soyasaponins can be metabolized by the intestinal microbiota into soyasapogenol and thus may take part in the regulation of the intestinal microbiota.[27,40] However, no study is available regarding the effects of soyasaponins on the composition of gut microbiota. In this study, we used the 16S rDNA amplicon sequencing high-throughput technique to detect the changes of gut microbiota caused by the SS-A_2 intervention in the MCD diet-induced NASH mice. *Lactobacillaceae* and *Lactobacillus* were significantly

increased in the caecum of the MCD diet-fed mice as compared to the MCS diet-fed mice. SS-A_2 (1 mg kg^{-1}•BW) significantly decreased *Lactobacillaceae* and *Lactobacillus* in the MCD diet-fed NASH mice. *Lactobacillus* can carry out bile acid deconjugation, esterify bile acids and convert the unconjugated primary bile acids (CDCA and CA) into the secondary bile acids (LCA and DCA) through 7α-dehydroxylation by CYP7A1.[7] *Lactobacillus* has been shown to exert beneficial effects on the host in human and animal studies.[56] However, the higher abundance of *Lactobacillales, Clostrodium*, and *Erysipelotrichaceae* rich with high BSH activity in intestine together with significantly increased 7-keto DCA levels in the liver of the male mice was closely associated with higher incidence of HCC in STZ-HFD induced NASH-HCC murine model.[57] Thus, SS-A_2 may exhibit beneficial effects by reducing *Lactobacillales* in the MCD diet-induced NASH mice.

Interestingly, the MCD diet did not result in change of *Unidentified_Clostridiales* in the caecum of mice as compared to the MCS diet. However, both OCA and SS-A_2 (100 mg kg^{-1}•BW) significantly increased the *Unidentified_Clostridiales* at the family and genus levels in the MCD diet-fed mice. The reason awaits further investigation.

The MCD diet caused significant increase of *Erysipelotrichaceae* and *Faecalibaculum* in the caecum of mice as compared to the MCS diet. Both OCA and SS-A_2 (1, 50, and 100 mg kg^{-1}•BW) markedly decreased *Erysipelotrichaceae* and *Faecalibaculum* in the MCD diet-fed NASH mice. The *Erysipelotrichia* class belongs to the *Firmicutes* phylum. The level of *Erysipelotrichia* is suggested to be important bacterial marker of susceptibility to choline deficiency-induced fatty liver disease.[58] High levels of *Erysipelotrichia* in human fecal microbiota are associated with hepatic steatosis and correlated to higher risk of developing fatty liver on low-choline diet.[59] It is reported that the attenuation of the MCD diet-induced NAFLD by the treatment of 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside (TSG) was seemingly associated with the reduced abundance of *Erysipelotrichaceae*.[60] *Faecalibacterium* genus belongs to the *Erysipelotrichaceae* family and is associated with liver health.[61,62] Therefore, the reduction in the abundance of *Erysipelotrichaceae* and *Faecalibaculum* in the MCD diet-induced NASH mice by SS-A_2 may partly contribute to its alleviative effects on steatohepatitis.

The MCD diet caused significant decrease in *Desulfovibrionaceae* and *Desulfovibrio* in the caecum of mice as compared to the MCS diet. This is in accordance with previous report by Ye et al. that the MCD diet-induced NASH mice had significantly reduced abundance of *Desulfovibrio*.[63] OCA and SS-A_2 (50 mg kg^{-1}•BW) significantly increased *Desulfovibrionaceae* and *Desulfovibrio* in the MCD diet-fed mice. *Desulfovibrionaceae* is known as the anti-inflammatory bacteria and sulfate-reducing bacteria that produce hydrogen sulfide (H_2S).[64] It has been shown that H_2S plays an important role in the gastrointestinal mucosal defense and repair and in reducing systemic inflammation.[65]

Third, SS-A_2 alters the profiles of BAs in liver, serum, and colon in the MCD diet-induced NASH mice. Although previous studies indicated that soyasaponins could promote the fecal excretion of BAs and interfere with the enterohepatic circulation of BAs by forming mixed micelles and thus effectively block the reabsorption of BAs from the terminal ileum,[27,28,38] no study is available regarding the effects of soyasaponins on the profiles of BAs and its interaction with the gut microbiota. In this study, we used the UPLC-TQMS high-throughput technique to detect the profiles of BAs in liver, serum, and colon and further analyze the association of BAs with the gut microbiota in order to understand the mechanisms underlying the preventive bioactivities of SS-A_2 against steatohepatitis in the MCD diet-induced NASH mice.

The BAs in liver consist of the newly synthesized BAs and the recycled BAs from the enterohepatic circulation. The accumulation of BAs in liver maybe because of the increased speed of BAs synthesis or the adaptive response of liver to TG accumulation therein. Health liver has high efficiency in clearing the BAs in the enterohepatic circulation. However, liver with impaired function cannot clear excessive BAs resulting in the existence of more BAs in the circulation.[66] Increased levels of total BAs and secondary BAs in the liver tissues have been observed in NASH patients.[67] In this study, the levels of total BAs, primary BAs, secondary BAs, conjugated BAs, and unconjugated BAs in liver tissues of the MCD diet-fed mice were not statistically different from that of the MCS diet-fed mice. However, the MCD diet-fed mice had reduced levels of THDCA, 6_ketoLCA, muroCA, HDCA, and TDCA, and increased levels of TβMCA and THCA in liver tissues as compared to the MCS diet-fed mice. OCA increased the hepatic levels of THDCA and decreased the levels of UDCA, CA, and THCA in the MCD diet-fed mice. SS-A_2 (1 mg kg^{-1}•BW) decreased the hepatic levels of TβMCA and THCA in the MCD diet-fed mice. Moreover, SS-A_2 (100 mg kg^{-1}•BW) elevated the levels of THDCA, TLCA, and TDCA, and reduced the level of THCA in live tissues of the MCD diet-fed mice. These results show that the MCD diet changes the profiles of BAs in liver tissues which can be modulated by both OCA and SS-A_2.

The hydrophobicity is an important factor in determining the biological effects of a particular bile acid. Hydrophobic BAs are the main nuclear receptor signal ligands and can also be used as toxic precursors leading to the direct activation of cell apoptosis and necrosis. According to the chromatographic analysis, the hydrophobicity of major BAs is LCA > DCA > CDCA > CA > UDCA > αMCA > βMCA > ωMCA.[68] It has demonstrated that hydrophobic BAs (LCA, DCA, and CDCA) were cytotoxic while hydrophilic BA (UDCA) and its aurine-conjugated derivative TUDCA had cytoprotective effects in the studies of BA-induced hepatocyte injury.[69] The hydrophobic BAs (DCA, LCA, CDCA, and TCDCA) have independent cytotoxic and cancer-promoting properties.[70] The intrahepatic accumulation of hydrophobic BAs such as DCA and CDCA has been proposed as a mechanism of cholestatic liver injury.[71] GCA, TCA, GCDCA, TCDCA, GDCA, DCA, and TDCA contribute to the development and progression of gastrointestinal and liver tumors.[72,73] THDCA is a natural hydrophilic bile acid and has been proposed for the treatment of liver diseases.[74] THDCA can induce the gene expression of apolipoprotein, bile secretion-related protein, and cytochrome P450 family, while inhibiting the expression of inflammatory genes in steatotic liver cells. In addition, THDCA can improve the accumulation of neutral lipids and insulin sensitivity by activating FXR in hepatocytes.[74] In this study, the levels of THDCA in liver tissues were significantly decreased in the MCD diet-fed mice as compared to that in the MCS diet-fed mice. OCA and SS-A_2 (100 mg kg^{-1}•BW) significantly increased the levels of THDCA in liver tissues of the MCD diet-fed mice. The reduction of THDCA level has been observed in liver

of HFD-induced NAFLD rats and considered as one of the reasons that promoting the hepatic lipids accumulation.[74] Therefore, the ability of SS-A_2 to increase the THDCA level in the MCD diet-fed mice may contribute to its protective effect against steatohepatitis.

The BAs in serum are mainly from the reabsorbed BAs in portal veins. In this study, the MCD diet-induced NASH mice had elevated levels of total BAs, primary BAs, secondary BAs, conjugated BAs, and unconjugated BAs in serum than the MCS diet-fed mice. It has been reported that the NASH patients had increased levels of total BAs and hydrophobic secondary BAs in fasting and postprandial serum. The elevated levels of BAs in serum was probably associated with the liver impairment and pathogenesis of NAFLD.[75] Metabonomics analysis revealed that NASH patients had elevated levels of GCA, TCA, and GCDCA in serum as compared to health individuals.[66] The BAs in fasting plasma were also elevated in both NAFL and NASH patients because of the increases in primary BAs and conjugated BAs.[13,66] It has demonstrated that the histological changes of liver in NASH patients was associated with the changed profiles of BAs in serum. High levels of plasma GCA or CA was positively associated with lobular inflammation.[13] Low TUDCA level and high TLCA level was related to the inflammation of portal vein.[76] Moreover, high levels of CA, CDCA, GCA, TCA, GCDCA, DCA, and GLCA were related to the hepatocyte ballooning.[13,76] In the HFD-induced NAFLD rats, serum TCA was markedly increased and suggested as sensitive biomarker for diagnosing NAFLD.[74] In the present study, OCA and SS-A_2 (1 and 100 mg kg^{-1}•BW) decreased the serum levels of total BAs, primary BAs, secondary BAs, and unconjugated BAs in the MCD diet-induced NASH mice. SS-A_2 (50 mg kg^{-1}•BW) reduced the serum levels of total BAs, primary BAs, conjugated BAs, and unconjugated BAs in the MCD diet-induced NASH mice. These results indicate that SS-A_2 can decrease the accumulation of BAs in serum of the MCD diet-induced NASH mice, which might contribute to the alleviative effects of SS-A_2 against steatohepatitis. It is worth noting that the THDCA was the only BA of which level in serum was significantly decreased in the MCD diet-induced NASH mice and SS-A_2 intervention could reverse its level.

In the enterohepatic circulation, about 95% of BAs which reaches the terminal ileum are reabsorbed into the portal vein and then transported back to the liver. The rest of BAs enter into the colon and are deconjugated and dehydroxylated by microbiota to the secondary BAs. In this study, the levels of total BAs, secondary BAs, conjugated BAs, and unconjugated BAs in colon contents were decreased in the MCD diet-induced NASH mice. Both OCA and SS-A_2 (100 mg kg^{-1}•BW) increased the levels of total BAs, primary and secondary BAs, and conjugated and unconjugated BAs in the colon contents of the MCD diet-induced NASH mice, suggesting that they promoted the excretion of BAs in feces. Previous studies have shown that soyasaponins can increase the fecal excretion of cholesterol and BAs.[27,28] Soyasaponins combine with cholesterol to form insoluble complexes and inhibit the absorption of endogenous and exogenous cholesterol in the intestine.[38] In addition, soyasaponins also affect the hepatoenteral circulation of BAs through forming a mixed micelle with BAs and effectively blocking the reabsorption of BAs at the end of the ileum.[27,38] We previously found that soyasaponins (A_1, A_2, and I) improved serum lipid profiles in HFD-fed obese mice at least partly by enhancing fecal excretion of BAs.[35] The results in this study further confirmed that soyasaponin could increase the fecal excretion of total BAs including primary and secondary BAs and conjugated and unconjugated BAs in the MCD diet-induced NASH mice. The enhancement of fecal excretion of BAs by soyasaponin may contribute to the decrease of BAs levels in liver and serum as observed in this study. One interesting point is that the THDCA level in liver and serum in the MCD diet-induced NASH mice was still increased although its fecal excretion was largely increased. On one side, it has been shown that soyasaponins could directly combine with BAs and promote its excretions in feces. That might be one of the possible reasons resulting in elevated THDCA level by SS-A_2 in colon contents. On the other side, SS-A_2 reduced the bile salt hydrolase (BSH)-producing bacteria (*Erysipelotrichaceae* and *Faecalibaculum*) and thus decreased the hydrolysis of conjugated BAs in colon. THDCA, as conjugated BA, thus increased in colon. Meanwhile, increased THDCA level in colon might promote itself reabsorption back into the serum and liver, contributing to the increased level of THDCA in serum and liver. Anyway, the underlying mechanism is worth further detailed investigating. Collectively, our results here suggest that SS-A_2 not only increases the fecal excretion of BAs but also changes the profile of BAs in liver and serum in the MCD diet-induced NASH mice. THDCA is probably a potential targeted bile acid by which SS-A_2 exhibits the alleviative effect against the steatohepatitis in the MCD diet-induced NASH mice.

The crosstalk between BAs and gut microbiota has been shown to play important role in the development and progression of NAFLD/NASH. In this study, we further analyzed the association between gut microbiota and the levels of BAs in liver, serum, and colon contents by using the Spearman correlation analysis. SS-A_2 (1, 50, 100 mg kg^{-1}•BW) and OCA decreased the relative abundance of *Faecalibaculum* in the MCD diet-induced NASH mice. The relative abundance of *Faecalibaculum* is positively correlated with the hepatic THCA level and the serum levels of CA, ACA, 7_DHCA, CDCA, and UDCA, and negatively correlated with the serum THDCA level and the colonic levels of TCA, muroCA, Tβ-MCA, and GCA. These results indicate that the increase of serum THDCA, the decrease of CA and 7_DHCA in the serum, and the increase of TCA and muroCA in the colon caused by the intervention of SS-A_2 and OCA is probably associated with their abilities to reduce the abundance of *Faecalibaculum*. In addition, the relative abundance of *Desulfovibrio* is negatively correlated with the levels of CDCA, UDCA, and muroCA in liver. This suggests that the reduction of the levels of UDCA and muroCA in liver caused by OCA intervention is possibly associated with its ability to increase the abundance of *Desulfovibrio*.

The last finding of this study is that SS-A_2 regulates the signaling of BA synthesis and transport in ileum of the MCD diet-induced NASH mice. FXR maintains the homeostasis of BAs by regulating the synthesis, transport, and metabolism of BAs in the liver and intestine.[72] BAs activates FXR and its synthesis reversely receives the negative feedback control of FXR. FXR is highly expressed in liver and ileum. In liver, BAs-activated FXR induces the expression of small heterodimer partner (SHP). SHP combines with liver receptor homolog-1 (LRH-1) and thus inhibits the gene expression of CYP7A1 which is the rate-limiting

enzyme of BAs synthesis. Hepatic FXR activation also promotes BSEP expression which increases the efflux of BAs into biliary tract, and inhibits NTCP expression which reduces the hepatic BAs accumulation by decreasing the BAs uptake from the portal vein. In this study, the MCD diet-induced NASH mice had significantly reduced expression of CYP7A1 and NTCP in liver tissues as compared to the MCS diet-fed mice. However, the hepatic expression of FXR in the MCD diet-induced NASH mice was not statistically different from that in the MCS diet-fed mice although it had the tendency to decrease. The decreased NTCP expression along with the increased serum BAs levels observed in this study is consistent with previous report by Tanaka et al. which showed that the elevation of BAs in serum was related to the decreased NTCP expression in the MCD diet-induced NASH mice.[77] In this study, OCA and SS-A_2 did not affect the expression of CYP7A1, FXR, NTCP, and BSEP in the liver tissues of the MCD diet-induced NASH mice. This suggests that SS-A_2 does not influence the expression of BAs receptors in liver which is also reflected by the unchanged total BAs levels in liver following SS-A_2 intervention in the MCD diet-induced NASH mice.

In intestine, FXR can be activated by BAs in the terminal ileum and further induce the expression of FGF 15/19. FGF15/19 goes into the liver through portal vein and combines with the fibroblast growth factor receptor 4 (FGFR4), resulting in the activation on signaling transduction of C-Jun N-terminal kinase 1/2 (JNK 1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) and the inhibition of CYP7A1 expression and hepatic BAs synthesis.[78,79] Intestinal FXR has been suggested to be the potential therapeutic target for NAFLD treatment.[80,81] Studies indicated that the intestinal FXR activation could improve metabolic diseases. Oral administration of fexaramine (Fex), an intestine specific FXR agonist, robustly induced the enteric expression of FGF15 leading to alterations in the composition of BAs while did not activate the FXR in the liver.[82] Fex reduced the diet-induced weight gain, systemic inflammation, and hepatic glucose production. Meanwhile, Fex promoted adipose tissue browning and reduced obesity and insulin resistance.[82] Fex was also shown to protect mice from ethanol-induced liver injury by regulating the bile acid-FXR-FGF15 signaling.[83] Supplementation of *Lactobacillus rhamnosus GG* reduced the hepatic BAs accumulation by increasing the intestinal FXR-FGF15 signaling pathway-mediated suppression of BAs de novo synthesis and enhancing BAs excretion, thus prevented the excessive BAs-induced liver injury and fibrosis in mice.[84] In this study, the MCD diet-induced NASH mice had decreased expression of FXR and FGF-15 in intestine. SS-A_2 (1 and 100 mg kg^{-1}•BW) increased the intestinal expression of FXR and FGF-15 in the MCD diet-induced NASH mice. These results suggest that SS-A_2 may regulate the BAs balance through enhancing the FXR-FGF-15 signaling in intestine of the MCD diet-induced NASH mice.

In summary, this study shows that SS-A_2 alleviates steatohepatitis in the MCD diet-induced NASH mice possibly through regulating the bile acids profiles and gut microbiota composition. In addition, SS-A_2 increases the expression of FXR and FGF15 in ileum when submitted to the same diet for mice. These results, for the first time, provides the direct evidences supporting the preventive bioactivities of soyasaponin against NASH and uncovering its underlying mechanism via the possible regulation of the crosstalk between bile acids and gut microbiota.

4. Experimental Section

Reagents: Soyasaponin monomers A_2 was prepared and purified by using the methods as previously described.[37] OCA was purchased from Meilunbio (cat no. MB6084, Dalian, China). Primary antibodies for β-actin and all secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies for CYP7A1 (cat no. ab65596) and NCTP (cat no. ab131084) were all purchased from Abcam (Cambridge, MA, UK). BSEP (cat no. sc-74500), FXR (cat no. sc-25309) and FGF-15 (cat no. sc-398338) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Soyasaponins Intervention on MCD Diet-induced NASH Mice: This study involving animals and their care was approved by the Southern Medical University Experimental Animal Ethics Committee (No. SMUA2019322). Sixty 4-week-old male C57BL/6J (B6) mice were purchased from Guangdong Medical Lab Animal Center (cat no. 44 007 200 050 562, Guangzhou, China). Animals were housed in standard cages in a room kept at 22±1 °C on a 12h/12h light/dark cycle. All mice were first adaptively fed with the methionine and choline supplemented (MCS) diet (cat no. A02082003B, ReadyDietech, Shenzhen, China) for 2 weeks and then used for soyasaponins intervention experiment lasting for 16 weeks. The body weight (BW), body length (from the apex nasi to anus), and feed intake were recorded weekly. The feed consumption was then calculated in both g d^{-1} $mice^{-1}$ and kcal d^{-1} $mice^{-1}$. The Lee's index was calculated by using the formula of $(\sqrt[3]{\text{body weightg}} * \frac{1000}{\text{body length (cm)}})$.

After an 8-h overnight fast at the end of intervention experiment, mice were weighed and sedated with pentobarbital (50 mg kg^{-1} body weight) by peritoneal injection and euthanized by cervical dislocation.

Dosage Information/Dosage Regimen: All mice were randomly assigned to six groups ($n = 10$ each). The 1st group served as a control in which mice were fed with the MCS diet. Mice in the 2nd group were used as the positive control and fed with the MCD diet (cat no. A02082002B, ReadyDietech, Shenzhen, China). Mice in the 3rd group were fed with MCD diet supplemented with 0.4 mg/kg•BW of OCA. Mice in the 4th, 5th and 6th groups were fed MCD diet supplemented with 1 mg kg^{-1}•BW (low dosage soyasaponin, LSS), 50 mg/kg•BW (medium dosage soyasaponin, MSS) and 100 mg kg^{-1}•BW (high dosage soyasaponin, HSS) of SS-A_2, respectively. The choice of the dosage of OCA was according to the reference.[20] The intervention dosage of SS-A_2 was determined based on the study of Kamo et al. (2014), which reported that the estimated daily intake of total soyasaponins by the Japanese was about 50.3 μmol.[85] Thus, the estimated daily intake of soysaponins ranges from 0.8 to 1.0 mg kg^{-1}•BW for human with an average body weight of 60 kg. Based on this, the supplemental dosage of soyasaponins in the present animal trial was about 1, 50, and 100 times to that of the estimated daily intake in human. The composition of MCS and MCD diet was shown in Table S5 and did not contain any soy-based materials in order to avoid the disturbance by natural source of soyasaponins. Both OCA and SS-A_2 were premixed into the feed. The diet and water were provided ad libitum.

Serum Parameters Analysis: Following anesthetization, blood was collected from the retro-orbital plexus, and the serum was separated and stored at −80 °C. The levels of TC (cat no. A111-1-1), TG (cat no. A110-1-1), LDL-C (cat no. A113-1), HDL-C (cat no. A112-1), ALT (cat no. C009-2), and AST (cat no. C010-2) in serum were determined by commercial enzymatic kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) with the recommended procedures. The levels ofIL-6 (cat no. EM004) and TNF-α (cat no. EM008) were determined by commercially available enzyme-linked immuno sorbent assay (ELISA) kits from Genetimes ExCell Technology Company (Shanghai, China).

Hematoxylin-eosin (HE) and Masson's Trichrome Staining of Liver Tissues: Following euthanization, the mice liver was collected and fixed in 4% paraformaldehyde for 48–72 h. Liver tissues were embedded in the paraffin and then sliced into 4 μm thin sections. A total of 42 sections

($n = 7$) from liver tissue sample of each mouse were chosen at regular intervals for HE staining and Masson's trichrome staining. Hepatic steatosis ballooning and inflammation were assessed by HE staining. Hepatic fibrosis was assessed by using the Masson's trichrome staining. Five pictures per slide (four corners and the center area) were used for quantitatively scoring. Two technicians observed and photographed independently pathological changes of specimens by Olympus microscopy and imaging system, which scored for the severity of hepatocellular steatosis, ballooning, inflammation and fibrosis according to NAFLD activity score (NAS) method which is the NAS semi-quantitatively scoring system designed and validated by the Pathology Committee of the NASH Clinical Research Network.[86,87] The NAS score is defined as the unweighted sum of the scores for steatosis (0-3), ballooning (0-2), and lobular inflammation (0-3). Cases with NAS scores of > 5 were diagnosed as steatohepatitis. Fibrosis is not included as a component of the NAS score because less reversible and generally thought to be a result of disease activity.[87] Fibrosis score (0-4) is an independent scoring standard which is defined as the unweighted sum of the scores for none (0), perisinusoidal or periportal (1), perisinusoidal and portal/periportal (2), bridging fibrosis (3), cirrhosis (4).[86]

BAs Quantitation by UPLC-TQMS: The BAs in serum, liver, and colon contents were measured by ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-TQMS) from Waters Corp (Waters, Milford, MA, USA) according to the reported protocol.[88,89,72] All data analyses were performed by using A Waters ACQUITY ultra performance LC system coupled with a Waters XEVO TQ-S mass spectrometer with an ESI source controlled by MassLynx 4.1 software (Waters, Milford, MA, USA). Chromatographic separations were performed with an ACQUITY BEH C18 column (1.7 µm, 100 mm × 2.1 mm internal dimensions) (Waters, Milford, MA, USA). UPLC-MS raw data was performed using the QuanMET v1.0 software of Metabo-Profile to obtain the quantitative concentration of each BA in the samples (Shanghai, China).

The 16S rDNA Amplicon Sequencing: Fresh caecum contents were stored at −80 °C until use. The DNA of caecum contents were extracted by using the cetyltrimethylammonium bromide (CTAB) method. The purity and concentration of DNA were determined by using agarose gel electrophoresis. Then, proper amount of sample DNA was transferred in a centrifuge tube and diluted to 1 ng μL^{-1} by using sterile water. The diluted genomic DNA was used as the template. The specific primers with Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Inc., MA, USA), and high efficiency and high-fidelity enzymes were used for PCR to ensure amplification and accuracy. The 16S rDNA genes of distinct regions V4 were amplified by T100 Thermal Cycler (Bio-Rad Laboratories, Inc., WS, USA) based on specific primers (515F: GTGCCAGCMGCCGCGGTAA; 806R: GGACTACHVGGGTWTCTAAT). Sequencing libraries were generated using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific Inc., MA, USA) and were analyzed by the high-throughput sequencing using the IonS5XL platform (Thermo Fisher Scientific Inc., MA, USA) by Novogene Bioinformatics Technology Co., Ltd (Beijing, China). Sequences of all samples were clustered into Operational Taxonomic Units (OTUs) with 97% similarity in order to annotate species.

Western Blotting: The proteins were extracted from tissues of liver and terminal ileum by PBS containing 1% phenylmethylsulfonyl fluoride (PMSF) and radio-immunoprecipitation assay (RIPA) lysate containing 1% PMSF with 0.4% phosphatase inhibitor. The protein concentration was determined by using the bicinchoninic acid (BCA, BioTeke Corporation, cat no. PP1001, Beijing, China) method. Western blotting was then performed as previously described.[53] Briefly, tissue protein samples and marker were sequentially added to each lane at a loading of 20–40 µg in a 1.0% thick Tris glycine gel. Electrophoresis was carried out using a voltage of 80–120V. The protein strip of the gel was then transferred to a nitrocellulose membrane (Bio-Rad) at a voltage of 100V. Proteins were quantified by immune-blotting with primary and secondary antibodies. Fluorescent bands were visualized and photographed using an Odyssey infrared imaging system (Odyssey Fc, LI-COR).

Statistical Analysis: Statistical analyses were performed using one-way analysis of variance (one-way ANOVA), Kruskal-Wallis test or Mann-Whitney U test by using the SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). Spearman correlation analysis was applied to evaluate the interactions between gut microbiota and BAs levels in the liver, serum, and feces. All results were expressed as means ± standard error of mean (Means ± S.E.M.). A p value less than 0.05 ($p < 0.05$) was considered to be statistical significance.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (NSFC) (No.81872621, No.81573125, and No.82073532).

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

F.X., Z.Z., and L.X. contributed equally to this work. The contributions of the authors to this study are as follows: L.Z. was responsible for the study concept and design; F.X., Z.Z., L.X., C.S., J.C., X.G., J.T., Y.Z., and H.L. performed the experiments and collection of data; F.X., Z.Z., L.X., C.S., and L.Z. carried out the statistical analysis and interpretation of data; F.X. and L.Z. wrote the manuscript; All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

bile acid, farnesoid X receptor (FXR), gut microbiota, non-alcoholic steatohepatitis (NASH), soyasaponin

Received: January 22, 2021
Revised: April 21, 2021
Published online: June 10, 2021

[1] N. Chalasani, Z. Younossi, J. E. Lavine, M. Charlton, K. Cusi, M. Rinella, S. A. Harrison, E. M. Brunt, A. J. Sanyal, *Hepatology* **2018**, *67*, 328.
[2] V. T. Samuel, G. I. Shulman, *Cell Metab.* **2018**, *27*, 22.
[3] S. L. Friedman, B. A. Neuschwander-Tetri, M. Rinella, A. J. Sanyal, *Nat. Med.* **2018**, *24*, 908.
[4] M. Eslam, L. Valenti, S. Romeo, *J. Hepatol.* **2018**, *68*, 268.
[5] E. Buzzetti, M. Pinzani, E. A. Tsochatzis, *Metabolism* **2016**, *65*, 1038.
[6] F. de Faria Ghetti, D. G. Oliveira, J. M. de Oliveira, L. E. V. V. de Castro Ferreira, D. E. Cesar, A. P. B. Moreira, *Eur. J. Nutr.* **2018**, *57*, 861.
[7] W. Jia, G. Xie, W. Jia, *Nat. Rev. Gastro. Hepat.* **2018**, *15*, 111.
[8] S. Fiorucci, E. Distrutti, *Trends Mol. Med.* **2015**, *21*, 702.
[9] Q. Yu, Z. Jiang, L. Zhang, *Pharmacol. Therapeut.* **2018**, *190*, 81.

[10] A. Wahlström, S. I. Sayin, H. Marschall, F. Bäckhed, *Cell Metab.* **2016**, *24*, 41.
[11] J. P. Arab, S. J. Karpen, P. A. Dawson, M. Arrese, M. Trauner, *Hepatology* **2017**, *65*, 350.
[12] M. D. Chow, Y. Lee, G. L. Guo, *Mol. Aspects Med.* **2017**, *56*, 34.
[13] P. Puri, K. Daita, A. Joyce, F. Mirshahi, P. K. Santhekadur, S. Cazanave, V. A. Luketic, M. S. Siddiqui, S. Boyett, H. K. Min, D. P. Kumar, R. Kohli, H. Zhou, P. B. Hylemon, M. J. Contos, M. Idowu, A. J. Sanyal, *Hepatology* **2017**, *67*, 534.
[14] J. Boursier, O. Mueller, M. Barret, M. Machado, L. Fizanne, F. Araujo-Perez, C. D. Guy, P. C. Seed, J. F. Rawls, L. A. David, G. Hunault, F. Oberti, P. Calès, A. M. Diehl, *Hepatology* **2016**, *63*, 764.
[15] L. Wang, Y. Lee, D. Bundman, Y. Han, S. Thevananther, C. Kim, S. S. Chua, P. Wei, R. A. Heyman, M. Karin, D. D. Moore, *Dev. Cell* **2002**, *2*, 721.
[16] B. Goodwin, S. A. Jones, R. R. Price, M. A. Watson, D. D. McKee, L. B. Moore, C. Galardi, J. G. Wilson, M. C. Lewis, M. E. Roth, P. R. Maloney, T. M. Willson, S. A. Kliewer, *Mol. Cell* **2000**, *6*, 517.
[17] T. Inagaki, M. Choi, A. Moschetta, L. Peng, C. L. Cummins, J. G. McDonald, G. Luo, S. A. Jones, B. Goodwin, J. A. Richardson, R. D. Gerard, J. J. Repa, D. J. Mangelsdorf, S. A. Kliewer, *Cell Metab.* **2005**, *2*, 217.
[18] B. Cariou, *Diabetes Metab* **2008**, *34*, 685.
[19] E. S. Friedman, Y. Li, T. D. Shen, J. Jiang, L. Chau, L. Adorini, F. Babakhani, J. Edwards, D. Shapiro, C. Zhao, R. M. Carr, K. Bittinger, H. Li, G. D. Wu, *Gastroenterology* **2018**, *155*, 1741.
[20] B. A. Neuschwander-Tetri, R. Loomba, A. J. Sanyal, J. E. Lavine, M. L. Van Natta, M. F. Abdelmalek, N. Chalasani, S. Dasarathy, A. M. Diehl, B. Hameed, K. V. Kowdley, A. McCullough, N. Terrault, J. M. Clark, J. Tonascia, E. M. Brunt, D. E. Kleiner, E. Doo, *Lancet* **2015**, *385*, 956.
[21] S. Mudaliar, R. R. Henry, A. J. Sanyal, L. Morrow, H. U. Marschall, M. Kipnes, L. Adorini, C. I. Sciacca, P. Clopton, E. Castelloe, P. Dillon, M. Pruzanski, D. Shapiro, *Gastroenterology* **2013**, *145*, 574.
[22] S. Paul, A. M. Davis, *JAMA, J. Am. Med. Assoc.* **2018**, *320*, 2474.
[23] F. Zhou, J. Zhou, W. Wang, X. J. Zhang, Y. X. Ji, P. Zhang, Z. G. She, L. Zhu, J. Cai, H. Li, *Hepatology* **2019**, *70*, 1119.
[24] B. van de Wier, G. H. Koek, A. Bast, G. R. M. Haenen, *Crit. Rev. Food Sci.* **2017**, *57*, 834.
[25] A. B. Ross, J. Godin, K. Minehira, J. P. Kirwan, *Int. J. Endocrinol.* **2013**, *2013*, 1.
[26] O. Guclu-Ustundag, G. Mazza, *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 231.
[27] B. Singh, J. P. Singh, N. Singh, A. Kaur, *Food Chem.* **2017**, *233*, 540.
[28] J. Kang, T. M. Badger, M. J. J. Ronis, X. Wu, *J. Agr. Food Chem.* **2010**, *58*, 8119.
[29] H. Kuzuhara, S. Nishiyama, N. Minowa, K. Sasaki, S. Omoto, *Eur J. Pharmacol.* **2000**, *391*, 175.
[30] K. Sasaki, N. Minowa, H. Kuzuhara, S. Nishiyama, *Bioorg. Medicin. Chem.* **2005**, *13*, 4900.
[31] X. Yang, C. Dong, G. Ren, *J. Agr. Food Chem.* **2011**, *59*, 1138.
[32] J. Kinjo, T. Hirakawa, R. Tsuchihashi, T. Nagao, M. Okawa, T. Nohara, H. Okabe, *Biol. Pharm. Bull.* **2003**, *26*, 1357.
[33] H. Kuzuhara, S. Nishiyama, N. Minowa, K. Sasaki, *J. Nat. Med.* **2006**, *60*, 113.
[34] J. Hong, S. Kim, H. S. Kim, *J. Medicin. Food* **2016**, *19*, 549.
[35] Q. Xie, X. Gu, J. Chen, M. Liu, F. Xiong, X. Wu, Y. Zhang, F. Chen, H. Chen, M. Li, S. Sun, X. Chu, L. Zha, *Mol. Nutr. Food Res.* **2018**, *62*, 1800205.
[36] Y. Zhang, F. Chen, J. Chen, *Mol. Nutr. Food Res.* **2016**, *60*, 1532.
[37] L. Y. Zha, L. M. Mao, X. C. Lu, H. Deng, J. F. Ye, X. W. Chu, S. X. Sun, H. J. Luo, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2415.
[38] D. Oakenfull, *J. Nutr.* **2001**, *131*, 2971.
[39] D. Oakenfull, G. S. Sidhu, *Eur. J. Clin. Nutr.* **1990**, *44*, 79.
[40] J. Hu, Y. L. Zheng, W. Hyde, S. Hendrich, P. A. Murphy, *J Agr. Food Chem.* **2004**, *52*, 2689.
[41] M. Varela-Rey, N. Embade, U. Ariz, S. C. Lu, J. M. Mato, M. L. Martínez-Chantar, *Int. J. Biochem. Cell Biol.* **2009**, *41*, 969.
[42] A. Gottlieb, A. Canbay, *Cells* **2019**, *8*, 1358.
[43] O. Kucera, *World J. Gastroentero.* **2014**, *20*, 8364.
[44] T. Yan, H. Wang, L. Cao, Q. Wang, S. Takahashi, T. Yagai, G. Li, K. W. Krausz, G. Wang, F. J. Gonzalez, H. Hao, *Drug Metab. Dispos.* **2018**, *46*, 1310.
[45] T. Goto, M. Itoh, T. Suganami, S. Kanai, I. Shirakawa, T. Sakai, M. Asakawa, T. Yoneyama, T. Kai, Y. Ogawa, *Sci. Rep.-Uk* **2018**, *8*, 8157.
[46] T. Ikeda, M. Udayama, M. Okawa, T. Arao, J. Kinjo, T. Nohara, *Chem. Pharm. Bull. (Tokyo)*. **1998**, *46*, 359.
[47] J. Kinjo, M. Imagire, M. Udayama, T. Arao, T. Nohara, *Planta Med.* **1998**, *64*, 233.
[48] J. X. Xiao, G. H. Peng, S. H. Zhang, *Acta Nutr. Sin.* **2005**, *27*, 147.
[49] S. O. Lee, A. L. Simons, P. A. Murphy, S. Hendrich, *Exp. Biol. Med.* **2005**, *230*, 472.
[50] Q. Xie, F. Xiong, X. Wu, J. Chen, X. Gu, C. Su, L. Xiao, Z. Zheng, Y. Wei, H. Ullah, L. Zha, *Food Funct.* **2020**, *11*, 253.
[51] J. M. Ntambi, M. Miyazaki, J. P. Stoehr, H. Lan, C. M. Kendziorski, B. S. Yandell, Y. Song, P. Cohen, J. M. Friedman, A. D. Attie, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11482.
[52] P. Jha, A. Knopf, H. Koefeler, M. Mueller, C. Lackner, G. Hoefler, T. Claudel, M. Trauner, *Biochim. Biophys. Acta.* **2014**, *1842*, 959.
[53] Y. Zhang, F. Chen, J. Chen, *PLoS One* **2014**, *9*, e107655.
[54] J. Chen, H. Ullah, Z. Zheng, X. Gu, C. Su, L. Xiao, X. Wu, F. Xiong, Q. Li, L. Zha, *BMC Complement. Med. Ther.* **2020**, *20*, 167.
[55] L. Ding, L. Zhang, H. Shi, C. Wang, X. Jiang, C. Xue, T. Yanagita, T. Zhang, Y. Wang, *J. Agric. Food Chem.* **2020**, *68*, 5339. https://doi.org/10.1021/acs.jafc.9b08296.
[56] D. D. Heeney, M. G. Gareau, M. L. Marco, *Curr. Opin. Biotechnol.* **2018**, *49*, 140.
[57] G. Xie, X. Wang, A. Zhao, J. Yan, W. Chen, R. Jiang, J. Ji, F. Huang, Y. Zhang, S. Lei, K. Ge, X. Zheng, C. Rajani, R. A. Alegado, J. Liu, P. Liu, J. Nicholson, W. Jia, *Sci. Rep.-Uk* **2017**, *7*, 45232.
[58] Y. Jung, I. Kim, M. Mannaa, J. Kim, S. Wang, I. Park, J. Kim, Y. S. Seo, *Food Sci. Biotechnol.* **2019**, *28*, 261.
[59] M. D. Spencer, T. J. Hamp, R. W. Reid, L. M. Fischer, S. H. Zeisel, A. A. Fodor, *Gastroenterology* **2011**, *140*, 976.
[60] M. Han, T. Zhang, W. Gu, X. Yang, R. Zhao, J. Yu, *Exp. Ther. Med.* **2018**, *16*, 1087.
[61] L. M. Cox, J. Sohn, K. L. Tyrrell, D. M. Citron, P. A. Lawson, N. B. Patel, T. Iizumi, G. I. Perez-Perez, E. J. C. Goldstein, M. J. Blaser, *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1247.
[62] E. Munukka, S. Pekkala, P. Wiklund, O. Rasool, R. Borra, L. Kong, X. Ojanen, S. M. Cheng, C. Roos, S. Tuomela, M. Alen, R. Lahesmaa, S. Cheng, *J. Hepatol.* **2014**, *61*, 132.
[63] J. Ye, L. Lv, W. Wu, Y. Li, D. Shi, D. Fang, F. Guo, H. Jiang, R. Yan, W. Ye, L. Li, *Front. Microbiol.* **2018**, *9*, 1967.
[64] P. C. Konturek, J. Koziel, W. Dieterich, D. Haziri, S. Wirtz, I. Glowczyk, K. Konturek, M. F. Neurath, Y. Zopf, *J. Physiol. Pharmacol.* **2016**, *67*, 859.
[65] J. L. Wallace, J. P. Motta, A. G. Buret, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2018**, *314*, G143.
[66] S. C. Kalhan, L. Guo, J. Edmison, S. Dasarathy, A. J. McCullough, R. W. Hanson, M. Milburn, *Metabolism* **2011**, *60*, 404.
[67] M. M. Aranha, H. Cortez-Pinto, A. Costa, S. I. Da, M. E. Camilo, M. C. de Moura, C. M. Rodrigues, *Eur. J. Gastroenterol. Hepatol.* **2008**, *20*, 519.
[68] C. Thomas, R. Pellicciari, M. Pruzanski, J. Auwerx, K. Schoonjans, *Nat. Rev. Drug Discovery* **2008**, *7*, 678.

[69] J. D. Amaral, R. J. Viana, R. M. Ramalho, C. J. Steer, C. M. Rodrigues, *J. Lipid Res.* **2009**, *50*, 1721.
[70] H. Bernstein, C. Bernstein, C. M. Payne, K. Dvorak, *World J. Gastroenterol.* **2009**, *15*, 3329.
[71] B. L. Woolbright, H. Jaeschke, *World J. Gastroenterol.* **2012**, *18*, 4985.
[72] G. Xie, X. Wang, F. Huang, A. Zhao, W. Chen, J. Yan, Y. Zhang, S. Lei, K. Ge, X. Zheng, J. Liu, M. Su, P. Liu, W. Jia, *Int. J. Cancer* **2016**, *139*, 1764.
[73] J. J. Rafter, P. Child, A. M. Anderson, R. Alder, V. Eng, W. R. Bruce, *Am. J. Clin. Nutr.* **1987**, *45*, 559.
[74] Y. Tang, J. Zhang, J. Li, X. Lei, D. Xu, Y. Wang, C. Li, X. Li, Y. Mao, *Biochim. Biophys. Acta. Mol. Cell Biol. Lipids* **2019**, *1864*, 1293.
[75] B. C. Ferslew, G. Xie, C. K. Johnston, M. Su, P. W. Stewart, W. Jia, K. L. Brouwer, *Dig. Dis. Sci.* **2015**, *60*, 3318.
[76] C. Caussy, C. Hsu, S. Singh, S. Bassirian, J. Kolar, C. Faulkner, N. Sinha, R. Bettencourt, N. Gara, M. A. Valasek, B. Schnabl, L. Richards, D. A. Brenner, A. F. Hofmann, R. Loomba, *Aliment Pharmacol. Ther.* **2019**, *49*, 183.
[77] N. Tanaka, T. Matsubara, K. W. Krausz, A. D. Patterson, F. J. Gonzalez, *Hepatology* **2012**, *56*, 118.
[78] E. Halilbasic, T. Claudel, M. Trauner, *J. Hepatol.* **2013**, *58*, 155.
[79] Y. Chen, X. Song, L. Valanejad, A. Vasilenko, V. More, X. Qiu, W. Chen, Y. Lai, A. Slitt, M. Stoner, B. Yan, R. Deng, *Hepatology* **2013**, *57*, 1530.
[80] C. Jiang, C. Xie, F. Li, L. Zhang, R. G. Nichols, K. W. Krausz, J. Cai, Y. Qi, Z. Fang, S. Takahashi, N. Tanaka, D. Desai, S. G. Amin, I. Albert, A. D. Patterson, F. J. Gonzalez, *J. Clin. Invest.* **2015**, *125*, 386.
[81] A. Rao, A. Kosters, J. E. Mells, W. Zhang, K. D. Setchell, A. M. Amanso, G. M. Wynn, T. Xu, B. T. Keller, H. Yin, S. Banton, D. P. Jones, H. Wu, P. A. Dawson, S. J. Karpen, *Sci. Transl. Med.* **2016**, *8*, 357ra122.
[82] S. Fang, J. M. Suh, S. M. Reilly, E. Yu, O. Osborn, D. Lackey, E. Yoshihara, A. Perino, S. Jacinto, Y. Lukasheva, A. R. Atkins, A. Khvat, B. Schnabl, R. T. Yu, D. A. Brenner, S. Coulter, C. Liddle, K. Schoonjans, J. M. Olefsky, A. R. Saltiel, M. Downes, R. M. Evans, *Nat. Med.* **2015**, *21*, 159.
[83] P. Hartmann, K. Hochrath, A. Horvath, P. Chen, C. T. Seebauer, C. Llorente, L. Wang, Y. Alnouti, D. E. Fouts, P. Stärkel, R. Loomba, S. Coulter, C. Liddle, R. T. Yu, L. Ling, S. J. Rossi, A. M. DePaoli, M. Downes, R. M. Evans, D. A. Brenner, B. Schnabl, *Hepatology* **2018**, *67*, 2150.
[84] Y. Liu, K. Chen, F. Li, Z. Gu, Q. Liu, L. He, T. Shao, Q. Song, F. Zhu, L. Zhang, M. Jiang, Y. Zhou, S. Barve, X. Zhang, C. J. McClain, *Hepatology* **2020**, *71*, 2050.
[85] S. Kamo, S. Suzuki, T. Sato, *Food Sci. Nutr.* **2014**, *2*, 289.
[86] D. E. Kleiner, E. M. Brunt, M. Van Natta, C. Behling, M. J. Contos, O. W. Cummings, L. D. Ferrell, Y. Liu, M. S. Torbenson, A. Unalp-Arida, M. Yeh, A. J. McCullough, A. J. Sanyal, *Hepatology* **2005**, *41*, 1313.
[87] E. M. Brunt, *Semin. Liver Dis.* **2004**, *24*, 3.
[88] X. Wang, G. Xie, A. Zhao, X. Zheng, F. Huang, Y. Wang, C. Yao, W. Jia, P. Liu, *J. Proteome Res.* **2016**, *15*, 1126.
[89] G. Xie, W. Zhong, H. Li, Q. Li, Y. Qiu, X. Zheng, H. Chen, X. Zhao, S. Zhang, Z. Zhou, S. H. Zeisel, W. Jia, *FASEB J.* **2013**, *27*, 3583.