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Salvia miltiorrhiza extract may exerts an anti-obesity effect in high-fat diet-induced obese rat by modulating gut microbiome and lipid metabolism

Ai ZL *et al*. Sal regulate gut microbiome and lipid metabolism

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Abstract

BACKGROUND

Studies have shown that a high-fat diet (HFD) can alter gut microbiota homeostasis (GM) and participate in lipid metabolism disorders associated with obesity. Therefore, regulating the construction of GM with the balance of lipid metabolism has become essential for treating obesity. *Salvia miltiorrhiza* (Sal), a common traditional Chinese medicine, has been proven effective against atherosclerosis, hyperlipidemia, obesity, and other dyslipidemia-related diseases.

AIM

To investigate the anti-obesity effects of Sal on HFD-induced obesity rats, and corresponding mechanism by focusing on GM and lipid metabolism.

METHODS

Rats were induced by HFD for 7 wk, while Sal (0.675 g/1.35 g/2.70 g/kg/d) was administered to treat obese rats for 8 wk. The therapeutic effect was evaluated by body weight, body fat index, waistline, and serum lipid level. Lipid factors (cAMP, PKA, HSL) in liver and fat homogenates were analyzed by ELISA. Sal's effect on GM and lipid metabolism was assessed by 16S rRNA-based microbiota analysis and untargeted lipidomic analysis (LC-MS/MS), respectively.

RESULTS

Sal treatment markedly reduced weight, body fat index, serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein, glucose, free fatty acid, hepatic lipid accumulation and adipocyte vacuolation, increased serum high-density lipoprotein (HDL-C) levels in HFD rats. These effects were associated with increased concentrations of lipid factors such as cAMP, PKA, and HSL in the liver and adipose tissues, enhanced gut integrity, and improved lipid metabolism. GM analysis revealed that Sal could reverse HFD-induced dysbacteriosis by promoting the abundance of *Actinobacteriota* and *Proteobacteria*, and decreasing the growth of *Firmicutes* and *Desulfobacterita*. Furthermore, LC-MS/MS analysis indicated that Sal decreased TGs (TG18:2/18:2/20:4, TG16:0/18:2/22:6), DGs (DG14:0/22:6, DG22:6/22:6), CL (18:2/18:1/18:1/20:0), and increased Cers (Cer d16:0/21:0, Cer d16:1/24:1), OAHFA(18:0/14:0) in the feces of rat. Spearman's correlation analysis further indicated that TGs, DGs, and CL were negatively related to the abundance of *Facklamia*, *Dubosiella,* and positively correlated with *Blautia*, *Quinella*, while OAHFA and Cer were the opposite.

CONCLUSION

Sal has an anti-obesity effect by regulating the relation of GM and lipid metabolism.

Key Words: *Salvia miltiorrhiza* extract; Obesity; Gut microbiota; Lipid metabolism; High fat diet

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Core Tip: Obesity is a major public health issue today and an obesity-related change in gut microbiota composition and its metabolite profile has been demonstrated. As a commonly used traditional Chinese medicine, *Salvia miltiorrhiza* extract (Sal) has many pharmacological effects, including anticoagulant, anti-inflammation, antioxidation, anti-

fibrosis, anti-tumor, and organ protection. Although it hasn't been documented, Sal has a regulatory effect on obesity and may be related to gut microbiota. In the present study, we found that Sal plays a role in weight loss, lowering serum lipid levels and regulating gut microbiota, improving intestinal fecal metabolites in obese rats at the same time.

INTRODUCTION

Obesity, a disease whose incidence increases yearly, can lead to several chronic metabolic syndromes such as diabetes, hyperlipidemia, and atherosclerosis. According to 2016 epidemiological statistics, more than 1.9 billion adults worldwide suffer from obesity, and its prevalence reaches 70% in the United States[1]. According to the WHO, adults with a body mass index (BMI) > 25 are considered overweight, while adults with BMI > 30 are considered obese[2].

Gut microbiota (GM) is among the key regulators of metabolism[3]. Dysregulation of the GM is closely connected with obesity and its complications[4]. Under physiological circumstances, there is a symbiotic relationship between GM and the host, which keeps the intestinal dynamic equilibrium of the body and metabolism[5]. On the other hand, the GM-host imbalance[6] has been associated with the occurrence of many diseases, such as obesity, enteritis, and colitis. Recent data indicated that there are significant differences in the structural composition of the GM of obese patients and those of non-obese people. For example, fewer Bacteroides phylum (beneficial bacteria which metabolize oligosaccharides and polysaccharides, providing nutrition to the host) and more *Pachyderma* were found in the intestinal tract of obese patients[7]. Consistent findings suggest that obesity is associated with decreased abundance in some taxa, such as *Bifidobacterium*, *Christensenella*, and *Ackermannia*, which are considered beneficial microbes. Moreover, as a key regulator of host metabolism, GM can influence lipid metabolism and the levels of blood and tissues in humans and rats[8]. The GM has a major role in the fermentation of carbohydrates, fermenting carbohydrates and producing short-chain fatty acids (SCFAs), such as acetic, propionic acid, and butyric

acid, to prevent and treat obesity and its complications. As a result, mounting evidence suggests treating obesity or obesity-related disease by improving the structure of the GM balance to regulate metabolism, particularly lipid metabolism.

Currently, bioactive substances of natural drugs are becoming increasingly popular as a new approach with a highly safe and effective drug to prevent and treat obesity. *Salvia miltiorrhiza* extract (Sal) is a traditional Chinese medicine that includes water-soluble components such as Sal salyanolic acid, Sal aldehyde, and comfrey acid that can inhibit early adipogenesis[9] and alleviate lipid metabolism disorders[10]. Moreover, its Fat-soluble components, such as dihydrodanhinone I, *Salvia miltiorrhiza* ketone II_A, II_B, and cryptosanthoxylinone can reduce glycerol release[11], promote adipocyte differentiation, and can reduce the content of triglycerides and cholesterol[12] to treat fatty liver or coronary atherosclerosis[13]. Sal's main pharmacological effects include restraining the excitation of IκB-α and NF-κB, inhibiting the oxidation of LDL from regulating lipid metabolism processes and antioxidant effects, and improving the body's sensitivity to insulin by activating the AMPK pathway[14]. Sal is also used to treat atherosclerosis[15], hyperlipidemia, obesity, and other dyslipidemia-related diseases. Furthermore, Wang *et al*[16] found that the effect of Sal on regulating hepatic steatosis may be related to intestinal flora. Hence, this study further investigated the anti-obesity effects of Sal on high-fat diet (HFD)-induced obesity rats and a corresponding mechanism by focusing on GM and lipid metabolism.

MATERIALS AND METHODS

Animals

Sprague-Dawley (SD) male rats (7-8 wk, 160 g ± 20 g) were provided by the Hunan Silaike Jingda Experimental Animal Co. Ltd. (Changsha, China) (Animal Certificate Number: SCXK (Xiang) 2019-0004) and housed in SPF conditions (23.0 °C ± 2.0 °C ambient temperature, 50%-60% relative humidity, and 12/12h light/dark cycle) in Laboratory Animal Science and Technology Center of Jiangxi University of Traditional Chinese Medicine (Animal use license SYXK 2021-0007). The rats had free access to food

and water throughout the experiment. The protocol (Permit Number: JZLLSC2021-236) was approved by the Jiangxi University of Chinese Medicine Animal Care and Use Committee and performed by the guidelines prescribed by the committee. Experimental manipulation was performed after seven days of acclimatization.

Drug

Salvia miltiorrhiza (batch number: 200701) was supplied by Baishixin Chinese Herbal Pieces Co., Ltd. (Millizhou, China). Preparation of *Salvia miltiorrhiza* extract: The radix *Salvia miltiorrhiza* bunge was crushed into powder, dried at 58 °C, placed in 5000 mL round bottom flask, mixed with 75% ethanol (material-liquid ratio 1:10), soaked for 18 h, and placed in a water bath temperature 90 °C. A reflux extraction device was used to heat the reflux for 3 h, after which the filtrate was collected with four layers of gauze and left for 18 h after hot filtration.

The prepared Sal was freeze-dried with a vacuum freeze-dryer (Scientz-100F) and then smashed with zirconia beads using a mixer mill (MM 400, Retsch) at 30 Hz for 1.5 min. Then, 100 mg was dissolved with 1.2 mL of 70% methanol solution and vortexed for 30 s every 30 min 6 times, and the samples were placed in a refrigerator at 4 °C overnight. After centrifugation at 15984 g for 10 min, the extracts were filtered and then analyzed by UPLC-MS/MS. Analytical conditions and mass spectrometry data were based on Wang *et al*[17] and Chen *et al*[18], respectively. LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP) with the AB4500 QTRAP UPLC/MS/MS System operating in positive and negative ion mode (Figure 1A and B). For each period, a particular set of MRM transitions were observed by the metabolites eluted during this period (Figure 1C and D). Table 1 shows some of the metabolites identified in this study along with their metabolite numbers, integral values, and names.

Experimental design

According to previous researches[19-23], a HFD with purified ingredients and a total caloric value of 475 Kcal/100 g, with lard as the main source of fat (D12451, Research Diets Inc.) was used to induce obesity. The experiment scheme for the model of HFD-induced obesity in rats and drug administration is shown in Figure 2. At the initial phase, all the rats were divided into two groups: normal diet (control group; n = 8) and high-fat diet (HFD group; n = 40) for 7 wk and housed at 22.0 °C ± 1.0 °C ambient temperature. Then, the HFD groups were randomly subdivided into 5 groups: Control (HFD + normal saline), Sal_L (HFD + 0.675 g/kg/d Sal), Sal_M (HFD + 1.35 g/kg/d Sal), Sal_H (HFD + 2.70 g/kg/d Sal), and orlistat (HFD + 32.4 mg/kg/d) for 8 wk. The body weights were measured every 3 d and intake of food was measured per cage daily. After deducting the residual food from the initially supplied, the food intake (g/rat/wk) was determined. Rats were randomly selected from multiple cages, and cages were changed every two weeks to control for potential cage effects and sex/age differences.

Macroscopic observation

On the final day of the trial, all rats were euthanized with pentobarbital sodium (40 mg/kg intraperitoneally), weighed, and the liver and fat were quickly removed and weighed after blood was drawn from the abdominal aorta, and the body fat index (BFI) was calculated as follows: BFI = Total weight of fat/body weight of rat × 100%.

Histological analysis

Fresh liver and adipose tissue were fixed with 4% polyformaldehyde (PFA) at 4 °C, embedded in paraffin, and then cut into 4-μm thick slices. Samples were then stained with hematoxylin-eosin (H&E) and observed under a light microscope to examine the histopathology according to the published criteria by Yerian *et al*[24] and Liew *et al*[25].

Determination of serum biochemical parameters

The serum was centrifuged for 15 min at 999 g at room temperature. Serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), glucose (GLU), and free fatty acid (FFA) were measured by Beckmann COULTERAU480 automatic biochemical analyzer.

Enzyme-linked immunosorbent assay (ELISA)

To detect the content of cAMP in liver tissue, and HS and PKA in adipose tissue, parts of the liver and adipose tissue were homogenized under slow rotation (4 °C, 30 min) in 300 μL of RIPA buffer. The supernatant was obtained by centrifugation at 18759 g for 30 min. The concentration of cAMP, HSL, and PKA was determined by commercial ELISA kits (Thermo Fisher Scientific, Waltham city, MA, United States), and the absorbance was measured at 450 nm with a microplate reader (Thermo, Varioskan, MA, United States).

Microbial diversity analysis

Intestinal contents of all rats were collected in cryopreservation tubes and preserved at -80 °C. The microbial diversity analysis was conducted using I-sanger (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China; www.i-s anger.com). Microbial community genomic DNA was extracted from Intestinal contents using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States). The DNA extract was tested on a 1% agarose gel, and the concentration and purity of the DNA were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States). PCR reactions were run in triplicate.

PCR products were extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and quantitation using a Quantus™ Fluorometer (Promega, United States). Purified amplicons were equimolar and end-to-end sequenced on the MiSeq PE300 platform/NovaSeq PE250 (United States) under the

standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were uploaded to the NCBI Sequence Read Archive (SRA).

Multiplexing of raw 16S rRNA gene sequencing reads was performed, followed by quality filtering achieved with fast version 0.20.0 and merging with FLASH version 1.2.7. UPARSE version 7.1 for clustering operational taxonomic units (OTUs) with a 97 percent similarity cutoff. The RDP classifier version 2.2 was used for classification analysis of the 16S rRNA database with a confidence threshold of 70%. Calculation of the Shannon index, sobs diversity, and Principal coordinates analysis (PCoA) was used to assess alpha diversity using Mothur (version v.1.30.1). The Kruskal-Wallis H test and the Wilcoxon rank-sum test were used to identify taxa that significantly differed (biomarkers) between groups $P < 0.05$. I-sanger was used to perform community bar plot and heatmap analysis, correlation analysis, and co-occurrence network analysis.

LC-MS/MS analysis

A total of 50 mg of rat feces were mixed with 80 μL methanol and 400 μL MTBE for lipid extraction. The mixtures were vortexed for 30 s, followed by sonication frequency for 30 min and then precipitated at -20 °C for 30 min. The organic phase was separated by centrifugation at 18759 g for 15 min at 4 °C. Then, 350 μL lipid extracts contained in the upper phase were transferred to EP vials and dried in a vacuum concentrator. Consequently, the lipid extract was re-dissolved in 100 μL of isopropanol: Acetonitrile (1:1, v/v) solution, followed by 2 min vortexing and 5 min ultrasonication in an ice water bath. Finally, 80 μL of supernatant was transferred carefully to sample vials for LC-MS/MS analysis.

Mass spectral data were collected using a Thermo Q-Exactive Mass Spectrometer equipped with an electrospray ionization[26] source that can operate in either positive or negative ion mode. The raw data from the LC-MS analyses were imported into Lipid Search (Thermo, CA, United States) for peak detection, alignment, and identification. MS/MS fragments were used to identify the lipids. At least 80% of the liposomal features detected in any set of samples were retained. At least 80% of the lipidomic

features detected in any set of samples were retained. After filtering, minimum lipid values were performed for specific samples with lipid levels below the lower limit of quantification, and each lipid profile was summed and pooled. After pooling procedures and imputation, log-transformed data were statistically analyzed to determine significant differences in metabolite levels between comparable groups.

Statistical analysis

Most of the data and figures were statistically analyzed and produced using GraphPad Prism 7.0 software (San Diego, CA, United States). One-way ANOVA was performed on multiple groups, followed by Duncan's test to analyze statistical differences. For the data on the GM, we used the online platform of the Majorbio Cloud. The Wilcoxon rank-sum test was used to analyze alpha diversity. Weighted UniFrac distances were used to generate the PCoA plots. Kruskal-Wallis rank sum tests were used to analyze species differences between groups. All results were represented as mean ± SE. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of sal on body weight gain and fat accumulation in obese rats

After 8 wk of feeding, the body weight of the HFD rat was markedly higher ($P < 0.05$) compared to the control group ($n = 8$) (Figure 3A). However, the body weight decreased significantly in the Sal_H group ($n = 8$) from the 9th wk, and in Sal_L ($n = 8$) and Sal_M ($n = 8$) groups from the 10th wk compared with the HFD group (all $P < 0.05$). On the last day, the rat's final body weight (Figure 3B), waistline (Figure 3C), and body fat index (Figure 3D) in the HFD group were observably increased compared with that of the control group, while Sal supplementation notably reduced these indices without dose-dependence (all $P < 0.05$). No significant difference was found among these groups in food intake (Figure 3E) ($P > 0.05$). The above results suggest that Sal can reduce the indices of HFD-induced obesity without affecting appetite intake.

Effects of sal on serum lipid profiles, liver tissue, and adipose histopathology in hfd rats

A long-time HFD intake often results in abnormal lipid metabolism[27]. Serum lipid content can reflect the lipid metabolism in the body, among which TG, TC, LDL-C, and HDL-C are the most critical indicators to measure lipid metabolism. In our study, HFD feeding led to a significant increase in serum TG (Figure 4A), TC (Figure 4B), LDL-C (Figure 4C), GLU (Figure 4E), and FFA (Figure 4F) levels and a significant decrease in the HDL-C (Figure 4D) level (all $P < 0.05$), while Sal_L, Sal_M, and orlistat remarkably reduced TG and LDL-C levels without a dose-dependent relationship compared with the HFD group (all $P < 0.05$). However, Sal_H had no significant effect on TC and HDL-C ($P > 0.05$).

To assess the pathological injury in liver and fat tissue, pathological samples were prepared and stained using H&E staining. The liver tissue in the control group revealed that the hepatic lobule structure was ordered and tight (Figure 4G and H). By contrast, the H&E sample in the HFD group exhibited many infiltrations of pitting necrosis with balloon-like changes in hepatocytes, which revealed an obvious accumulation of lipid droplets in the livers of HFD-induced rats. However, Sal and orlistat treatment alleviated these pathological changes. These data further suggest that the HFD intervention for 7 wk leads to obesity, while Sal administration can effectively reduce dyslipidemia and hepatic lipid accumulation caused by HFD.

H&E of fat tissue was observed under a microscope, which showed that the outline of fat cells and volume became larger with the arrangement was loose in the HFD group (Figure 4I and J) compared with the control group. After treatment with Sal and orlistat, both the outline and volume of fat cells were reduced. The results showed that Sal alleviates hepatic steatosis and adipocyte hyperplasia in HFD rats.

Sal increases the activities of cAMP, PKA, and HSL in HFD rats

The cAMP-dependent protein kinase A (PKA) and HSL signaling system involved in promoting lipolysis as fat metabolic pathways are widely expressed and have a central

role in regulating metabolism in all organ systems affected by obesity. In the present study, the levels of cAMP (Figure 5A) in liver tissue, and HSL (Figure 5B) and PKA (Figure 5C) in adipose of the HFD group were markedly lower than those of the control group (all $P < 0.05$); contrary, these levels were significantly increased in HFD rat treated with Sal and orlistat treatment (all $P < 0.05$ *vs* HFD group). The results indicated that Sal regulates lipid metabolism by enhancing lipolysis in obese rats by HFD.

Sal improves the microbial composition of gut microbiome in HFD rats

Many studies have shown that GM dysbiosis has an essential role in the pathogenetic process of human obesity and animal obesity induced by HFD. Fecal samples from various groups were analyzed by 16S rRNA to investigate the regulatory effect of Sal on the GM composition in HFD rats. Twenty-four samples yielded a total of 1224808 sequencing reads. The Shannon index curve (Figure 6A), reflecting the alpha diversity of the intestinal flora, shows an adequate amount of sample sequencing data; the Venn diagram (Figure 6B) shows the overlap among the six groups at the OTU level. A total of 856, 810, 862, 889, 853, and 738 OTUs were identified in the control, HFD, Sal_L, Sal_M, and Sal_H groups, respectively. Statistical analysis of bioinformatics for OTUs at 97% similar levels found that 502 OTUs overlapped among groups. Compared with the control group, the number of OTUs in the GM was reduced in the HFD group, while Sal reversed the change. We think the main reason for the invalid effect of orlistat on OTUs of GM is that there was an anomalous sample in the orlistat group.

To determine which bacteria were improved through Sal and thus intervene in the disease progression of obesity, we analyzed the composition of GM in different groups. The community barplot analysis at the phylum levels (Figure 6C) showed that the relative abundance of *Proteobacteria*, *Actinobacteriota* decreased in the HFD group, and *Desulfobacterota* increased compared with that in the control, Sal, and orlistat groups. Compared with the control group at the genus level (Figure 6D), the relative abundances of *Aerococcus*, *Dubosiella*, *Psychrobacter*, and *norank_f_Lachnospiraceae* were significantly decreased, and the *Quinella* and *Turicibacter* were increased in the HFD

group. The community heatmap analysis at the genus level (Figure 6E) showed that the relative abundance of the *Blautia* was increased in the HFD group than in the control, Sal, and orlistat groups, while *Facklamia*, *Jeotgalicoccus*, *NK4A214_group*, and *Corynebacterium* were decreased.

Furthermore, β-diversity analysis, including non-metric multidimensional scaling (NMDS) and partial least squares discrimination analysis (PLS-DA), was used to assess the diversity variance among these six groups. NMDS (Stress = 0.15) (Figure 6F) revealed that the GM composition of the HFD group was completely separated from that of the control group. Although the aggregation of the Sal_L group was similar to that of the HFD group, the two groups were significantly separated, and the aggregation of the control group was significantly enhanced as the dose of Sal was increased.

The PLS-DA (Figure 6G) was further used to analyze the similarity and differences among the grouped sample. It demonstrated that the species distribution of the HFD group was separated from that of each dose group of Sal and the control and orlistat group, while the distance between Sal_M and the control group was shorter than that between the HFD and the control group. These findings suggest that Sal effectively improves GM composition in obese rats.

To further discover the regulatory effect of Sal on specific GM in obese rats, we analyzed species differences among the control, HFD, Sal_L, Sal_M, Sal_H, and orlistat groups. The genus-level Kruskal-Wallis H test bar plot (Figure 7A) showed that Sal and orlistat treatment markedly decreased the relative abundance of *Quinella* and *Blautia* in obese rats and significantly increased the abundance of *Facklamia*, *Corynebacterium*, *Psychrobacte* and *norank_f_Ruminococcaceae*. In addition, the Wilcoxon rank-sum test at the genus level showed that compared with the control, Sal_L, Sal_M, Sal_H, and orlistat groups, the relative abundance of these species in the HFD group was significantly decreased, including *Facklamia*, *Jeotgalicoccus*, *Aerococcus*, and *Dubosiella*, while *Lactobacillus*, *Turicibacter*, and *Quinella* increased significantly (Figure 7B-F). These

findings suggest that Sal is important in treating obesity by regulating the GM composition in obese rats.

Correlation analysis of gut microbiota

To further clarify the distribution between groups and species, we analyzed the correlation of species abundance information among different samples through network analysis to obtain the coexistence relationship of species in environmental factors. The association and model prediction by network analysis at the genus level (Figure 7G) showed that the top species were most closely related to these six groups according to a degree of weighting (Table 2), including *Romboutsia*, *Aerococcus*, *Turicibacter*, *Quinella*, *Facklamia*, *Corynebacterium* and *Desulfobacterota*. The evolution analysis by the Phylogenetic tree at the genus level (Figure 7H) indicated that the top species of the closest consanguinity relation were *Romboutsia*, *Lactobacillus*, *unclassified_Lachnospiraceae*, *Aerococcus*, *Turicibacter*, and *Quinella*, which is consistent with the conclusion of community Bar plot analysis, indicating that Sal exerts an anti-obesity role by regulating the above bacterial flora structure.

Finally, the functional prediction analysis (Figure 7I) revealed that these bacteria were primarily concentrated in energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism, biosynthesis, transport, and catabolism of secondary metabolites, lipid transport, and lipid oxidation, according to COG and KEGG orthology information and abundance. These findings suggest that the mechanism of Sal in obesity treatment involves energy and lipid metabolism.

Sal regulates lipid metabolism in obese rats

Lipid metabolism disorders are closely related to obesity and metabolic syndrome[28]. Here, non-targeted lipidomic studies of intestinal contents in rats were conducted to explore the effects of Sal on lipid metabolism. The number of differential metabolites between the control and HFD groups and between the HFD and the Sal groups is visualized in Figure 8A. In a statistically significant analysis, different metabolites were

identified where $P < 0.05$ and VIP > 1 were used to identify the different metabolites. In order to understand the metabolic differences between control, Sal, and HFD groups, the significant lipids data were analyzed using a heat map of metabolite cluster analysis, which showed the variation of each lipid in each group directly and illustrated the relative increase (red) or decrease (blue) tendency in the HFD compared with the control and Sal groups (Figure 8B). Interesting, the expression of the control group and the HFD group showed opposite trends; the Sal_M group was the closest to the control group trend in each Sal group, while the low and high dose groups of Sal showed the opposite trend when compared to control group which could be explained by the dose-response curve. Therefore, the Sal_M group was selected to make further comparisons of differential metabolites. Relative contents of identified lipids and fold changes in metabolites in the control and Sal_M groups compared with those in the HFD group were calculated to further investigate the magnitude of change in the significant lipids (Table 3). Increased TGs (TG18:2/18:2/20:4, TG16:0/18:2/22:6, TG16:0/14:0/22:6), DGs (DG14:0/22:6, DG22:6/22:6) and CL (18:2/18:1/18:1/20:0) were observed in the HFD rats. In contrast, the lipids including Cers (Cerd18:0/20:4, Cerd16:0/21:0, Cerd16:1/24:1), OAHFA (18:0/14:0), and Hex1Cers (Hex1Cer d18:0/16:0 + O, Hex1Cer d18:1/18:2 + 2O, Hex1Cer t18:0/16:0 + O, Hex1Cer t18:1/18:1 + 2O) showed a decreasing tendency in HFD rats. These results identified the up-regulated and down-regulated lipids in the obesity rat induced by HFD.

In order to further obtain the variation of the expression trend of the different metabolites among the control, HFD, and Sal_M groups, we performed VIP value analysis (Figure 8C-D). TG (16:0/14:0/22:6), TG (16:0/18:2/22:6), TG (18:2/18:2/20:4), Cer (d16:0/21:0), and DG (22:6/22:6) were the most significant metabolites among these groups. These metabolites are closely related to the development of obesity and might have an important role in the metabolizing dysfunction in HFD-induced obesity.

In pathway analysis, matched metabolic pathways were displayed based on the P value and KEGG pathway enrichment analysis (Figure 8E). A total of 7 pathways were found with $P < 0.05$, five of which were closely associated with obesity, including

regulation of lipolysis in adipocytes, insulin resistance, glycerolipid metabolism, fat digestion and absorption, and cholesterol metabolism. In general, these results demonstrate that Sal may exert an anti-obese effect by regulating lipid metabolism in obese rats.

The above results show that Sal can effectively regulate the blood lipid level and GM composition of obese rats. The distance-based redundancy analysis (db-RDA analysis) and spearman correlation heatmap were used further to investigate the correlation between lipid metabolism and the GM. Three sets of environmental factors were selected to establish the correlation between lipid metabolism and GM, including the physiological and biochemical indexes--FFA, weight, BFI, GLU, the key factors of lipid metabolism-cAMP, PKA, HSL, and the lipid molecules screened by lipidomics. According to the db-RDA analysis, the levels of FFA, Weight, BFI, and GLU of obese rats in the HFD group were opposite to the GM abundance in control, Sal_L, Sal_M, and Sal_H groups on the genus level (Figure 8G). Analysis of the Spearman-related heatmap (Figure 8F) at the genus level found that TGs, DGs, and Cers are positively correlated with *Quinella*, *Desulfovibrio*, *Blautia*, and *Turicibacter*, and negatively correlated with *Aerococcus*, *Dubosiella*, *Facklamia*, *Jeotgalicoccus*; cAMP, PKA, and HSL were positively correlated with *Corynebacterium*, *Aerococcus*, *Dubosiella*, and *Facklamia* and negatively correlated with *Desulfovibrio* and *Blautia* (Figure 8H), which agreed with our previous findings about the structure of the microbiome. The above analysis hinted that Sal participated in the regulation of the microbial community's structure and environmental characteristics, as well as the regulation of lipid metabolism, which helps to promote lipolysis and prevent adipogenesis.

DISCUSSION

In the present study, the weight loss effect of Sal on HFD rats is evident, which was proven by the decrease in weight and BFI without changing food intake compared to the HFD groups with developed increased weight, obesity indexes, and lipid profiles. Moreover, we observed that Sal significantly down-regulated the levels of TG, TC, LDL-

C, GLU, and FFA in the serum of obese rats. Additionally, H&E analysis revealed that the liver tissue from HFD-induced obese rats treated with Sal displayed reduced mean adipocyte size, increased smaller adipocytes, and alleviated hepatic steatosis. Sal can improve dyslipidemia and reduce obesity by preventing the buildup of excess lipid compositions in the liver that can cause fatty liver and dyslipidemia in obese people. Similarly, after Sal intervention, smaller adipocytes are observed in adipose tissue. These results suggest that Sal has promising anti-obesity effects associated with reducing HFD-induced body weight independent of food intake.

Salvia miltiorrhiza extract has been proven to have an anti-inflammation[29] and antioxidation effect, which is widely used to treat cardiovascular disease[30], diabetes[31], fatty liver disease[32], and other dyslipidemia diseases[33]. Jung *et al*[9] reported that TanI (Tanshinone), an ingredient in the Sal extract, can inhibit HFD-induced obesity by preventing early adipogenesis and improving lipid metabolism. Importantly, we found that Sal exerts anti-HFD-induced obesity by improving blood lipid levels, and regulating GM and metabolites, thus further suggesting the potential of Sal for clinical treatment of obesity.

As an environmental factor, the GM interaction with the host[34] has an essential role in the occurrence and development of obesity[35]. Previous studies reported lower GM diversity in obese mice than in the control group, and obese mice showed a decrease in *Bacteroides* and an increase in *Firmicutes*[36,37]. Armougom *et al*[38] found more *Methanobacter*, *Bacteroidetes*, and *Lactobacillus* in anorexic patients. GM imbalance is one of the pathogeneses of obesity. In this study, we found that obese rats have a different gut flora composition than lean rats, with a higher abundance of *Desulfobacterota*, *Quinella*, *Turicibacter* and a lower abundance of *Jeotgalicoccus*, *NK4A214*, *Aerococcus*, *Dubosiella*, *Psychrobactor*, *norank_f_Lachnospiraceae*, and *norank_f_Erysipelotrichaceae* at the genus level and *Proteobacteria*, *Actinomycetes* at the phylum level. Studies have suggested that *Firmicutes*, including *Lachnoaceae*, *Erysipelotrichaceae*, *Oscillospiraceae*, and *Ruminococcaceae*, can promote energy absorption in obese and diabetic rats[39]. In this study, we found that Sal decreased the abundance of *Firmicutes*, including

norank_f_Lachnospiraceae and *Dubosiella*, in HFD-induced obesity rats. Similar results were observed in HFD rats treated with polyphenols and procyanidins[40,41]. *Bacteroidetes*, a beneficial bacterium, have been reported to be negatively associated with obesity and hyperlipidemia. Previous studies have suggested that the proportion of *Bacteroidetes* and *Proteobacteria* is slightly higher than that of the control population[27]. Interestingly, when HFD successfully induced obesity in rats, we found an increased abundance of *Bacteroidetes* at the phylum level. Our results are consistent with Schwiertz's evidence of a significant increase in *Bacteroidetes* in overweight and obese subjects[42]. We believe this is because factors, such as heredity, diet, and environment, may have similar or even opposite effects on the flora. Lipid factors involved in metabolism may also interfere with the structure of GM through various mechanisms. Studies have shown that FXR agonists regulate lipid metabolism and GM by increasing the presence of *Ackermann* and decreasing the presence of *Desulfuricans* in obese mice[43], which is consistent with our findings.

One of the consequences of dyslipidemia is inefficient lipid metabolism, which in turn accelerates the development of obesity and dyslipidemia[44,45]. Lipids metabolism disorders provide the "first hit" in the progress of metabolic diseases such as obesity[46]. Hence, this study applied lipidomics to analyze the composition of lipid metabolites in obesity and observed the alteration of lipid metabolites by increasing the treatment of Sal to analyze the molecular mechanism of the anti-obesity and cholesterol-lowering effects of Sal, thus providing new insights into the treatment of obesity. A range of significant changes occurred in the lipidome with progressive obesity. Most notably, the contents of DGs, TGs, and CLs were increased in the HFD group compared to the control group, which was similar to previous studies[47]. Contrary, Sal decreased the levels of these lipid species in the lipidome profile, suggesting that Sal could improve the imbalance between HFD-induced lipid synthesis and catabolism. The concentration of FFA in serum is related to lipid metabolism and glucose metabolism, and diseases such as diabetes and obesity can increase FFAs' concentration. When energy metabolism is abnormal, FFA will accelerate the accumulation of TG in hepatocytes or

convert to lipid intermediates such as DGs, CLs, and Cer, depleting cellular functions and, in turn, leading to obesity and other metabolic disorder diseases[48]. Interestingly, we found that the supplementation of Sal effectively decreases the hepatic accumulation of TGs, DGs, and CLs. Evidence suggests that Cer is involved in obesity-induced metabolic disorders by various mechanisms, including inflammation, apoptosis, and autophagy. However, in our study, Cer was not entirely increased in the HFD-induced obese rat. We wondered whether Cer might interfere with obesity by participating in other pathways or whether the balance of sphingolipid (SP) metabolism, rather than Cer accumulation, is correlated with the development of obesity[49]. SP species are not only increased in obese patients but are also associated with hepatic oxidative stress, suggesting that these lipids may participate in the progression of obesity[50]. In general, triglyceride accumulation is a sign of obesity and is positively correlated with hexosylceramide (HexCer); yet, in our experiment, Hex1Cer, which acts as a sphingolipid, was reduced in the HFD rats and negatively correlated with triglycerides. Part of that has to do with the small number of animals analyzed, and the role of Hex1Cer in obesity is not quite clear yet. Our results were consistent with Eisinger's study[51]. Some researchers also believe that pharmacological blockage of glucosylceramide prevents obesity and liver steatosis[3,52,53]. In addition, we found that a few fatty acids, such as OAHFA, a novel lipid in structure with functions such as stimulating insulin secretion and improving glycolipid transport *in vivo*[54,55], were increased in HFD rats treated with Sal. These lipids are structurally novel lipids that are provided. It is worth mentioning that some beneficial lipids are important to the development of obesity, such as PC (phosphatidylcholines)[56], which can regulate lipids and cholesterol metabolism[57]. Although it did not appear in our study, it is also one of the focuses of our future research.

Pathway enrichment analysis can assess the biochemical pathways involved in significantly different metabolites and their functional classification. Significant pathways enriched by KEGG, including lipolysis in adipocytes, insulin resistance, glycerolipid metabolism, fat digestion and absorption, and cholesterol metabolism,

have been shown to be closely associated with the development of obesity, among which metabolism of cholesterol had the greatest significance, with *P*-values of 0.0016. Moreover, the dysfunction of glycerolipid metabolism may disturb the energy metabolism of hepatocytes and adipocytes. Furthermore, obese patients are often accompanied by hormonal dysregulation, which aggravates lipid metabolic disorders. For example, as one of the anti-lipolysis hormones, insulin has an important role in reducing lipolysis by reducing cAMP concentration in adipocytes. The increase of cAMP catalyzes the hydrolysis of triacylglycerol to fatty acids and glycerol and then completes the lipolysis process, and the protein kinase (PKA) with hormone-sensitive lipase (HSL) is involved[2]. Similarly, increases in FFAs can also cause insulin-mediated decreases in glucose oxidation and utilization, increase insulin resistance and cause hyperinsulinemia and obesity. In our study, the levels of cAMP, PKA, and HSL were decreased in the HFD group compared to the control group, while GLU and FFA were increased. Spearman-related heatmap analysis further showed that cAMP, PKA, and HSL had a negative correlation with the structure of the GM in the HFD group, while FFA, weight, BFI, and GLU were positively correlated with GM.

It is widely believed that the regulation of intestinal microbiota on lipid metabolism is closely related to bile acids and short-chain fatty acids (SCFA). According to Zhang *et al*[58], increasing the SCFA-producing genera *Blautia* and *Allobaculum* could enhance intestinal integrity and reduce the body weight in obese mice[59], thus rivaling obesity. *Allobaculum* is a member of *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae*, a family that affects host metabolism[60] and protects body weight gain from HFD. *Allobaculum* can produce butyric acid and propionic acidand[61,62]. In our research, analysis of the spearman-related heatmap showed that lipids including TGs, DGs, and CL that increased in the HFD group were inversely associated with *Allobaculum* and positively related to *Blautia*, which suggests that Sal may improve *Allobaculum* but not *Blautia* to producing SCFA and then regulating the GM and lipid metabolism. Acetate, propionate, and butyrate are examples of SCFA produced by bacteria, and research showed that SCFA-induced activation of the PPAR pathway could modulate lipid

metabolism by increasing energy consumption[63], reducing body weight, and decreasing liver triglyceride accumulation[64]. It should be noted that the genus *Blautia*, as a member of the *Lachnospiraceae* family, could produce SCFAs, which can regulate inflammation and metabolism[65]. Consequently, the observation in our research that Sal promoted a decrease in *Lachnospiraceae* and *Allobaculum* indicated that Sal likely confers beneficial effects *via* modulating the GM composition and host lipid metabolism with SCFA, which is consistent with previous reports[66,67]. However, whether *Lachnospiraceae* has a key role in the regulation of obesity and lipids still needs to be confirmed by accurate tests.

Desulfovibrio has been reported to be positively correlated with metabolism[68,69] and may increase after a high-fat diet[70,71]. Similar to our findings, *Desulfovibrio* was reported to be decreased by the intervention of Sal. We also found that the improvement of gut microecology resulting in the increase of SCFAs may be responsible for the cholesterol-lowering effects of obesity. There is a reciprocal relationship between bile acids and cholesterol metabolism, which is closely related to intestinal flora. Bile acids are synthesized from cholesterol in the liver and have a role in maintaining cholesterol homeostasis and promoting lipid absorption[72]. Intestinal flora regulates lipid metabolism in the host by affecting bile acid composition. GM could influence bile acid metabolism by performing structural modifications, including oxidation, deconjugation, or hydroxylation[73]. It has been confirmed that bile acids and bile acid signaling pathways are involved in the control of plasma lipid and lipoprotein levels and that hepatic bile acid synthesis has an important role in the regulation of plasma triglyceride levels in obese individuals[74], which is consistent with the present study that the cholesterol metabolism was most concentrated in KEGG pathway with the elevated cholesterol and triglyceride in the serum and the significant elevation of TGs in lipidomics, while Sal may regulate the relationship between bile acids and cholesterol metabolism by reducing cholesterol accumulation and regulating lipid metabolism.

The above experimental results suggest that HFD-induced obesity causes dyslipidemia and dysregulation of GM and metabolites, accompanied by weight gain, hepatic steatosis, and abdominal fat accumulation. Importantly, we found that Sal effectively improved blood lipids and reshaped the balance of GM and lipid metabolism in obese rats (Figure 9), reversing weight gain and fat accumulation. Correlation analysis further demonstrated that Sal exerted anti-obesity effects through lipid metabolites of intestinal flora, which laid a good foundation for the subsequent study. In the follow-up study, the key components of Sal's anti-obesity were further investigated by pharmacokinetic and high-performance liquid chromatography techniques. The key flora and metabolites of Sal's anti-obesity were further demonstrated by intestinal flora transplantation and lipid metabolite supplementation, and the key targets of Sal's anti-obesity were further explored by molecular docking or protein interaction and gene enrichment and editing techniques, which will become a promising way to find new targets for obesity.

CONCLUSION

Our results showed that Sal reduces body weight, body fat index, serum lipid level, hepatic lipid accumulation, and adipocyte vacuolation in HFD rats, which may be associated with enhanced gut integrity and improved lipid metabolism. 16s RNA analysis revealed that Sal could reverse HFD-induced dysbacteriosis while LC-MS/MS analysis indicated that Sal could improve the lipid composition of HFD rats, which provides research basis and evidence to study the mechanism of Sal in the treatment of obesity.

ARTICLE HIGHLIGHTS

Research background

Obesity is a world health problem. A growing number of studies have suggested that gut microbiota is an important regulator of host metabolism, and the dysregulation or imbalance of gut microbiota (GM) is closely related to obesity and its complications.

Mounting evidence suggests that improving the structure of the GM balance to regulate metabolism, particularly lipid metabolism, is a viable strategy for treating obesity or obesity-related disease.

Research motivation

Salvia miltiorrhiza (Sal) has shown good efficacy in experimental obese rats induced by a high-fat diet (HFD). Also, disturbances in gut microbiota have been observed in various diseases, including metabolic disease. However, few studies have explored the role of Sal on gut microbiota and lipid metabolism when treating obesity.

Research objectives

To investigate whether Sal can alleviate obesity induced by HFD by regulating gut microbiome and lipid metabolism.

Research methods

Rats were induced by the HFD (with purified ingredients and a total caloric value of 475 Kcal/100 g, with lard as the main source of fat) for 7 wk, while Sal (0.675 g/1.35 g/2.70 g/kg/d) was administered for 8 wk. Serum lipid test, liver and fat tissue histopathologic examination, ELISA, 16s RNA, and LC-MS/MS analysis were used to evaluate the efficacy of Sal on obesity.

Research results

Sal effectively improved blood lipids and reshaped the balance of gut microbiota and lipid metabolism in obese rats, reversing weight gain and fat accumulation caused by HFD. Correlation analysis further demonstrated that Sal exerted anti-obesity effects through lipid metabolites of intestinal flora, which laid a good foundation for the subsequent study.

Research conclusions

Sal extract may exert an anti-obesity effect in HFD-induced obese rat by modulating the gut microbiome and lipid metabolism.

Research perspectives

The manuscript addresses an important topic of the development of obesity, *i.e.*, the role of gut microbiota and lipid metabolism in the development of obesity, using an extract from a Chinese herb that has been found to have anti-obesity effects in various diseases.

Figure 1 UPLC-MS/MS analysis of *Salvia miltiorrhiza* extract. A: Total ions Current-N of QC_MS; B: Total ions Current-P of QC_MS; C: MRM detection of multimodal maps-N; D: MRM detection of multimodal maps-P.

Figure 2 *In vivo* experiment schemes. Sal: *Salvia miltiorrhiza*.

Figure 3 Therapeutic evaluation of *Salvia miltiorrhiza* ameliorates high-fat diet-induced obesity in rat. A: Body weight change; B: Rats final weight; C: Waistline; D: Body fat index; E: Food intake. Values are expressed as mean ± SE and analyzed by one-way ANOVA Duncan's multiple comparison test, n = 8. HFD: High-fat diet. Compared with the control group, $^{a}P < 0.05$, or $^{b}P < 0.01$; compared with the HFD group, $^{c}P < 0.05$ or $^{d}P < 0.01$.

Figure 4 Effects of *Salvia miltiorrhiza* on serum lipid profiles, glucose, free fatty acid levels and histopathological changes of the liver and adipose tissue in HFD-fed rat. A-D: Serum serum triglyceride, total cholesterol, low-density lipoprotein, and high-density lipoprotein levels; E: Serum glucose level; F: Serum free fatty acid level; G and

H: Representative images of hematoxylin-and eosin-stained (H&E) sections of liver tissue (400 ×) in the six groups (a: Lipid droplets accumulation; b: Punctate necrosis of hepatocytes with inflammatory cell infiltration; c: Balloon-like changes) (Scale bar: 20 μm); I and J: Representative images of H&E sections of adipose tissue (400 ×) in the six groups (Scale bar: 20 μm). Significance between every group was calculated using one-way ANOVA Duncan's multiple comparison test. Data are shown as mean ± SE. $^{*}P < 0.05$ or $^{**}P < 0.01$. Error bars represent standard error.

Figure 5 The levels of cAMP in liver tissue and HSL, PKA in adipose tissue of high-fat diet rat. A: cAMP expression; B: PKA expression; C: HSL expression. Data are presented as mean ± SE ($n = 8$). HFD: High-fat diet. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the HFD group.

Figure 6 Beneficial effects of *Salvia miltiorrhiza* are associated with the improved gut microbiome composition in high-fat diet rats. A: α-diversity analysis: Shannon curves at the operational taxonomic unit (OTU) level; B: Venn diagram; C: Community bar plot analysis at the phylum level; D: Community bar plot analysis at the genus level; E: Community heatmap analysis of 30 species at the genus level; F: Non-metric multidimensional scaling (NMDS) at the OTU level; G: Partial least squares discriminant analysis (PLS-DA) score on OTU level.

Figure 7 Effect of *Salvia miltiorrhiza* on intestinal microbiota composition in six groups of high-fat diet rats and some analysis results. A: Differential analysis among these six groups at the genus level; B-F: Differential analysis compared to control and high-fat diet (HFD) group, HFD and Sal_L group, HFD and Sal_M group, HFD and Sal_H group at the genus level ($^{*}P < 0.05$, $^{**}P < 0.01$); G: Network analysis on genus level; H: Phylogenetic tree on genus level; I: COG functional classification.

Figure 8 Lipidomics analysis and associations of gut microbial species with environmental factors. A: Numbers of differential metabolites between the control and high-fat diet (HFD) groups, HFD and Sal_L groups, HFD and Sal_M groups, and HFD and Sal_H groups (Venn diagram); B: Hierarchical clustering of metabolites in Control, HFD, Sal_L, Sal_M, and Sal_H groups; C: Heatmap of the VIP expression profile of the metabolite between control and HFD groups; D: Heatmap of the VIP expression profile of the metabolite between HFD and Sal_M groups; E: KEGG pathway enrichment; F: Spearman's correlation between metabolites and gut microbiota; G: Correlation between free fatty acid, Weight, body fat index, glucose, and microbial flora structure displayed by distance-based redundancy analysis (db-RDA analysis); H: Spearman's correlation between cAMP, PKA, HSL, and gut microbiota. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

Figure 9 Graphical abstract of the mechanism of action of *Salvia miltiorrhiza* to alleviate obesity. *Salvia miltiorrhiza* ameliorated obesity by reshaping the balance of gut microbiota, modulating the lipid metabolites and improving blood lipids. Sal: *Salvia miltiorrhiza*; HFD: High-fat diet; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein; LDL-C: Low-density lipoprotein; GLU: Glucose; FFA: Free fatty acid.

Table 1 The metabolite number, the integral value, and the corresponding metabolite name of some metabolites detected in the *Salvia miltiorrhiza* extract

Index	Compounds	Class I
pme2292	Putrescine	Alkaloids
pmf0096	Oxalic acid	Organic acids
Zmyn000268	2,3-Dihydroxypropanal	Others
pme2601	3-Hydroxypropanoic acid	Organic acids
MWS1787	2-Picoline; 2-Methylpyridine	Alkaloids
pma6298	3-Hydroxypyridine	Alkaloids
MWSmce460	2-Piperidone	Alkaloids
MWS1990	4-Pentenoic acid	Organic acids
MWSmce461	L-Azetidine-2-carboxylic acid	Alkaloids
MWStz073	5-Hydroxy-2-pyrrolidinone	Alkaloids

Table 2 Node name represent species nodes

Node name	Degree	Weighted degree
g_Romboutsia	6	36385.41667
g_Aerococcus	6	13881.41667
g_Turicibacter	6	10477.08333
g_Quinella	6	269.16667
g_Facklamia	6	6077.25000
g_Corynebacterium	6	4418.00000
g_Desulfovibrio	6	2716.58333

The degree is the degree of the node. Weighted degree, which means the degree weight of the node, is the number of species sequences corresponding to the node.

Table 3 Differential metabolites in feces after Sal treatment

NO	Metabolites	Rt (min)	m/z	Formula	VIP		FC		Trend	
					H *vs* C	S *vs* H	H *vs* C	S *vs* H	H *vs* C	S *vs* H
1	Cer (d18:0/20:4)	6.72	588.54	$C_{38}H_{70}O_3N_1$	1.34	1.67	1.07	0.90	↓[a]	↑[c]
2	DG (14:0/22:6)	6.62	630.51	$C_{39}H_{68}O_5N_1$	1.51	1.91	0.91	1.14	↑[a]	↓[d]
3	DG (22:6/22:6)	6.56	730.54	$C_{47}H_{72}O_5N_1$	1.54	2.01	0.91	1.15	↑[a]	↓[c]
4	Hex1Cer (d18:0/16:0 + O)	6.01	718.58	$C_{40}H_{80}O_9N_1$	1.07	1.01	1.03	1.01	↓[a]	↑[c]
5	Hex1Cer (d18:1/18:2 + 2O)	5.76	756.56	$C_{42}H_{78}O_{10}N_1$	1.13	1.12	1.04	1.12	↓[a]	↑[c]
6	Hex1Cer (t18:0/16:0 + O)	5.79	734.58	$C_{40}H_{80}O_{10}N_1$	1.26	1.20	1.05	1.20	↓[a]	↑[c]
7	Hex1Cer (t18:1/18:1 + 2O)	5.80	756.56	$C_{47}H_{72}O_5N_1$	1.13	1.14	1.04	1.14	↓[a]	↑[c]
8	MGDG (16:1/17:2)	5.70	756.56	$C_{47}H_{72}O_5N_1$	1.13	1.14	1.04	0.96	↓[a]	↑[c]
9	TG (6:0/9:0/18:2)	4.39	615.46	$C_{36}H_{64}O_6Na_1$	1.23	1.36	0.95	1.06	↑[a]	↓[c]
10	TG (4:0/14:1/18:3)	6.29	631.49	$C_{39}H_{67}O_6$	1.34	1.52	0.93	1.08	↑[a]	↓[c]
11	TG (12:1e/6:0/18:4)	5.82	615.50	$C_{39}H_{67}O_5$	1.82	1.86	0.90	1.12	↑[b]	↓[c]
12	TG (16:0/14:0/22:6)	12.21	868.74	$C_{55}H_{98}O_6N_1$	1.87	2.27	0.87	1.18	↑[a]	↓[d]

13	TG (16:0/18:2/20:5)	12.82	877.73	$C_{57}H_{97}O_6$	1.46	1.68	0.94	1.08	↑[a]	↓[c]
14	TG (15:0/18:2/22:6)	11.72	895.74	$C_{58}H_{96}O_6Li_1$	1.45	1.92	0.92	1.13	↑[a]	↓[d]
15	TG (18:2/18:2/20:4)	11.79	920.77	$C_{59}H_{102}O_6N_1$	1.83	2.22	0.87	1.19	↑[a]	↓[c]
16	TG (16:0/18:2/22:6)	12.36	925.73	$C_{59}H_{98}O_6Na_1$	2.24	3.01	0.80	1.36	↑[a]	↓[e]
17	TG (18:2/17:1/22:6)	11.60	921.75	$C_{60}H_{98}O_6Li_1$	1.49	1.78	0.91	1.12	↑[a]	↓[c]
18	CL (18:2/18:1/18:1/20:0)	5.57	741.53	$C_{83}H_{152}O_{17}P_2$	1.50	1.31	0.95	1.05	↑[a]	↓[c]
19	Cer (d16:0/21:0)	8.47	626.57	$C_{38}H_{76}O_5N_1$	2.14	1.89	1.12	0.88	↓[a]	↑[c]
20	Cer (d16:1/24:1)	9.02	664.59	$C_{41}H_{78}O_5N_1$	1.87	1.66	1.11	0.89	↓[a]	↑[c]
21	OAHFA (18:0/14:0)	5.70	756.56	$C_{47}H_{72}O_5N_1$	1.32	1.18	0.95	0.94	↓[a]	↑[c]

[a]$P < 0.05$, [b]$P < 0.01$ as compared to the control group.

[c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.01$ as compared to the high-fat diet (HFD) group.

Control, HFD, and Sal_M (n = 8 per group) groups. ↑, metabolite content increased; ↓, metabolite content decreased; *vs*: *Versus*; C: Control group; H: High-fat diet group; S: Sal_M group.

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