Hepatic Lipidomics Reveals the Homeostasis and Profile of Sphingomyelin from Yak Butter in Normal-Fat Diet-Fed Rats

Xin Luo¹, Wancheng Sun¹, and Yihao Luo¹

¹College of Agriculture and Animal Husbandry, Qinghai University

Address for correspondence:
Prof. Yihao Luo
College of Agriculture and Animal Husbandry, Qinghai University Ning Da 251, Qinghai Province, Xining 810016, China
E-mail: sun0108@163.com
Tel.: +86 13519780623
Abstract

Background: Dietary sphingomyelin was showed to inhibit the uptake of lipids in mice fed with a high-fat diet, however, the effect of sphingomyelin on normal diet was on reported. The current study aims to examine the effects of sphingomyelin extracts from yak butter on hepatic steatosis and inflammation in C57/B6J mice fed with a normal diet.

Methods: A UHPLC-QTOF-MS based lipidomics method was utilized to screen the liver metabolites and predict the dominant potential metabolic pathways after sphingomyelin feeding.

Results: The results showed that sphingomyelin extracts reduced the accumulation of lipid droplets, suppressed the expression of pro-inflammatory factors IFN-γ, IL-6 and TNF-α, synchronously, promoted the expression of anti-inflammatory factors IL-10, IL-4 and IL-1Ra. In addition, sphingomyelin extracts exhibited the modulation on liver lipid metabolism when supplement sphingomyelin in normal diet for one month and five months. Specifically, 16, 68 different metabolites and 2, 6 metabolic pathways were identified by quantitative lipidomics, respectively. Six CERs including Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d17:1/22:0), Cer(d17:1/24:1), Cer(d17:1/24:0) and Cer(d17:0/26:1), six SMs including SM(d15:0/24:1), SM(d14:0/26:1), SM(d14:1/24:1), SM(d15:1/22:0), SM(d15:1/24:1) and SM(d19:1/26:1), and PS(18:1/22:6) were identified and can be used as potential biomarkers of steatosis and inflammation.

Conclusions: This study highlighted the effects of yak butter sphingomyelin on hepatic steatosis, tissue inflammation and lipid metabolism of mice under a normal diet.

Keywords: sphingomyelin, lipidomics, steatosis, inflammation, lipid metabolism
Introduction

Yak butter was a kind of butter like a dairy product, which was the fat extracted from yak milk. It was one of the main dietary manner for herdsmen to eat fat in Qinghai Tibet plateau area. The special plateau climate endowed yak milk with higher nutritional value than ordinary milk [1]. The Tibetan people exhibited adaptive capacity under environment at altitude of 3000 - 5000 m, which was related to their regular intake of yak butter [2]. It not only provided sufficient energy for organismal metabolism, but possessed favourable nutritional and health care effect [3]. Yak butter contained several vitamins, which showed bright yellow in spring-produced, turned light yellow in winter-produced. Compared with common cream, it has higher nutritional value, higher sodium carbonate and nearly eight times and twice of trans oleic acid and conjugated linoleic acid, respectively. These polyunsaturated fatty acids have the effect of lowering blood lipids and reducing inflammatory reaction [4].

Sphingomyelin (SM) consisting of ceramide and phosphorylcholine moieties belonged to the sphingolipid analogue of phosphatidylcholine, which was vital precursor of intracellular mediators [5]. SM was also a component of microdomains and lipid rafts, which played the functional role in several immune responses and intercellular signaling [6]. Numerous foods contain different types of SM. In some fruits and vegetables, the content of SM was less than 100 μmol/kg, while it was more than 2000 μmol/kg in eggs and dairy products [7]. In recent years, studies was showed that sphingomyelin was closely related to some diseases caused by fatty acids, cholesterol and mycotoxins in human body. Therefore, sphingomyelin in food has gradually attracted people's attention [8]. Dietary sphingomyelin, a sphingolipid found exclusively in animals, was showed to dose-dependently reduce the absorption of cholesterol, triglyceride and fatty acids both in vitro [9,10] and in rat models [11,12]. In addition, it effective prevented diet-induced obesity, adipose tissue inflammation and hepatic steatosis [13]. SM from different dietary sources has different amide-linked fatty acids and sphingosine bases [14,15], which may have different biological effects on lipid metabolism in vivo [11,16]. Nowadays, few studies have evaluated the effects of chronic dietary SM intake on lipid metabolism in high fat, except for eggs and milk [16-19].

Lipidomics was a branch of metabolomics, which was the methodology for qualitative and quantitative analysis of lipid compounds and for understanding their functions and changes under different physiological and pathological conditions. New analytical techniques applied to lipidomics have greatly promoted the development of lipidomics. The main methods of lipidomics included nuclear magnetic resonance (NMR), gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS), and GC-MS [20]. Among these methods, LC-MS with high resolution and high throughput has become the most commonly used technique in the analysis of lipidomics [21]. It could better identify low-abundance lipids and achieve high-throughput and quantitative detection of hundreds of lipids [22]. Via LC-MS/MS based lipidomics technologies, masses of
researchers analyzed the lipid components in the serum and liver of experimental animals, and then identified potential biomarkers and revealed the process of disease occurrence and development [23]. It brings us inspiration to study the biological activity of sphingomyelin by lipidomics.

The current study evaluated the effects of dietary SM from yak butter on steatosis, tissue inflammation and lipid metabolism in C57BL/6J mice. The aims of this study was to observe the characteristics of lipid metabolism in mice based on a quantitative lipidomics approach, screen related differential metabolites, analyze the lipid metabolism status and the relationship with diseases through the change of relative content of lipid metabolites, so as to reveal the regulatory mechanism of sphingomyelin on diseases from the metabolic level. Our data may lay a theoretical foundation for further development and utilization of dietary sphingomyelin.

Methods

Animals and diets

Four-week-old male mice (Hos: C57BL/6J, SPF level, Veterinary Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China) were used in this study. All mice were housed in plastic cages (five mice/cage) in the animal facility of Medical College of Qinghai University under a 12 hr light-dark cycle. After acclimatization for one week, mice were randomized into two groups (control group and treatment group, n = 10/group). The control group was given a maintenance diet, while the other group was given a maintenance diet with 1.2% of SM added as previously described [17]. Detailed composition of the diets is listed in Table 1. The analysis was carried out after one month and five months, respectively. All of the animal experiments in this study were approved by The Institutional Animal Care and Use Committee of Qinghai Province.

Preparation of sphingomyelin: Yak butter was melted by constant temperature oscillation at 45 °C. Neutral lipids were eluted by adding n-hexane. Oil liquid was obtained by rotating evaporation apparatus (50 °C). Then, acetone was added, the sample was left overnight in refrigerator at 4 °C. After vacuum filtration and drying, powdered phospholipids insoluble in acetone were obtained. In the dried phospholipid compound, ether was added (a ratio of material to liquid was 1:10), extracted at 30 °C for 25 min, and centrifuged at 3,500 rpm for 10 min to obtain crude sphingomyelin. Chloroform: methanol (2:1, V/V, a ratio of material to liquid was 1:10) was added into the dried crude sphingomyelin, which was dissolved, concentrated and dried in vacuum. The dried solid was purified sphingomyelin. All reagents were provided by Tianjin Fuyu Fine Chemical Co., Ltd. (Tianjin, China).

Fresh food was provided daily and mice were allowed free access to the diet and water. Body weights and food intake were recorded on a weekly basis. After one month and five months on their respective diets, mice were fasted for 8-10 h prior to
blood collection by eyeball. Blood was allowed to clot at room temperature for 30 min before serum was isolated by
centrifugation (2,000 rpm for 10 min at 4 °C) and then stored at -80 °C. The adipose of abdominal, scapular, perirenal and liver
tissues were collected from animals. Then, snap-frozen in liquid nitrogen and stored at -80 °C. The liver was harvested for
histology and lipid analysis. Liver and adipose tissues were fixed in 4% paraformaldehyde (Biosharp, China) for at least 48 h
prior to histological processing.

**Serum biochemical analysis**

Total serum cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL) and fasting
glucose were measured using kits supplied by Nanjing Jiancheng Bio-Engineering Institute Co., Ltd. (Nanjing, Jiangsu, China).

**Tissue histology**

Paraformaldehyde-fixed liver, and abdominal and perirenal adipose tissues were embedded in paraffin and cut into 5-μm
sections prior to staining with hematoxylin and eosin (H&E). All histological procedures were conducted at the Super Biotech
Co., Ltd. (Nanjing, Jiangsu, China). The stained tissue sections were viewed under Olympus BX 45 bright field microscopy.

**Determination and analysis of fatty acids**

Methylation: (1) Preparation of free fatty acid: take 0.1 g of sample into glass tube, add 10 ml of methanol and 1 g of potassium
hydroxide, seal the tube mouth, and shake at 65 °C for 2 h.

(2) Acidification: after cooling, add concentrated hydrochloric acid to acidify to pH 2-3.

(3) Extraction: add 10 ml of n-hexane, shake it, leave it for 10 min to separate layers, take n-hexane layer and put it into another
drying tube.

(4) Drying: dry n-hexane with nitrogen to obtain dry free fatty acids.

(5) Methyl esterification: take the dried fatty acid and add 1 ml of BF3 whose mass fraction is 15%, seal the tube mouth, and
take a water bath at 90 °C for 2 h.

(6) Extraction and demulsification: after cooling, add 2 ml of n-hexane into the centrifuge tube, shake, add 2 ml of saturated
NaCl solution, seal and shake, centrifuge for 2 min at 3,500 rpm, and take n-hexane layer into another centrifuge tube.

(7) Drying: add 1 g anhydrous sodium sulfate for dehumidification, shake, centrifugation at 3,500 rpm for 2 min, take n-hexane
layer and put it into another drying tube, dry with nitrogen to less than 1 ml.

(8) Sample collection: Dissolve 2 times with 300 μl n-hexane, wash and collect the fat on the tube wall and put it into a brown
vial for GC-MS analysis.
GC-MS analysis: Column model DB-5MS 60 m×0.25 mm×0.25 μm (Guangzhou fenigan Instrument Co., Ltd.), chromatographic column: column model ZB-5MS 60 m x 0.25 mm x 0.25 μm. Keep the initial temperature at 60 °C for 1 min, rise to 180 °C at 10 °C/min, rise to 280 °C at 4 °C/min, keep for 20 min, rise to 300 °C at 20 °C/min, keep for 2 min. The injection hole temperature is 280 °C. Carrier gas: high purity He (99.999 %), flow rate of 1 ml/min. The temperature of connecting rod is 285 °C. The temperature of ion source is 250 °C. The solvent delay time was 7 min. Quality scanning range: 40 ~ 550 amu.

The data processing system of the chemical workstation was used to search the spectrum library, analyze the spectrum and confirm the chemical structure of each fatty acid. The relative content of fatty acids was calculated by peak area normalization.

**RNA preparation and quantitative real-time PCR**

Total RNA from the liver was extracted using RNA-solv reagent (OMEGA Bio-Tek, USA) in accordance with the manufacturer's instructions. The quality and purity of total RNA were assessed using a Nano Drop 2000 spectrophotometer (ThermoFisher Scientific, Shanghai, CN, China). cDNA was synthesized from 2 μl total RNA using cDNA Reverse Transcription Kit (Accurate Biotechnology Co., Ltd, China). Reaction conditions were as follows: 37 °C for 15 min followed by 85 °C for 5 s. Quantitative real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) on a CFX 96 real-time-PCR detection system (Bio-Rad). Reactions were incubated in a 96-well optical plate at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s, 95 °C for 10 s, 65 °C for 5 s and 95 °C for 5 s. Relative expression levels of genes were normalized to the geometric mean of the reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the 2^−ΔΔCT method. Primer sequences are listed in Table 2.

**Lipidomics analysis**

**Metabolite extraction**

The procedure for metabolite extraction was performed as follows: First, 20 mg of sample was weighed to an EP tube, and homogenized with 400 μl water. Transfer 50 μl of homogenate to a new EP tube (A) and dilute to 400 μl with water. Next, 960 μl extraction liquid (MTBE:methanol = 5:1, v/v), including 9 μl of 10 μg/ml IS1, 9 μl of 10 μg/ml IS2, 9 μl of 100 μg/ml IS3, was added to each sample. The homogeneously mixed sample was vortexed for 30 s, sonicated for 10 min in a 4 °C water bath, and then centrifuged for 15 min at 3,000 rpm and 4 °C. The supernatant (500 μl) was transferred to a new EP tube (B), and 500 μl MTBE was added from the original EP tube (A); the sample was then vortexed for 30 s, sonicated for 10 min, and centrifuged for 15 min at 3,000 rpm and 4 °C. The supernatant (500 μl) was transferred to a new EP tube (C). Next, 500 μl MTBE was added from EP tube A, and the sample was vortexed for 30 s, sonicated for 10 min, and centrifuged for 15 min at
3000 rpm and 4 °C. The supernatant (500 µl) was then transferred to a new EP tube (D). The liquid in EP tubes B, C, and D were dried in a vacuum concentrator at 37 °C, and add 100 µl extraction liquid (methanol:dichloromethane = 1:2, v/v) in a ice bath for 10 min, then centrifuged for 15 min at 13,000 rpm and 4 °C for recombination. The samples (75 µl) were then transferred to a fresh LC/MS glass vial for ultra-high-performance liquid Chromatography-Q Exactive orbitrap-MS (UHPLC-QTOF-MS) analysis.

**Chemicals and QC samples**

The chemicals used in lipidomics analysis were as follows: methanol (67-56-1, CNW Technologies); MTBE (1634-04-4, CNW Technologies); dichloromethane (75-09-2, Merck); internal standards IS1, IS2, IS3 (Avanti); ammonium acetate (631-61-8, Merck); and ammonium hydroxide (1336-21-6, Merck). The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

**LC-MS/MS Analysis**

LC-MS/MS analyses were performed using a UHPLC system (1290; Agilent Technologies, Santa Clara, California, USA) with a Phenomen Kinetex 1.7 u C18 100 A column (2.1 × 100 mm) coupled with a Triple TOF 6600 (Q-TOF; AB Sciex). The mobile phase consisted of A (10 mM HCOONH4+ 40% H2O + 60% acetonitrile [can]) and B (10 mM HCOONH4 + 10% acetonitrile + 90% isopropanol), and the elution gradient was as follows: 0~12 min, 40% B; 12 ~13.5 min, 100% B; 13.5 ~13.7 min, 100% B; 13.7 ~18 min, 40% B, column temperature 45 °C. The automatic injection temperature was 4 °C, and the injection volumes was 2 µl (pos) or 2 µl (neg), respectively. A triple TOF mass spectrometer was used due to its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Analyst TF1.7; AB Sciex) continuously evaluated the full-scan survey MS data during collection and triggered the acquisition of MS/MS spectra depending on preselected criteria. In each cycle, 12 precursor ions whose intensity was greater than 100 were chosen for fragmentation at a collision energy (CE) of 45 eV (12 MS/MS events with a production accumulation time of 50 msec each). ESI source conditions were as follows: ion source gas 1, 60 psi; ion source gas 2, 60 psi; curtain gas, 30 psi; source temperature, 600 °C; declustering potential as 100 V, ion spray voltage floating (ISVF), 5000 V or -4500 V in positive and negative modes, respectively.

**Data processing and annotation**

An in-house program, Lipid Analyzer, was developed using R for automatic data analysis. The raw data files (.wiff format) were converted to files in mzXML format using the “msconvert” program from ProteoWizard (version 3.0.6150). The mzXML files were then loaded into LipidAnalyzer for data processing. Peak detection was first applied to the MS1 data. The CentWave
algorithm in XCMS was used for peak detection. With the MS/MS spectrum, lipid identification was achieved through a spectral match using an in-house MS/MS spectral library. The absolute content of lipid in samples was calculated according to the peak area, stable isotope-labeled internal standard, and relevant fragment information.

**Data analysis of lipidomics**

After a series of preparation and collation of the original data, the final dataset was imported to SIMCA15.0.2 software package (Sartorius Stedim Data Analytics AB, Umeå, Sweden) for multivariate analysis. Principal component analysis (PCA) was used for unsupervised data analysis to investigate the clustering of data in each group. In order to maximize the separation between groups, the supervised data analysis was carried out by using orthogonal projects to latent structures-discriminate analysis (OPLS-DA). Finally, a Variable Importance in Projection (VIP) value of greater than 1 was selected as a potential biomarker, and compounds with statistical differences of p-value of less than 0.05 were evaluated as differential metabolites for identification.

**Statistical analysis**

All statistical analyses were conducted using SPSS Statistics 25.0 software. Data were reported as the mean ± SD. Statistical significance between groups was denoted by *p < 0.05, **p < 0.01, and ***p < 0.001.

**Results**

**Yak butter SM causes weight gain**

A month of SM-containing diets, there was no significant difference in body weight compared to control, while after five months, the body weight of mice in the treatment group increased by 4% compared with the control. No differences were observed in food intake between two groups (Figure 1).

**Yak butter SM increased serum lipids and fasting glucose**

Serum lipids and glycemic markers are presented in Table 3. Total serum cholesterol (+23%, +31%), LDL (+16%, +40%) and HDL (+30%, +50%) were significantly increased with dietary SM relative to control after one and five months, respectively. No significant differences were detected in serum TG and fasting glucose between two groups.

**Yak butter SM attenuated hepatic steatosis**

The fixed tissue is often dehydrated, embedded in paraffin, sectioned and stained with H&E, it was found that the liver tissues and structures of the two groups were normal and intact after one month of adding SM diets. Hepatocytes were arranged radially along the central vein, and no degeneration or necrosis was seen. No inflammatory cell infiltration and fibrosis were found in the stroma. Adipose of abdominal: The structure of the adipose tissue in the control group was complete, the adipose cells were
arranged densely, the shape was generally circular, and the size was uniform, no obvious pathological changes were found. While in the treatment group, mild focal/multifocal neutrophil infiltration was seen from abdominal adipose. Perirenal adipose: The adipose tissue structures of the two groups were intact, the adipose cells were arranged densely, the shape was generally circular, and the size was uniform. After five months on diets, there was no obvious pathological change for abdominal adipose in the control group, but the treatment group showed slight infiltration of monocytes and neutrophils; H&E-stained livers showed extensive lipid droplet accumulation in the control group. In contrast, SM extracts in yak butter can reduce the accumulation of lipid droplet in the liver and prevent hepatic steatosis (Figure 2D.).

The composition of fatty acids in scapular adipose tissue

The composition of fatty acids in the adipose tissue of mouse scapula was analyzed. The results are shown in Table 4. As it can be seen from the table, the content of UFA was significantly increased, especially MUFA (+9%), and the content of SFA decreased (-9%) compared with the control group after a month with dietary SM. The content of MUFA was 34.38% in the control group and 37.34% in the treatment group, and the SFA was 36.58% and 33.15%, respectively; in contrast, the content of PUFA (-14%) was significantly decreased and SFA increased with dietary SM relative to control after five months.

The effects on the expression of genes related to lipid metabolism and inflammation

Fat metabolism is an important and complex biochemical reaction in the body, which refers to the process of digestion, absorption, synthesis and decomposition of fat in the body with the help of various related enzymes, processing into the substances needed by the body, ensuring the normal physiological function operation, which is of great significance for life activities. The main enzymes related to metabolism are fatty acid synthase (FAS), fatty acid desaturase 2 (FADS2), stearoyl-CoA desaturase 1 (SCD1), 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGCR), fatty acid desaturase 1 (FADS1), carnitine palmitoyltransferase 1 (CPT1) and so on.

Hepatic mRNA expression was determined using real-time PCR and the 2−ΔΔCT value of mRNA in the control group was set to 1 for threshold comparison. After a month of feeding SM diets, relative expressions of FAS, FADS2, SCD1, glycerol-3-phosphate acyltransferase mitochondrial (GPAM), FADS1, CPT1, HMGCR and glycerol-3-phosphate acyltransferases 4 (GPAT4) genes in the test group were higher than controls, which belonged to the up-regulated genes; among them, the relative expressions of FAS, FADS2, CPT1, HMGCR, SCD1, FADS1 and GPAM genes were significantly increased by 523%, 406%, 183%, 175%, 123%, 123%, and 96%, respectively, while the relative expressions of thyroid-hormone responsive (THRSP), adipocyte fatty acid binding protein (A-FABP), fatty acid binding protein 1 (FABP1) and acetyl-CoA carboxylase alpha (ACC-a) genes were lower than control, especially THRSP (-80%) and FABP1 (-62%), which belonged to the down-regulated genes. After five months of feeding SM, the relative expression levels of all genes in the treatment group were higher than those in the
control group, especially FAS (+601%), THRSP (+728%), FADS1 (+641%), CPT1 (+1264%), HMGCR (+498%), ACC-a (+241%) and GPAT4 (+1055%) genes showed high expression (Figure 3).

Ceramide and sphingosine, the metabolites of sphingomyelin, play an important role in modulating the immune system. After a month of feeding SM diets, the expression levels of pro-inflammatory factors interferon-Y (IFN-Y), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the liver were lower than the control group. Anti-inflammatory factor interleukin-1 receptor antagonist (IL-1Ra) was highly expressed in the treatment group, the expression of interleukin-10 (IL-10) and interleukin-4 (IL-4) were relatively low. While after five months, the relative expression levels of IL-1Ra, IL-10, and IL-4 were significantly higher than the control group.

Analysis of lipid metabolism in liver samples of mice

Lipid pattern recognition analysis of mouse liver samples

The resultant data involving the peak number, sample name, and normalized peak area were processed by the SIMCA15.0.2 software package for PCA and OPLS-DA analysis. Figure 4A&D show the distribution of origin data, the closer the distribution of the degree of aggregation and dispersion between groups, the closer the composition and concentration, on the contrary, the farther the distribution, the greater the difference. And all the mouse liver samples in the PCA score plots were within the 95% Hotelling T2 ellipse. Furthermore, due to the usage of several models, information regarding between-class differences is not easily accessible, which hampers the quality of interpretation of the classification model [24]. In order to visualize group separation and find significantly changed metabolites, supervised orthogonal projections to latent structures-discriminate analysis (OPLS-DA) was applied. Then, a 7-fold cross-validation was performed to calculate the value of R2 and Q2. R2 indicates how well the variation of a variable is explained and Q2 means how well a variable could be predicted. To check the robustness and predictive ability of the OPLS-DA model, a 200-time permutations was further conducted. Afterward, the R2 and Q2 intercept values were obtained. The R2 and Q2 intercept values were (0, 0.91) and (0, -0.67) (Figure 4B). Here, the intercept value of Q2 represents the robustness of the model, the risk of overfitting and the reliability of the model, which will be the smaller the better. Our results show that our OPLS-DA model is robust, without overfitting. Based on the OPLS-DA, the value of variable importance in the projection (VIP) and P-value of the first principal component in OPLS-DA analysis were obtained. It summarizes the contribution of each variable to the model. The metabolites with VIP >1 and p < 0.05 (Student’s t test) were considered as significantly changed metabolites. The OPLS-DA scores plot of two groups is shown in Figure 4C&F. The differences in metabolic profiles obtained in the experiment reflect the biological differences between the samples. It can be seen from the picture, noticeable separations were found between the two groups, demonstrating that the PCA and OPLS-DA models could be used to identify the differences between two groups. In addition, exogenous addition of sphingomyelin
significantly affects liver metabolism in mice.

**Screening of differential metabolites**

VIP value of OPLS-DA model (VIP>1) and P value of Student's t test (P<0.05) were used to screen differential metabolites. In the database, the retention time and other conditions are matched with the substances in the database to determine the nature of differential metabolites. The volcano map of differential metabolite screening is shown in **Figure 5**. Overall, 16 and 68 significantly differential lipid species were identified in the control group versus the treatment group at one month and five months, respectively (Figure 5). These species include 5 diacylglycerols (DG), 10 triacylglycerols (TG), 3 cardiolipins (CL), 25 glycerophosphatidylcholines (PC), 3 glycerophosphatidic acids (PA), 19 glycerophosphatidylethanolamines (PE), 4 glycerophosphatidylglycerols (PG), 1 glycerophosphatidylinositol (PI), 1 glycerophosphatidylserine (PS), 6 ceramides (Cer), and 6 sphingomyelins (SM) (**Table 5 and 6**).

**Content analysis of differential lipid species in the liver**

The differential metabolites obtained through the above screening are often biologically similar/complementary in results and functions, or are positively/negatively regulated by the same metabolic pathway, showing similar or opposite expression characteristics among different experimental groups. To further investigate whether the lipid biomarkers are responsible for the bioactivities of SM, we analyzed the differences in metabolites among the experimental groups based on the lipidomics approach. As shown in **Figure 6A-D**, a total of 14 lipid classes were identified, but the contents of hexosylceramide (HexCer), dihexosylceramide (Hex2Cer), and sphingosine were not affected between control and treatment groups. Of the 84 identified significantly differential metabolites, the levels of 17 lipid species from three major lipid classes in the treatment group, including SMs, CERs and PSs, were markedly enhanced as compared with the control group. In addition, the differences in the levels of the potential lipid biomarkers among two groups were displayed in **Figure 6E & F**. The contents of six CERs (Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d17:1/22:0), Cer(d17:1/24:1), Cer(d17:1/24:0) and Cer(d17:0/26:1)), six SMs (SM(d15:0/24:1), SM(d14:0/26:1), SM(d14:1/24:1), SM(d15:1/22:0), SM(d15:1/24:1) and SM(d19:1/26:1)), and PS(18:1/22:6) in the treatment group were markedly enhanced in contrast to the control group (p < 0.05). Thus, these results indicated that these 13 lipids included 6 CERs, 6 SMs, and 1 PS might be the representative potential biomarkers responsible for the effects of SM on attenuated hepatic steatosis and tissue inflammation.

**Analysis of enriched metabolic pathways**

In order to elucidate alterations in metabolic pathways of mouse liver lipids after feeding sphingomyelin and the effects of lipid content on metabolic pathways, significantly different lipids were imported into software for comparison of two groups (**Figure 7**). These pathways included glycerolipid metabolism, sphingolipid metabolism, glycerophospholipid metabolism, linoleic acid
metabolism, alpha-linoleic acid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis and arachidonic acid metabolism.

Discussion

It has been shown that diets linked with the development of human diseases. Sphingolipids are dietary bioactives shown to reduce lipid absorption and alter lipid metabolism both in vitro and in rodent models [9-12,16-19]. In this study, C57BL/6J mice were fed with SM derived from yak butter for one and five months, respectively, and then studied for the effects on steatosis, tissue inflammation and lipid metabolism. Surprisingly, SM can reduce the accumulation of liver fat and prevent steatosis. In addition, neutrophil infiltration was seen adipose of abdominal. It is a kind of immune cell with very strong phagocytic function. Neutrophil infiltration can not only produce bactericidal substances with immunoregulatory effects, but also it can synthesize and secrete highly active multifunctional protein polypeptides-cytokines, thereby affecting the immune process and regulating the intensity of inflammatory responses. Further analysis of the expression of inflammation related genes confirmed the anti-inflammatory effect of SM. Lipidomics analysis of liver tissue revealed that sphingomyelin changed the metabolism of lipids in the liver of mice. Overall, the current findings showed that dietary SM derived from yak butter can attenuate steatosis and tissue inflammation, and influence liver lipid metabolism in mice.

In this study, short term consumption of SM derived from yak butter could reduce body weight gain. SM increased TC, TG, LDL, HDL and fasting glucose in serum compared to the control group. Generally speaking, the change of blood lipids often indicates the change of fatty synthesis and decomposition of the liver. As the main site of lipid metabolism, the liver plays an important role in the absorption, synthesis, decomposition and transport of lipids. The expression of fatty acid metabolism related enzymes in the liver can affect the metabolism and storage of lipids in the liver.

Some studies have shown that milk SM can reduce the blood lipids and liver triglyceride of C57BL/6J mice induced by high-fat diet [16] and that of egg SM fed mice increased significantly [17]. Hepatic cholesterol biosynthesis may have increase with SM treatment, which is related to the expression of related genes [11]. Previous studies have shown that the mRNA of HMGCR in the liver of milk SM fed mice increased by 190%, egg SM increased SCD1 mRNA in liver (189%, compared with the control) [16]. HMGCR is the rate limiting enzyme of cholesterol synthesis and regulates cholesterol synthesis. SCD1 mainly catalyzes stearoyl and palmitoyl COA (C16:0) to synthesize palmitoleic acid and oleic acid. Palmitoleic acid and oleic acid are important substrates for the synthesis of various lipids, including triglycerides, phospholipids, cholesterol and triglycerides. SCD1 is involved in de novo lipogenesis, enhancing hepatic triglyceride formation [25]. We also found that the proportion of long-chain fatty acids in the scapular fat, such as palmitic acid, stearic acid, oleic acid and arachidonic acid, increased in the experimental group compared with the control group after five months. The results showed that the intervention of dietary
sphingomyelin in yak butter not only increased the expression of HMGCR and SCD1, but also increased the expression of GPAM and GPAT4. Our study further confirmed that SM can promote the gene expression of fatty acid synthase and cause fatty acid accumulation. GPAM and GPAT4 belong to GPATs, which catalyze the reaction of glycerol-3-phosphate and fatty acyl CoA, and are the key enzymes in the first step of glycerol synthesis. GPAM is located in mitochondria, GPAT4 is located in endoplasmic reticulum, GPATs and DGAT are key enzymes in the phosphoglycerin pathway, which is the main pathway for most cells to synthesize triglycerides. Although the up-regulation of HMGCR gene can inhibit cholesterol synthesis, the genes related to triglyceride synthesis are up-regulated, which further shows that the addition of yak butter sphingomyelin leads to the increase of TG concentration. In addition, we found that sphingomyelin supplementation significantly increased the expression of FAS, FADS1 and FADS2. FAS is a multifunctional enzyme system, which catalyzes the formation of fatty acids from acetyl CoA, malonyl CoA and nicotinamide adenine dinucleotide (NADPH), and plays a central role in lipid biosynthesis [26]. The higher the expression level of FAS in the liver, the more fat deposition in the body [27], the more likely to cause obesity [28]. There was a positive correlation with the increase in mouse weight compared with the control group after five months of feeding SM. FADS1 and FADS2 genes are important regulatory genes of metabolic rate limiting enzymes in the process of polyunsaturated fatty acid synthesis [29], and their expression quantity determines the ability of the body to synthesize unsaturated fatty acids. In this experiment, we found that sphingomyelin can effectively improve the expression quantity of FADS1 and FADS2 genes, induce the body to produce more fatty acid desaturase, so as to regulate the saturated fat combined with the carrier Fatty acids or monounsaturated fatty acids form more double bonds on the acyl chain, which ultimately increases the degree of unsaturated fatty acids in sphingomyelin group, and the unsaturated fatty acids have the effect of lowering blood lipids [30].

Although studies have shown that SM and its hydrolytic byproducts have been shown to reduce the intestinal absorption of cholesterol, triglycerides and fatty acids in rodents [9-11,18,31]. However, these effects were not observed in this study, which may be related to differences in study design, such as animal model and diet. There is research evidence that the ratio of cholesterol and SM in lipid emulsions is known to impact the magnitude of cholesterol absorption in vitro and in vivo [9]. Cholesterol and SM mutually inhibit each other's absorption [10]. As little as 0.1% (w/w) dietary milk SM was shown to reduce cholesterol absorption by 20% in mice on a chow diet [9]. Egg SM (0.2% w/w) supplemented over 6 weeks to a high-fat, high-cholesterol diet lowered serum lipids in APOE*3-Leiden mice [18]. Egg SM supplementation of a high-fat diet for 4 weeks, without added cholesterol, was able to increase serum and hepatic lipids in mice [16]. Sphingolipid supplementation in rodents consuming large amounts of dietary cholesterol typically reduces serum cholesterol and hepatic lipids, whereas in the absence of large doses of cholesterol, supplementation with sphingolipids resulted in increased serum and hepatic lipids in some studies
In the current study, SM has not reduced serum lipids, although it can significantly increase serum HDL-C content, this may be related to diet. Further studies are needed to determine the optimal dietary combination of sphingomyelin to reduce serum and liver lipids.

The most striking observations between dietary SM-supplemented group and control group were the histological differences in hepatic steatosis and adipose tissue inflammation. Previously, eparguli Abula et al [33] studied the effect of sphingomyelin extract from Xinjiang Baicheng oil chicken on the liver histopathology of hyperlipidemia mice. The results showed that the sphingomyelin extract of Baicheng oil chicken could protect the liver cells and improve the fatty degeneration of the liver. Chung et al [17] also found that egg SM, when added to a high-fat diet, can effectively reduce the level of liver cholesterol in a dose-dependent manner. SM has a potential beneficial effect on diet induced liver steatosis by reducing the absorption of intestinal cholesterol. In this study, after five months of diet, H&E staining of liver tissue showed that lipid droplets were widely gathered in the control group, and there were cavities of different sizes in the cytoplasm. The liver tissues and structures of the treatment group was normal and intact, the results showed that yak butter sphingomyelin could prevent hepatic steatosis. SM can also reduce the development of adipose tissue inflammation induced by high fat diet in obese mice [13]. After yak butter sphingomyelin extract was fed to mice, mild focal neutrophil infiltration was found in abdominal fat. Neutrophils play an anti-inflammatory effect. It has been found that by activating peroxisome proliferator activated receptor (PPAR)-γ, neutrophils can change from pro-inflammatory phenotype to anti-inflammatory phenotype [34]. When inflammation occurs in the tissue, it can gather around the infection focus and play the role of phagocytosis and digestion of pathogenic bacteria [35]. Therefore, we speculate that yak butter sphingomyelin can reduce inflammation of adipose tissue. In order to determine the role of sphingomyelin in tissue inflammation, we further studied the expression of related inflammatory factors, the low expression of pro-inflammatory factors and high expression of anti-inflammatory factors in the treatment group confirmed this hypothesis.

The liver lipid metabolites of the two groups of mice were measured by quantitative lipidomics. The study found that there were differences between the two groups. A total of 84 different lipids were found. 58 lipids were down regulated and 26 lipids were up regulated in the treatment group. The most significant differences were Cers, SMs, PCs, PEs, TGs and DGs. Sphingomyelins is the most abundant sphingolipids in the cell, which play an important role in cell membrane structure, energy supply and signal transduction [36]. Their metabolic abnormalities can lead to inflammation, tumor, immune disease, cardiovascular disease and so on [37]. Ceramide is a synthesis precursor of sphingolipids and a metabolite of sphingolipids. As an important part of eukaryotic cell membrane, ceramide not only provides cell structural integrity, but also plays a second messenger role in the process of cell signal transmission [38]. Research [39] found that the level of ceramide in obese patients
was significantly higher than that in non-obese patients, and ceramide played an important role in the occurrence and
development of obesity, insulin resistance, atherosclerosis and other diseases. In this study, five kinds of SMs and seven kinds of
Cers were identified by feeding sphingomyelin.

The results provide a basis for further understanding the effect of sphingomyelin on the lipid composition and biological
function of liver. Additionally, PC and PE are important phospholipid components in cell membrane, and their distribution on
cell membrane is an important regulatory factor for cell membrane integrity and material transport across membrane. The
decrease of PC/PE ratio will increase the permeability of cell membrane and lead to the exudation of hepatocyte contents. The
change of relative abundance of PEs and the damage of PE metabolism may affect hepatic function via multiple mechanisms,
especially in hepatic steatosis. The increase of PEs promoted the combination of lipid droplets, and then increased the size of
lipid droplets. When contrasted with the control group, the relative intensities of 19 PEs were dramatically reduced in the
treatment group, which might be related to improve hepatic steatosis due to prevent coalescence of the lipid droplets. The
increase of TG in the liver is related to the decrease of PC. PC in the liver can promote the metabolism of TG in the liver and
reduce the deposition of TG in the liver. At the same time, the emulsifying property of PC can reduce the deposition of
triglyceride and cholesterol on the vessel wall, so as to regulate the lipid disorder. Kulkarni et al [40], believed that the decrease
of PC was an important reason for the disorder of lipid metabolism. In this study, we found that compared with the normal group,
the PC of all subtypes of SM basically showed a downward trend. In addition, DG and TG lipids were also found in this study.
DG is one of the main lipid subclasses in organism and the second messenger in various cell activities. It has been proved that
DG can accelerate the β-oxidation of fatty acids and affect the expression of lipid metabolism related genes [41,42], thus
reducing the TG level in serum and liver [43]. The results of this study showed that the content of TG in serum and liver of mice
fed with sphingomyelin was up-regulated compared to control group. It was speculated that the reason was that sphingomyelin
affected the metabolism of DG and TG in liver and increased the efflux of TG. This finding was further verified by the analysis
of lipidomics. The composition of TG(17:0/17:0/17:1), TG(18:2/18:2/19:0), TG(18:1/20:4/22:5), TG(18:2/18:3/22:6),
TG(18:2/20:5/20:5), TG(18:2/20:5/22:6), TG(18:3/18:3/20:5), TG(20:4/20:5/20:5), DG(17:2/18:2), DG(22:4/17:0), and
DG(19:0/20:5) in the liver was up regulated compared with the control group obviously.

By means of quantitative lipidomics, we not only identified the significantly different lipids between the two groups, but
also studied the lipid metabolism pathways involved in these lipids. Based on the analysis of lipidomics pathway, there are 7
metabolic pathways for eleven major lipid classes, glycerophospholipid metabolism was the pathway with the most significantly
different lipid involvement. SM and Cer are involved in these seven lipid metabolism pathways. The contents of SM and Cer in
the control group were lower than those in the treatment group. Therefore, our results support the hypothesis that the change of
sphingomyelin addition may affect glycerolipid metabolism, sphingolipid metabolism, glycerophospholipid metabolism, linoleic acid metabolism, alpha-linoleic acid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis and arachidonic acid metabolism, consequently leading to increase in certain lipids, such as Cers and SMs. Through the analysis, it is helpful for us to further understand the mechanism of sphingomyelin supplementation altered the important differential lipid species in the liver.

**Conclusion**

In the current study, we found that the addition of SM extract of yak butter to mice with a normal diet reduced the accumulation of liver lipid droplets, which could prevent hepatic steatosis and tissue inflammation. This conclusion is confirmed by quantitative lipidomics analysis of liver lipids and detection of gene expression related to lipid metabolism and inflammation in liver. Yak butter sphingomyelin can down regulate the expression of pro-inflammatory factors IFN-γ, IL-6, TNF-α, and up regulate the expression of anti-inflammatory factors IL-10, IL-4 and IL-1Ra. Meanwhile, the genes related to fat metabolism, such as CPT1, SCD1, FAS, FADS1, FADS2 and HMGCR, were also significantly up-regulated, which affected the lipid metabolism in the liver. Further analysis of the differential metabolites between the two groups using lipidomics revealed that there were 84 differential lipids in total after one and five months of feeding. Six CERs (Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d17:1/22:0), Cer(d17:1/24:1), Cer(d17:1/24:0) and Cer(d17:0/26:1)), six SMs (SM(d15:0/24:1), SM(d14:0/26:1), SM(d14:1/24:1), SM(d15:1/22:0), SM(d15:1/24:1) and SM(d19:1/26:1)), and PS(18:1/22:6) was highly expressed in the experimental group. Therefore, it can be used as potential biomarkers of hepatic steatosis and inflammation, providing practical information for the development of yak butter sphingomyelin.

**Authors’ contributions**

**LX**: research design, collection and assembly of data, data analysis and interpretation, writing the article, final approval of article.

**SWC**: performing and analyzing genetic tests, writing the article, critical re- vision of the article, final approval of article.

**LYH**: research concept and design, data analysis and interpretation, critical revision of the article, final approval of article.

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**Availability of data and materials**

On request.
Ethics approval and consent to participate

The study protocol conforms to the ethical guidelines of the Declaration of Basel and was approved by the Institutional Animal Care and Use Committee of Qinghai Province. (approval number 2019070801).

Consent for publication

All authors of the manuscript have read and agreed to its content and are accountable for all aspects of the accuracy and integrity of the manuscript in accordance with ICMJE criteria. The article is original, has not already been published in a journal, and is not currently under consideration by another journal. We agree to the terms of the BioMed Central Copyright and License Agreement, where applicable, Open Data policy.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author details

1 College of Agriculture and Animal Husbandry, Qinghai University, Qinghai Province, Xining, China
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Figure Legends

Figure 1: Effect of SM supplementation on body weight and food intake. SM increases body weight with no change in food intake after five months. Body weight was assessed monthly. Food intake was measured weekly, and daily food intake was calculated. Each value represents the mean ± SD. for n=5 mice for two groups.

Figure 2: The pathological changes of liver, adipose tissues in two groups of mice. A. control group (one month; liver; X 40) B. treatment group (one month; liver; X 40) C. control group (five months; liver; X 200) D. treatment group (five months; liver; X 200) E. control group (one month; abdominal adipose; X 40) F. treatment group (one month; abdominal adipose; X 40) G. control group (five months; abdominal adipose; X 200) H. treatment group (five months; abdominal adipose; X 200) I. control group (one month; perirenal adipose; X 40) J. treatment group (one month; perirenal adipose; X 40)

Figure 3: Effects of SM supplement on the expression levels of hepatic lipid metabolism and inflammation genes. Data are presented as means ± SD (n = 5). The relative mRNA expression levels of genes were analyzed referring to the 2−ΔΔCT method and normalized to the GAPDH gene.

Figure 4: PCA score plot (A, D), PLS-DA (B, E), and OPLS-DA score plots (C, F). In figure A and D, the abscissa PC[1] and the ordinate PC[2] represent the scores of the first and second principal components respectively. In Figure B and E, the abscissa represents the replacement retention of the replacement test (the proportion consistent with the order of Y variables of the original model, the point where the replacement retention is equal to 1 is the R²Y and Q² values of the original model). The ordinate represents the value of R² Y or Q², the green dot represents the R² Y value of the replacement test, and the blue square point represents the Q² value obtained by displacement test, and the two dotted lines represent the regression lines of R² Y and Q² respectively. In Figure C and F, the abscissa t[1]P represents the predicted principal component score of the first principal component, and the ordinate t[1]O represents the orthogonal principal component score. Scatter shape and color represent different experimental groups. Cyan and orange square point represents treatment group, and red dot represents control group.

Figure 5: Volcanic map analysis of lipid profiles in liver. In the volcanic map, each point represents a metabolite, the abscissa represents the multiple change of the group compared with each material (the logarithm based on 2), the ordinate represents the p-value of the student's t-test (the negative number based on 10), the scatter size represents the VIP value of opls-da model, and the larger the scatter, the greater the VIP value. The scatter color represents the final screening result. The significantly up-regulated metabolites are shown in red, the significantly down regulated metabolites are shown in blue, and the non significantly different metabolites are shown in gray. As shown that there are more up-regulated and down regulated
metabolites, but the 84 differential metabolites shown in Table S3 and S4 are finally screened based on the comprehensive multiple change and significance.

**Figure 6:** Lipid plot of lipid differential metabolites for control versus treatment group in mouse liver. Each column represents a class of metabolites (A: one month; C: five months). Each point in the bubble represents a metabolite (B: one month; D: five months). The size of the point represents the p-value of the student's t-test (take the negative number of the base 10 logarithm). The larger the point, the smaller the p-value. The gray dots represent the non significant foreign bodies with p-value no less than 0.05, and the color dots represent the significant foreign bodies with p-value less than 0.05 (different colors are marked according to lipid classification). The abscissa (A, C) and the ordinate (B, D) represent the lipid classification information. The ordinate (A, C) and the abscissa (B, D) represent the relative change percentage of the content of each substance in the comparison group. The relative change percentage of the content is zero, which means that the content of the substance in the two groups is the same; the relative change percentage of the content is a positive number, which means that the content of the substance in control group is higher; the relative change percentage of the content is a negative number, which means that the content of the substance in treatment group is higher. In Figure E (one month) &F (five months), the abscissa represents different experimental groups (A: control group; F: treatment group), the ordinate represents different metabolites compared with the group, and the color blocks at different positions represent the relative expression of metabolites at corresponding positions.

**Figure 7:** Metabolomic view map of the significant metabolic pathways of mouse liver lipids. Significantly changed pathways based on the enrichment and topology analysis are shown (A: one month; B: five months). The X-axis represents pathway enrichment, and the Y-axis represents the pathway impact. The circular picture in the figure is the expression of the influence value combined with the P value. The smaller the P value, the bigger the -ln (p) and the greater the influence, the more important the path is. The color changes from blue to red. Dark colors and large sizes represent the high pathway impact values and major pathway enrichment, respectively.
Table 1: Diet Composition (g / kg of diet)

| Diet component       | (g / kg) |
|----------------------|----------|
| Casein               | 140      |
| L-Cystine            | 1.8      |
| Corn starch          | 465.692  |
| Maltodextrin         | 155      |
| Sucrose              | 100      |
| Soybean oil          | 40       |
| Cellulose            | 50       |
| Mineral mix          | 35       |
| Vitamin mix          | 10       |
| Choline bitartrate   | 2.5      |
| t-Butylhydroquinone  | 0.008    |
| **Total**            | **1000** |

Table 2: Primer sequences information

| Primer | Upstream sequence (5'-3') | Downstream sequence(5'-3') |
|--------|---------------------------|-----------------------------|
| ACC-α  | ACGTGAATGCTTGACCAGGG      | AGGCAAGCCTCCAGTAAGC         |
| FAS    | GTGAGTCTATCTGCCTCC        | GTCGATGAGGGCAATCTGGA        |
| FADS1  | CTGGATCGACCCGGAAGG        | ATGCCACAAAGGATCGTGG         |
| FADS2  | AGCCCCCTGAGATGCGAAG       | ATAGTAGCTGATGGCCCAAGC       |
| GPAT4  | CTGGTGCGTAAAGGCGTAC       | AGAAGGGGTCACCAAAACTGA       |
| GPAM   | AGCTTCTAAGTCCCAACAC       | TTGCCAGGCTCTGATAAATAG       |
| THRSP  | TCTGAAAGATCGCTTTACACGAGA | GATGCACATCAGGGAGGACG        |
| HMGCR  | AGAGAACAAAGGGGGTCACCAGCC | CCTTGAGATCCCAAGCCGA         |
| SCD1   | CTGAACACCCATCCCGAGAG      | AACTGAGATCTCTGAGAAGC        |
| CPT1   | TCCGCTCCTCATCTCCG         | TGCCATTCTGTAATGGAGATGACT    |
| FABP1  | TGAAGGCAATAGGCTGCCC       | GTCATGCTCTCAGTTGCAGA        |
| A-FABP | GTGCGATGGAAAGTCAGCCA      | CATAACACATACCACACCAGC       |
| IL-1Ra  | GCGCTTTACCTCATCCG         | GGACGGTCAGCCTTACTAGTG       |
| IFN-γ   | CTTCAGCAACAGCAAGGCGA      | CATTGAATGCTTGGCGTGG         |
| IL-10   | TGGGTGCAACAGGCTTATCG      | TCAGCTTCTACCCAGGGAA         |
| IL-4    | AGTGGCTCCTGCTTAGGGC       | CAGGCTACAAAGGCCCCAAG        |
| IL-6    | CCACTTCACATGGAGGAGGC      | TCTGCAATGCTCATGCTTGT        |
| TNF-α   | GCCAACGGCGATGGATCTCAA     | TAGCAAAATCGGCTGACG        |
Table 3: Serum Markers of C57BL/6J Mice with Dietary SM Diets

| Parameter      | One month |          | Five months |          |
|----------------|-----------|----------|-------------|----------|
|                | Control group | Treatment group | Control group | Treatment group |
| TC (mmol / L)  | 5.24±0.12 | 6.47±0.54* | 5.52±0.64 | 7.21±0.56* |
| TG (mmol / L)  | 1.48±0.05 | 1.61±0.12 | 1.53±0.08 | 1.65±0.20 |
| LDL (mmol / L) | 2.80±0.26 | 3.24±0.04** | 2.84±0.02 | 3.98±0.49** |
| HDL (mmol / L) | 2.08±0.07 | 2.72±0.45** | 2.48±0.10 | 3.71±0.22** |
| Fasting glucose (mmol / L) | 4.57±0.15 | 5.90±0.64 | 4.87±0.18 | 6.00±0.74 |

Each value represents the mean ± SD of the values for n= 5 mice. Superscripts with different symbols indicate differences at p <0.05 using post-hoc comparisons. *: p < 0.05; **: p < 0.01.
| Fatty Acids    | One month          | Five months        |
|---------------|--------------------|--------------------|
|               | Control group      | Treatment group    | Control group | Treatment group |
| C12:0         | 0.13±0.03          | 0.17±0.00          | NA            | 0.11±0.03       |
| C14:1         | 0.11±0.02          | NA                 | NA            | NA              |
| C14:0         | 2.52±0.54          | 1.67±0.43          | 1.88±0.12     | 2.23±0.36       |
| C15:0         | 0.35±0.07          | 0.31±0.08          | 0.29±0.03     | 0.26±0.07       |
| IsoC16:0      | 0.31±0.04          | 0.21±0.05          | 0.29±0.01     | 0.19±0.04       |
| C16:2         | 0.11±0.03          | NA                 | 0.07±0.00     | NA              |
| C16:1n-7      | 0.89±0.32          | 0.43±0.20          | 0.70±0.04     | 0.35±0.07       |
| transC16:1    | 5.82±0.59          | 3.58±0.61          | 5.13±0.55     | 4.73±0.23       |
| C16:0         | 18.50±0.67         | 20.72±0.08         | 20.38±0.08    | 21.50±0.74      |
| isoC17:0      | 0.18±0.05          | 0.14±0.04          | 0.11±0.01     | 0.12±0.03       |
| AnteisoC17:0  | 0.08±0.03          | 0.14±0.00          | 0.15±0.06     | 0.10±0.01       |
| C17:1n-7      | 0.44±0.16          | 0.27±0.10          | 0.26±0.01     | 0.21±0.03       |
| C17:0         | 0.61±0.17          | 0.48±0.14          | 0.39±0.03     | 0.36±0.09       |
| C18:3         | 0.17±0.05          | 0.14±0.00          | 0.10±0.00     | NA              |
| C18:2n6       | 19.22±2.84         | 23.78±1.07         | 24.04±1.26    | 21.24±1.05      |
| C18:1n6       | 16.98±2.06         | 24.09±2.85         | 22.03±0.88    | 23.70±1.69      |
| C18:1n9       | 5.10±0.82          | 6.99±1.57          | 5.83±0.78     | 5.83±0.81       |
| transC18:1n9  | 0.21±0.05          | NA                 | 0.07±0.00     | NA              |
| C18:0         | 12.41±2.43         | 8.75±2.24          | 7.98±0.86     | 9.62±0.57       |
| CLA           | 2.12±0.00          | 1.12±0.38          | 1.47±0.00     | 1.01±0.30       |
| C19:0         | 0.20±0.11          | NA                 | 0.10±0.01     | 0.12±0.00       |
| C19:2         | NA                 | NA                 | 0.23±0.07     | 0.16±0.00       |
| C19:1         | NA                 | NA                 | 0.24±0.11     | NA              |
| C20:0         | 0.94±0.31          | 0.56±0.12          | 0.49±0.01     | 0.64±0.12       |
| C20:1         | 3.77±1.61          | 1.51±0.74          | 2.49±1.09     | 1.79±0.38       |
| C20:2         | 1.21±0.66          | 1.36±0.00          | 0.35±0.05     | 0.32±0.07       |
| C20:3         | 1.00±0.61          | 0.64±0.00          | 0.40±0.01     | 0.30±0.00       |
| C20:4         | 2.41±1.33          | 1.30±0.38          | 2.00±0.57     | 2.06±0.28       |
| C20:5         | 0.32±0.02          | NA                 | NA            | NA              |
| C22:0         | 0.34±0.03          | NA                 | 0.18±0.00     | 0.29±0.00       |
| C22:1         | 0.89±0.63          | 0.48±0.20          | 0.37±0.00     | 1.23±0.43       |
| C22:6         | 2.47±1.17          | 1.16±0.30          | 1.72±0.43     | 1.20±0.11       |
| C24:0         | NA                 | NA                 | 0.24±0.00     | 0.32±0.00       |
| C24:1         | 0.17±0.00          | NA                 | NA            | NA              |
| BCFA          | 0.58±0.12          | 0.50±0.09          | 0.55±0.08     | 0.41±0.08       |
| MUFA          | 34.38±1.26         | 37.34±1.65         | 37.12±1.47    | 37.85±1.94      |
| PUFA          | 29.04±2.71         | 29.51±1.75         | 30.40±2.39    | 26.28±1.51      |
| SFA           | 36.58±1.48         | 33.15±1.78         | 32.49±2.22    | 35.86±2.06      |

BCFA, branched chain fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Iso-branched chain, Anteiso-trans branched chain, NA, not detected.
Table 5: List of differential metabolites of two groups in one month

| Lipid species | Lipid metabolites | Retention time | Mass to charge ratio | VIP | P-Value | Fold change | LOG-Fold change | Significance of difference |
|---------------|-------------------|----------------|----------------------|-----|---------|-------------|-------------------|--------------------------|
| PC            | PC(P-18:0/2:0)    | 143            | 550                  | 1.8334 | 0.0179 | 1.2259     | 0.2939            | *                        |
|               | PC(P-20:0/13:0)  | 441            | 732                  | 1.7739 | 0.0147 | 0.7627     | -0.3908           | *                        |
|               | PC(P-20:0/16:0)  | 524            | 774                  | 1.8252 | 0.0089 | 0.5883     | -0.7654           | **                       |
|               | Cer(d18:1/18:0)  | 464            | 566                  | 1.8415 | 0.0126 | 0.5787     | -0.7891           | *                        |
|               | Cer(d18:1/20:0)  | 501            | 594                  | 1.5678 | 0.0386 | 0.7226     | -0.4687           | *                        |
| Cer           | Cer(d17:1/22:0)  | 519            | 608                  | 1.7649 | 0.0157 | 0.7513     | -0.4126           | *                        |
|               | Cer(d17:1/24:1)  | 519            | 634                  | 2.0024 | 0.0021 | 0.7329     | -0.4483           | **                       |
|               | Cer(d17:1/24:0)  | 550            | 636                  | 2.0617 | 0.0041 | 0.7099     | -0.4944           | **                       |
| SM            | SM(d15:0/24:1)   | 523            | 773                  | 1.4560 | 0.0346 | 0.8500     | -0.2343           | *                        |
|               | SM(d14:0/26:1)   | 548            | 787                  | 1.6873 | 0.0387 | 0.7786     | -0.3610           | *                        |
|               | DG(17:2/18:2)    | 467            | 620                  | 1.4788 | 0.0425 | 0.6760     | -0.5649           | *                        |
| DG            | DG(18:2/18:2)    | 434            | 634                  | 1.7219 | 0.0204 | 0.6447     | -0.6333           | *                        |
|               | DG(19:0/20:5)    | 550            | 674                  | 1.8250 | 0.0196 | 0.7724     | -0.3725           | *                        |
|               | TG(17:0/17:0/17:1)| 717          | 764                  | 2.1105 | 0.0019 | 0.6727     | -0.5719           | **                       |
| TG            | TG(18:2/18:2/19:0)| 709         | 914                  | 1.4056 | 0.0450 | 0.7955     | -0.3301           | *                        |
|               | TG(18:0/18:0/20:0)| 734         | 936                  | 1.7956 | 0.0136 | 1.5472     | 0.6297            | *                        |
Table 6: List of differential metabolites of two groups in five months

| Lipid species | Lipid metabolites | Retention on time | Mass to charge ratio | VIP Value | P-Value | Fold change | LOG-Fold change | Significance of difference |
|---------------|-------------------|------------------|----------------------|-----------|----------|-------------|---------------------|---------------------------|
| PE(16:1/18:2) | 312               | 712              | 1.5521               | 0.0496    | 1.2267   | 0.2948      |                     | *                         |
| PE(16:1/18:3) | 285               | 710              | 1.8269               | 0.0286    | 1.6373   | 0.7113      |                     | *                         |
| PE(18:0/20:1) | 463               | 772              | 1.7250               | 0.0108    | 1.4456   | 0.5317      |                     | *                         |
| PE(18:1/22:6) | 302               | 788              | 1.7261               | 0.0175    | 1.5123   | 0.5968      |                     | *                         |
| PE(18:1/18:2) | 358               | 740              | 1.6588               | 0.0248    | 1.1795   | 0.2382      |                     | *                         |
| PE(18:2/18:2) | 323               | 738              | 1.8504               | 0.0009    | 1.5472   | 0.6297      |                     | **                        |
| PE(18:2/20:4) | 316               | 762              | 1.7446               | 0.0110    | 1.3631   | 0.4469      |                     | *                         |
| PE(18:3/0:0)  | 68                | 474              | 1.6749               | 0.0446    | 1.6885   | 0.7557      |                     | *                         |
| PE(18:3/18:2) | 288               | 736              | 1.8705               | 0.0004    | 2.3033   | 1.2037      |                     | **                        |
| PE(18:3/22:6) | 270               | 784              | 1.8926               | 0.0061    | 2.5215   | 1.3343      |                     | **                        |
| PE(19:0/18:2) | 417               | 756              | 1.7097               | 0.0238    | 1.3588   | 0.4423      |                     | *                         |
| PE(20:0/18:2) | 436               | 770              | 1.7618               | 0.0034    | 1.5678   | 0.6488      |                     | **                        |
| PE(20:0/20:3) | 432               | 796              | 1.6392               | 0.0471    | 2.4782   | 1.3093      |                     | *                         |
| PE(20:0/20:4) | 431               | 794              | 1.7982               | 0.0051    | 1.8168   | 0.8614      |                     | **                        |
| PE(20:1/18:0) | 436               | 772              | 1.7710               | 0.0052    | 1.5157   | 0.6000      |                     | **                        |
| PE(20:3/18:2) | 274               | 764              | 1.8207               | 0.0022    | 1.6948   | 0.7611      |                     | **                        |
| PE(20:4/20:4) | 302               | 786              | 1.7784               | 0.0090    | 1.5816   | 0.6614      |                     | **                        |
| PE(22:6/20:0) | 420               | 818              | 1.7802               | 0.0110    | 2.1100   | 1.0773      |                     | *                         |
| PE(22:6/20:4) | 292               | 810              | 1.5764               | 0.0383    | 1.2959   | 0.3740      |                     | *                         |
| SM(d14:1/24:1) | 375               | 757              | 1.5844               | 0.0337    | 0.7226   | -0.4686     |                     | *                         |
| SM(d15:1/22:0) | 391               | 745              | 1.5937               | 0.0323    | 0.7554   | -0.4045     |                     | *                         |
|          |         |       |       |       |       |       |       |       |
|----------|---------|-------|-------|-------|-------|-------|-------|-------|
| SM(d15:1/24:1) | 392     | 771   | 1.5069| 0.0398| 0.6656| -0.5870| *     |
| SM(d19:1/26:1) | 532     | 855   | 1.6639| 0.0160| 0.5137| -0.9608| *     |
| PC(16:0/18:3)  | 315     | 800   | 1.6161| 0.0384| 1.1498| 0.2014| *     |
| PC(16:1/18:2)  | 269     | 800   | 1.8276| 0.0017| 1.2784| 0.3543| **    |
| PC(18:0/17:0)  | 413     | 820   | 1.6359| 0.0263| 0.8433| -0.2457| *     |
| PC(18:0/20:2)  | 396     | 858   | 1.5962| 0.0434| 1.5531| 0.6352| *     |
| PC(18:0/22:6)  | 366     | 878   | 1.7161| 0.0097| 1.3534| 0.4366| **    |
| PC(18:2/0:0)   | 72      | 520   | 1.6561| 0.0207| 1.3335| 0.4153| *     |
| PC(18:2/18:2)  | 309     | 826   | 1.7009| 0.0200| 1.4317| 0.5177| *     |
| PC(18:2/20:4)  | 301     | 850   | 1.5683| 0.0454| 1.2274| 0.2956| *     |
| PC(18:3/18:2)  | 274     | 824   | 1.8312| 0.0030| 2.1499| 1.1043| **    |
| PC(19:0/18:2)  | 406     | 844   | 1.8187| 0.0046| 1.4651| 0.5510| **    |
| PC(19:0/20:3)  | 417     | 870   | 1.8060| 0.0054| 1.2942| 0.3721| **    |
| PC(20:0/0:0)   | 153     | 552   | 1.7921| 0.0048| 1.8196| 0.8636| **    |
| PC(20:0/18:2)  | 426     | 858   | 1.8427| 0.0016| 2.1436| 1.1000| **    |
| PC(20:0/20:4)  | 420     | 882   | 1.8099| 0.0025| 1.8864| 0.9156| **    |
| PC(20:2/0:0)   | 92      | 548   | 1.7141| 0.0171| 1.4898| 0.5751| *     |
| PC(P-20:0/22:6) | 427    | 846   | 1.7224| 0.0108| 1.3383| 0.4204| *     |
| PC(20:5/0:0)   | 53      | 542   | 1.6432| 0.0173| 1.4363| 0.5224| *     |
| PC(22:0/18:2)  | 464     | 886   | 1.6939| 0.0340| 1.9274| 0.9466| *     |
| PC(22:6/18:2)  | 289     | 874   | 1.6654| 0.0292| 1.4573| 0.5433| *     |
| PC(22:6/18:3)  | 260     | 872   | 1.7974| 0.0070| 1.9106| 0.9340| **    |
| PC(22:6/20:1)  | 368     | 904   | 1.5662| 0.0280| 1.3360| 0.4179| *     |
| PC(22:6/20:4)  | 279     | 898   | 1.6836| 0.0302| 1.3964| 0.4817| *     |
| TG(18:1/20:4/22:5) | 625 | 972 | 1.6914 | 0.0316 | 2.2670 | 1.1808 | *     |
| TG(18:1/22:6/22:6) | 608 | 994 | 1.7000 | 0.0167 | 0.6735 | -0.5701 | *     |
| Metabolite | Log2FoldChange | FDR   | p-value | p-value | FoldChange |
|------------|----------------|-------|---------|---------|------------|
| TG(18:2/18:3/22:6) | 1.6714 | 0.0337 | 1.7838 | 0.8349 | *          |
| TG(18:2/20:5/20:5) | 1.6768 | 0.0203 | 1.4125 | 0.4982 | *          |
| TG(18:2/20:5/22:6) | 1.7062 | 0.0211 | 1.5185 | 0.6026 | *          |
| TG(18:3/18:3/20:5) | 1.6796 | 0.0478 | 2.5501 | 1.3505 | *          |
| TG(20:4/20:5/20:5) | 1.7133 | 0.0153 | 1.9061 | 0.9306 | *          |
| PG(16:1/18:1) | 1.6638 | 0.0207 | 0.7771 | -0.3636 | *          |
| PG(17:0/18:1) | 1.7711 | 0.0075 | 0.6933 | -0.5282 | **         |
| PG(20:1/20:3) | 1.8501 | 0.0012 | 2.1143 | 1.0802 | **         |
| PG(23:0/19:0) | 1.6688 | 0.0227 | 1.3735 | 0.4578 | *          |
| DG(20:5/20:2) | 1.6751 | 0.0330 | 0.7069 | -0.5004 | *          |
| DG(22:4/17:0) | 1.7133 | 0.0149 | 1.3633 | 0.4471 | *          |
| PS(18:1/22:6) | 1.7863 | 0.0051 | 0.5073 | -0.9790 | **         |
| Cer(d17:0/26:1) | 1.6942 | 0.0109 | 0.7359 | -0.4423 | *          |
| Cer(d18:1/18:0) | 1.6668 | 0.0338 | 0.6482 | -0.6254 | *          |
| CL(16:0/16:0/18:0/20:3) | 1.6927 | 0.0374 | 1.6321 | 0.7067 | *          |
| CL(16:0/20:2/20:3/22:6) | 1.5465 | 0.0498 | 1.1601 | 0.2143 | *          |
| CL(16:1/18:0/18:2/18:0) | 1.6854 | 0.0384 | 1.6203 | 0.6962 | *          |
| PI(17:0/20:3) | 1.8059 | 0.0044 | 1.8877 | 0.9166 | **         |
| PA(18:0/22:1) | 1.7778 | 0.0112 | 1.4345 | 0.5206 | *          |
| PA(18:1/23:0) | 1.7276 | 0.0134 | 1.1361 | 0.1841 | *          |
| PA(22:5/26:0) | 1.7498 | 0.0039 | 1.7312 | 0.7918 | **         |

LOG-Fold change, The positive sign indicates the increase of this kind of metabolite content in the control group, and the negative sign indicates the decrease. It can be seen from the table that the content of 2 and 56 metabolites is higher in the control group than in the treatment group after one and five months, respectively, and the content of 14 and 12 differential metabolites is lower than in the treatment group. *represents p<0.05, **represents p<0.01.
Figure 1:

Monthly Body Weight

![Bar graph showing monthly body weight comparison between control and treatment groups.]

Daily Food Intake

Final Body Weight

![Bar graphs showing daily food intake and final body weight for control and treatment groups.]

- Bar graph for daily food intake with 0.0 to 3.5 food intake (g/day) and two bars for control and treatment.
- Bar graph for final body weight with 0.0 to 30.0 body weight (g) and three bars for control, one month, and five months.
Figure 2:
Figure 3:

Liver mRNA of a month

Liver mRNA of five months
Figure 4:

Figure 5:
Figure 6:
Figure 7: