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Integrating lipidomics and gut microbiota to study the anti-hyperlipidemia effect of *Sargentodoxae Caulis* extract

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Author contributions

Zhang XX, Jia L and Ngina C were responsible for primary data analysis and writing the manuscript. Zhan XJ was involved in the preparation of SC samples and the analysis of MS. Zhang QR was involved in the preparation of SC samples and formal analysis. Li XG and Jiang QB were involved in animal experiments. Jiang MM and Bai S designed and coordinated the work. All authors read and approved the final version.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

SC, *Sargentodoxa Caulis*; TC, total cholesterol; TG, triglyceride; OxTG, oxidized triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; D-LA, D-Lactic acid; C, Control group; M, Model group; Y, Positive group; L, SC extract low-dose group; Z, SC extract medium-dose group; H, SC extract high-dose group; FFA, free fatty acid; TNF-α, tumor necrosis factor; PPAR-α, serum peroxisome proliferator-activated receptor alpha; PPAR-γ, serum peroxisome proliferator-activated receptor gamma; QC, quality control; PG, phosphatidylglycerol; GPs, glycerophospholipids; LPS, lipopolysaccharide; FA, fatty acids; DG, diacylglycerol; STs, sterol lipids; LDA, linear discriminant analysis; PE, phosphatidyl ethanolamine; F/B, Firmicutes/Bacteroidetes; SM, sphingomyelin.

Citation

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Abstract

Background: *Sargentodoxae Caulis* (SC) is the vine stem of *Sargentodoxa Cuneata* (Oliv.) Rehd. & E. H. Wilson in C. S. Sargent, and it in traditional Chinese medicine has been known for promoting blood circulation and removing blood stasis as recorded in the ancient book "*Illustrated Classics of Materia Medica*". It has been used effectively to treat blood stasis too in modern clinical practice. However, the anti-hyperlipidemia effect of SC is not fully understood. This paper aims at exploring the use of SC stems to improve the balance of blood lipids in the body, and its new role in treating hyperlipidemia. **Methods:** The effects of SC extract on hyperlipidemia were explored by combining lipidomics and gut microbiota. Secondly, we explored the potential mechanism of SC in treating hyperlipidemia by pathway analysis. **Results:** The results showed that the stem extract of SC could restore the physiological and biochemical indices of hyperlipidemia in mice, as well as repair the morphological and structural damage to tissues. Compared to the Model group, the SC extract significantly reduced the liver index, epididymal fat index, and Lee's index. It also significantly decreased serum levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol, peroxisome proliferator-activated receptor gamma (PPAR-γ), D-lactate, and free fatty acids, while significantly increasing the relative content of peroxisome proliferator-activated receptor alpha (PPAR-α). These changes were statistically significant. Non-targeted lipidomics, based on LC-MS, were utilized to investigate the lipid metabolism characteristics in serum, liver, and epididymal fat of the subjects. It was observed that, compared to the blank group, the Model group exhibited significant changes primarily in glycerol lipids and glycerophospholipids. The treatment group also displayed alterations in these lipids. A total of 38, 81, and 27 differential lipids were identified in serum, liver, and epididymal fat samples, respectively. Among these, 14 common differential lipids were found in both serum and liver samples, and their KEGG enrichment pathways were largely consistent. Among them, the sphingolipid signaling pathway and the glycerophospholipid metabolic pathway were identified as key metabolic pathways that were regulated. Our gut microbiota analysis revealed that SC diminishes the abundance of Actinobacteria by altering the cecal flora in mice. **Conclusion:** This alteration leads to the downregulation of genes involved in triglyceride metabolism, which in turn changes lipid processing and reduces triglyceride levels. Consequently, SC effectively combats hyperlipidemia. Notably, SC impacts key metabolic pathways, including the sphingolipid signaling and glycerophospholipid metabolism. These findings underscore SC's therapeutic potential, positioning it as a promising alternative for reducing the health risks associated with hyperlipidemia.

Keywords: hyperlipidemia; *Sargentodoxae Caulis*; lipidomics; intestinal flora

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Highlights

This study, by integrating lipidomics and gut microbiota analysis, reveals that the extract of *Sargentodoxae Caulis* can treat hyperlipidemia by affecting key metabolic pathways, including the sphingolipid signaling pathway and the glycerophospholipid metabolic pathway. This research provides a theoretical basis for the use of *Sargentodoxae Caulis* extract in the treatment of hyperlipidemia.

Medical history of objective

Sargentodoxae Caulis is a plant used in traditional Chinese medicine. In the "*Tujing Bencao*" compiled by Su Song and others during the Song Dynasty, *Sargentodoxa* is mentioned, with descriptions of its morphological characteristics and the time for collection. In traditional ancient Chinese medicine, it is often used to promote blood circulation and remove blood stasis. Modern pharmacological research indicates that the stem of *Sargentodoxa* possesses antimicrobial properties, can inhibit platelet aggregation, increase coronary blood flow, suppress thrombosis, enhance ischemic tolerance, dilate the coronary arteries, and reduce the size of myocardial infarction.

Background

Hyperlipidemia is a disorder of lipid metabolism characterized by elevated levels of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C), as well as decreased levels of high-density lipoprotein cholesterol (HDL-C) in plasma [1]. With the improvement of the economic level, changes in diet structure, and a sedentary lifestyle, there has been a significant increase in the number of patients with hyperlipidemia. It has become one of the major diseases troubling human health [2]. Effective prevention and treatment of hyperlipidemia are crucial for preventing cardiovascular and cerebrovascular diseases [3, 4].

The treatment of hyperlipidemia encompasses diet control, exercise, and pharmacotherapy. Pharmacological agents, including statins, fibrates, cholesterol absorption inhibitors, fibric acid derivatives, bile acid sequestrants, PCSK9 inhibitors, and other chemical drugs, are widely used in clinical practice [5]. Although these drugs demonstrate significant efficacy in treating hyperlipidemia, as research progresses, some adverse effects have been reported, such as nausea, vomiting, and muscle pain. Prolonged use of these synthetic drugs may even result in rhabdomyolysis, diabetes, damage to the liver and kidneys, and the development of new tumors [6, 7]. Consequently, finding safe and effective treatments for hyperlipidemia is of utmost importance.

Sargentodoxae Caulis (SC) is the vine stems of *Sargentodoxa Cuneata* (Oliv.) Rehd. et Wils. In ancient Chinese traditional medicine, it is often used to promote blood circulation and remove blood stasis. Its anti-hyperlipidemia effect has not been reported yet. However, modern pharmacological studies have shown that SC total phenolic acids can inhibit myocardial ischemia in mice apoptosis and oxidative stress [8]. It has also been reported to improve cerebral ischemia-reperfusion injury in rat brain tissue levels of oxidative stress and reduce the expression of inflammatory factors in brain tissue [9, 10]. The above content to some extent shows that SC has the effect of reducing inflammation and oxidative stress injury and has a protective effect on cardiovascular disease. In summary, this study speculated that SC may prevent the occurrence of hyperlipidemia to some extent.

Lipidomics, by comparing the overall changes in lipid metabolic networks, elucidates the characteristics of lipid metabolism under various physiological and pathological conditions. It identifies key biomarkers and reveals the relationships between different lipids, diseases, and the associated signaling pathway mechanisms [11]. In addition, compounds in traditional Chinese medicine can improve the secretion of metabolites by intestinal microorganisms, by regulating the abundance of related intestinal flora, thereby antagonizing hyperlipidemia [12, 13]. The intestinal flora is a complex and vast ecosystem, often referred to as the “second gene pool” of humans [14]. In recent years, an increasing number of studies have shown a close relationship between intestinal flora and blood lipids.

Diet is the primary factor influencing the diversity of intestinal bacteria. A high-fat diet can alter the composition of the intestinal flora and increase the incidence of lipid metabolism disorders. The main pathway implicated involves the intestinal flora and its metabolites, which can compromise the integrity of the intestine and alter its permeability. This, in turn, can lead to the proliferation of potentially pathogenic bacteria and the release of inflammatory factors, disrupting immune homeostasis [15]. Conversely, a balanced diet can regulate the balance of lipid metabolism by modulating cholesterol metabolism in the liver, enhancing lipid oxidation in the muscles, and regulating lipid storage in adipose tissue [16]. Therefore, maintaining the homeostasis of the intestinal microbial community is essential for preserving the balance of lipid metabolism.

In this study, we investigated the effect of SC extract on lipid mass spectrometry in hyperlipidemic mice by non-targeted lipidomics and screened the related differential metabolites in serum, liver, and epididymal adipose tissue for enrichment pathway analysis, we also evaluated the anti-hyperlipidemic effect of SC extract in combination with intestinal flora.

Materials and methods

Reagents and materials

SC was purchased from Beijing Tongrentang Pharmaceutical Co., Ltd. (Beijing, China, No. 2020808) and identified as the vine stem of *Sargentgloryvine* of the genus *Sargentgloryvine* of the Mangnolaceae family by associate researcher Dr. Wu Honghua (Tianjin University of Chinese Medicine). The voucher specimen was preserved in the author’s laboratory of Tianjin University of Traditional Chinese Medicine (Tianjin, China, No. TJUTCCM-SS-202008). The vine stems of SC were extracted by a combination of methanol cold soak and 70% ethanol-water heating reflux. The Folin-Ciocalteu method was used to determine the total polyphenol content of the SC extract.

Simvastatin (S24506) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). 10% paraformaldehyde (G2160) was purchased from Beijing Solebold Technology Co., Ltd. (Beijing, China). TG (A110-1-1), TC (A111-1-1), HDL-C (A112-1-1), LDL-C (A113-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). PPAR-α ELISA kit (JM-02532M1), PPAR-γ ELISA kit (JM-11521M1), FFA ELISA kit (M-02609M1), D-LA ELISA kit (JM-11669M1) were purchased from Jiangsu Jingmei Biotechnology Co., Ltd. (Yancheng, China). Methanol, methyl tert-butyl ether, ethanol, petroleum ether, ethyl acetate, and dichloromethane were all analytically pure and purchased from Tianjin Kangkede Technology Co., Ltd. (Tianjin, China). Isopropanol (HPLC grade) was purchased from Fisher company (Fair Lawn, NJ, USA). Ammonium formate (A800015-50g, LC-MS grade) was purchased from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China).

Animals and experiments procedure

All protocols used in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (Ethics approval number TCM-LAEC2021178). Male C57BL/6J mice (specific pathogen free, 6 weeks, 220 ± 10 g) were obtained from SPF Biotechnology Co., Ltd. (Beijing, China; license number: SCXK (Jin) 2019-0008). After 7 days of adaptive feeding in an environment of 50 ± 10%, 23 ± 2 ˚C humidity, alternating light and dark for 12 h, 48 mice were divided into 6 groups (8 mice in each group), except for Control group (group C), the rest were fed with high-fat diet. The group C and the Model group (group M) were gavaged with equivalent saline. The Positive group (simvastatin, Y, 20.00 mg/kg), SC extract low-dose (L, 0.3 g/kg/d), medium-dose (Z, 0.6 g/kg/d), and high-dose (H, 1.2 g/kg/d)

groups, the dose was calculated according to the clinical dose of *Chinese Pharmacopoeia*. All groups drank water freely, except the blank group which was fed with ordinary feed, and the other groups were fed with high-fat feed. After 8 weeks of administration, the mice were fasted overnight and anaesthetized with 0.3%, 0.1 mL/10 g pentobarbital, and a sample of liver, serum, and epididymal fat were harvested.

Histopathological and biochemical analysis

Tissue blocks were placed in 10% formalin solution, embedded in paraffin wax, and cut into sections for dyeing. Serum TG, serum TC, serum HDL-C, serum LDL-C, serum peroxisome proliferator-activated receptor alpha (PPAR-α), serum peroxisome proliferator-activated receptor gamma (PPAR-γ), serum D-Lactic acid (D-LA) and free fatty acid (FFA) were detected using an automatic biochemical analyzer, and the liver biochemical indexes including lipopolysaccharide (LPS) and tumor necrosis factor (TNF-α) were also analyzed by ELISA kits.

Hepatic lipidomics analysis

The serum samples (100 μL) were mixed with methanol (300 μL) and methyl tert-butyl ether (1 mL) in a 2 mL centrifuge tube. After vortexing for 30 s, the mixture was centrifuged at 13,680 g for 10 min. The supernatant was dried with nitrogen and re-dissolved with 100 μL methanol. After centrifugation, the supernatant was collected into water injection vials for analysis. A quality control (QC) sample was prepared by pooling an equal volume of all samples.

The liver/fat sample (100 mg) was ground with 3 abrasive beads and 435 μL water for 40 s. After adding 1.5 mL dichloromethane/methanol (2:1, v/v) and vortex oscillation for 30 s, it was centrifuged at 3,000 rpm for 15 min. The next layer of 0.5 mL liquid was placed in a new tube and dried with nitrogen. After dissolution in 200 μL isopropanol/methanol (1:1, v/v), the samples were centrifuged after vortex oscillation for 30 s and the supernatant was analyzed. A QC (QC for liver & QC for fat) sample was prepared by pooling equal volumes of all samples.

Detection used a Q Exactive™ Q-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) coupled with an UltiMate® 3000 UHPLC system. Chromatographic separation was achieved on Hypersil GOLD C18 (100 × 2.1 mm, 1.9 μm; Thermo Scientific, Waltham, MA, USA) maintained at 45 °C using a binary mobile phase which consisted of acetonitrile: water (60:40, v/v) solution containing 5 mM ammonium formate and 0.1% formic acid (A) and acetonitrile: 2-propanol (10:90, v/v) solution containing 5 mM ammonium formate and 0.1% formic acid (B). The mobile phase at a flow rate of 0.35 mL/min according to an optimal gradient eluting program: 0–14.5 min 40%–100% (B), 14.5–16.5 min 100% (B), 16.50–16.51 min 100–40% (B) and 16.51–20 min 40% (B). An injection volume of 4 μL was set. High-resolution MS data were recorded on a Q Exactive™ Q-Orbitrap mass spectrometer in the positive and negative ESI mode. The source parameters were as follows: Spray voltage, −3.5 kV/+2.8 kV; Capillary temperature, 350 °C; Gas temperature, 350 °C; Sheath Gas flow rate, 35 arb; Aux Gas flow rate: 15 arb; and Sweep Gas, 1 arb. Data acquisition and processing were controlled and performed by the Xcalibur 4.1 software.

The original spectral data was converted into ABF format using ABF Converter software and subsequently imported into MS-DIAL software for peak extraction, identification, alignment, and normalization. Lipid identification was conducted based on precursor and fragment ions, utilizing the built-in Lipid Blast database and MS-Finder database. Compounds were identified when their molecular weights and fragment ions matched those in the database. Ultimately, a three-dimensional data matrix was derived, encompassing sample information, identification results for each substance peak, lipid categories, branched chain details, retention times, mass-to-charge ratios, and mass spectrometry response intensities (peak areas).

Intestinal flora analysis

Under sterile conditions, the cecal tissues were extruded using sterile tweezers. The contents of the tissues were then squeezed into sterile EP tubes. The tubes were quenched with liquid nitrogen, placed in dry ice, and sent to Suzhou Panomik Biomedical Technology Co., Ltd. for microbial community diversity composition spectroscopic study.

The specific process and operation of high throughput sequencing experimental procedure and bioinformatics analysis methods are shown in Supplementary Figure S1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 and Origin Pro 2021 software. Data was presented as mean ± SD. Significance was indicated by ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$. Differential lipids were screened for FC > 3 and non-parametric test $P < 0.05$ using Metaboanalyst 5.0 (https://www.metaboanalyst.ca/). FC was calculated based on the peak area of lipids between the two groups. FC (M vs C) > 3 represented up-regulated lipid metabolites caused by group M, and FC (M vs C) < 1/3 represented down-regulated lipid metabolites caused by the M group.

Result

High-fat diet-induced biochemical index and histopathological changes in mice are modulated by SC extracts

The weight gain rate of the mice in the group M was significantly higher than that in the treatment group, as shown in Table 1. Lee's index measures the degree of obesity, while the organ index is used to assess the growth of mice during intragastric administration. The liver plays a crucial role in in vivo lipid metabolism, regulating both lipid synthesis and lipolysis. The results showed that treatment with SC extracts improved weight gain induced by a high-fat diet and effectively mitigated fatty liver conditions, as detailed in Table 2. Furthermore, the low-dose group exhibited a more pronounced inhibitory effect on fat accumulation.

In obese mice induced by a high-fat diet, SC extracts reduced TG, TC, and LDL, and increased HDL significantly ($P < 0.05$). The ELISA kit results indicated that the PPAR-α content in group M decreased significantly while the PPAR-γ content increased significantly. Each treatment group reversed these indicators, showing the potential for SC extracts to improve hyperlipidemia by regulating PPAR-related pathways. In comparison to group M, each treatment group significantly reduced serum D-LA and FFA levels ($P < 0.05$), suggesting that SC extract could regulate lipid metabolism to some extent (Figure 1A).

Table 1 The effect of SC extractive on mice weight

Group	Initial weight (g)	Final weight (g)	Weight growth rate (%)
C	22.73 ± 0.4	27.71 ± 0.71*	22 ± 4.45*
M	22.7 ± 0.68	29.8 ± 1.84	31.49 ± 10.76
Y	22.51 ± 0.51	26.5 ± 1.38*	17.73 ± 5.81*
L	22.65 ± 0.71	25.81 ± 1.31**	14.05 ± 6.64**
Z	22.54 ± 0.33	26.01 ± 1.23**	15.4 ± 4.76*
H	22.49 ± 0.38	25.75 ± 1.59**	14.54 ± 7.48*

The "*" in the same column indicates a significant difference compared with the group M ($P < 0.05$). The "**" in the same column indicates a significant difference compared with the group M ($P < 0.01$). C, Control group; M, Model group; Y, Positive group; L, SC extract low-dose group; Z, SC extract medium-dose group; H, SC extract high-dose group.

Table 2 The effect of SC extractive on obesity index in mice

Group	Lee's index	Liver index	Epididymal fat index
C	0.3043 ± 0.0036***	0.3258 ± 0.0004**	0.0109 ± 0.0018***
M	0.3326 ± 0.0066	0.4818 ± 0.0033	0.0232 ± 0.0038
Y	0.3147 ± 0.0086**	0.3932 ± 0.0023*	0.0177 ± 0.0027*
L	0.3218 ± 0.0075*	0.3937 ± 0.0021*	0.0174 ± 0.0046**
Z	0.3284 ± 0.0059	0.3930 ± 0.0013*	0.0207 ± 0.0056
H	0.3264 ± 0.0054	0.4148 ± 0.0020*	0.0217 ± 0.0055

The "*" in the same column indicates a significant difference compared with the group M ($P < 0.05$). The "**" in the same column indicates a significant difference compared with the group M ($P < 0.01$). C, Control group; M, Model group; Y, Positive group; L, SC extract low-dose group; Z, SC extract medium-dose group; H, SC extract high-dose group.

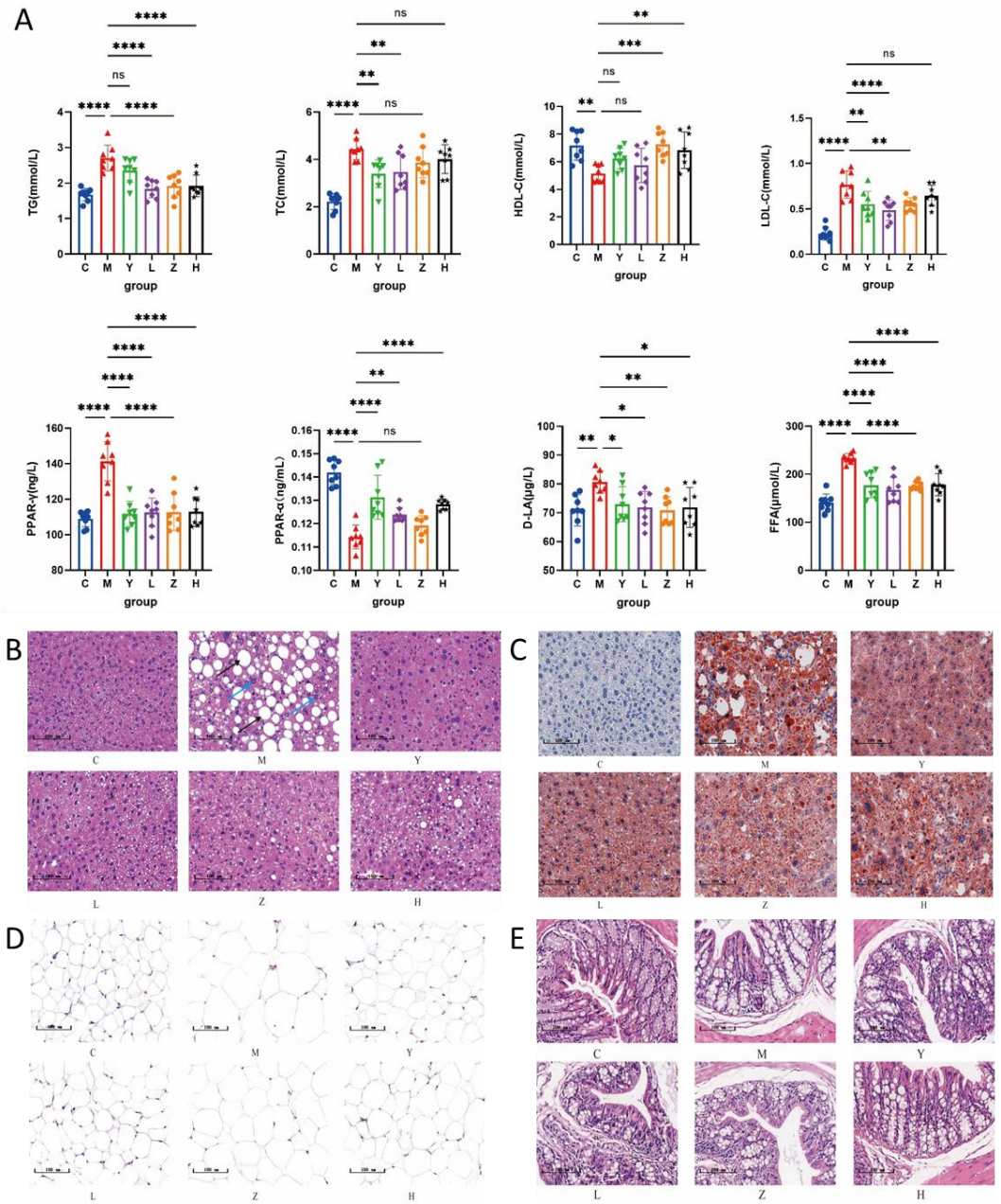

Figure 1 Statistical and pathological observations of the effects of SC extract on serum indices and tissue in mice. (A) Statistical diagram of the effect of the extract of SC on serum index content in mice. (B) H&E pathological observation of hepatic tissue in mice (×100). (C) Oil red staining pathological observation of hepatic tissue in mice (×100). (D) H&E pathological observation of epididymal adipose tissue in mice (×100). (E) H&E pathological observation of colonic tissue in mice (×100). Scale bar = 100 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (n = 6). ns, no significance; C, Control group; M, Model group; Y, Positive group; L, SC extract low-dose group; Z, SC extract medium-dose group; H, SC extract high-dose group; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; D-LA, D-Lactic acid; FFA, free fatty acid; PPAR-α, serum peroxisome proliferator-activated receptor alpha; PPAR-γ, serum peroxisome proliferator-activated receptor gamma.

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In mice fed with a high-fat diet, intracellular lipid accumulation, liver aggravation, and hepatocyte balloon expansion (magnified 100×) were observed (Figure 1B–1E). However, treatment with SC extract and positive drugs alleviated liver inflammation, improved hepatocyte balloon formation, and inhibited fat accumulation and degeneration in various tissues. This was consistent with lower levels of TC and TG in the treatment group. The low-dose group of SC had a significant effect on improving histological tissue and biochemical determination, so it was chosen for further study.

SC improved lipid metabolism in high-fat diet mice

All 10 QC sample data underwent unsupervised overall principal component analysis (PCA) to assess instrument and method stability. Both positive and negative ionic mode QC samples were within 2std, indicating a reliable experimental method and stable instrument. Results are presented in Supplementary Figure S2. ABF Converter and MS-DIAL software were used to process lipid identification data from serum, liver, and epididymal fat. The data matrix was imported into SIMCA-P 14.1 software and processed by Par formatting before carrying out the corresponding analysis.

Blood lipid levels differed significantly between the blank and model controls, suggesting substantial changes in lipid metabolites in the serum, liver, and epididymis of mice on a long-term high-fat diet. Following treatment with SC, up- or down-regulated lipids were significantly altered. Supplementary Table S1–S4, S6, S7 showed the distribution of species and differential lipids. This study found that SC extracts primarily reversed the up-regulation of lipids caused by hyperlipidemia, mainly TG, oxidized triglyceride (OxTG), and phosphatidylglycerol (PG). The number of down-regulated lipoproteins caused by hyperlipidemia was minimal, mainly glycerophospholipids (GPs) and glycerol (Supplementary Figure S5). Based on the results of volcanic (Supplementary Figure S7A–S7C), Venn (Supplementary Figure S4), heat (Figure 2A–2C) analysis of differential metabolites, differential lipids indicated hyperlipidemia lesions. SC prevented and improved hyperlipidemia by regulating lipids.

Results of combined analysis of serum, liver, and epididymal fat lipidomics

The data of serum (M vs C-S, M vs L-S), liver (M vs C-L, M vs L-L), and epididymal fat group (M vs C-E, M vs L-E) shows changes in lipids in serum, liver, and epididymal fat group samples. This lipid might be a

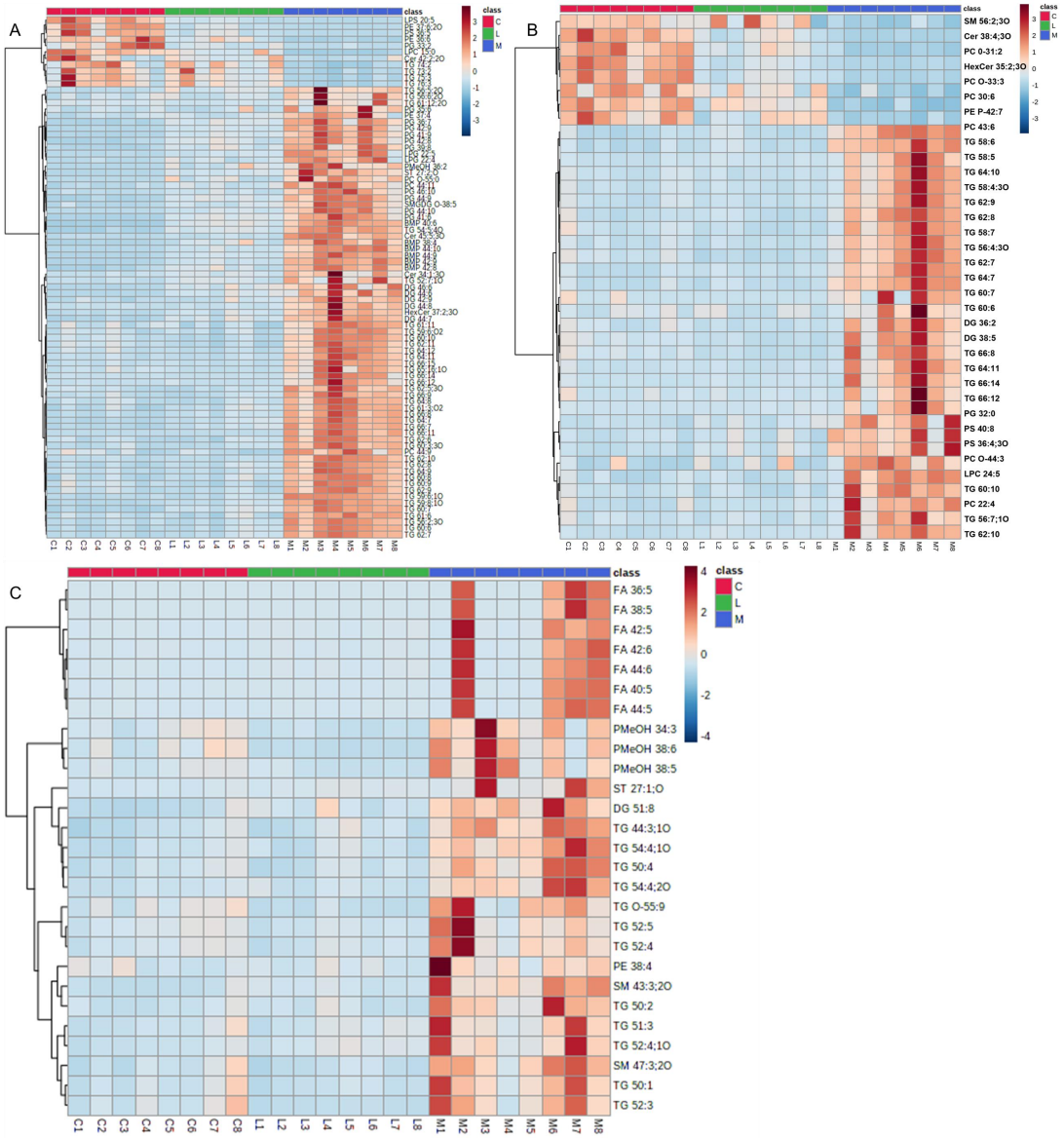

Figure 2 Analysis of differential lipids in liver, serum, and epididymal fat by heat map. (A) Heat map of differential lipids in liver. (B) Heat map of differential lipids in serum. (C) Heat map of differential lipids in epididymal fat. C, Control group; M, Model group; L, SC extract low-dose group; FA, fatty acids; DG, diacylglycerol; PMeOH, phosphatidylmethanol; STs, sterol Lipids; PE, phosphatidyl ethanolamine; SM, sphingomyelin.

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key marker of dyslipidemia and is involved in the regulation of SC extract. The Up-Set diagram (Figure 3A, 3B) indicates that the liver plays an important role in the whole lipid metabolism. The study analyzed 12 datasets and found that glycerol lipids and GPs were the most affected lipids in serum, liver, and epididymal fat, followed by sphingolipids and fatty acids (FAs). All identified lipids in group C, group M, and group L were categorized, summed, and plotted on a box plot to reveal the trends of various lipids in serum, liver, and epididymal fat in the three groups. The box plot is shown in Supplementary Figure S3. The serum, liver, and epididymal fat contents of various lipids are significantly increased in mice in Group M compared to Group C, however, the serum contents of sphingomyelin (SM), ether phosphatidylcholine, ether phosphatidyl ethanolamine (PE), ceramides_HDS and hexosylceramides_HS are significantly decreased. After drug intervention, serum lipids other than ether phosphatidylcholine, ceramides_HDS, and hexosylceramides_HS were significantly reduced. In the liver of mice, compared with group C, the contents of TG, OxTG, diacylglycerol (DG), FA, phosphatidylmethanol (PMeOH), sterol lipids (STs), SM, and PE in group M were significantly increased. The content of EtherTG also increased, but there was no significant difference. Compared with group M, the lipid content of group L decreased significantly except ST. There were 15 categories of lipids upregulated in epididymal fat of mice, which were extremely high compared with group C, with significant differences, while only 14 were significantly reversed after administration. Compared with group C, there were four categories of lipids significantly downregulated in group M, and only one lysophosphatidyl choline was significantly reversed after administration.

Due to the similarity in the broad-spectrum chemical properties of lipid compounds, the lipid metabolism pathways identified so far are complex, often with a given lipid shuttling between several different pathways. And a change in the membership level of a lipid not only affects the other members of the class, but also the other members of the class. Therefore, the analysis of the relation between differential lipids is shown in Figure 4.

To visualize the differential metabolites screened out by the lipid group, this experiment used LIPEA (https://lipea.biotec.tu-dresden.de/home) for analysis of pathway enrichment to study the related pathway functions of differential lipids. The results showed that there were only 6 common differential lipids between serum and liver (Figure 5A), all of which were TG, namely TG 18:1/18:1/24:5, TG 18:1/22:5/24:5, TG 18:1/18:1/28:5, TG 22:4/22:5/22:6, TG 20:4/22:5/24:5, TG 18:1/24:5/24:6 (Figure 5C). The specific content diagram is shown in Figure 5B. Pathway enrichment bubble maps were created for eligible pathways screened at $P < 0.05$. Details of the

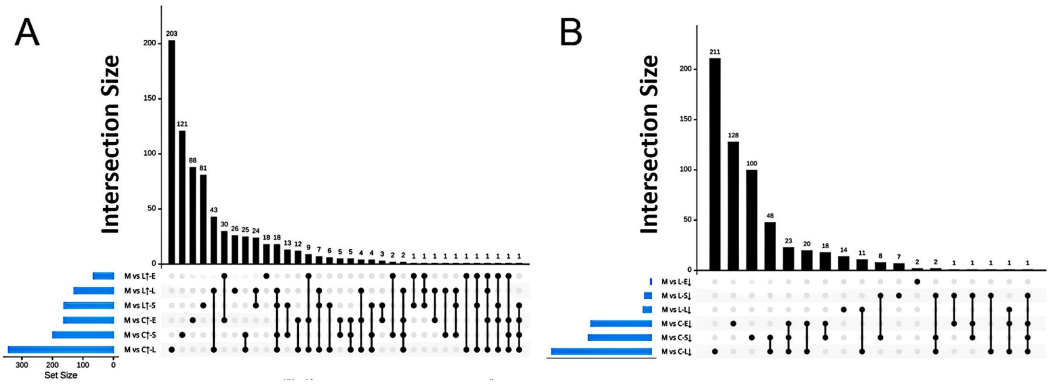

Figure 3 UpSet diagram analysis of up- and down-regulated lipids. (A) UpSet diagram of up-regulated lipids. (B) UpSet diagram of down-regulated lipids.

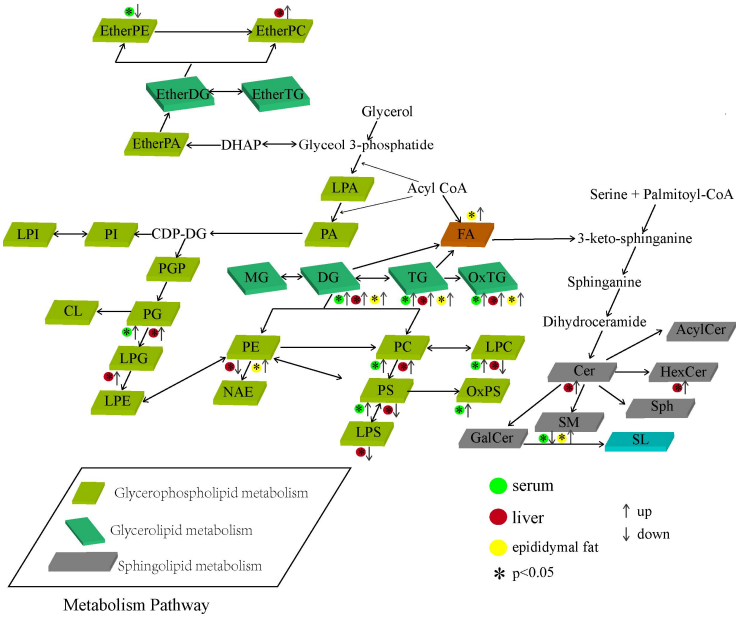

Figure 4 Possible metabolic pathway maps associated with the lipid biomarkers. TG, triglyceride; OxTG, oxidized triglyceride; PG, phosphatidylglycerol; LPS, lipopolysaccharide; FA, fatty acids; DG, diacylglycerol; PE, phosphatidyl ethanolamine; LPI, lysophosphatidylinositol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; PGP, phosphatidylglycerol phosphate; CL, cardiolipin; NAE, N-acylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; MG, ponoglyceride; PA, phosphatidic acid; LPA, lysophosphatidic acid; SL, sphingolipids; SM, sphingomyelin.

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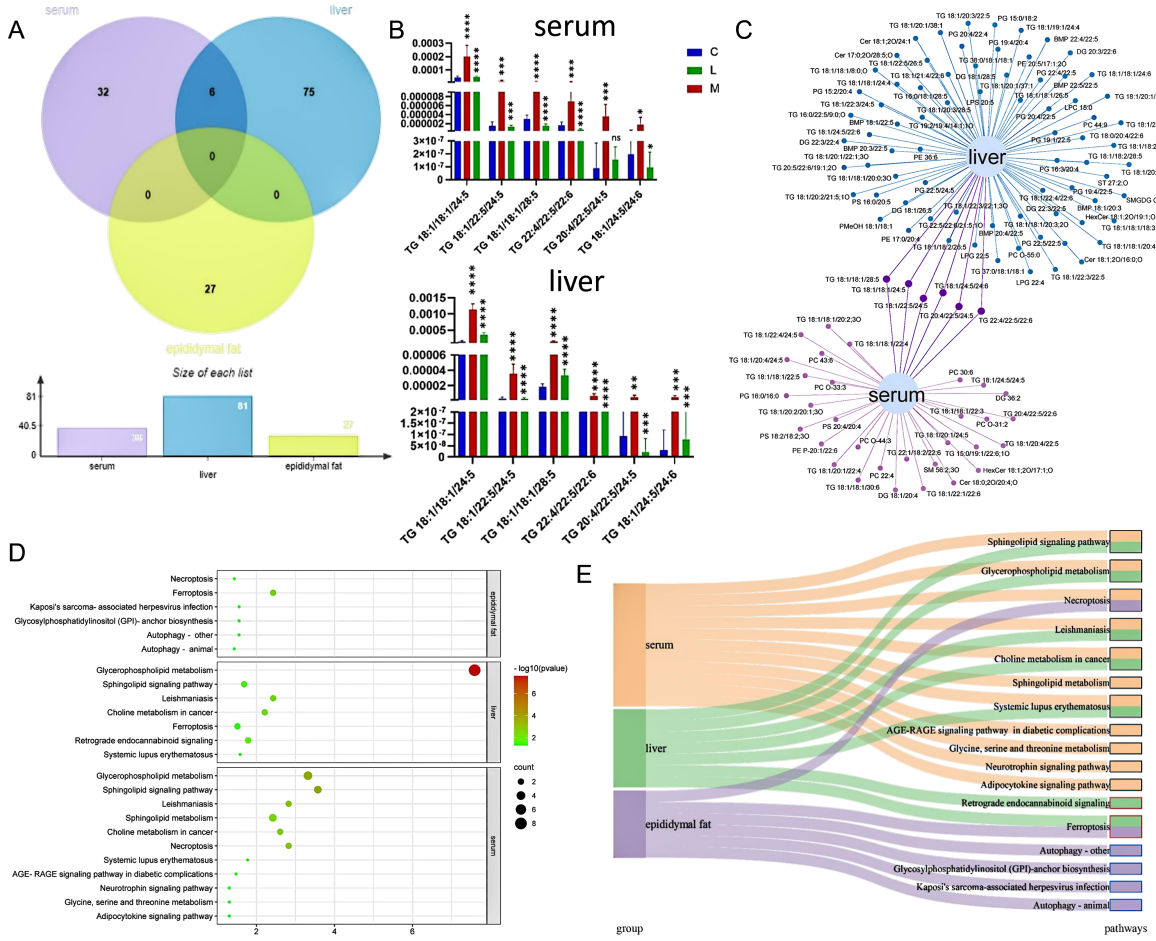

Figure 5 Lipidomics analysis: differential lipid profiles, KEGG pathway enrichment, and tissue comparison. (A) Venn diagram of differential lipid. (B) The relative contents of 14 different lipids. (C) Bipartite diagram of serum and liver differential lipids. (D) KEGG enrichment pathway of differential lipids. (E) KEGG reciprocal diagram of serum, liver, and epididymal fat. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (n = 6). ns, no significance; TG, triglyceride; C, Control group; M, Model group; L, SC extract low-dose group.

11 pathways associated with differential lipids in serum, 7 pathways in the liver, and 6 pathways in epididymal fat are given in Supplementary Table S3. Both liver and serum mainly used the glycerol-phospholipid metabolism pathway. Figure 5E was included for clarity. Although 81 differential lipids were found in the liver, the enrichment pathways were mostly consistent with those found in serum. The lipid enrichment pathway of epididymal fat closely resembles that of serum and liver, except in scrofula cases.

SC improved intestinal microbial disorders in mice on a high-fat diet

The study used seven indicators (Chao1, Observed species, Shannon, Simpson, Faith's PD, Pielou's evenness, and Good's coverage) to assess alpha diversity and found that a high-fat diet reduced species richness and diversity in rats' gut flora (Figure 6A). Extracts of SC did not increase gut flora diversity (Figure 6C), but they were able to reduce the distance between the model and blank groups, suggesting that they could improve gut flora differences (Figure 6B).

The levels of gut bacteria in groups L and M were significantly impacted by a high-fat diet. Group C was dominated by Bacteroidetes and Firmicutes. After eight weeks, there was a significant increase in Actinobacteria in group M, while Bacteroidetes decreased ($P < 0.05$). This change was not observed in group C, as shown in Figure 7A, 7C, Supplementary Table S8. At the genus level (Figure 7B, 7D), there was an increase in the number of species of flora after administration, as compared to group C and group M. *Akkermansia*, *Allobaculum*, and *Oscillospira* accounted for a larger proportion of the three groups. The abundance of *Allobaculum*, unclassified-Lachnospiraceae, and unidentified-Coriobacteriaceae increased in group M, which was significantly different from that in group C, whereas *Akkermania* decreased. After administration, the genus L group showed improvement, but there was a significant difference ($P < 0.001$) in the unidentified-Coriobacteriaceae.

An analysis of gut bacteria in mice found that SC extract can regulate species composition and richness in the intestines, improving hyperlipidemia in mice on a high-fat diet. The SC extract increased the abundance of certain bacteria like *Akkermansia*, *Ruminococcus*, and *Burkholderia* while decreasing the abundance of *Allobaculum*, *Subdoligranulum*, and *Olsenella* (Figure 8A). It also showed the taxonomic level distribution of significantly enriched species and marker species in each group as in Figure 8B, 8C. Linear discriminant analysis (LDA) > 4, S24_7, Bacteroidales, Bacillales, Bacteroidetes, and Bacteroidia in group C, Coriobacteriaceae, Coriobacteriale, *Olsenella,* Coriobacteriia, and Actinobacteria in group M, *Adlercreutzia* and *Ruminococcus* in group L showed a significant difference ($P < 0.05$).

Discussion

According to traditional Chinese medicine theory, hyperlipidemia is associated with "the blood flow in the body is impeded" and "the impaired excretion of metabolic products and secretions within the cavities or conduits of tissues and organs". SC is commonly used to treat conditions related to blood stasis, suggesting its potential efficacy in treating hyperlipidemia. Studies have indicated that SC extracts may treat hyperlipidemia by reducing body weight and blood

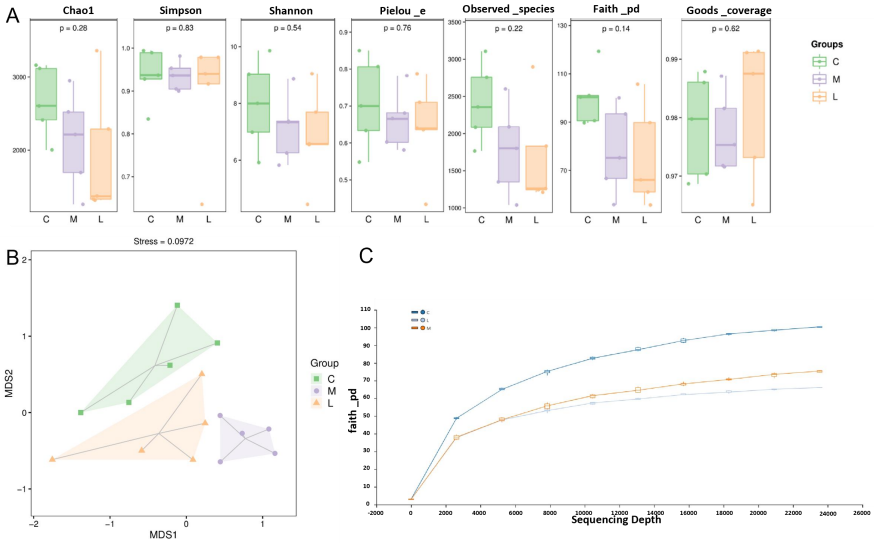

Figure 6 Comprehensive assessment of alpha diversity index boxplot, NMDS cluster analysis, and rarefaction curve. (A) Grouping boxplot of Alpha diversity index. (B) NMDS cluster analysis diagram. (C) Rarefaction curve. C, Control group; M, Model group; L, SC extract low-dose group.

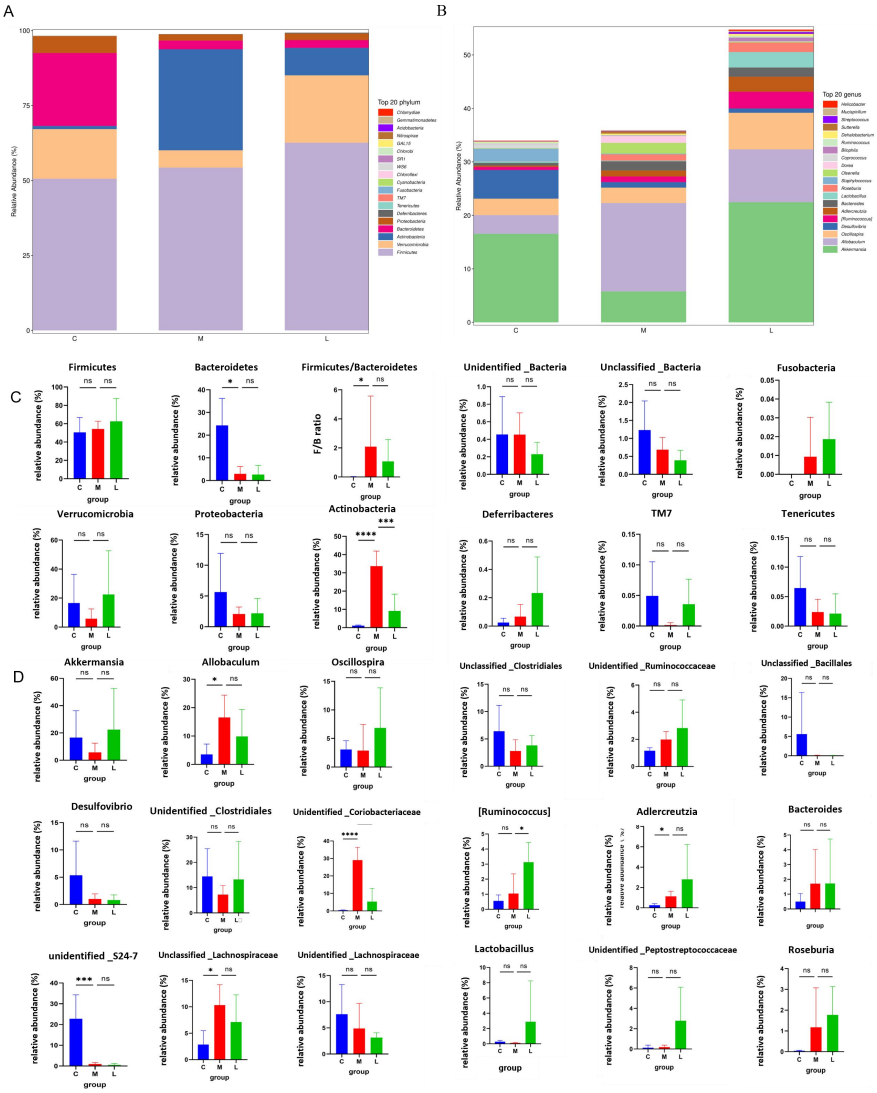

Figure 7 Gut microbiota composition and relative abundance analysis at phylum and genus levels. (A) The species composition of gut microbiota at the phylum level. (B) The species composition of gut microbiota at the genus level. (C) Analysis of the relative abundance of gut microbiota at the phylum level. (D) Analysis of the relative abundance of gut microbiota at the genus level. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (n = 6). ns, no significance; C, Control group; M, Model group; L, SC extract low-dose group.

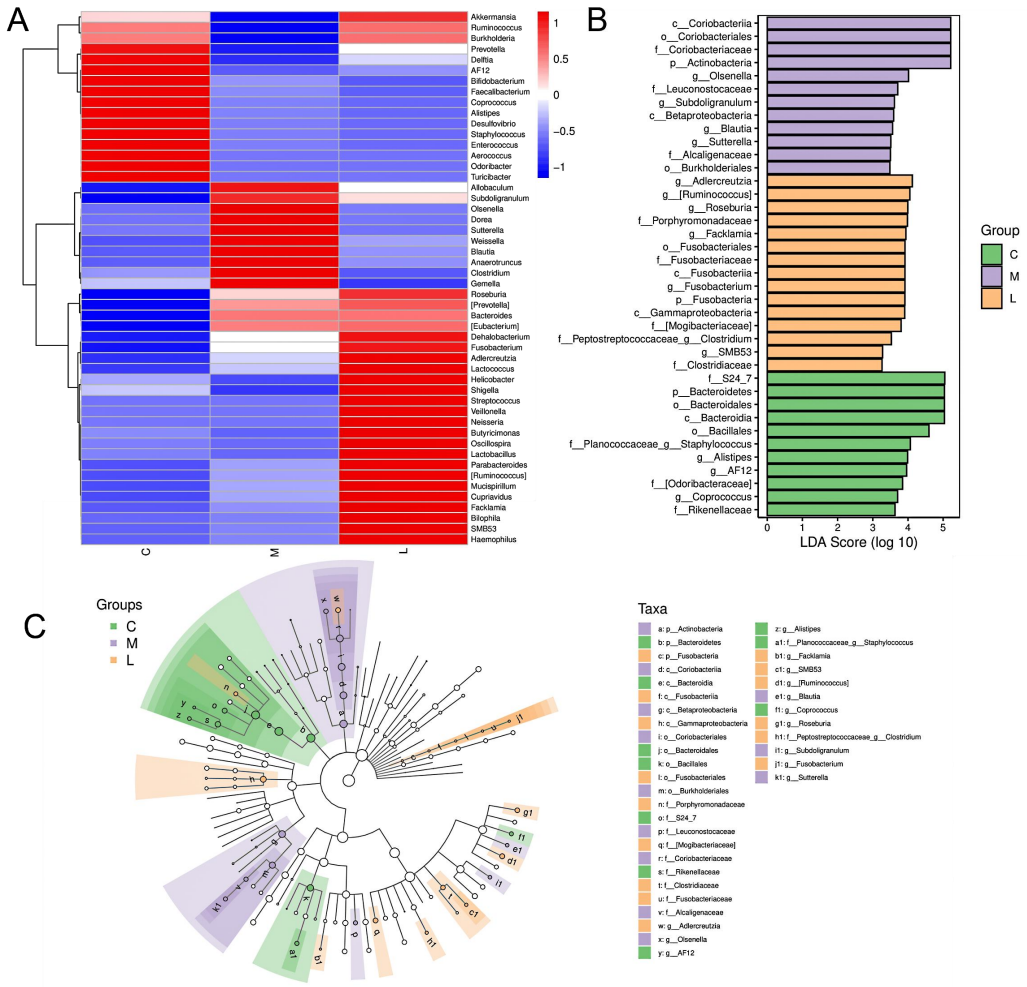

Figure 8 Gut microbiota analysis: genus-level species composition heatmap, LDA effect size histogram, and LEfSe analysis. (A) Heatmap of the species composition of gut microbiota at the genus level. (B) Histogram of LDA effect values of marked special. (C) LEfSe analysis. C, Control group; M, Model group; L, SC extract low-dose group.

lipid levels in mice on a high-fat diet. They can also regulate abnormal lipid metabolism and contain active components that help reduce inflammation and prevent the formation of reactive oxygen species [17, 18]. However, the primary active ingredient of SC in treating hyperlipidemia is still unclear. The research focused on the low-dose group of SC extracts, as high doses may inhibit the peristalsis of gastrointestinal smooth muscle.

Hyperlipidemia is characterized by increased levels of TC, TG, and LDL-C, and reduced levels of HDL-C [19]. High levels of TG and TC indicate a higher fatty acid content. In this experiment, SC extracts reduced the serum levels of TC, TG, and LDL-C in mice on a high-fat diet and significantly increased the level of HDL-C. This suggests that SC possesses some anti-hyperlipidemia activity. PPAR-α is an upstream transcription factor in the fatty acid oxidation process, and PPAR-γ is the primary regulator of adipocyte gene expression and insulin cell signal transduction. Both factors can regulate the expression of genes related to lipid metabolism. Additionally, SC is absorbed in the intestine and exerts a therapeutic effect by modulating the immune response of the intestinal mucosa and reestablishing the intestinal microbiota [20]. In this study, diamine oxidase (DAO) and D-LA were used as markers of intestinal permeability [21]. The results showed that liver lipid deposition was pronounced in the group M, and the serum D-LA content was significantly elevated. This suggests a certain relationship between steatosis and intestinal permeability, as increased intestinal permeability is observed in patients with steatosis [22]. TG is a type of blood lipid that can trigger atherosclerosis. SC treatment reduced various types of TG in hyperlipidemic mice, suggesting its potential in regulating TG in the liver [23, 24]. DG is a type of glyceride that can inhibit fat accumulation and improve insulin sensitivity [25]. Low-dose SC extracts increased DGs in serum, liver, and epididymal fat. The glycerolipid-free fatty acid cycle may be related to the mechanism of SC extract regulating glycerolipid metabolism.

In the screening of differential lipids, we found that, in addition to glycerides, GPs were the most varied in both serum and liver, followed by fatty acids in the epididymal fat. Numerous animal studies have shown that metabolic disorders of GPs can lead to diseases such as obesity, insulin resistance, and dyslipidemia, indicating that GPs are associated with hyperlipidemia [26]. In mice fed a high-fat diet, SC effectively reversed the differential lipids, including PC in serum, PG, and bis(monoacylglycero)phosphate in the liver. A decrease in the level of PC may be related to atherosclerosis and non-alcoholic fatty liver disease [27]. It has been reported that treatment with SM leads to increased apoptosis of human intestinal epithelial cells. The proposed mechanism is that sphingolipids amplify endothelial cell apoptosis through a pathway that connects ceramide-activated cathepsin D with the mitochondrial apoptosis TNF pathway [28]. In this experiment, SC extracts could reverse some abnormal sphingolipid levels. This finding, along with pathway analysis, has shown that SC extracts have a certain effect on sphingolipid metabolism and programmed apoptosis, although the interaction between the two requires further research for elucidation.

The results of 16S rRNA analysis showed that SC could reduce hyperlipidemia by affecting the Beta diversity and species composition

of intestinal flora. It is worth noting that at the species level, Verrucomicrobia, Actinobacteria, Firmicutes, and *Akkermansia* are the main changes in intestinal microbes during hyperlipidemia in mice, and the therapeutic effect of SC. In this experiment, the ratio of Bacteroidetes, Actinobacteria, and Firmicutes/Bacteroidetes (F/B) in cecal contents of mice induced by a high-fat diet increased significantly, but the low-dose group of SC extracts did not reverse the ratio of F/B but significantly decreased the content of Actinobacteria. The claim that changes in the F/B ratio are key biomarkers for assessing steatosis and obesity is subject to uncertainty and requires further investigation, which was proved by the results of this study [29]. We found that one unidentified Coriobacteriaceae genus had significant changes before and after treatment. Similarly, experiments have shown that the combined use of quercetin and resveratrol can significantly inhibit the increase in the relative abundance of Coriobacteriaceae caused by a high-fat diet [30]. There was no significant difference between *Akkermansia* and *Allobaculum*. Studies have shown that *Akkermansia* is a kind of Verrucomicrobia. The decrease in its level will destroy the function of the intestinal wall, leading to the increase of plasma endotoxin, and eventually leading to low-grade inflammation and metabolic disorder, which was consistent with the results of serum L-DA level and lipidomics [31]. Abnormally changing flora abundance was found to be associated with changes in lipid levels. It was shown that *Actinobacteria* was positively correlated with TC and LDL-C [32]. The abundance of the Coriobacteriaceae and TG, LDL, and cholesterol ester levels were positively correlated and were consistent with our experimental findings [33]. In addition, some pathogenic bacteria in the phylum Actinobacteria can cause inflammatory responses, which is related to the abnormally increased PC lipids in the study. PC can promote proliferative growth and programmed cell death, while lysophosphatidyl choline is a pro-inflammatory lipid mediator involved in pro-inflammatory processes in acute injury or chronic diseases [34]. *Oscillospira*, a genus of beneficial bacteria, was negatively associated with TG, TC was negatively correlated, thus indicating that *Oscillospira* abundance increased and TG, TC content decreased in the treatment group. As it is known, gut microorganisms are known as the “second gene pool” of the human body. Based on the relationship between the abundance of bacterial flora and lipid levels in the experiment, we hypothesized that *Oscillospira* interfered with the diversity and species composition of gut microorganisms in hyperlipidemic mice. This regulated lipid-expressed gene to control the lipid levels and influence lipid metabolism, to achieve the purpose of treating hyperlipidemia. However, the specific upstream genes regulated were not clear and need to be verified by subsequent studies.

Conclusion

SC has the effect of treating hyperlipidemia, and its mechanism may be to play a lipid-lowering role by regulating different subtypes of PPAR and its related pathways to improve hyperlipidemia. The results of biochemical indexes and pathological morphology also showed that SC extracts could reduce fat accumulation and improve liver injury to a certain extent, so that the external morphology and internal structure of each tissue could be improved and restored accordingly. The results of non-targeted lipidomics analysis showed that the extract of SC could interfere with the lipid mass spectrum of hyperlipidemia, mainly concentrated in the metabolism of glycerol lipids and GPs, and SC may produce anti-inflammatory effects by regulating the content of inflammation-related lipids such as PC and PG and promote the normal level of lipid metabolism. In addition, it also involves cell death, including necrosis, autophagy, ferroptosis, etc. Finally, the intestinal flora was analyzed, and it was found that the extract of SC could interfere with the occurrence and development of hyperlipidemia by affecting the Beta diversity and species composition of intestinal flora.

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