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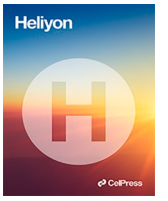

Research article

Lipidomics study of Liujunzi decoction in hyperlipidemia rats with spleen deficiency based on UPLC-Q-TOF/MS

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ABSTRACT

Hyperlipidemia refers to the abnormal levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL-C) and high-density lipoprotein (HDL-C) in peripheral blood circulation. It is a predominant risk factor underlying cardiovascular and cerebrovascular diseases, including coronary heart disease and atherosclerosis. Furthermore, it is also one of the most prevalent chronic diseases globally. Liujunzi Decoction is the basic prescription for the treatment of spleen and stomach diseases. It can tonify the spleen and qi, remove dampness, and reduce turbidity. Moreover, it is also clinically used for the treatment of spleen deficiency hyperlipidemia. However, its metabolites and therapeutic effect on spleen deficiency hyperlipidemia have not been comprehensively determined in vitro and in vivo. This study established a rat model of spleen deficiency hyperlipidemia by inducing starvation and satiety disorders, exhaustion swimming, and intragastric administration of the fat emulsion. To identify related metabolite changes and serum lipid composition, UPLC-Q-TOF-MS, PCA, and OPLS-DA lipidological methods were performed. The results demonstrated significant changes in rat's signs during the modeling process, which were consistent with the criteria for the syndrome differentiation of spleen deficiency in traditional Chinese medicine. Furthermore, this study identified 100 potential biomarkers in rat serum, of which 52 were associated with lipid synthesis, such as LPC, PC, PI, PE, PA, Cer, SM, etc. The pathways involved were glycerol phospholipid, sphingomyelin, and glycerol ester metabolisms. After the Liujunzi decoction intervention, 56 potential biomarkers were observed in the high-dose group, alleviating the metabolic spectrum imbalance by reducing metabolite levels. In addition, metabolic pathway disturbances were markedly improved. This study provides references for future studies on Liujunzi decoction and furnishes essential data for assessing the relationships between chemical constituents and pharmacological activities of Liujunzi decoction.

1. Introduction

Hyperlipidemia is abnormal lipid concentrations in peripheral blood circulation [1]. It is one of the primary risk factors for the

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progression of cardiovascular and cerebrovascular diseases such as coronary artery disease and atherosclerosis [2–4]. Currently, it has been identified as a prevalent chronic disease globally. The unhealthy eating habits of modern people such as high sugar, high fat, high protein, and excessive energy intake are increasing the incidence of hyperlipidemia every year [5], resulting in various metabolic disorders of lipids which significantly affect people's quality of work and life [6]. According to the World Health Organization (WHO), the global incidence of hyperlipidemia is about 25 % [7]. Traditional Chinese medicine believes that "spleen deficiency" is the basis of hyperlipidemia, and the spleen is the biochemical basis of qi and blood of the whole body [8]. Furthermore, spleen deficiency is associated with abnormal spleen function, especially its "transformation" activity is insufficient, because of which the body lipids cannot be normalized and metabolized, resulting in their excessive accumulation in blood, in turn causing dyslipidemia [9–11]. Therefore, revitalizing the spleen and modulating the spleen and stomach are essential therapeutic approaches for individuals with hyperlipidemia.

Liujunzi decoction is a typical TCM prescription for invigorating the spleen and stomach [12]. Clinically Liujunzi decoction is often used for the treatment of spleen and stomach deficiencies. When combined with Qingre Quzhuo capsule, Liujunzi decoction can effectively improve glucose and lipid metabolism [13]. Furthermore, modified Liujunzi decoction can improve lipid metabolism and is therefore effective for hyperlipidemic rats with a deficient spleen. Additionally, it significantly regulates the reverse transport of cholesterol, thereby correcting the dysfunction of high-density lipoprotein [14,15]. However, the changes and mechanisms of lipid metabolism of Liujunzi decoction in the treatment of spleen deficiency and hyperlipidemia have not been sufficiently detailed. Therefore, this study aimed to construct a spleen deficiency hyperlipidemia rat model to explore the treatment mechanism of Liujunzi Decoction in spleen deficiency and hyperlipidemia. This research provides a new direction for the clinical research of spleen deficiency hyperlipidemia.

2. Materials and methods

2.1. Experimental material

2.1.1. Chemicals and reagents

Tween-80 (analytical grade), cholestenone (purity >97.0 %), propylthiouracil (purity >98.0 %), sodium deoxycholate (SDC, purity 99.0 %), Methyl *tert*-butyl ether (MTBE, analytical grade), Methanol (analytical grade) was purchased from Shanghai Baiye Biotechnology Center; Lard oil (homemade); GAS, TG, CHO, HDL-C, LDL-C kits were obtained from Zhongsheng Beikong Biotechnology Co., Ltd.; D-xylose kit was obtained from Nanjing Jiancheng Bioengineering Institute; Ammonium acetate (analytical grade), dichloromethane (analytical grade), ammonia (analytical grade), and isopropanol (analytical grade) were purchased from Merck, Germany.

2.1.2. Preparation of Liujunzi decoction

The pericarpium citri reticulatae, ginseng, pinellia ternata, poria cocos, atractylodes macrocephala, radix and glycyrrhizae were all purchased from Harbin Tonrentang Pharmacy (Harbin, China), all of which were identifed by Prof. Lv Shaowa, school of Pharmacy, Heilongjiang University of Traditional Chinese Medicine. The laboratory of Heilongjiang University of Chinese Medicine Pharmacy houses specimens of these medicinal materials (Catalog number: 2020091011, 2020091012, 2020091013, 2020091014, 2020091015, 2020091016).

According to the Medical Zhengzhuan [16]: take 12 g each of ginseng, Poria cocos, fried licorice, tangerine peel, 18 g each of Atractylorhizoma atractylorhizoma and pinellia, put into a casserole with 10 times the amount of water, soak for 1.5 h at room temperature, decoction twice, each time for 60 min, and then strain. The liquid was concentrated to a crude drug content 0.84 g mL^{-1} to obtain the stock solution of Liujunzi decoction. Additionally, the usual dosage of Liujunzi Decoction in adults is 84 g·day^{-1} (Adult weight: 70 KG) and according to "The algorithm of drug dosage exchange between different kinds of animals" (the algorithm of drug dosage exchange between human and rat is 6.3), the medium dose of Liujunzi Decoction in rat was 7.56 g kg^{-1}, the low dose and high dose were determined to be 3.78 g kg^{-1} and 15.52 g kg^{-1}.

2.2. Animals

Clean grade (SPF) SD rats, half male and half female, weight (200 ± 20) g, provided by Liaoning Changsheng Biotechnology Co., Ltd. with laboratory animal production permit number: SCXK (Liao) 2015-0001. The animals were bred at Heilongjiang University of Traditional Chinese Medicine and then acclimatized for 10 days before formal experiments. The rats were provided ad libitum standard pellet feed and water, which were refreshed daily. The housing conditions included natural light, 21 ± 2 °C temperature, and 70 ± 5 % relative humidity. All animal protocols were approved by the ethics committee of the Heilongjiang University of Chinese Medicine (Approved No: 2023032927; Harbin, China), and disposal methods were in accordance with animal ethics standards.

2.3. Establishment of a model of spleen deficiency hyperlipidemia

2.3.1. Preparation of high-fat feed and fat emulsion

The fat emulsion consists of lard (30 %), Tween-80 (20 %), cholesterol (10 %), propylthiouracil (1 %), and sodium deoxycholate (5 %). Briefly, lard (30 g) was weighed, transferred in a 200 mL beaker, and heated in a water bath at 50 °C with constant slow mixing using a magnetic blender until the lard dissolved. Then, 10 g of cholesterol was added slowly to this mixture along the wall of the cup

and stirred with a glass rod until no crystalline white solid remained. Afterwards, 1 g of propylthiouracil and 20 mL of Tween-80 were added mix thoroughly and homogeneously as an oil phase. Then in another beaker, 50 mL of distilled water was heated in a water bath to about 60 °C, and 5 g of sodium deoxycholate was added and stirred thoroughly with a glass rod until the solid dissolved into the water phase. This mixture of water and oil is called a fat emulsion. Before dosing, the fat emulsion was heated to 37 °C in a water bath. The dose was converted based on the conversion factor for human and animal surface area.

2.3.2. Experimental animal groupings and model preparation

A total of 48 SD rats were randomly categorized into the following groups: blank control (n = 12), hyperlipidemic (n = 6), and splenic deficient hyperlipidemic (n = 30) groups. The blank control group received 2 mL of normal saline twice daily and a standard granular diet for 4 weeks. The splenic hyperlipidemic groups were subjected to a combined method of starvation and satiety disorders, exhaustion swimming, and intragastric fat emulsion administration. Rats were fed the above high-fat diet on odd days without restriction and fasted on even days, were allowed to drink freely, and swam daily in warm water at 25 ± 1 °C until exhaustion (criterion = the rat must be submerged for 5 s) [17], and were given 2 mL of intragastric fat emulsion once a day. Furthermore, to elucidate the effect of spleen deficiency on the formation of hyperlipidemia and spleen deficiency hyperlipidemia groups were formed for comparison. The hyperlipidemic group received a high-fat diet plus 2 mL of intragastric fat emulsion once daily. High-fat diet formula: 10 % lard, 10 % egg yolk powder, 10 % cocoa powder, 10 % cholesterol, 0.5 % cholic acid, and the rest of basic rat food.

2.3.3. Detection index

2.3.3.1. Assessment of general obvious signs. Rats were measured on days 0, 3, 6, 9, 12, 18, 21, 24, and 27 days to record changes in body weight and physiological status of over the previous four weeks. For instance, visible signs, including hair, strabismus, and anal washing, were evaluated according to the criteria listed in Table 1.

2.3.3.2. D-xylose absorption experiment. On day 30 of modeling, 6 rats were randomly selected from each group for the treatment of 5 % D-xylose solution (1 mL·100 g^{-1}) after 12 h of fasting. Then their blood was collected from the suborbital veins and centrifuged. Subsequently, 50 μL of serum, D-xylose solution (50 μg mL^{-1}), distilled water, and 5 mL of phloroglucinol chromogenic agent were mixed and placed in boiling water for 5 min. The absorbance of this mixture was acquired at 554 nm in a UV spectrophotometer. The D-xylose content in rat serum was calculated using distilled water as a blank.

2.3.3.3. Detection of blood lipids. After taking blood from the suborbital veins, rats were anesthetized with 10 % chloral hydrate according to body weight. Blood was drawn from the abdominal aorta and placed in test tubes containing anticoagulants and allowed to stand at room temperature for 30 min. The sample was then centrifuged at 3000 r·min-1 to acquire serum, which was then transferred to EP tubes and stored at 20 °C for testing. TG, TC, LDL-C, and HDL-C indices were measured on an automated biochemical analyzer.

2.3.3.4. Pathological observation of liver, stomach, and small intestinal mucosa. The rat's liver, stomach, and small intestine were dissected, washed with normal saline, fixed in a 10 % formaldehyde solution, and sliced for HE staining. The stained samples were analyzed with the Image-proExpress pathological visual analysis system [18].

2.3.3.5. Statistical analysis. The statistical methods of SPSS 21.0 software were used to quantitatively analyze the acquired data, which was expressed as x ± s. The intergroup statistical data were assessed by independent samples *t*-test, and $p < 0.05$ was considered statistically significant.

2.4. Serum lipidomics analysis

2.4.1. Sample preparation

Successful model rats were randomly divided into a splenic deficient hyperlipidemic group, a low-dose Liujunzi decoction group, a medium-dose group, a high-dose group, and a blank control group (n = 6/group). The blank control group was given 1 mL·100g-1 of

Table 1
Assessment of common physical signs in animals.

Project	0	1	2
General state	Good spirit, free movement, quick and agile action	The mental state is slightly poor, the movement is not very agile, slightly slow。	Poor spirit, basically no activity, lack of energy.
Squint, bow back	Basically, no squint, no bow back	Occasionally squint, occasional bow back tendency	Often squint, or even squint for a long time, there is a bow back movement
Perianal cleanliness	Basically, no squint, no bow back	White, perianal hair slightly fascicled	Feces in the anus, and the perianal fur is sticky and contaminated
Fur	The hair is white, supple and shiny	Yellowish, with slightly dim hair	Yellow, dry, rough and dull

normal saline every day and all the dose groups were provided varying concentrations of Liujunzi Decoction for 14 days, and the splenic deficient hyperlipidemic group was continuously perfused with fat emulsion. After 14 days of continuous administration, the rats fasted for 24 h. Then, abdominal aorta blood (5 mL) was collected into EP tubes, left for 1 h, and centrifuged at 3000 rpm for 10 min. The supernatant was collected into a new EP tube and stored in an 80 °C freezer. For the experiment, all serum samples were reconstituted at 4 °C, then 100 μL of the sample and 480 μL of extract (methyl *tert*-butyl ether: methanol = 5:1) were mixed, sonication in an ice bath for 5 min, and allowed to stand at 40 °C for 1 h. The samples were then centrifuged again at 3000 rpm at 4 °C for 15 min, and 350 μL of the supernatant was taken into an EP tube and vacuum dried. For reconstitution, 100 μL of solution (DCM: MeOH = 1:1) was added to the dried sample, vortexed for 30 s, and sonicated in an ice water bath for 10 min. After centrifugation for 15 min at 13,000 rpm at 4 °C, 75 μL of supernatant was transferred to an injection bottle. All samples were mixed with 10 μL of supernatant to make a QC sample for detection.

2.4.2. UPLC–MS analysis

For chromatographic analysis, ExionLC (AB Sciex) ultra-high performance liquid chromatograph with Phenomen Kinetex C18 (2.1 * 100 mm, 1.7 μm) column was used. Mobile phase A was 40 % water and 60 % acetonitrile solution (containing 10 mmol/L ammonium formate) and mobile phase B was 10 % acetonitrile and 90 % isopropanol solution, and 50 mL 10 mmol/L ammonium formate aqueous solution added every 1000 mL. Gradient elution was used as follows: 0–12.0 min, 40 % 100 % B; 12.0–13.5 min, 100 % B; 13.5–13.7 min, 100 % 40 % B; 13.7–18.0 min, 40 % B. Mobile phase flow rate: 0.3 mL·min-1, column temperature: 45 °C, sample plate temperature: 4 °C, injection volume: positive ion 2 μL, negative ion 2 μL.

For mass spectrometry: Triple TOF 5600 high-resolution mass spectrometer, the collision-induced dissociation energy was 45 eV, and the accumulation time of each second-order spectrum was 50 ms. The ion source parameters are GS1: 60 psi, GS2:60 psi, CUR: 30 psi, TEM: 600 °C, DP: 100 V, ISVF: 5000 V (Pos)/-3800 (Neg).

2.4.3. Data processing and analysis

The original MS data were converted to mzXML format by ProteoWizard (V3.0) software. Then, retention time correction, peak recognition, peak extraction, and peak integration were then performed in XCMS (V3.2). Minfrac was set to 0.5 and the cutoff to 0.3. Furthermore, LipidSearch software (V4.1, Thermo Fisher Scientific - US) was used to extract and identify the data, normalize the data, and finally organize the data into a two-dimensional data matrix format, and Simca software (V14.1, Sartorius Stedim Data Analytics AB, Umea, Sweden) was used to perform principal component analysis (PCA). Multidimensional statistical analyses such as PCA and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using Simca software. Moreover, the OPLS-DA analysis of variables revealed a more significant variable importance plot (VIP) in the project than one was selected as biomarkers. Retention time and mass/nuclear ratio information were obtained and screened for biologically significant lipids differences between the groups (VIP >1.0, $p < 0.05$, FC > 1.5) using multidimensional OPLS-DA and one-dimensional analysis (Student's t-test). Furthermore, based on accurate molecular weight and mass ratio data, and fragment ion information, the ChemSpider database was searched to identify metabolites.

3. Results

3.1. Model evaluation

3.1.1. Assessment of general obvious signs

In the rat model experiments, apparent signs and specific changes before and after the rat model experiments were observed and recorded based on the research principles described above (Table 2). Scoring results indicated that the blank and model groups scored higher in the hyperlipidemia and splenic hyperlipidemia groups for general physical conditions, including hair, squint, perianal cleanliness, and the total score ($p < 0.01$). Perianal cleanliness and total score were significantly higher in the spleen-deficient hyperlipidemic group than in the hyperlipidemic group ($p < 0.01$). During the modeling process, rats in the blank and hyperlipidemic groups gained weight, while those in the spleen-deficient hyperlipidemic group indicated decreased body weight and swimming endurance (Fig. 1).

3.1.2. D-xylose adsorption test and detection of blood lipids

The levels of TG, TC, and LDL-C in the modeling group were significantly increased, and the level of HDL-C was significantly decreased compared to the blank control group. Compared to the hyperlipidemia group, serum D-xylose absorption was significantly lower in the splenic deficient hyperlipidemia group (Table 3).

Table 2
Effect of modeling on scores of common body symbols ($\bar{X} \pm s$, n = 6).

Group	General physical signs	Squint, arching back	Perianal cleanliness	Hair	Total score
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hyperlipidemia	1.14 ± 0.37**	1.08 ± 0.64**	0.63 ± 0.37**	1.24 ± 0.48**	4.09 ± 0.72**
spleen deficiency hyperlipidemia	1.23 ± 0.55**	1.17 ± 0.22**	1.78 ± 0.36**##	1.53 ± 0.45**	5.71 ± 0.86**##

Note: $p < 0.01$ is indicated by **compared to the control group. Compared to the hyperlipidemia group, $p < 0.01$ is indicated by ##.

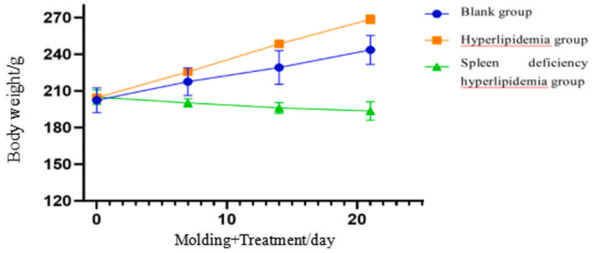

Fig. 1. Weight changes during modeling for each group of rats.

3.1.3. *Pathological observation of liver, stomach, and small intestine mucosa*

As shown in Fig. 2, the gastric mucosa and intestinal wall in the blank group and the hyperlipidemia group were intact and transparent (Fig. 2(A1, A2, B1, B2)), while the integrity of gastric mucosa and small intestine in the spleen deficiency hyperlipidemia group was destroyed. Thinning and partial exfoliation of the gastric mucosa, infiltration of inflammatory cells, and edema were observed (Fig. 2(A3)), as were disruption of the intestinal mucosa, infiltration of inflammatory cells, and edema of the lamina propria (Fig. 2(B3)). The hyperlipidemia group and the spleen deficiency hyperlipidemia group showed varying degrees of fat deposition and the formation of fat vacuoles (Fig. 2(C2, C3)) and a small amount of inflammatory cell infiltration(Fig. 2(C3)).

3.2. *Lipidomics of Liujunzi decoction on spleen deficiency hyperlipidemia rats*

3.2.1. *Stability and reproducibility of lipidomic analysis*

To evaluate the stability and repeatability of the experiment, the samples of each group were evenly mixed with the quality control samples (QC). The total ion chromatograms (TIC) of QC samples obtained by analysis were compared by spectral overlap, and it was found that the retention time and peak area overlap of QC samples TIC were significant, indicating that the instrument had high stability. All QC and experimental samples were extracted and subjected to unsupervised PCA analysis after Pareto-scaling, and QC samples were found to be tightly clustered, indicating that the lipidomics analysis was highly reproducible (Additional file 1).

3.2.2. *Multivariate statistical analysis of serum lipid molecules*

The lipidomics analysis of rat serum demonstrated that each group's samples were well-clustered, while there was a significant separation between the groups. This finding provides evidence for the impact of a high-fat diet on rat metabolism and highlights the ability of Liujunzi decoction to improve lipid metabolism in spleen deficiency and hyperlipidemia rats (Fig. 3). Subsequently, the OPLS-DA test was performed on each group. For OPLS-DA modeling analysis, first principal component was utilized and the quality of the model was tested by 7-fold cross validation. Moreover, R^2Y and Q^2 obtained after cross-validation were used to evaluate the effectiveness of the model. Finally, the permutation test was performed to randomly alter the permutation order of categorical variable Y for multiple times to obtain different random Q^2 values. Additionally, model's validity was further assessed, and the score plot and permutation test results of the OPLS-DA model under different groups were obtained, which indicated a high level of fitting accuracy for the model. Furthermore, 200 random permutation tests were performed on the model, demonstrating that there was no overfitting (Q^2 intercept <0) and confirming its reliability (Fig. 4).

3.2.3. *Identification of differential lipid metabolites in serum of spleen deficiency hyperlipidemia rats*

Under the OPLS-DA model, VIP values > 1 and p-values <0.05 were used as the standard for differential metabolites screening for assessing the significant differences in liver between the spleen deficiency hyperlipidemia and the blank groups. Based on the accurate information on molecular weight and mass-charge ratio as well as fragment ion, significant differences in lipids were detected by www.lipidmaps.org. Compared with the blank group, 100 differential lipids were detected in the spleen deficiency hyperlipidemia group, as shown in Fig. 5. Among them, 52 substances such as LPC, PC, PI, PE, PA, Cer, SM, etc. Play an important role in lipid synthesis, 11 substances are found in the positive ion mode, respectively: CerNS (d18:1/16:0), PC (14:0–22:5), PC (18:0–18:3), PC (18:0–22:5), PC (18:0–18:0), PC (16:0–22:1), PC (18:1–18:2), PC (18:2–20:4), LPC (24:0), PC (18:2–18:3), PC (22:6–22:6), 41 substances are found in

Table 3
Effects on serum biochemical indices and serum D-xylose absorption in rats. ($\bar{X} \pm s$, $n = 6$).

Group	TG /mmol·L^{-1}	TC /mmol·L^{-1}	HDL-C /mmol·L^{-1}	LDL-C /mmol·L^{-1}	D-xylose absorption
Blank control group	0.82 ±0.42	1.36 ±0.14	1.50 ±0.13	1.23 ±0.50	6.74 ±0.51
Hyperlipidaemia group	0.48 ±0.33**	2.59 ±0.23*	1.17 ±0.06*	1.64 ±0.22*	6.67 ±0.58
Spleen deficiency hyperlipidemia group	0.46 ±0.24*	2.67 ±0.71*	1.19 ±0.08*	1.62 ±0.42*	4.65 ±0.44*##

Note: $p < 0.05$ compared to the control group is indicated by *. $p < 0.01$ compared to the hyperlipidemia group is indicated by ##.

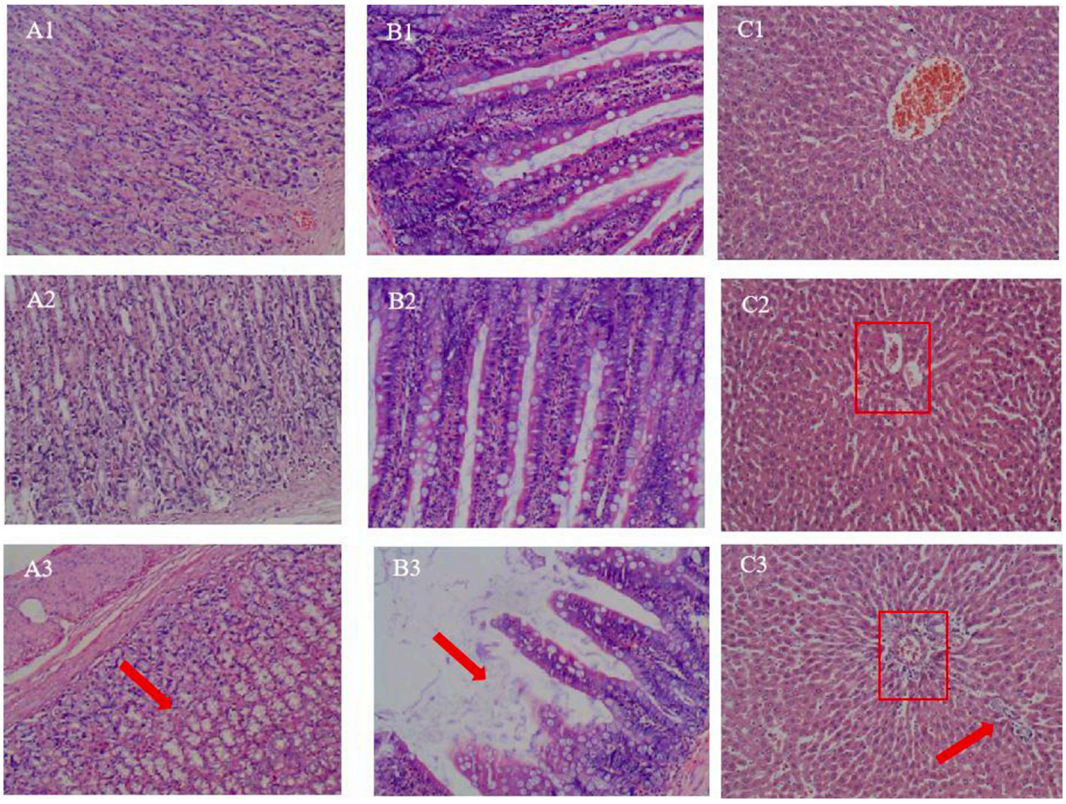

Fig. 2. Comparison of the morphology of stomach, small intestine, and liver (100x HE staining).
Note: A1: Gastric tissue of blank control group, complete gastric mucosa with clear layers. B1: Small intestinal tissue of the blank control group, the structure of each layer of the small intestinal wall was intact. C1: Liver tissue of the blank control group, hepatocytes had normal morphology. A2: Gastric tissue of the hyperlipidemia group, with intact gastric mucosa and clear layers. B2: Small intestinal tissue of the hyperlipidemia group, the structure of each layer of the small intestinal wall was intact. C2: Liver tissue of the hyperlipidemia group, mild steatosis and fat vacuoles. A3: Gastric tissue of the spleen deficiency hyperlipidemia group, the gastric mucosa inflammatory cells infiltrated. B3: Small intestinal tissue of spleen deficiency hyperlipidemia group, a large number of inflammatory cell infiltration, intestinal mucosa propria edema. C3: Liver tissue of spleen deficiency hyperlipidemia group, steatosis, fat vacuoles, and a small amount of inflammatory cell infiltration. Red arrows indicate inflammatory cell infiltration. Red squares indicate the presence of fat vacuoles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the negative ion mode, respectively: PI (16:0–16:0), LPC (18:3), PC (18:2–18:3), PC (16:1–18:2), PC (18:2–20:4), PI (20:2–20:2), PI (18:0–18:2), PI (18:2–18:2), PE (20:0–18:1), PE (18:0–18:0), PC (16:0–18:0), PC (18:0–20:1), PE (18:0–18:2), PC (16:0e/18:2), PI (16:0–18:1), PI (16:0–18:2), SM (d18:1/22:0), SM (d18:1/23:0), PI (18:0–18:3), PC (16:1e/18:2), PC (18:0–18:0), PE (18:0–20:0), PC (15:0–16:0), PC (18:1–20:1), SM (d18:1/20:0), SM (d18:1/18:0), PC (22:0–18:2), PE (16:0–18:2), PI (18:0–16:1), HexCer-NS (d18:1/16:0), PC (14:0–16:0), Cer-NS (d18:1/24:0), Cer-NS (d18:1/23:0), PA (18:1–18:1), PC (16:1–22:6), LPC (22:0), PA (16:0–22:6), PA (16:0–18:2), PC (18:1–20:4), LPC (20:4), LPC (16:1). They showed a significant upward trend in the splenic hyperlipidemia group (FC $>$ 1). The above results suggest that the disorders of lipid metabolism such as LPC, PC, PI, PE, PA, Cer and SM are the causes of spleen deficiency hyperlipidemia.

3.2.4. 3.2.4regulation of lipid difference metabolites in high dose group of Liujunzi decoction

A bubble diagram and cluster analysis heat map were used to characterize lipid difference metabolites. In the bubble diagram of the lipid group, each point represents a metabolite, gray points represent non-significant differences with a p-value of $>$ 0.05, colored points represent significant differences with a p-value of $<$ 0.05 (marked in different colors according to the lipid classification), and abscisxes represent the relative percentage change in the level of each substance in a group. The ordinate represents the classification information of lipids. The black line segment at the bottom shows the distribution density of metabolites. The cluster analysis heat map grouped the metabolites with the same characteristics into one class and revealed variable characteristics between the experimental groups. Furthermore, the abscissa illustrates different experimental groups, the ordinate shows the differential metabolites in this group, and the color blocks at different positions represent the relative expression levels of metabolites at corresponding positions.

Fig. 6 shows the bubble diagram, and cluster analysis heat map of lipid differential metabolites in spleen deficiency hyperlipidemia and Liujunzi Decoction high-dose group in positive ion mode (Fig. 6(A)) and negative ion mode (Fig. 6(B)). Compared with the spleen deficiency hyperlipidemia group, the high-dose Liujunzi decoction group could significantly reverse 56 lipid metabolism differences.

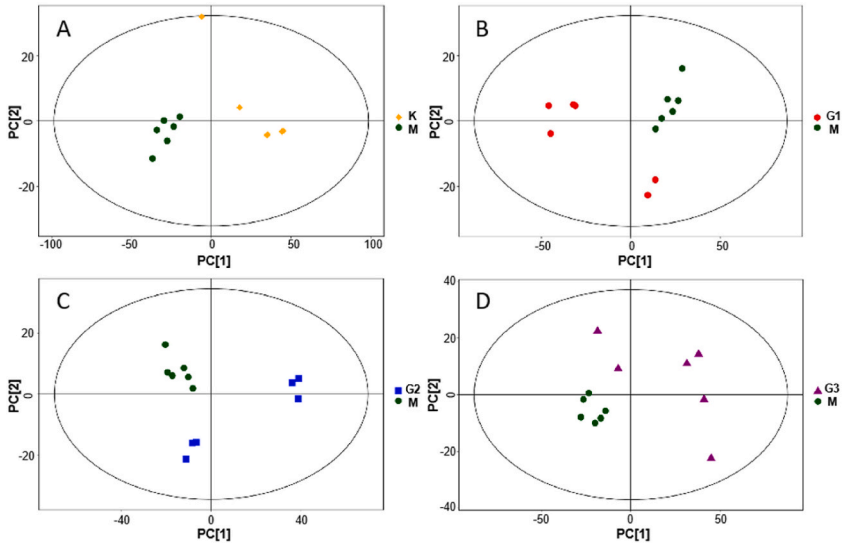

Fig. 3. The PCA score plot derived from UPLC-Q-TOF/MS profiles of serum sample between the two groups
Note: A: model group (M, green color)-blank (K, yellow color), B: low-dose group (G1, red color)-model group (M, green color), C: medium-dose group (G2, blue color)-model group (M, green color), D: high-dose group (G3, purple color)-model group (M, green color). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Among them, 10 indicators are up, and 46 indicators are down. TG (16:0/16:0/21:0), LPE (20:4), LPC (20:4/0:0), PE (18:0/22:6), TG (16:0/16:0/16:0), L-Palmitoylcarnitine, TG (18:0/18:0/22:5), DG (18:2/18:2/0:0) were highly significantly upregulated ($p < 0.01$); DG (18:1/22:6/0:0), DG (16:0/18:2/0:0) were significantly upregulated ($p < 0.05$); PC (18:0/18:3), GlcCer (d18:1/22:0), GlcCer (d18:1/16:0), PC (15:0/16:0), PG (18:0/18:1), TG (12:0/12:0/14:0), PI (18:2/18:2), TG (12:0/12:0/19:0), CE (18:3), PC (18:0/18:0), PC (18:0/18:1(9Z)), TG (15:0/15:0/15:0), PC (18:0/20:1), SM (d18:1/18:0), PI (18:0/18:2), Gpcho (16:1/18:2), PC (16:0/22:1), TG (12:0/12:0/20:0), SM (d18:1/20:0), TG (14:0/14:0/14:0), PC (O-14:0/16:1(9Z)), PI (16:0/18:1), PI (16:0/18:2), TG (12:0/12:0/22:0), PC (18:2/18:3), CE (20:3), PE (20:0/18:1), PE (20:1/20:1), SM (d18:1/22:0), PC (22:0/18:2), LPC (24:0/0:0), LPC (18:3/0:0), TG (12:0/12:0/12:0), CE (18:2), PC (18:0/20:2), LPC (22:0/0:0), SM (d18:1/16:0), PC (18:1/18:2), SM (d18:1/23:0), GlcCer (d18:1/18:0), PC (18:1/18:1), TG (14:1/20:5/20:5), PE (18:0/18:0) were highly significantly down-regulated ($p < 0.01$); PC (14:0/22:5), PC (14:0/16:0), Cer (d18:1/22:0) were significantly down-regulated ($p < 0.05$). These results suggest that Liujunzi decoction has a significant effect on spleen deficiency hyperlipidemia, and its mechanism may be related to the regulation of these different lipids.

4. Discussion

4.1. Replication of a model of spleen deficiency and hyperlipidemia

The high-fat diets have been widely used in previous studies because of their hyperlipidemia-forming characteristics; however, modeling takes a prolonged time and has poor uniformity. The intragastric administration of the same volume of fat emulsion can ensure the consistency of fat emulsion intake in each rat and make up for the deficiency of high-fat diet feeding. However, this can induce higher stimulation in animals, causing a state of prolonged stress, which markedly increases the incidence of rat death, thereby influencing the experimental results [19,20]. This study used a combined method of starvation and satiety disorders combined with fatigue swimming and intragastric fat emulsification for a well-sustained model to avoid the above shortcomings. During the modeling period, physical signs of spleen deficiency and hyperlipidemia were observed, such as the hair was loose, erect, gaunt, and lacking luster. In traditional Chinese medicine, the criteria for differentiating spleen deficiency symptoms include weight loss, being underweight, mental fatigue, wooziness, squinting, and warping. The rats met these criteria; however, those in the hyperlipidemia group did not have such characteristics.

The decrease in D-xylose excretion rate is a clinical indicator of spleen deficiency syndrome, and the degree of spleen deficiency is inversely proportional to the urinary D-xylose excretion rate [21], which is consistent with the significant decrease of D-xylose absorption in the hyperlipidemia group of spleen deficiency in this experiment. Previous literature has indicated that the levels of TC, TG, LDL-C, and HDL-C are important indicators of hyperlipidemia. Furthermore, abnormal lipid metabolism can cause fat deposition, especially triglyceride (TG) accumulation, in the internal organs or subcutaneous tissues. Increased TG levels reduce HDL content in the body while increasing sLDL, and secondary inflammatory reactions [22]. HDL-C is a medium-carrying cholesterol, which interferes with the three processes of cholesterol efflux, esterification, and conversion into bile acid by reversing the cholesterol transport mechanism. LDL-C is the main substance of the sterol hormone, bile acid, and cell membrane, jointly regulating the lipid balance in the body. Moreover, it has been observed that decreased HDL-C levels are accompanied by increased LDL-C content, causing lipid

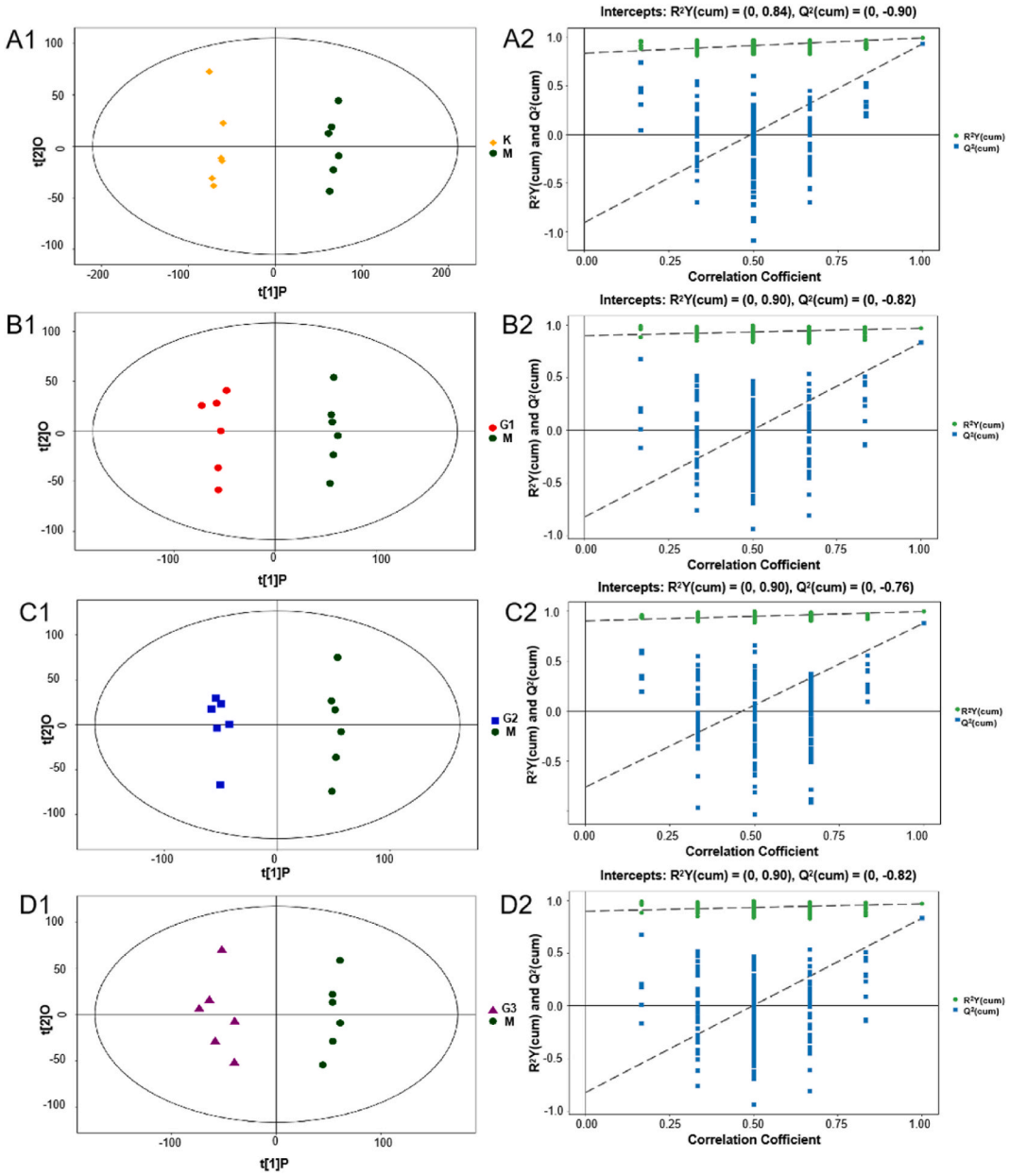

Fig. 4. Score plot and permutation test results of OPLS-DA model
Note: A1: Score plot of model group (M, green color)-blank (K, yellow color), A2: Permutation test of model group (M, green color)-blank (K, yellow color); B1: Score plot of low-dose group (G1, red color)-model group (M, green color), B2: Permutation test of low-dose group (G1, red color)-model group (M, green color); C1: Score plot of medium-dose group (G2, blue color)-model group (M, green color), C2: Permutation test of medium-dose group (G2, blue color)-model group (M, green color); D1: Score plot of high-dose group (G3, purple color)-model group (M, green color), D2: Permutation test of high-dose group (G3, purple color)-model group (M, green color). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

deposition [23,24]. This study showed that the levels of TG, TC, and LDL-C in the hyperlipidemia group were significantly increased, while that of HDL-C was markedly decreased. Additionally, foam cell formation, dyslipidemia, and lipid deposition were observed, which indicated that this method could make rats dyslipidemia and form hyperlipidemia. Li et al. [25] observed the changes associated with spleen deficiency and found that it may aggravate dyslipidemia and liver lipid deposition in hyperlipidemia rats by affecting the expression of related proteins related to RCT response, consistent with the further aggravation of spleen deficiency and dyslipidemia observed in the hyperlipidemia group of this study compared with just the hyperlipidemia group. All the above results indicate that this method can successfully construct a stable rat model of spleen deficiency hyperlipidemia, laying the foundation for the subsequent intervention of Liujunzi decoction on spleen deficiency hyperlipidemia.

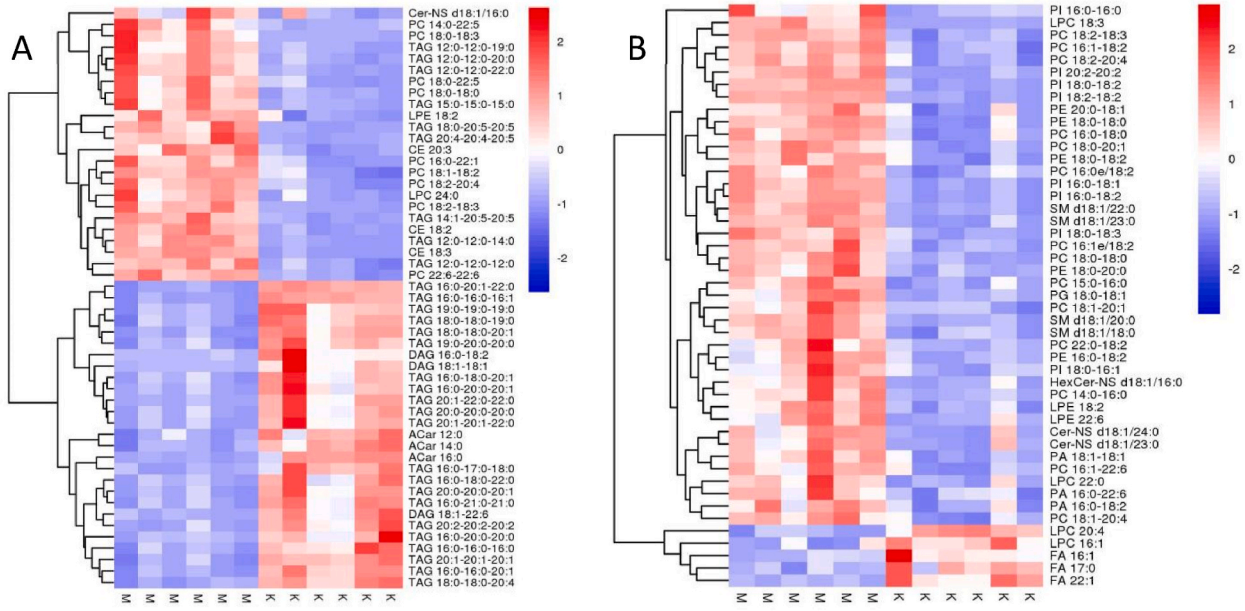

Fig. 5. Cluster analysis of serum metabolites(A: ESI+, B: ESI-).

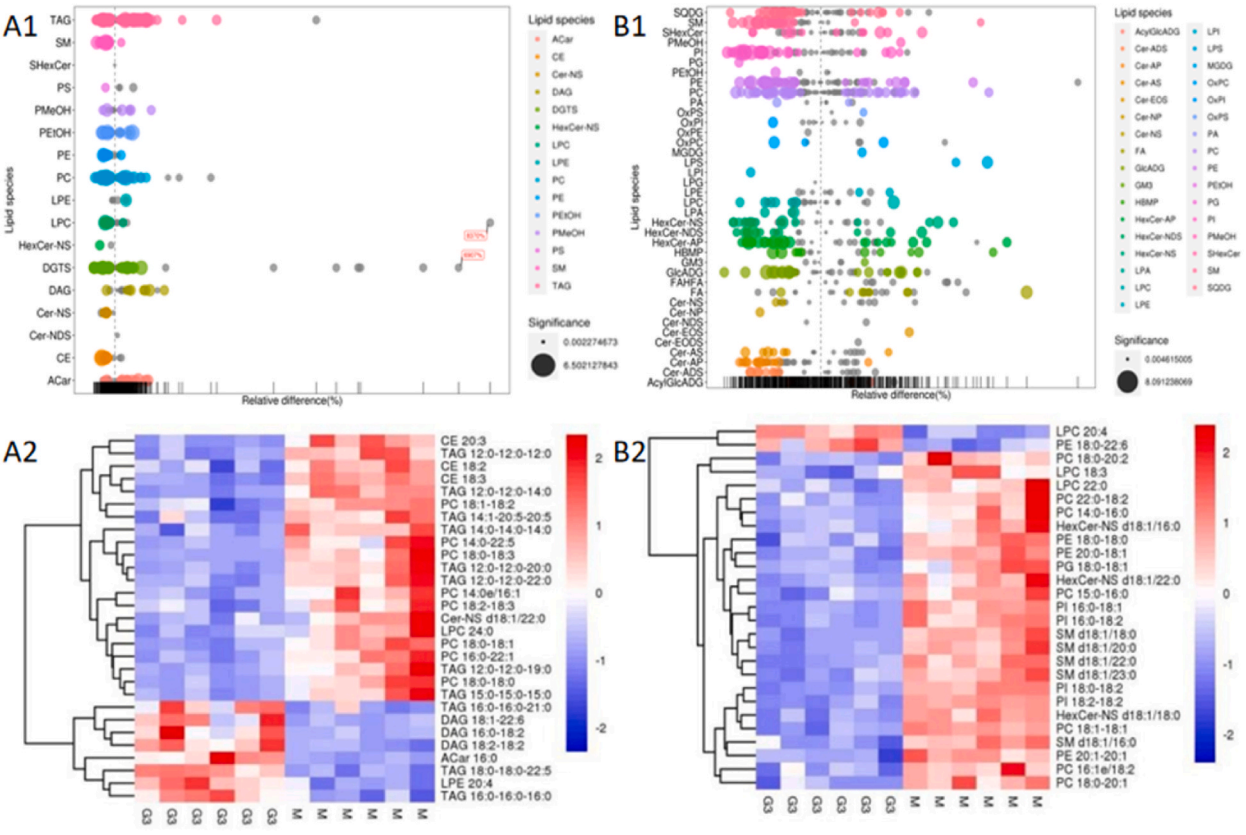

Fig. 6. The difference in Metabolite Screening for high-dose group versus M Group lipid bubble diagram, and cluster analysis heat map.
Note: A1: ESI+, Bubble diagram of high-dose group (G3)-model group (M), A2: ESI+, Cluster analysis heat map of high-dose group (G3)-model group (M); ESI-, Bubble diagram of high-dose group (G3)-model group (M), A2: ESI-, Cluster analysis heat map of high-dose group (G3)-model group (M).

4.2. Intervention effect of Liujunzi decoction on lipid metabolism in spleen deficiency hyperlipidemia

Liujunzi Decoction originated from Medical Zhengzhuan in the Ming Dynasty and has a long history of application. Traditionally it has been prescribed for the treatment of various diseases caused by spleen and stomach weakness. Previous studies have found that Liujunzi decoction can improve gastrointestinal motility disorders caused by spleen deficiency, increase the number of immune cells in the body, and enhance intestinal mucosal immune function [26]. Furthermore, it has also been therapeutically used for treating spleen deficiency hyperlipidemia. Traditional Chinese medicine believes that Liujunzi decoction can treat hyperlipidemia by invigorating qi and strengthening the spleen [27]; however, the research on its underlying mechanism of action has not been comprehensively studied. This paper discussed the mechanism of Liujunzi decoction in the treatment of spleen deficiency and hyperlipidemia. The results indicated that after Liujunzi Decoction treatment, 56 serum metabolites of lipid metabolism were changed in rats, including TG, PC, LPC, DG, SM, PE, etc. Furthermore, the levels of PC, DG, PI, SM, and PE related to lipid synthesis were significantly down-regulated. Moreover, lipid content, lipid synthesis, and fat accumulation were also reduced. In conclusion, Liujunzi Decoction has obvious therapeutic and relieving effects on spleen deficiency hyperlipidemia, and its mechanism includes improving the metabolic pathway disorder by modulating the levels of related metabolites, in which sphingolipid metabolism, glycerol phospholipid metabolism and triglyceride metabolism are the core metabolic modes.

Diglycerides (DG) are the main components of glyceride metabolism and are associated with improved liver fat metabolism and the expression of hypothalamic genes that control appetite, thereby affecting the balance of fat metabolism [28]. Triglycerides (TG) also called triacylglycerols are the main component of vegetable oils and animal fats, as well as very low-density lipoprotein (VLDL) and kairos particles [29]. Furthermore, TG acts as an energy source, and dietary fat transporter, and plays an essential role in metabolism. Hypertriglyceridemia is a common form of hyperlipidemia. Anthraquinone derivatives in rhubarb have been found to repair the gastrointestinal mucosal barrier, promote gastrointestinal peristalsis, reduce cholesterol absorption, and effectively lower total cholesterol and triglyceride levels in the body [30]. Moreover, Du Yuzhong [31] indicated that tangerine peel alcohol extract significantly reduced TG in hyperlipidemic rats, possibly due to an increase in PPARγ-LPL/ATGL and FXR-HL glycerol. Additionally, Luo Jiaxian [32] revealed that DG has a specific optimizing effect on blood lipids, increased serum HDL-C, and significantly reduced hepatic cell lipidosis in hypercholesterolemic rats. In addition, DG can inhibit enzymes related to TG synthesis and upregulate fatty acid oxidation and fat accumulation in the liver [33]. Here, it was revealed that the content of some TGs in the splenic deficient hyperlipidemia group was significantly higher than in the blank control group. In hepatic lipidosis, TG content accumulated more in the lipid droplets of hepatocytes, and decreased after Liujunzi decoction intervention.

Glycerol phospholipids are among the most abundant and complex molecules in the body and regulate many cellular processes. Furthermore, glycerol phospholipid metabolism is associated with humoral metabolic pathways via phospholipase hydrolysis. It has been observed that spleen deficiency affects phospholipase synthesis, decreases its content, and promotes metabolic disturbances of numerous phospholipid metabolites as well as their accumulation [34]. Zhang [35] and colleagues found that Ginkgo biloba reduces uric acid by regulating PC and LPC levels, suggesting that the therapeutic mechanism may be related to decreased PLA2 activity. The present study found that PC content was significantly increased in a model of splenic deficiency and hyperlipidemia. Furthermore, Liujunzi decoction reduced the serum levels of LPC and PC in spleen-deficient hyperlipidemic rats, accelerated the rate of phospholipase hydrolysis, increased the content of PI, and further affected the production of uric acid in vivo. These effects cause swelling of the legs, dampness, and cloudy fat accumulation in the rats, thereby further proving that Liujunzi decoction promotes dampness, and and activate the spleen, and lower lipids.

Sphingomyelin is a significant component of biological membranes, including animal cell membranes, and plays a significant role in intracellular signal transduction [36]. Ceramide (Cer) is a highly hydrophobic substance and an essential product of sphingomyelin metabolism. Depending on the fatty acid chain, there are at least 50 different ceramides [37], which are hydrolyzed from sphingomyelin by the action of sphingomyelinase and broken down into sphingosine and free fatty acids [38]. Furthermore, sphingomyelin signaling is activated by various stimuli and mediators and has been shown to affect cell proliferation, apoptosis, and inflammatory cell activity. In the development of atherosclerotic lesions, atherogenic lipoproteins such as VLDL and LDL are rich in sphingomyelin [39]. Moreover, ceramide is an essential metabolite of sphingomyelin synthesis and can cause spleen edema. Liujunzi decoction was found to be able to regulate ceramide Cer (d18:1/22:0), Cer (d18:1/18:0), sphingomyelin, and glucose ceramide. Therefore, Liujunzi decoction may affect the sphingomyelin metabolic pathway by regulating ceramide, thereby improving the abnormal lipid metabolism in splenic hyperlipidemic rats, and enhancing the immunomodulatory capacity by modulating splenic hyperlipidemia.

Based on the aforementioned data, the Liujunzi decoction intervention can alleviate spleen deficiency hyperlipidemic rat metabolic spectrum disorders, decrease the content of metabolites, improve metabolic disorders, and attenuate serum lipid metabolism. These therapeutic effects might be linked with glycerol phospholipid, sphingolipid, and glyceride metabolisms and their metabolites.

5. Conclusions

This experiment established an animal model of spleen deficiency hyperlipidemia by the combination of starvation and satiety disorders combined with fatigue swimming and intragastric fat emulsificatio for subsequent studies. In addition, the preliminarily explored the metabolic mechanism of the Liujunzi decoction intervention in spleen deficiency and hyperlipidemia, which provided a basis for the diagnosis of spleen deficiency and hyperlipidemia and the therapeutic mechanism of Liujunzi decoction in the future.

CRediT authorship contribution statement

Shuang Sun: Conceptualization. **Hongli Guo:** Writing – review & editing, Writing – original draft, Investigation. **Eryu Shang:** Validation. **Qiaoxin Guo:** Writing – review & editing, Data curation. **Aixia Ju:** Validation. **Yalun Li:** Data curation. **Yawen Feng:** Data curation. **Yuyan Guo:** Data curation. **Dayu Yang:** Data curation. **Shaowa Lü:** Conceptualization.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31710.

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