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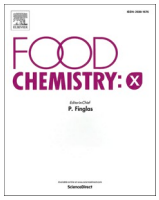

UPLC-Q-TOF/MS-based metabonomics reveals mechanisms for *Holothuria leucospilota* polysaccharides (HLP)-regulated serum metabolic changes in diabetic rats

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ABSTRACT

This study aimed to use metabolomic methods to explore how *Holothuria leucospilota* polysaccharides (HLP) improved metabolism disorders in the liver of Goto-Kakizaki (GK) rats with spontaneous type 2 diabetes. The results showed that HLP effectively improved the metabolic disorder. Based on KEGG functional analysis, five key biomarkers associated with bile acid metabolism were detected and screened ($P < 0.05$). The results of serum total bile acid levels and liver damage in diabetic rats further showed the regulatory effects of HLP on bile acid metabolism. The results of bile acid-related gene expression in the liver showed that HLP inhibited liver farnesoid X Receptor - small heterodimer partner (FXR-SHP) signalling and increased the expression of bile acid synthesis genes ($P < 0.05$). Our results explored the underlying mechanisms by which HLP accelerated cholesterol consumption to anti-hypercholesterolemia and anti-diabetic by inhibiting liver FXR-SHP signaling. HLP's effect on bile acid regulation provides insights into treating T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM), a chronic metabolic disease, is associated with abnormal glucose metabolism and metabolic disorders caused by pancreatic β-cell defects (Guariguata et al., 2014). Changes in lifestyle and greater availability of low nutritional value and high caloric food have led to the highest rates of obesity in history (Prasad et al., 2020). At the same time, the number of patients with T2DM has increased substantially. The International Diabetes Federation (IDF) Diabetes Atlas Tenth Edition 2021 assessed approximately 537 million adults (20–79 years) were living with diabetes, and the total number of people living with diabetes was projected to rise to 643 million by 2030 and 783 million by 2045 (Ogurtsova et al., 2022). Sulfonylureas, α-glycosidase inhibitors, and biguanides are the main drugs used to control blood glucose in diabetic patients (Hu & Jia, 2019). Dietary intake and nutritional conditioning also play essential roles in improving T2DM indicators (Lee et al., 2021). Complications of T2DM include retinopathy, nephropathy, and neuropathy. All of these complications reduce life expectancy among diabetic patients (Artasensi et al., 2020). With the increasingly severe complications and the increasing incidence of T2DM, finding functional food ingredients that effectively regulate T2DM has become one of the new research hot spots (Li et al., 2021).

Holothuria leucospilota is an edible sea cucumber widely distributed in the Indo-Pacific region (Dai et al., 2015). The main components of extracts from *Holothuria leucospilota* polysaccharide (HLP) are primarily *N*-acetylgalactosamine, fucose, and glucuronic acid (Yuan et al., 2019, Yuan et al., 2019). HLP has many physiological activities, such as antioxidation, antithrombosis, anticoagulation, and antitumor properties (Wang et al., 2021, Wang et al., 2021). Previous studies showed that HLP reduced glycosylated protein, triglycerides (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) and increased

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high-density lipoprotein cholesterol (HDL-C) in diabetic rats' serum (Zhao et al., 2020). At the same time, HLP prevents liver damage by alleviating oxidative stress, relieving inflammation and playing a role in regulating the structure of the gut microbiome in diabetic rats (Zhao et al., 2021).

Metabolomics has been used to identify the overall changes in endogenous substances in the biological system under the stimulation of external conditions (physical, chemical, environmental). It is an effective way to analyze the physiological and pathological changes in the organism. In pharmacology, the change in endogenous markers reflects the disorder of the physiological variations (Nicholson, Lindon & Holmes, 1999). Zhang et al. applied metabolomics to reveal changes in metabolites in T2DM rats affected by Xiexin Decoction (Zhang et al., 2021).

As an essential organ, the liver participates in many metabolic processes, including glucose metabolism, protein amino acid metabolism, and lipid metabolism. Liver damage leads to blood glucose and lipid metabolism disorders, while disorders in liver fat metabolism result in nonalcoholic fatty liver disease (Wang et al., 2021, Wang et al., 2021). Previous studies have focused on polysaccharides' effects on changes in diabetes-related enzymes and the gut microflora (Zhao et al., 2020). The intervention of oyster mushrooms and shiitake mushrooms reduced fasting blood glucose, and the activity of α-glucosidase and angiotensin-converting enzymes in diabetic rats (Agunloye & Oboh, 2021). *Laminaria japonicapolysaccharides* (LP) regulated the structure of intestinal microflora and the content of short-chain fatty acid in gastric-type diabetes mellitus (GDM) patients (Lin et al., 2021). These studies proposed the improving effect of nutrients on the intestines and metabolism. However, metabolic mechanisms by which HLP improves hepatic lipid metabolism have not been proposed.

In this study, to understand the potential mechanism of HLP's ameliorating effect on T2DM rats, UPLC-Q-TOF/MS was applied to detect serum metabolites in Goto-Kakizaki (GK) rats. Differential markers were obtained by metabolomics multivariate analysis methods, while the function of biomarkers was obtained based on KEGG. The relevant metabolic pathways in the liver affected by HLP were inferred from the selected biomarkers, and the conjecture was further validated with related biological methods. This study provided new insight for an in-depth understanding of the mechanism of action of HLP's antidiabetic properties.

2. Materials and methods

2.1. Extraction of HLP

Dried *Holothuria leucospilota* (8.46 ± 1.17 cm long and 22.25 ± 3.31 g weight) was acquired from Market Property Development Co, Ltd (Haikou, China) (Zhao et al., 2020). The species of *Holothuria leucospilota* was identified by Professor Yongqin Feng (Hainan University, Haikou, China). The extraction of HLP was based on previously published methods (Yuan et al., 2019, Yuan et al., 2019). HLP was defatted by immersion in acetone (Shanghe Biotechnology, Haikou, China) and was digested by papain (Qiyuan Biotechnology, Haikou, China) for 24 h. After centrifugation, the enzyme exudate was added to Cetylpyridinium Chloride (CPC) (Aladdin Bio-Chem Technology, Shanghai, China) to precipitate polysaccharide sulfate. The polysaccharide precipitate was further sedimented with ethanol (Aladdin Bio-Chem Technology, Shanghai, China). Then the precipitate was deproteinized by trichloroacetic acid (TCA) (Aladdin Bio-Chem Technology, Shanghai, China). The deproteinized part was extensively dialyzed in distilled water and lyophilized to obtain polysaccharides. Previous research has shown that the content of total sugar, protein, sulfate group and ash in HLP were 55.64%, 9.22%, 26.19% and 24.93%, respectively (Zhao et al., 2021).

2.2. Animal experiments

The laboratory animals were raised in accordance with the laboratory animal care and use guidelines issued by the US National Institutes of Health. The procedures were approved by the Animal Ethics Committee of Hainan Medical University.

Goto-Kakizaki (GK) rats (male) and Wistar rats (male) were supplied by Tianqin Biotechnology Co, Ltd (Changsha, China), and the Certificate number was SCXK (Xiang) 2014–0011. Rats were adaptively fed basal diets in random groups. The rat diet (Guangdong medical laboratory animal center, Guangdong, China) consisted of imported fish meal, wheat, corn, soybean meal, bran, vegetable oil, alfalfa powder, vitamins, minerals and essential amino acids. Rats were adapted to the laboratory condition with an ambient temperature (23 ± 1 °C) and a 12:12 h light/dark cycle. After seven days, GK rats were randomly distributed to 3 groups (n = 10), which were regarded as diabetes mellitus (DM, distilled water intake) model control group; low and high dose HLP (HLP-L, 100 mg/kg HLP; HLP-H, 200 mg/kg HLP) groups, respectively. Wistar rats (n = 10) were fed as the normal control (NC, distilled water intake) group. All rats were cage fed in groups with 5 rats per cage, and HLP groups received daily oral HLP in addition to the usual basal diet for four weeks.

After fasting for 10 h, all rats were anesthetized by subcutaneous injection of 5% Chloral hydrate (0.5 mL/100 g) and then sacrificed by cervical vertebral dislocation. The rat's whole blood was obtained, chilled, and centrifuged (4000*g*, 4 °C, 15 min). The serum located in the upper layer of the blood was collected. Samples from animal experiments, such as serum and tissue, were taken immediately and stored at −80 °C until biochemical and metabolomics experiments.

2.3. UPLC-Q-TOF/MS-based serum metabolite analysis

Serum samples were stored at 4 °C in the refrigerator. Serum samples (150 μL) were mixed with 600 μL of acetonitrile and deionized water (4:1, acetonitrile: deionized water) and centrifuged. Serum mixtures were centrifuged (10000*g*, 4 °C, 20 min), and the supernatants were transferred into vials. The mass spectrometry injection volume was 3 μL/needle. The instrument used for mass spectrometry analysis was an Agilent 1290 Infinity II with an Agilent 6530 Accurate-Mass Q/TOF-LC/MS system (Agilent Technologies, CA). The chromatographic column used in the experiment was an Agilent ZORBAX RRHT Eclipse Plus C18 (Agilent Technologies). The flow phase of the system consisted of 0.1% formic acid in water (A) and 100% acetonitrile (B). The elution gradient used in the analysis was as follows: 5–30% B, 0–8 min; 30–65% B, 8–16 min; 65% B, 16–20 min; 65–90% B, 24–26 min; 90–5% B, 26–30 min. The electrospray ionization mass spectrometry system was used to analyze molecular properties. With the instrument in positive/negative ion mode, the following parameters were set: fragment voltage was 150 V; capillary voltage was 3500/−3500 V; skimmer voltage was 60 V; the pressure of the nebulizer is 40 psig (Zhu et al., 2016).

2.4. Bioinformatics analysis of ion fragments

The initial mass spectrometry result was processed using the Mass Hunter Qualitative Analysis B 7.0 software (Agilent Technologies). Results were processed using Mass Profiler Professional DVD 14.8 software (Agilent Technologies). The standardized data were analyzed using SIMCAP (version 14.0, UmetricsAB, Sweden) for Partial Least Squares Discriminant Analysis (PLS-DA), and consensus orthogonal partial maximum squared discriminant analysis (OPLS-DA). R^2X, R^2Y and Q^2 were used to represent the statistical significance of OPLS-DA and PLS-DA models. The validity mode was tested with 200 permutations. In addition, the data significance test $P < 0.05$ and the fold change > 2 were used as the critical factor in screening biomarkers. Molecular Structure Correlator (Agilent Technologies) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to confirm the accurate mass

data and the structure of potential biomarkers. The clustering of filtered potential markers was processed using MetaboAnalyst 3.0 (https://www.metaboanalyst.ca). At the same time, metabolic pathway analysis of biomarkers was performed in conjunction with the KEGG database (https://www.kegg.jp).

2.5. Measurements of total bile acid (TBA) in liver tissues

The total bile acid of the serum was measured according to the instructions of the kit (Changchun huili Biotech, Jilin, China). The fully automatic biochemical instrument (Shenzhen Leidu Life Technology) was set with the appropriate parameters (37 °C, 405 nm). Taking 150 μL per serum sample and automatically determining it on the fully automated biochemical instrument.

2.6. Western blotting of liver tissue

The expression levels of Caspase-3 in the liver were determined according to Western blotting. Protein was extracted from the liver using lysis buffer (Beyotime, Shanghai, China) and centrifuged (12000*g*, 4 °C, 10 min). The BCA assay kit determined the protein concentration (Beyotime, Shanghai, China). Equal amounts of denatured protein were separated through 12% SDS-PAGE, transferred into PVDF membranes, blocked by skim milk, and incubated with specific primary antibodies overnight. Following washing with Tris-buffered saline containing 0.1% Tween 20 (TBST), the membranes were incubated with HBR-conjugated secondary antibodies and further washed by TBST. The blots were detected by ECL chemiluminescence detection kit (Beyotime, Shanghai, China) and visualized using the ImageQuant LAS4000mini system (GE, Boston, USA).

2.7. RT- qPCR of liver tissue

The RT-qPCR method was required to detect the mRNA amplification level shifts of farnesoid X Receptor (FXR), small heterodimer partner (SHP), bile acyl-CoA synthetase precursor (BACS), cholesterol 7α-hydroxylase (CYP7A1), bile salt-export pump (BSEP) and Na^+-taurocholic acid co-transporting polypeptide (NTCP) in the liver. The relative expression levels were standardized by β-actin and analyzed by the formula $2^{-\Delta\Delta CT}$. Primer sequences used in the study are shown in Table.1 (Sangon Biotech, Shanghai, China).

2.8. Statistical analysis

The experimental data were analyzed by SPSS Statistics Version 24.0 software (SPSS, Inc, Chicago, IL). The mean value was compared using the standard error of the mean, and the variance significance test was performed using the S—N—K test ($P < 0.05$). The figures were acquired through OriginPro 2020 (OriginLab, Northampton) and Adobe Illustrator CC 2018 (Adobe Systems Incorporated, San Jose, USA).

3. Results

3.1. HLP regulated the total bile acid level in the serum of T2DM rats

The total bile acid in serum samples has been detected in this study. Bile acids are produced by cholesterol metabolism and are related to the regulation of lipids. Bile acid plays a critical role in regulating diseases such as lipid metabolism, glucose metabolism and inflammation. (Dossa et al., 2016). Compared with the NC group (10.87 μmol/L), the total bile acid content of the DM group (19.92 μmol/L) increased significantly ($P < 0.05$). Meanwhile, the HLP-L group (12.68 μmol/L) and the HLP-H group (9.96 μmol/L) resulted in a significant reduction in total bile acid content in GK rats ($P < 0.05$) (Fig. 1A). Similarly, studies have declared that T2DM individuals exhibited higher levels of fasting bile acid in the peripheral cycle, mainly due to increased levels of deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA). The above results suggested that HLP regulated the bile acid level of T2DM rats and alleviated the abnormality of bile acid.

3.2. HLP regulated liver damage in T2DM rats

The results of western blotting revealed that the relative expression of Caspase-3 in the liver of the DM group was higher than that in the NC group ($P < 0.05$) (Fig. 1B, C). This result indicated diabetic rats suffered liver damage. After the treatment of HLP, the relative expression of Caspase-3 was reversed. Previous studies have also expressed that HLP reduced oxidative stress in the liver. The liver tissue damage that occurred during the development of diabetes in diabetic rats was obtained by liver histopathology analysis (Zhao et al., 2021). Through the treatment of HLP, the liver damage and hepatocyte structure in rats were also relieved.

3.3. Effects of HLP on serum bile acid levels

The results of RT-qPCR demonstrated that the mRNA amplification levels of FXR, SHP, BACS and NTCP in the livers of the DM group were significantly higher than those of the NC group (Fig. 2A, B, D, F). Furthermore, these expressions were significantly reduced in the HLP groups ($P<0.05$). Nevertheless, the expression level of CYP7A1 and BSEP in the DM group was significantly lower than in the NC group (Fig. 2C, E). After treatment with HLP, the expression of these genes increased significantly ($P < 0.05$).

3.4. HLP improved endogenous metabolic differences in GK rats

The ion chromatography of serum has been shown in supplementary information in Fig. 1S. The results of the Principal component analysis (PCA) (Fig. 3A) have shown that the total variances of PC1 and PC2 in positive ion mode were 75% and 15.8%, respectively. The total variances of PC1 and PC2 in negative ion mode were 58.8% and 23.0%, respectively (Fig. 3B). The NC, HLP and DM groups showed a clear discrete trend, indicating differences in metabolic profiles in these groups.

The PLS-DA multivariate discriminant analysis plot further demonstrates the trend. The stoichiometry method proposes that R^2Y and $Q^2 >$

Table 1
Primers used in study.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
FXR	TCTCCTCCTCGTCCTATTATTCCAACC	GCATTCGCCTGAGTTCATAGAGTCC
SHP	GGCACTATCCTCTTCAACCCAGATG	GGGCTCCAGGACTTCACACAATG
CYP7A1	AGGTCTCTGAACTGATCCGTCTACG	GAGAATAGCGAGGTGCGTCTTGG
BACS	CGCCTTAACAGACATCCTCCAGAAC	AGCCCTCAGACCCAGTACACAC
BSEP	GAGTGGTGGTCAGAAGCAAAGAGTAG	GAGGTAGCCATATCCAGAAGCAAGATC
NTCP	TCTGGCTTTCTGATGGGTTACATTCTC	GGAAGGTCACATTGAGGATGGTAGAAC
β-actin	GTCAGGTCATCACTATCGGCAAT	AGAGGTCTTTACGGATGTCAACGT

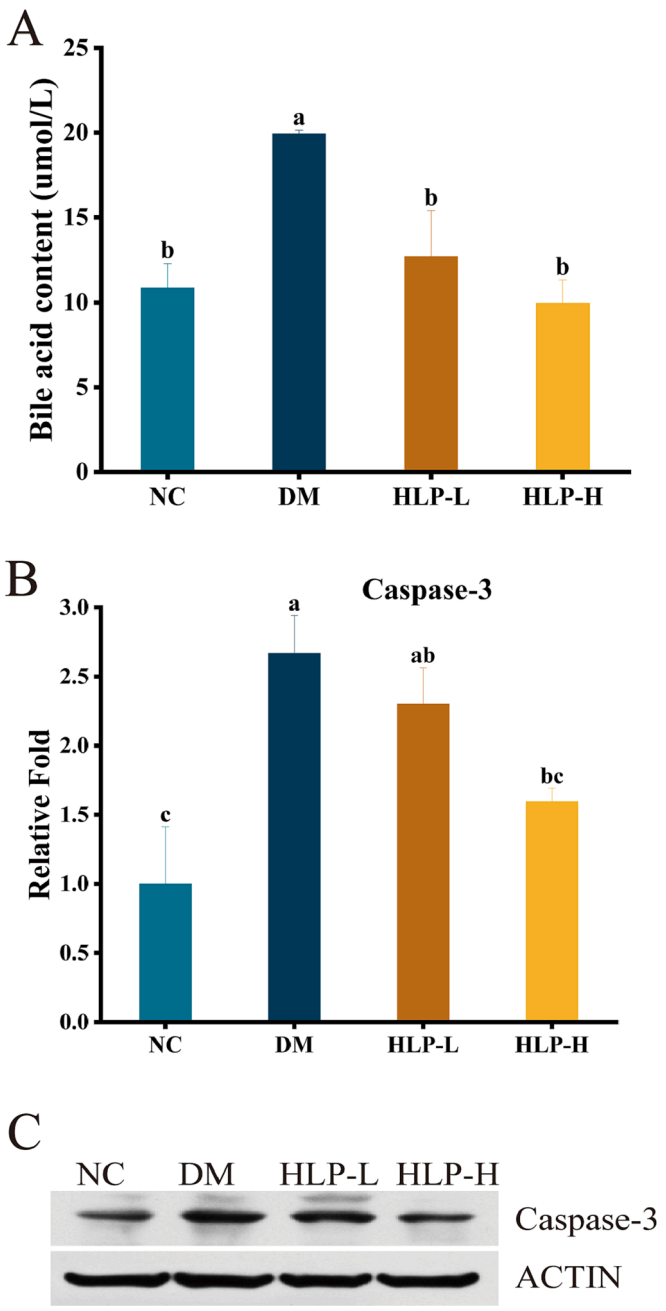

Fig. 1. The relative content of total bile acids in different groups of serum samples (A). The western blot bands and relative grayscale of Caspase-3 (B, C). Values with different letters of the same indicator are significantly different ($P < 0.05$). NC: non-diabetes mellitus control group; DM: untreated diabetes mellitus group; HLP-L: DM + 100 mg/kg HLP; HLP-H: DM + 200 mg/kg HLP.

0.5 are ideal models, and > 0.9 are excellent models in the OPLS-DA and PLS-DA analysis. PLS-DA in positive ($R^2Y = 0.74$, $Q^2 = 0.663$) and negative ($R^2Y = 0.921$, $Q^2 = 0.84$) ion modes for all samples conformed to the requirement (Fig. 3C, D). The plausibility of the PLS-DA model requires that the intercept of Q^2Y is < 0.05 and the intercept of R^2Y is < 0.4. The 200 validations in the positive (intercept of R^2 Y = 0.0567, intercept of Q^2 Y = −0.36) and negative (intercept of R^2 Y = 0.117, intercept of Q^2Y = −0.389) ion modes were reliable (Fig. 3E, F). The OPLS-DA method was used to compare the DM group with other groups (Supplementary information Fig. 2S.). Among these results, the HLP groups and DM group were evidently separated, and the HLP groups were closer to the NC group, suggesting that HLP's intervention improved the serum metabolic profile of T2DM rats (Fig. 1C, D).

3.5. HLP callback core metabolites and metabolic pathway in GK rats

Variable important in projection (VIP) value is commonly used in metabolomics strategy analysis to screen for statistically significant serum differential metabolites. Larger VIP values indicated that the compound contributed more to the differences between the two groups. Combined with the folding coefficient (FC > 2), p-value ($p < 0.05$), and VIP value (VIP > 1) screening, 15 differential metabolites were finally screened in rats' serum. As shown in the heat map (Fig. 3G), cholic acid, glycocholic acid (GCA), Glycochenodeoxycholic acid (GCDCA), Glycodeoxycholic acid (GDCA), and lysophosphatidyl ethanolamine (LysoPE) were elevated in the serum of T2DM rats. Comparative analysis of the NC group with the MC group suggested that serum metabolites in T2DM rats were disturbed. After the HLP intervention, the effect improved distinctly.

Based on information such as retention time, accurate quality, MS/MS fragments, combined with the KEGG database, five differential metabolites were interpreted as biomarkers for HLP-regulated diabetes in rats. HLP expressed the positive regulation of these potential biomarkers (Table 2). Five metabolic pathways: urea cycle and metabolism of amino groups, primary bile acid biosynthesis, secondary bile acid biosynthesis, bile secretion, cholesterol metabolism were used as detection indicators to evaluate the effect of HLP on the metabolism of GK rats, and to explore its anti- diabetes mellitus.

4. Discussion

4.1. Biomarker analysis

Metabonomics provides a powerful way to explore the basic mechanism of disease and treatment methods. Indole contains a 2,3-phenylpyrrole structure formed by the pyrrole ring and benzene complexation. Indoleacrylic acid is an indole compound with critical biological functions in animals. The result of this study confirmed that indoleacrylic acid is a biomarker in diabetic rats, and HLP reduced the concentration of indoleacrylic acid and alleviated the metabolic disorder in diabetic rats. Yue et al. reported that deoxycholic acid, cholic acid, 3-indole acrylic acid, and melatonin were discovered in the serum of rats that consumed a high-sugar diet, and these metabolites were negatively correlated with the abundances of *Bacteroides acidifaciens* and *Staphylococcus saprophyticus subspecies* (Yue et al., 2019). Our previously published research has shown that the relative abundance of *Staphylococcus* increased in T2DM rats and decreased after intragastric administration of HLP (Zhao et al., 2020).

Cholic acid is a primary bile acid produced by cholesterol metabolism and is transported to the intestines to promote intestinal fat absorption. When bile acid content is overloaded, cholic acid acted as a hepatic and metabolic toxin, damaging liver cells and adversely affecting health (Delzenne et al., 1992). The presence of excess bile acids in the blood led to liver damage and fat malabsorption. High-fat rats fed bile acids were more likely to cause liver fibrosis (Fukuda et al., 2019). The heat map demonstrated that HLP reduced cholic acid in serum in T2DM rats, suggesting that HLP relieved lipid metabolism and liver injury in T2DM rats (Fig. 3G).

Glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA) are converted from cholesterol in liver cells under different catalytic enzymes. GCA and GCDCA are formed by the combination of glycine and cholic acid. As primary bile acids, GCA and GCDCA were related to bile acid and cholesterol metabolic pathways (Table 2). Bile acids are bound to amino groups of carboxylic acids in liver cells. GCA is involved in emulsifying fat and absorbing soluble fat, and regulating bile flow and lipid secretion. GCA also promotes fat-soluble vitamin absorption and regulates the synthesis of all key enzymes in cholesterol homeostasis (Xie et al., 2021). However, the accumulation of GACs in the liver

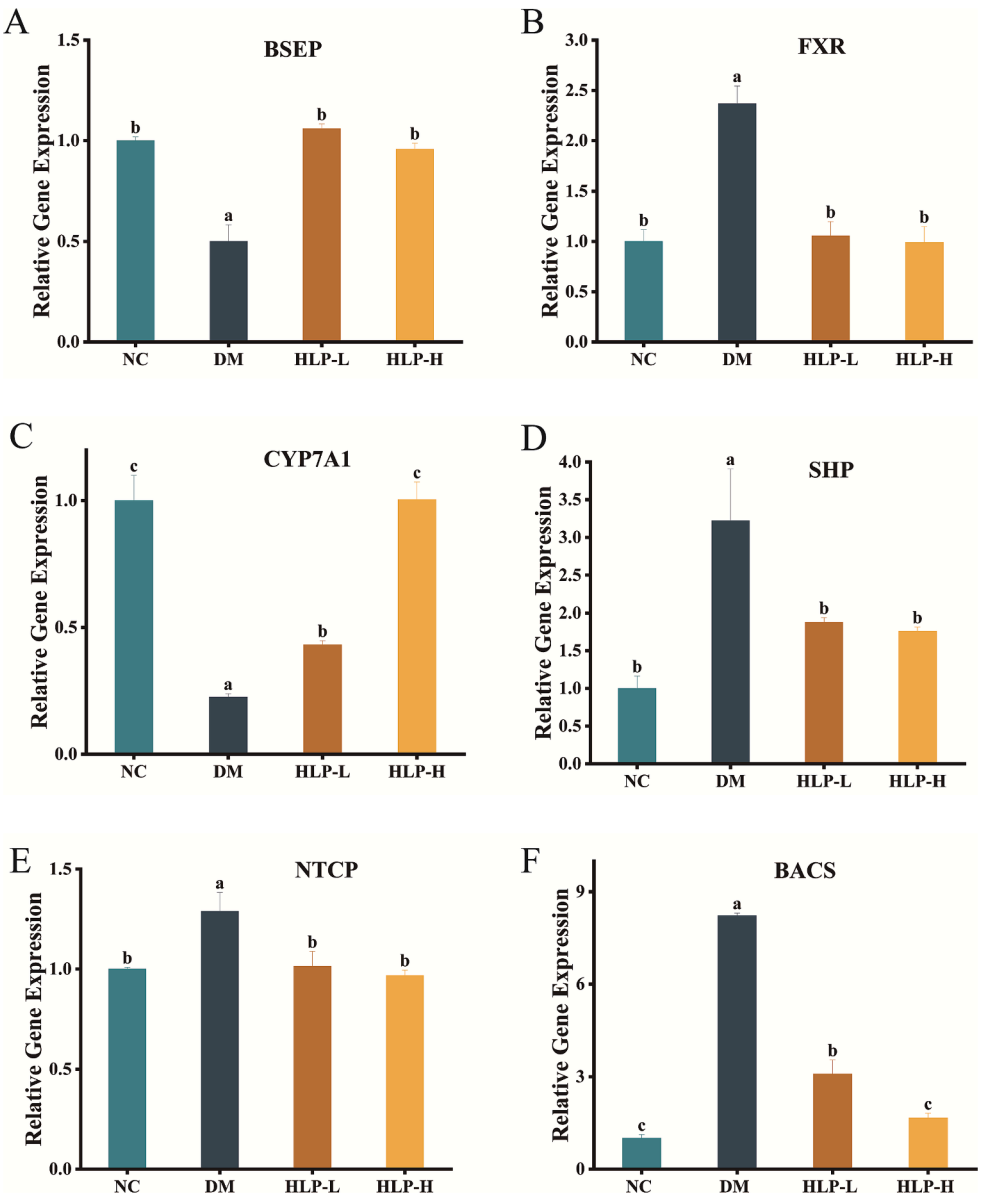

Fig. 2. Effects of HLP on mRNA expression levels of BSEP (A), FXR (B), CYP7A1 (C), SHP (D), NTCP (E), BACS (F) in the liver tissue of diabetes mellitus rats. Data are expressed as the means ± SEM (n = 10). Values with different letters are significantly different ($P < 0.05$). FXR, farnesoid X Receptor; SHP, small heterodimer partner; BACS, bile acyl-CoA synthetase precursor; CYP7A1, cholesterol 7α-hydroxylase; BSEP, bile salt-export pump; NTCP, Na + -taurocholic acid co-transporting polypeptide. NC: non-diabetes mellitus control group; DM: untreated diabetes mellitus group; HLP-L: DM + 100 mg/kg HLP; HLP-H: DM + 200 mg/kg HLP.

destroyed the cell membranes of hepatocytes (Claudel, Staels & Kuipers, 2005). GCDCA causes defects in autophagy removal of lysosomal bodies, leading to human hepatocyte death. GCA was also one of the potential biomarkers of serum metabolites in studies of the improving effects of *Ganoderma lucidum* polysaccharides in T2DM rats (Zhu et al., 2016). Disorders in cholesterol metabolism and bile acid metabolism impaired liver function in GK rats. Treatment of HLP distinctly reduced GCDCA and GCA serum levels in diabetic rats (Fig. 3G). This result corroborated previous studies that HLP alleviated liver damage in diabetic rats (Zhao et al., 2021). HLP regulated the disorder of bile acid metabolism in diabetic rats.

Glycodeoxycholic acid (GDCA) is a secondary bile acid metabolized by enzymatic action in the colonic environment. The accumulation of pure GDCA causes a huge toxic effect on cells (Delzenne et al., 1992). GDCA has the effect of emulsifying fats, promoting the absorption of lipids and fat-soluble vitamins. Metabolic surgery studies have found that only an increase in GDCA was significantly associated with insulin sensitivity of all the bound secondary bile acids (Ahlin et al., 2019). The cystic fibrosis (CF) bile acid metabolism profile showed that GDCA separated patients with non-cirrhosis and patients with undetected cirrhosis and was a potential biomarker for CF-related liver disease (Drzymala-Czyz et al., 2022). This study showed that GDCA was a potential biomarker in T2DM rats and participated in the pathways related to bile acid and cholesterol metabolism. The serum GDCA level in the DM group was elevated, indicating disturbances in the metabolic function of bile acids and cholesterol and accompanied by damage to organs and tissues in T2DM rats. After intake of HLP, the relative abundance of GDCA in the serum decreased, indicating that cholesterol and bile acid metabolic dysregulation caused by diabetes attenuated.

Analysis of potential biomarkers suggested that deregulated bile acid and lipid metabolism accompanied liver damage in GK rats. HLP regulated the metabolic levels of indole acrylic and bile acid, improving bile acid metabolism and alleviating liver damage. These potential biomarkers were essential indicators of HLP relieving symptoms in T2DM rats.

4.2. *Expression analysis of bile acid metabolism genes*

FXR is an essential receptor involved in bile acid metabolism and is usually expressed in the liver and colon. Intestinal FXR is mainly responsible for bile acid reabsorption, and liver FXR regulates bile acid synthesis (Honda et al., 2020). Overexpressed FXR inhibited

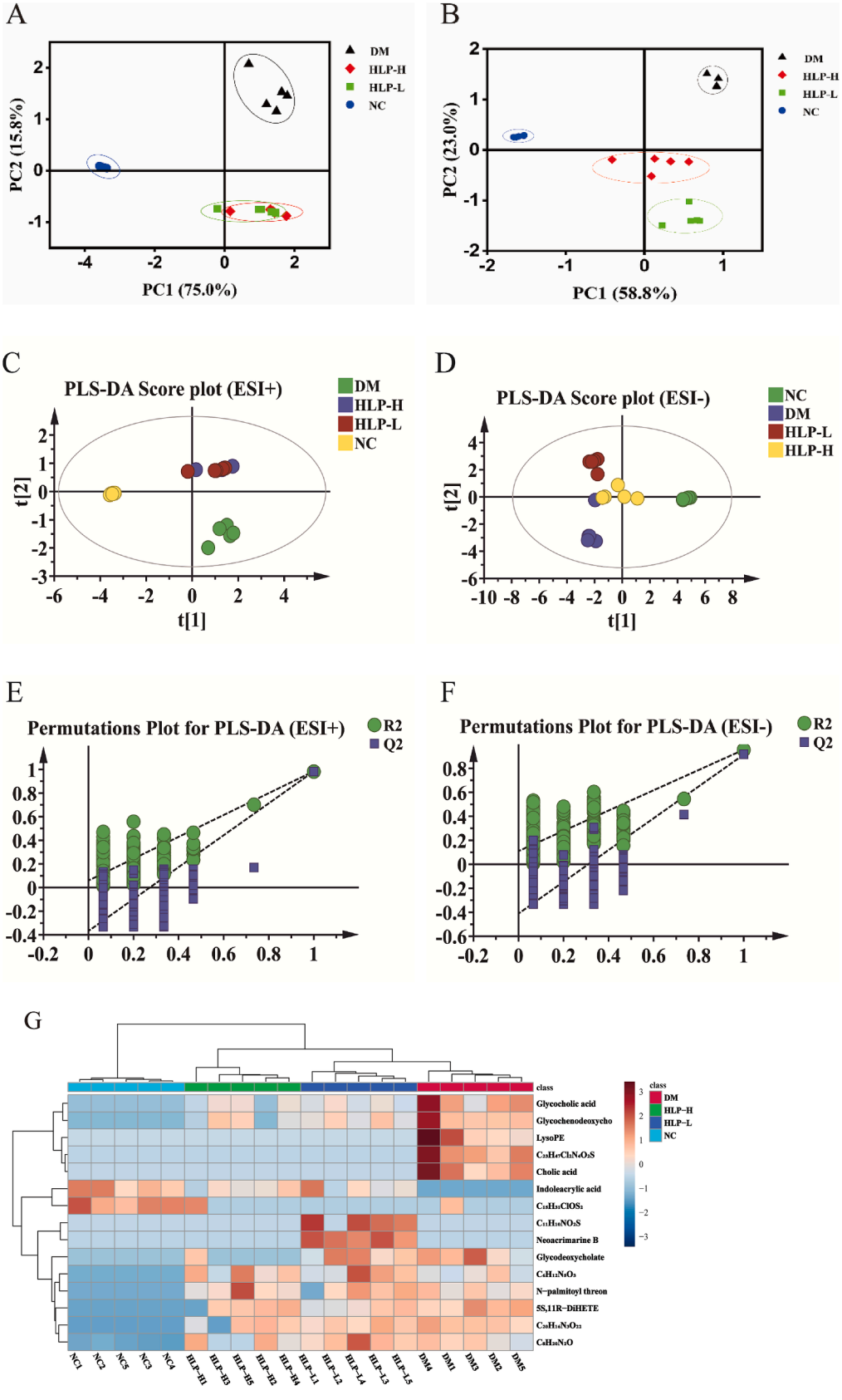

Fig. 3. Multivariate analysis of serum metabolomics. Score plots of PCA for samples in positive (A) and negative (B) mass spectrometry modes. Score plots of PLS-DA in positive (C) and negative (D) modes and plausibility verification in positive (E) and negative (F) modes. Heatmap (G) of differential biomarkers among different groups in the positive and negative modes. NC: non-diabetes mellitus control group; DM: untreated diabetes mellitus group; HLP-L: DM + 100 mg/kg HLP; HLP-H: DM + 200 mg/kg HLP.

Table 2
Effects of HLP on biomarkers in diabetic rats.

Metabolites	Structure	Molecular formula	Molecular weight	Related pathway	Log (NC vs DM)	Log (HLP-H vs DM)
Indoleacrylic acid		$C_{11}H_9NO_2$	187.0635	Urea cycle and metabolism of amino groups	14.4089	0.7794
Cholic acid		$C_{24}H_{40}O_5$	408.2872	Primary bile acid biosynthesis Secondary bile acid biosynthesis Bile secretion	−10.7095	−10.7095
Glycocholic acid		$C_{26}H_{43}NO_6$	465.3087	Primary bile acid biosynthesis Secondary bile acid biosynthesis Bile secretion Cholesterol metabolism	−14.3430	−3.2986
Glycodesoxycholic acid		$C_{26}H_{43}NO_5$	449.3143	Primary bile acid biosynthesis Secondary bile acid biosynthesis Bile secretion Cholesterol metabolism	−12.0958	−15.1852
Glycochenodeoxy-cholic acid		$C_{26}H_{43}NO_5$	449.3139	Primary bile acid biosynthesis Secondary bile acid biosynthesis Bile secretion Cholesterol metabolism	−13.9747	−3.1935

glucose-induced glucose progenitor expression and glucagon-like peptide-1 (GLP-1) secretion (Wang et al., 2021, Wang et al., 2021). Furthermore, the activation of FXR induced SHP to regulate the expression of CYP7A1 (Jiao et al., 2018). CYP7A1 is the main enzyme that converts cholesterol into bile acids in the liver. Recent studies have reported that the expression of CYP7A1 was significantly upregulated in the Astragalus Radix (TFA) treatment of diabetic mice, and FXR played a pivotal role in modulating bile acid and lipid metabolism (Wang et al., 2021, Wang et al., 2021). The increase in CYP7A1 metabolized more cholesterol, allowing more bile acid to be excreted through feces, reducing cholesterol in the serum. (Zhao et al., 2021). BACS was associated with bile acid and glycine synthesis. FXR induced BACS expression during glycine bile acid-binding (Zhang et al., 2021). FXR activated the expression of the bile acid output transporter BSEP while inhibiting the input of bile acids by down-regulating NTCP.

Extrapolating from these results, the hypoglycemic and hypolipidemic effects of HLP might be in part by modulating FXR-SHP mediated cholesterol and bile acid metabolism. The treatment of HLP caused diabetic rats to metabolize more cholesterol. At the same time, the intake of HLP limited the expression of genes related to the bile acid cycle; more bile acids were excreted from the body to reduce the toxic effect of bile acids on the body.

4.3. Potential metabolic mechanism of HLP regulating T2DM

Based on the published data, this study further revealed the mechanism of HLP regulated the secretion of bile acids and cholesterol in diabetic rats (Fig. 4). Our previous research has shown that HLP regulated the levels of TC, TG, LDL-C, HDL-C and GLP-1; decreased the abundance of *Bacteroides, Roseburia, Bifidobacterium*, *Blautia* and elevated the concentrations of SCFAs in diabetic rats (Zhao et al., 2020). At the same time, previous research has also shown that HLP alleviated

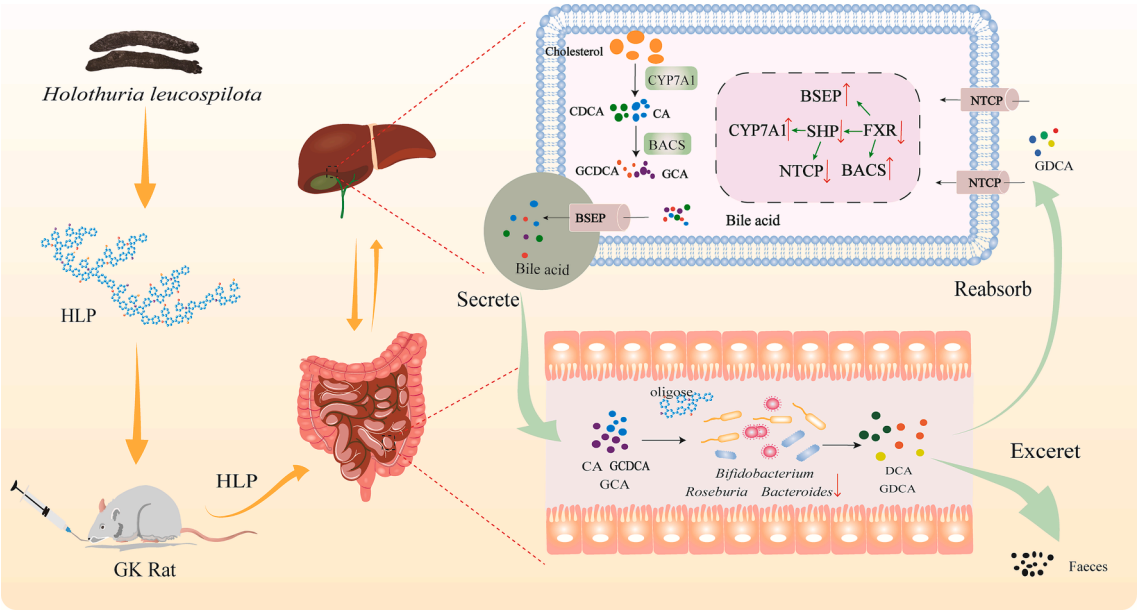

Fig. 4. Potential mechanism of HLP regulating the bile acid metabolism. HLP, *Holothuria leucospilota* polysaccharide; CA, Cholic acid; CDCA, Chenodeoxycholic acid; GCA, Glycocholic acid; GCDCA, Glycochenodeoxy-cholic acid; DCA, Deoxycholic acid; GDCA, Glycodesoxycholic acid; FXR, farnesoid X Receptor; SHP, small heterodimer partner; BACS, bile acyl-CoA synthetase precursor; CYP7A1, cholesterol 7α-hydroxylase; BSEP, bile salt-export pump; NTCP, Na^+-taurocholic acid co-transporting polypeptide.

liver injury in T2DM rats (Zhao et al., 2021). In this study, HLP regulated the relative content of bile acid metabolites (cholic acid, GCA, GCDCA and GDCA) in serum of diabetic rats by UPLC-Q-TOF/MS. HLP regulated the FXR-SHP signaling pathway affected the synthesis (CYP7A1), glycineization (BACS), excretion (BSEP), and reabsorption (NTCP) of bile acids; accelerated the consumption of cholesterol and the excretion of bile acids in diabetic rats. The Intestinal flora (*Blautia*, *Bifidobacterium, Bacteroides, Roseburia*, *Faecalibacterium* and *Ruminococcus*) had the ability to secrete bile salt hydrolases (BSHs) and hydroxysteroid dehydrogenases (HSDHs) to produce secondary bile acid (GDCA) (Ballan & Saad, 2021). The toxic bile acids (GCA and GCDCA) accumulated in the liver were excreted through feces and urine, which relieved liver damage (Beysen et al., 2012). Therefore, HLP reduced cholesterol accumulation, regulated bile acid metabolism and alleviated liver damage in diabetic rats by inhibiting FXR-SHP signal, regulating the composition of intestinal flora and improving intestinal metabolism.

5. Conclusion

This study proposed a potential mechanism by which HLP regulated bile acid balance and accelerated cholesterol metabolism in T2DM rats. Fragmentation information of HLP-regulated diabetic rat metabolites was analyzed by UPLC-Q-TOF/MS strategies. Based on the KEGG database, the analysis of crucial biomarkers (indoleacrylic acid, cholic acid, GCA, GCDCA and GDCA) deduced that HLP regulated bile acid metabolic pathways. At the same time, this study showed that HLP regulated the expression of genes (FXR, SHP,CYP7A1, BACS, BSEP and NTCP) related to bile acid metabolism. Our published research has demonstrated that HLP regulated the levels of TC, TG, LDL-C, HDL-C and GLP-1 and the structure of gut microbiota, alleviated liver injury in T2DM rats (Zhao et al., 2020; Zhao et al., 2021). Therefore, the mechanism by which HLP regulated cholesterol and bile acid metabolism in diabetic rats was proposed. After HLP intervention, the intestinal flora related to bile acid metabolism (*Blautia*, *Bifidobacterium*, *Bacteroides*, *Roseburia*, *Faecalibacterium* and *Ruminococcus*) were reduced and the content of bile acid (cholic acid, GCA, GCDCA and GDCA) were regulated. Meanwhile, HLP regulated liver FXR-SHP signaling, affected the the expression of genes (CYP7A1, BACS, BSEP and NTCP) related to bile acid and cholesterol metabolism, and eventually reduced cholesterol, accelerated liver bile acid excretion and maintained bile acid metabolism balance in diabetic rats. This study provided a new idea for preventing and treating clinical diabetes and its complications.

CRediT authorship contribution statement

Xin Zhang: Investigation, Writing – original draft, Data curation. **Fuqiang Zhao:** Data curation, Investigation. **Tingting Ma:** Methodology. **Yuanping Zheng:** Resources. **Jun Cao:** Resources. **Chuan Li:** Writing – review & editing, Supervision, Conceptualization. **Kexue Zhu:** Resources.

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.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100741.

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