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Exploring the effects of Qijiao Shengbai capsule on leukopenic mice from the perspective of intestinbased on metabolomics and 16S rRNA sequencing

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ABSTRACT

Qijiao Shengbai capsule (QJSB) is formulated according to the traditional Chinese medicine formula, its function is to nourish Qi and blood, improve the body's immunity. Leukopenia has been treated with it in clinical settings. However, the mechanism of leukopenia from the perspective of intestinal tract has not been reported. This study combined metabolomics and 16S rRNA sequencing technologies to investigate the mechanism of QJSB on leukopenia from the intestine. As a result of cyclophosphamide induction in mice, the results demonstrated that QJSB may greatly increase the quantity of peripheral leukocytes (including neutrophils). Meanwhile, QJSB had a restorative effect on the colon of leukopenic mice; it also increased the level of IL-2, IL-6 and G-CSF in the intestine, further enhancing the immunity and hematopoietic function of mice. Metabolic studies showed that QJSB altered 27 metabolites, most notably amino acid metabolism. In addition, QJSB had a positive regulatory effect on the intestinal microbiota, and could alter community composition by improving the diversity and abundance of the intestinal microbial, which mainly involved 6 related bacterial groups, and primarily regulates three associated SCFAs (acetic acid, butyrate acid and valeric acid). Therefore, this study suggests that QJSB can improve hematopoietic function, enhance the immune system, relieve leucopenia and improve the gut in leucopenic mice by modulating metabolic response pathways, fecal metabolites and intestinal microbiota.

1. Introduction

The intestine contains the largest proportion of immune cells in the body. It is constantly exposed to new antigens and immune

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Abbreviations

QJSB	Qijiao Shengbai capsule
XRS	Buerger Lespedeza Root
HQ	Astragalus Membranaceus
EJ	Equus asinus L.
DG	Radix Angelicae Sinensis
DZ	Fructus Ziziphi Jujubae
KS	Sophora Flavescens
YYH	Epimedium
ICR	Institute of Cancer Research
HE	Hematoxylin-eosin
N2	Nitrogen
NE	Neutrophil
QC	Quality control
UHPLC	Ultra High Performance Liquid Chromatography
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
G-CSF	Granulocyte colony-stimulating factor
EPO	Erythropoietin
PCA	Principal component analysis
OPLS-DA	Orthogonal partial least squares discriminate analysis
CTAB	Cetyltrimethylammonium Bromide
PCR	Polymerase Chain Reaction
NMDS	Non-metric multidimensional scaling
Csf3	Colony-stimulating factor 3
BCAA	Branched-chain amino acid
CA	Cholic acids
UA	Ursolic acid
IBD	Inflammatory bowel disease
CRC	Colorectal cancer

stimuli from the diet, microbiota and intestinal pathogens [1]. As an indispensable "metabolic organ", it plays a vital role in maintaining the homeostasis of the body's internal environment, including digestion, nutrient absorption, energy supply, immune regulation, etc. [2]. The intestinal microbiota, including bacteria, archaea, fungi, viruses and phages, inhabits the gastrointestinal tract. This symbiotic microbiota helps to regulate the host immune response and homeostasis in vivo. And alterations in the gut flora have been found in many immune-related diseases [3]. Although the vast majority of these microorganisms play a crucial role in maintaining the health of the host, they still pose a threat to the homeostasis of the gut and are prone to adverse outcomes such as inflammation and sepsis [4]. Thus, the immune system regulates the homeostasis of microbial communities and has evolved the adaptive capacity to work synergistically with the gut microbiota, which can maintain a symbiotic relationship between host and microbiota by controlling the microbiota and ensuring a mutually beneficial relationship [5]. Metabolomics, which is the systematic analysis of small molecules (metabolites) in an organism or biological sample, offers the opportunity to comprehensively study the interactions between metabolism and immunity in physiology and disease. Intergrating metabolomics into systemic immunology can explore the interaction of multiple layers of features in biological systems and the regulatory mechanisms of these features. Meanwhile, metabolic processes also affect immune regulation [6]. Therefore, gut microbiota and metabolomics techniques can provide a more comprehensive understanding of changes in gut microbes and metabolites after disease onset and treatment and can be used as an effective method to study the mechanisms of disease onset and improvement.

Clinical studies and animal experiments have proved that herbal medicines can maintain the balance of the intestinal microecosystem [7]. It is usually used as a prebiotic to regulate intestinal microbiota structure and host metabolites, and further as a new way to target intestinal microbiota for disease treatment [8]. QJSB is a well-known Hmong medicine composed of seven ingredients that can be used to improve leukopenia, including Buerger Lespedeza Root (XRS), Astragalus Membranaceus (HQ), Equus Asinus L. (EJ), Radix Angelicae Sinensis (DG), Fructus Ziziphi Jujubae (DZ), Sophora Flavescens (KS) and Epimedium (YYH) in the ratio of 3:6:2:2:2:2:3 [9]. The combination of the herbs in the formula has the effect of benefiting qi and tonifying the blood [10]. The related literature reported that QJSB capsule can regulate the immune system and treat leukopenia.

Chemotherapy is widely used to treat cancer, but it also has many side effects, including hematologic toxicity and neurotoxicity [11–13]. Leukopenia is a kind of hematotoxicity, and can be as a common marker of hematopoietic disease, which causes serious damage to the body's hematopoietic function, gastrointestinal and immune systems. These results, in turn, lead to delay,

discontinuation or even failure of oncology treatment, which ultimately threatens human health [14,15]. Cyclophosphamide is a common chemotherapeutic drug, that is widely used as an anticancer agent and immunosuppressant agent in clinic. While killing cancer cells, it also significantly damages rapidly proliferating normal cells, seriously damaging the immune system and hematopoietic function, resulting in immune hypofunction and leukopenia [16–19]. Thus, cyclophosphamide-induced leukopenia is closely associated with impairment of immune and hematopoietic functions. However, the mechanism of the QJSB capsule improving leukopenia and the relationship between leukopenia and intestinal microbiota and metabolites have not been reported. Based on this, from the perspective of fecal metabolomics and 16S rRNA gene sequencing analysis, we explored the effect of QJSB on the intestinal tract of leukopenia mice and its relationship with immune and hematopoietic function.

2. Materials and methods

2.1. Materials and reagents

Qijiao Shengbai capsule (Guizhou, China) purchased from Guizhou Dechangxiang Pharmaceutical, lot number Z2522012. Cyclophosphamide for injection was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd., batch number HJ20160467. Mouse ELISA kits, including IL-2, IL-6 and G-CSF, purchased from Qingdao Bozhou Biotechnology Co. (Qingdao, China). Tiangen Magnetic Bead Extraction Kit from QIAGEN Ltd (California, USA). CTAB was purchased from Beijing Noblelight Technology Co. (Beijing, China). Phusion® High-Fidelity PCR Master Mix, GC Buffer and Ph,fusion® high-fidelity DNA polymerase were purchased from Annoron Biotechnology Co., Ltd (Beijing, China). The Universal DNA Purification Recovery Kit (Beijing, China, Catalog #: DP214) used in the construction of the library was purchased from TIANGEN Biochemical Technology Co. Library construction kits were purchased from Gene Biotech International Trading (Shanghai, China) Co. NEB Next® Ultra DNA Library Prep Kit for library construction. (Catalog #: E7370L). Other reagents and solvents were of analytical grade and were not otherwise purified prior to use.

2.2. Mice and treatments

A total of 30 male (ICR) mice (18 ± 2 g) were provided by Guizhou Medical University (animal license number SCXK (Xiang) 2022 - 0011) and kept in a standard animal laboratory with regulated temperature (25 ± 1 °C), humidity (60 ± 5%), and 12 h light/dark cycle with free access to water and food. All animal experiments were reviewed and approved by the animal ethics committee of the institution and were performed in strict accordance with the Guizhou Medical University Animal Care and Use Guidelines. After one week of acclimatization feeding, 30 mice were randomly divided into three groups (n = 10). Normal, Model and QJSB groups. All mice were grouped and labelled and then evenly mixed and housed together. Modeling. Normal group mice were injected intraperitoneally with 0.1 mL of 0.9% saline for 3 consecutive days. Mice in the Model group were injected intraperitoneally with 100 mg kg^{-1} d^{-1} of CTX 0.1 mL for 3 consecutive days. Correspondingly, the QJSB group was modeled in the same way as the Model group, but QJSB solution (1.0 g kg^{-1} d^{-1} for 14 days) 0.3 mL was administered by gavage starting from the 4th day after modeling. however, mice in the Normal and Model group mice were administered with the same solvent (i.e. distilled water) according to the QJSB group's administration method and dose.

2.3. Sample collections and preparation

At the end of modeling (i.e., day 4 of the experiment), 50 μL of blood was taken from the inner orbital canthus of 6 mice in each group at random for the detection of leukocytes including neutrophils on a blood cell analyzer (IDXX, USA). Three groups of mice were simultaneously executed on day 18 of the experiment. Blood samples were collected from the inner corner of the eye for haematological testing. Cecum contents were collected and stored in sterile tubes filled with liquid nitrogen (N2) at −80 °C for fecal metabolomics and 16S rRNA gene sequencing analysis. Colon tissues were collected in duplicate for each sample, one stored at −80 °C for cytokine assays and the other fixed in 4% paraformaldehyde for histopathological analysis, they were embedded in paraffin, cut into 3 mm tissue sections using a microtome, stained with hematoxylin-eosin (HE) and photographed with a light microscope.

2.4. Measurement of leukocytes including neutrophils

Using capillary tubes, 50 μL of whole blood was drawn from the inner corner of the eye of six mice randomly selected from each group, loaded into K_2 anticoagulation tubes, shaken slowly to prevent clotting, and placed on a ProCyte Dx fully automated hematocrit analyzer (IDXX, USA) for testing.

2.5. Measurement of cytokines

The cytokines IL-2, IL-6 and G-CSF were measured strictly according to the instructions of the ELISA kit (Qingdao, China) and subsequently placed on an enzyme marker at 450 nm to determine the relative content of the colonic tissue cytokines.

2.6. Fecal metabolomics analysis

The fecal samples were thawed at room temperature from the −80 °C refrigerator, 6 samples were randomly taken from each group

and weighed 50 mg, added 500 μL of pre-chilled water: acetonitrile: methanol (1:2:2), vortexed (MIX-3000, Hangzhou Mirabu Co., Ltd., China) for 5 min, sonicated for 10 min, and centrifuged at 15000 r min-1 under a high-speed centrifuge at 4 °C. The samples were centrifuged at 15000 r min-1 for 15 min at 4 °C, and the supernatant was passed through a 0.22 μm filter membrane. Aspirate 10 μL of each of the above samples and mix together, vortexed for 2 min, placed in a high-speed centrifuge at 4 °C at 15,000 r min-1, and the supernatant was aspirated. quality control (QC) samples were obtained by mixing 18 equal volumes of samples, and after every 10 samples, a mixture of all samples would be injected for quality control. All samples were run on a Vanquish horizon UHPLC system (Thermo Fisher Scientific, USA) and a Q-Exactive Plus system (Thermo-Fisher Scientific, USA). Samples were injected into Hypersil Gold (2.1 × 100 mm, 1.9 μm) and eluted with acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid in a gradient at a flow rate of 0.30 mL min-1 for 19 min. Evaporator and capillary temperatures were set to 350 °C and 320 °C, respectively, ms/ms spectra of metabolites in *m/z* were scanned in the range of 70–1050.

The raw data obtained from the mass spectra were imported into Compound Discoverer 3.1 software (Thermo Fisher Scientific) for pre-processing such as missing value filling, normalization and background deduction. The data were imported into SIMCA-P 14.1 (Umetrics, Umea, Sweden) software for unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminate analysis (OPLS-DA). Then the validation of the permutation test was performed to determine the reliability of the model.

Differential metabolites were screened by FC $\geq$ 1.2 or FC $\leq$ 0.6, $p < 0.05$ and VIP >1. The data were imported into SPSS 22.0 software for statistical analysis to test whether they conformed to normal distribution, and data conforming to normal distribution were subjected to independent sample *t*-test to obtain P values, and VIP values were obtained by SIMCA-P 14.1 (Umetrics. Umea, Sweden) software was calculated to obtain. The screened differential metabolites were matched with the Human Metabolome Database (HMDB) to identify the differential metabolites. After that, the screened differential metabolites were imported into the MetaboAnalyst database for pathway enrichment analysis.

2.7. 16S rRNA gene sequencing analysis

The cecum contents obtained after the execution of ICR mice were transferred into sterile centrifuge tubes, rapidly stored in liquid nitrogen, and then transferred to −80 °C for storage.

Genomic DNA was extracted by the CTAB or SDS method, and the purity and concentration of DNA were checked by agarose gel electrophoresis.Using the diluted genomic DNA as a template, PCR was performed using specific primers with Barcode, Phusion® High-Fidelity PCR Master Mix with GC Buffer from New England Biolabs, high-performance and high-fidelity enzymes according to the selection of sequencing regions to ensure amplification efficiency and accuracy. The primers correspond to primers corresponding to regions. 16S V4 region primers (515F and 806R). identification of bacterial diversity; 18S V4 region primers (528F and 706R). identification of eukaryotic microbial diversity; ITS1 region primers (ITS5-1737F and ITS2-2043R). identification of fungal diversity; In addition, the amplified regions include. 16S V3–V4/16S V4–V5/16SV5–V7; Archaea 16S V4–V5/Archaea 16S V8; 18S V9 and ITS2 region.

The PCR products were detected by electrophoresis using a 2% agarose gel; the PCR products that passed the test were purified by magnetic beads, quantified by enzyme labeling, mixed in equal amounts according to the concentration of PCR products, and then detected by electrophoresis using a 2% agarose gel, and the target bands were recovered using the gel recovery kit provided by Qiagen.

Libraries were constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit. After the libraries were quantified by Qubit and Q-PCR, the libraries were qualified and sequenced using NovaSeq6000.

2.8. Determination of short-chain fatty acids (SCFA) in cecal contents

200 mg of cecum contents and 1 ml of ultrapure water were mixed thoroughly and then centrifuged (ALLEGRA-64R, Beckman Coulter, Inc., USA) at 1000 rpm for 10 min to obtain the supernatant, which was then filtered and 7 μL of an extract of 50% sulfuric acid solution and 1 ml of ethyl ether solution were added, and the extracted ethyl ether layer was placed in a sample vial and assayed by the GC system.

GC analyses were performed using an Agilent gas chromatograph (Agilent 7980A, Agilent Technologies, Santa Clara, USA) with a flame ionization detector (FID) equipped with an InertCap WAX GC column (30 m × 0.53 mm i.d. film thickness 1 μm, Agilent) and high-purity nitrogen as carrier gas. And a non-shunt injection was used with an injection volume of 2 μL.The initial column temperature was maintained at 100 °C for 1 min, and then ramped up to 150 °C at a rate of 5 °C for 7 min.The FID detector was maintained at 280 °C.The content of SCFA was determined using an external standard method.

2.9. Correlation profiling between the metabolites and intestinal microbiotas

Spearman correlation analysis ($p < 0.05$) was performed between differential metabolites screened from fecal metabolomics and the top ten gut microbial genera screened from 16S rRNA gene sequencing analysis. Ultimately, a heat map of significantly correlated metabolites and gut microbial genera was obtained ($*p < 0.05$, or $**p < 0.01$). Red colors in the graph indicate positive correlations and blue colors indicate negative correlations.

3. Results

3.1. Effect of QJSB on leukopenic mice

As can be seen in Fig. 1A. After three days of modeling, the number of leukocytes in the model group was significantly ($p < 0.001$) decreased compared with the normal group, proving the success of modeling. Secondly, the leukocyte count (Fig. 1B) and neutrophil count (Fig. 1C) of each group on day 18 indicated that QJSB could significantly ($p < 0.001$) increase the leukocyte and neutropenia induced by cyclophosphamide ($p < 0.001$). Histopathological analysis (Fig. 1D) showed that the colonic tissue of mice in the model group had severe intestinal mucosal injury, and manifested obvious cellular infiltration and edema. In contrast, QJSB group showed improvement in colon pathophysiology to some extent.

3.2. Effect of QJSB on colonic tissue cytokine concentrations

As shown in Fig. 2(A-C), QJSB markedly increased IL-2 ($p < 0.05$), IL-6 ($p < 0.05$) and G-CSF levels; especially the level of G-CSF was similar to that of the normal group.

3.3. Metabolic regulation of QJSB on leukopenia mice

In order to assess the distribution of intestinal tissue samples, principal component analysis (PCA) was performed in Fig. 3A. The results revealed that the Normal, Model, and QJSB groups could be clearly separated. In the absence of molecular selection, the metabolomic data of mice in the QJSB group and model group were well analyzed, indicating that QJSB has a significant modulatory effect on the intestinal metabolic abnormalities of leukopenia mice. As shown in Fig. 3B, the reliability of the model was identified using supervised model OPLS-DA analysis to determine the degree of fit and reliability of the model from the 200 alignment tests of OPLS-DA. It can be seen from Fig. 3C that the regression curve of Q2 intersects negatively with the vertical axis, which proves that the model is reliable and suitable for predicting metabolites without overfitting.

In Fig. 4A, a multivariate statistical analysis of feces from QJSB-treated leukopenic mice identified 27 differential endogenous metabolites (VIP >1, $P < 0.05$)(see Table 1) (including pantothenic acid, ursolic acid, diethyl phosphate, taurine, thymidine, thymidine, cholic acid, adenine, ursolic acid, N-Acetylmuramic acid, L-aspartate, serine, valylproline, N,N-dimethylglycine,

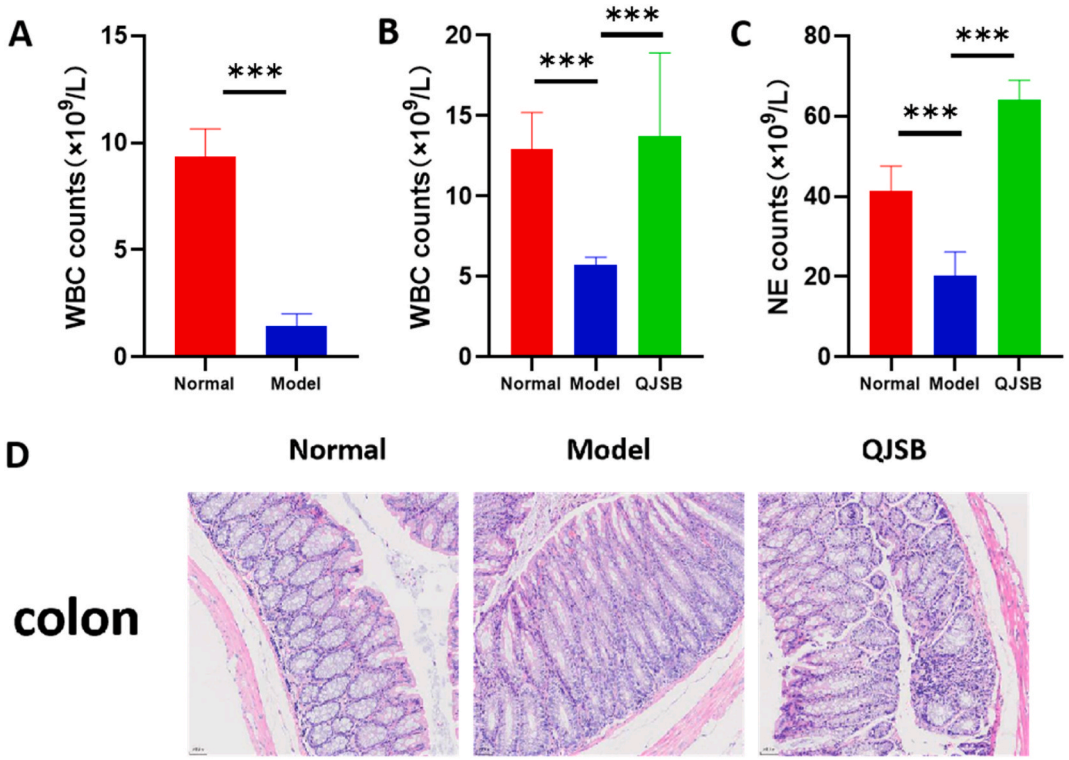

Fig. 1. Comprehensive evaluation index of the therapeutic effect of QJSB on leukopenic mice (n = 6). (A) Leukocyte counts in normal and model groups (including the QJSB group) after 3 days of modeling. (B) Leukocyte counts among groups on day 18 of the experiment. (C) Neutrophil counts among groups on day 18 of the experiment. ***$P < 0.001$ compared with the model group. (D) Pathological changes of colonic tissue in QJSB-induced leukopenia mice. (H&E staining, original magnification × 50).

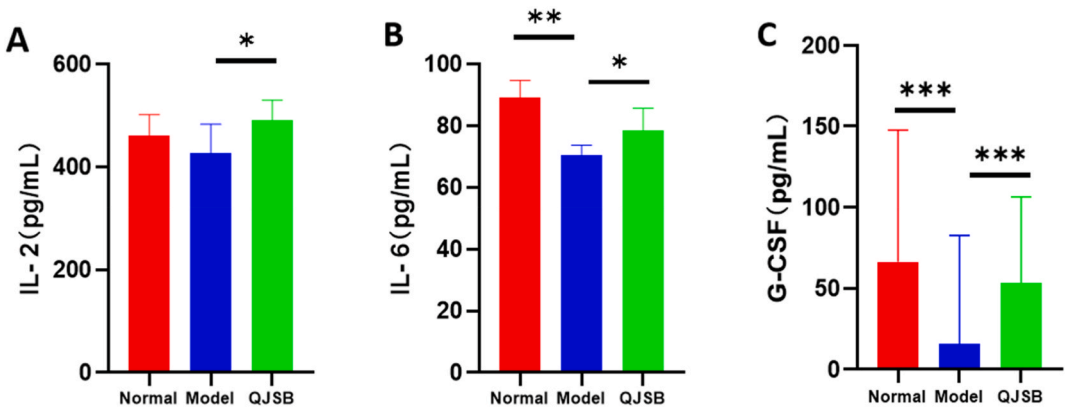

Fig. 2. Effect of QJSB on cytokines in leukopenic mice (n = 8). (A) Levels of IL-2 in each group (B) Levels of IL-6 in each group (C) Levels of G-CSF in each group. Compared with the model group, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

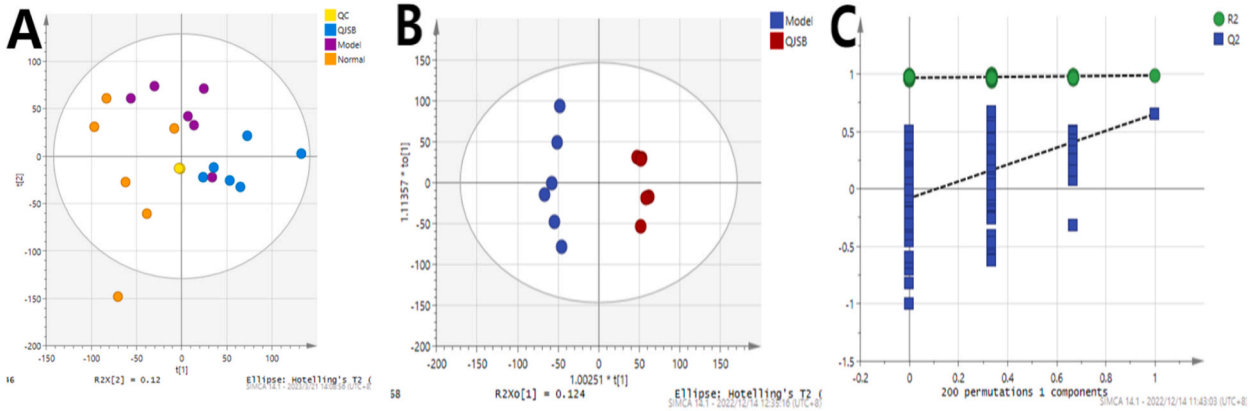

Fig. 3. Fecal metabolomic indexes of QJSB on leukopenic mice (n = 6). (A) PCA plots of Normal, Model and QJSB groups (B) OPLS-DA score plots of Model and QJSB groups (C) Displacement test of Model and QJSB groups.

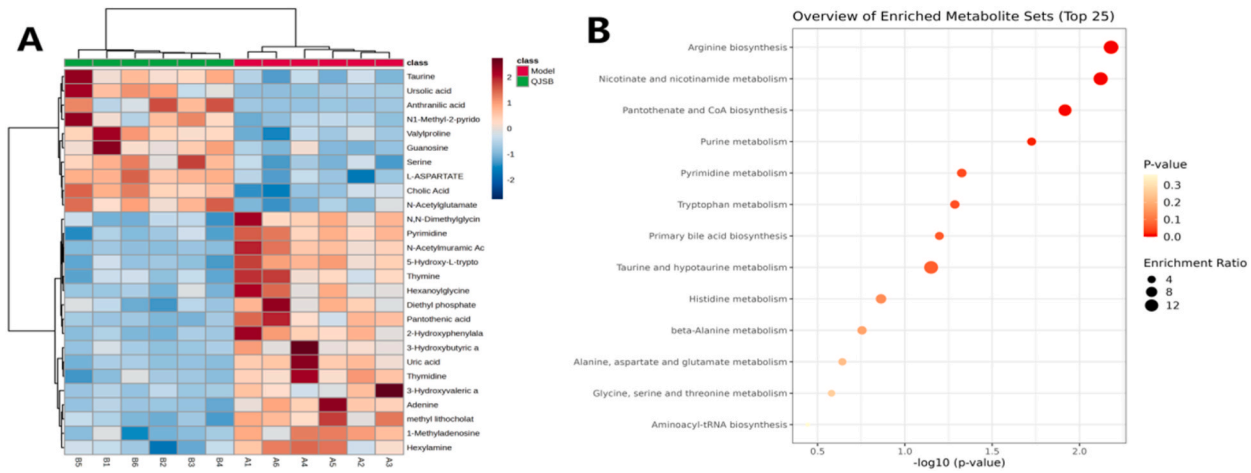

Fig. 4. Metabolomic indices of QJSB on leukopenic mice (n = 6). (A) Heat map of differential metabolites in the model and QJSB groups (B) Correlation pathways of differential metabolite regulation.

Table 1
The different metabolites of mice in the QJSB group compared with the Model group.

Metabolites	Formula	*m/z*	Retention Time (min)	VIP	*p* Value	Change Trend
Pantothenic acid	$C_9 H_{17} N O_5$	219.11081	5.342	2.07804	0.006	↓
Uric acid	$C_5 H_4 N_4 O_3$	168.02749	1.087	2.05627	0.020	↓
Diethyl phosphate	$C_4 H_{11} O_4 P$	154.03926	8.019	2.0074	0.022	↓
Taurine	$C_2 H_7 N O_3 S$	125.01348	0.973	1.99693	0.048	↑
Thymidine	$C_{10} H_{14} N_2 O_5$	242.09015	4.309	1.97952	0.040	↓
Thymine	$C_5 H_6 N_2 O_2$	126.04312	1.695	1.92674	0.007	↓
Cholic Acid	$C_{24} H_{40} O_5$	408.28705	11.495	1.92026	0.037	↑
Adenine	$C_5 H_5 N_5$	135.05466	1.836	1.91303	0.001	↓
Ursolic acid	$C_{30} H_{48} O_3$	456.35997	13.213	1.90249	0.039	↑
N-Acetylmuramic Acid	$C_{11} H_{19} N O_8$	293.11104	1.520	1.86029	0.000	↓
L-ASPARTATE	$C_4 H_7 N O_4$	133.03635	1.119	1.84362	0.009	↑
Serine	$C_3 H_7 N O_3$	105.04278	1.010	1.76337	0.032	↑
Valylproline	$C_{10} H_{18} N_2 O_3$	214.13174	1.751	1.73148	0.016	↑
N,N-Dimethylglycine	$C_4 H_9 N O_2$	103.06389	1.032	1.71647	0.002	↓
Pyrimidine	$C_4 H_4 N_2$	80.03781	1.072	1.71009	0.032	↓
3-Hydroxyvaleric acid	$C_5 H_{10} O_3$	118.06196	5.519	1.56777	0.034	↓
Anthranilic acid	$C_7 H_7 N O_2$	137.04772	4.054	1.55148	0.002	↑
2-Hydroxyphenylalanine	$C_9 H_{11} N O_3$	181.07396	7.464	1.51288	0.029	↓
N-Acetylglutamate	$C_7 H_{11} N O_5$	163.195	1.712	1.45374	0.031	↑
Hexanoylglycine	$C_8 H_{15} N O_3$	173.10553	5.083	1.40737	0.006	↓
3-Hydroxybutyric acid	$C_4 H_8 O_3$	104.04614	2.245	1.33664	0.019	↓
1-Methyladenosine	$C_{11} H_{15} N_5 O_4$	281.11125	1.061	1.15622	0.045	↓
5-Hydroxy-DL-tryptophan	$C_{11} H_{12} N_2 O_3$	220.08486	1.592	1.14375	0.048	↓
methyl lithocholate	$C_{25} H_{42} O_3$	390.31297	15.669	1.12551	0.003	↓
Hexylamine	$C_6 H_{15} N$	101.12074	1.070	1.07525	0.013	↓
N1-Methyl-2-pyridone-5-carboxamide	$C_7 H_8 N_2 O_2$	152.05857	1.424	1.01508	0.042	↑
Guanosine	$C_{10} H_{13} N_5 O_5$	283.09155	2.036	1.01114	0.019	↑

pyrimidine, thymidine, 3-Hydroxyvaleric acid, Anthranilic acid, 2-hydroxyphenylalanine, N-Acetylglutamate, hexanoylglycine, 3-hydroxybutyric acid, 1-methyladenosine, 5-hydroxy-DL-tryptophan, methyl cholate, hexylamine, N1-methyl-2-pyridone-5-carboxamide, guanosine). Ten of these metabolites (taurine, cholic acid, ursolic acid, L-aspartate, serine, valylproline, anthranilic acid, N-acetylglutamate, N1-methyl-2-pyridone-5-carboxamide, and guanosine) were upregulated, and 17 metabolites other than these were downregulated. These metabolites mediate six major metabolic pathways (Fig. 4B), namely arginine biosynthesis, niacotinate and nicotinamide metabolism, pantothenate and CoA biosynthesis, purine metabolism, pyrimidine metabolism, and tryptophan metabolism.

3.4. 16S rRNA detection

3.4.1. Alpha and Beta diversity analysis

As shown in the NMDS (Non-metric Multidimensional scaling) plot of Fig. 5A, the separation of gut microbial composition curves

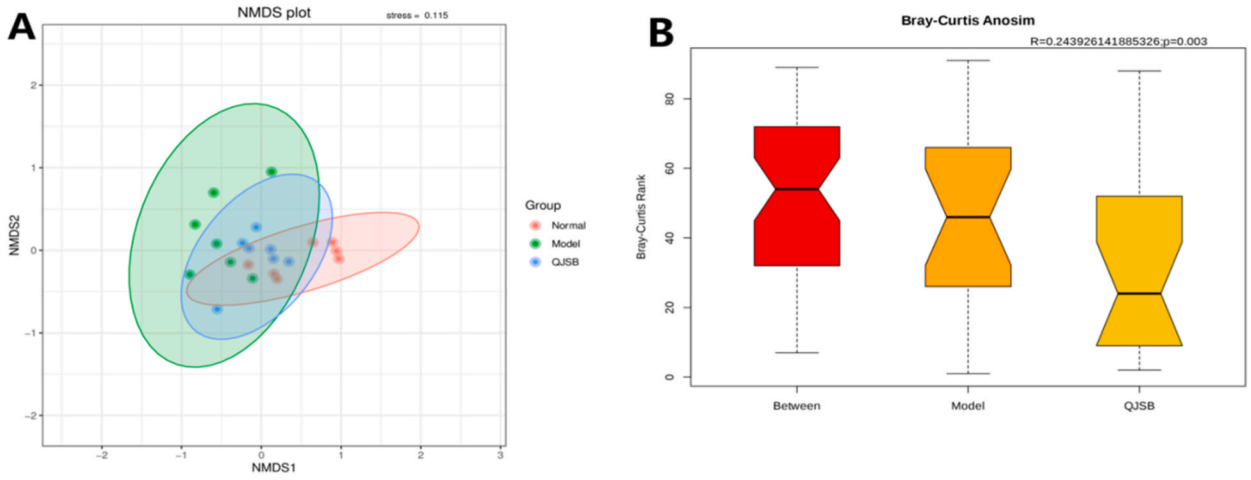

Fig. 5. QJSB for 16S rRNA-related indicators in leukopenic mice (n = 7). (A) NMDS plot of the Model and QJSB groups. (B) Bray-Curtis Anosim plot of the Model and QJSB groups.

between the Normal, Model and QJSB groups is obvious, indicating that there is a difference between the groups, and the QJSB group is between the Normal and Model groups, indicating that QJSB has a certain regression on the gut microbes of mice suffering from leukopenia, proving that it has a certain therapeutic effect on leukopenic mice. And from the Bray-curtis Anosim plot (Fig. 5B), it can be seen that the longitudinal coordinates of the notch in the first box are higher than the others, indicating that the difference between the samples between groups is greater than the difference between the samples within groups ($R > 0$).

Fig. 6(A-D) shows the Ace, Chao1, Shannon, and Observed_otus indices, each of which improved compared to the Model group and more closely resembled the Normal group. This demonstrates that the administration of QJSB can regulate the disorder of intestinal microbiota caused by cyclophosphamide and make the composition and abundance of the community more similar to those of the Normal group.

3.4.2. Analysis of gut microbiota at phylum level and genus level

Fig. 7A represents the results of species annotation analysis at the phylum level for the three groups revealed by 16S rRNA sequencing. The graph shows the top ten bacteria in terms of abundance at the phylum levels, with the remaining species combined as "other" and the unclassified species representing species that are not taxonomically annotated.

At the phylum level, the mouse intestinal microorganisms were mainly composed of *Bacteroidetes*, *Fimicutes*, *Proteobacteria*, *Verrucomicrobiota* and Actinobacteria. Compared with the Model group, the QJSB group showed an increase in *Fimicutes* and *Proteobacteria*, and a decrease in *Bacteroidetes*, *Verrucomicrobiota*, and *Actinobacteria*. Fig. 7B is a graph of UPGMA cluster analysis at the phylum level showing a smaller OUT between the normal and QJSB groups, which suggests that the differences between the two groups are relatively small, and presumably QJSB can move the gut microbiota of the diseased mice in a healthy direction.

At the genus level, QJSB mainly reversed *Bacteroides*, *Alloprevotella*, *Lactobacillus*, *Alistipes*, *unidentified_Enterobacteriaceae* and *Staphylococcus* which are genera, and the results are shown in Fig. 8(A-F). Among them, *Lactobacillus*, *Alistipes*, and *Alloprevotella* were upregulated, and *Bacteroides*, *Staphylococcus*, and *unidentified_Enterobacteriaceae* were downregulated.

3.4.3. Effect of QJSB on the SCFAs in the cecum content

To investigate the effects of QJSB on the mouse intestine, the concentrations of acetic, propionic, butyric and valeric acids in the contents of the cecum were determined (Fig. 9). QJSB significantly increased the concentrations of acetic, butyric and valeric acids compared to the Model group ($p < 0.01$), but there was no significant difference in the concentration of propionic acid. These data suggest that QJSB can affect the production of SCFAs in mice.

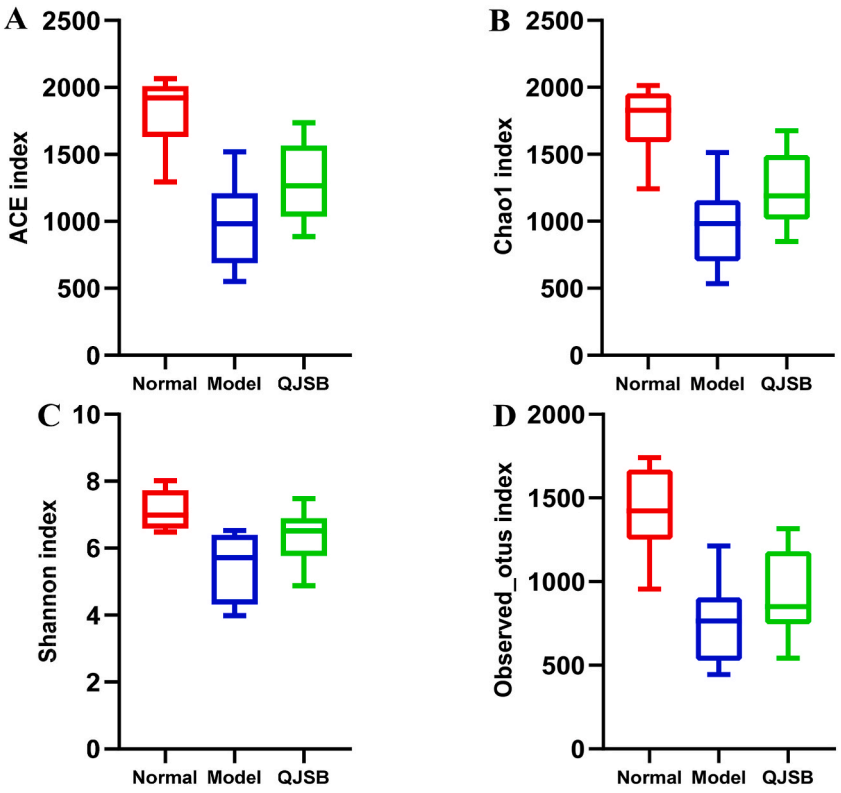

Fig. 6. QJSB for 16S rRNA-related indicators in leukopenic mice (n = 7). (A) ACE index (B) Chao1 index (C) Shannon index (D) Observed index.

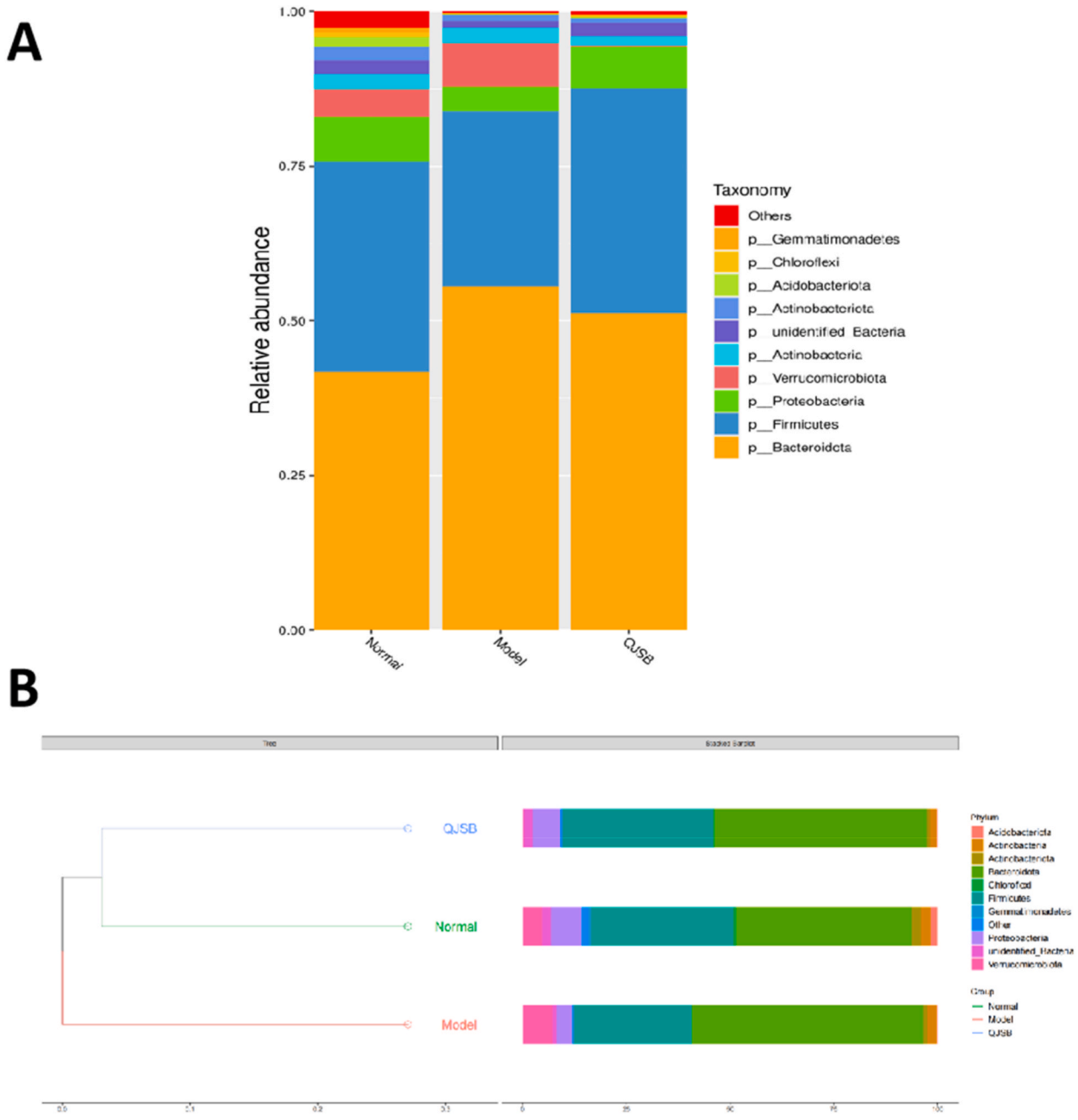

Fig. 7. QJSB for 16S rRNA-related indicators in leukopenic mice (n = 7). (A) Top ten colonies at the phylum level. (B) UPGMA cluster analysis plot at the phylum level.

3.4.4. Correlation analysis between metabolites and gut microbiota

The results in Fig. 10 show that serine, anthranilic acid, taurine, valylproline, ursolic acid and cholic acid were significantly positively correlated ($p < 0.05$) with *unidentified-clostridia*. Serine was also significantly positively correlated ($p < 0.05$) with *Alistipes*; guanosine was significantly positively correlated ($p < 0.05$) with *Parasutterella*, N1-methyl-2-pyridone-5-carboxamide was significantly positively correlated ($p < 0.01$) with *Alistipes*; Anthranilic acid was also positively correlated ($p < 0.01$) with *Alistipes*, *Alloprevotella* ($p < 0.05$); N-Acetylglutamate was significantly and positively correlated with *Alistipes* and *Lactobacillus* ($p < 0.05$). 11 differential metabolites (taurine, cholic acid, ursolic acid, L-aspartate, serine, valylproline, anthranilic acid, N-Acetylglutamate, N1-methyl-2-pyridone-5-carboxamide, guanosine) were all negatively correlated with *unidentified_Enterobacteriaceae*.

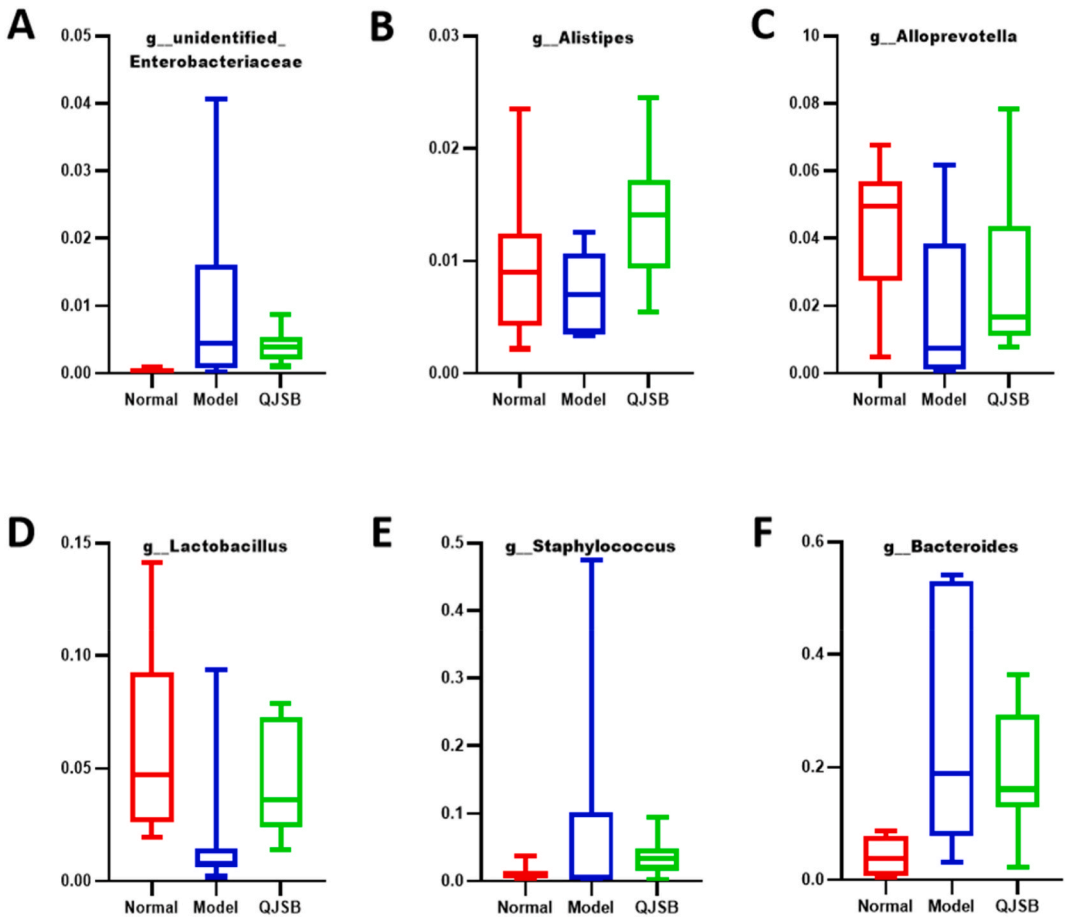

Fig. 8. QJSB for 16S rRNA-related indicators in leukopenic mice (n = 7). The genus-level bacterial flora of the reversal effect.

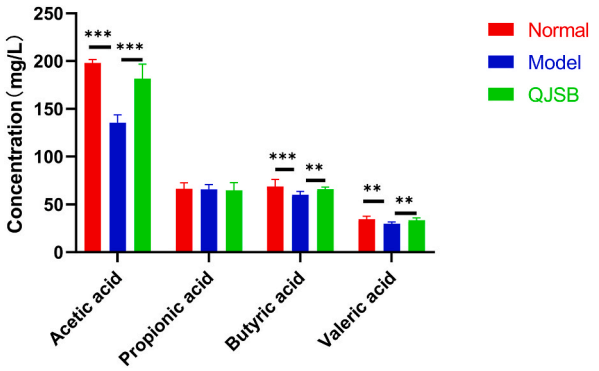

Fig. 9. SCFAs concentration in cecum contents. $^{***}P < 0.001$, $^{**}P < 0.01$.

4. Discussion

Human hematopoietic function is regulated by cytokines, such as stem cell factor (SCF), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and erythropoietin (EPO), which have positive regulatory effects [20]. Among them, IL-2 can regulate hematopoiesis by promoting the secretion of positive regulatory factors that promote hematopoiesis by NK cells and lymphocytes, and can play a key role in immune tolerance by affecting regulatory T (Treg) cell function and survival [21–23]. IL-6 can regulate hematopoiesis in both directions. Cyclophosphamide induces bone marrow suppression. Bone marrow hematopoietic disorders such as cyclophosphamide and radiation-induced myelosuppression cause the

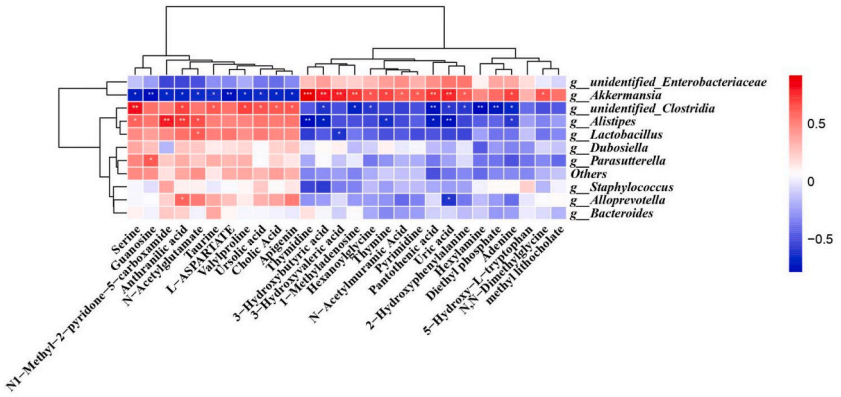

Fig. 10. Correlation analysis of metabolites and gut microbiota. Red represents positive correlation; blue represents negative correlation. And the darker color means the more significant the correlation. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

body to compensate for IL-6 secretion to maintain hematopoietic homeostasis, promote the growth of B cells and immunoglobulin expression, effectively enhance immunity, reduce intestinal injury and improve intestinal barrier function [24,25]. G-CSF, also known as colony-stimulating factor 3 (Csf3), is the main hematopoietic growth factor involved in the control of neutrophil development. G-CSF can directly affect the number of neutrophils. Neutrophils are an important component of the defense system. A decrease in neutrophils leads to a decreased ability to fight non-specific infections, which increases the risk of serious infections. There is growing evidence that G-CSF also plays an important regulatory role in hematopoietic and natural immune responses [26].

The side effects of chemotherapy as an effective treatment for cancer are immunosuppression and myelosuppression, which lead to a decrease in leukocyte count and function. Currently, the following methods are commonly used clinically to increase the number of leukocytes, including component blood leukocyte transfusion; oral western drugs, such as leucine, inosine and vitamin B_4 and granulocyte colony-stimulating factor (G-CSF), but these measures have no significant enhancement on leukocyte function [27]. In this study, QJSB increased the levels of IL-2, IL-6, and G-CSF in the intestinal tract, which enhanced hematopoiesis and increased the number of neutrophils, further playing an important role in the treatment of leukopenia. A mixture of the most potent amino acids including aspartate, leucine and glycine have been reported to specifically increase the concentration of the cytokine IL-2 [28]. L-aspartate was upregulated in the QJSB group in this study, presumably contributing positively to IL-2 expression.

Immunodeficiency is usually associated with abnormal amino acid metabolism. At the same time, the development of leukopenia is also correlated with amino acid metabolism mediated by intestinal microorganisms [29]. In this study, we analyzed the metabolomics of the feces of mice with leukopenia [30,31]. The results show that amino acids are one of the main metabolites in the intestine of mice with leukopenia, accounting for more than 30% of the differential metabolites, including taurine, L-aspartate, serine, valylproline, N, N-dimethylglycine, 2-hydroxyphenylalanine, N-acetylglutamate and hexanoylglycine. Taurine, serine and valylproline were significantly upregulated after taking QJSB ($P < 0.05$). Taurine is a β-amino acid that not only reduces inflammation and enhances immunity but also treats leukopenia. It can enhance intestinal epithelial tight junctions in mice for the treatment of intestinal mucositis induced by chemotherapeutic agents such as cyclophosphamide. It also regulates the stability of the internal environment and enhances immunity in the intestinal tract of immunosuppressed mice [32]. Taurine deficiency impairs the growth of the body and triggers immune deficiency. It can promote the recovery of hematopoietic function to a certain extent and has a direct therapeutic effect on leukopenia [33,34]. Taurine not only enhances the tumor-suppressive effect of cyclophosphamide, but also increases the number of leukocytes and leukocyte function after cyclophosphamide chemotherapy, and reduces the side effects of cyclophosphamide such as myelosuppression and immunosuppression [27]. And in the pharmacodynamic and metabolomic results, QJSB significantly increased the number of leukocytes including neutrophils in peripheral blood ($P < 0.001$), and the taurine content in the feces of mice in the QJSB group was significantly increased ($P < 0.05$), which proved that it could improve the number and function of leukocytes and hematopoietic function in mice after cyclophosphamide chemotherapy, reduce the effect of cyclophosphamide on immune function, restore intestinal mucosal damage and maintain membrane stability, and it was hypothesized that QJSB could play a leukocyte-raising role through taurine. Valylproline is a branched-chain amino acid (BCAA) that is equally important in the regulation of energy homeostasis, nutrient metabolism, intestinal health, immunity and disease in the body. Reported to be produced by the fermentation of *Lactobacillus* [35,36]. QJSB metabolizes valylproline in the intestine, thereby replenishing intestinal nutrition, maintaining intestinal health, and enhancing the immune system. In summary, it is hypothesized that QJSB administration can mediate the associated amino acid metabolism, which is consistent with our findings and confirms that QJSB administration can improve amino acid metabolism disorders in leukopenic mice, thus providing a therapeutic effect on the leukopenic. Amino acid utilization is a determinant factor affecting the distribution of intestinal metabolites. These findings may provide new insights into microbe-gut interactions and provide guidance for gut health.

In addition to amino acid modulation, this study found that some other metabolites play a crucial role in immune regulation, including cholic acid and ursolic acid. Cholic acids (CA) are bile acids that directly or indirectly regulate the microbial composition of the gut and maintain intestinal homeostasis by activating innate immune genes in the small intestine [37]. In this study, cholic acid was

significantly upregulated in the QJSB group ($P < 0.05$). Therefore, cholic acids are also one of the key metabolites that regulate the immune system in leukopenic mice. Ursolic acid (UA) is a natural pentacyclic triterpenoid [38], with multiple intracellular and extracellular targets that play beneficial roles in apoptosis, metastasis, angiogenesis and inflammatory processes [39]. In recent years, several studies have shown that UA can reduce the risk of pathological intestinal injury, alleviate intestinal dysfunction, and restore intestinal barrier function. Its beneficial effects on intestinal injury and diseases, including inflammatory bowel disease (IBD) and colorectal cancer (CRC) [40]. UA can also increase the abundance of beneficial bacteria in the intestine and inhibit the growth of harmful bacteria. It inhibits intestinal inflammation by promoting intestinal nutrient absorption and tight junction protein expression [41]. Combined with the experimental results, it is inferred that QJSB acts against apoptosis and intestinal inflammation and regulates intestinal microflora through metabolizing ursolic acid, thus showing some improvement in leukopenic mice. In summary, after administration of QJSB, leukopenic mice can be treated with bile acids and ursolic acid in the intestine together with immune enhancing effects.

The metabolic pathways mediated by QJSB include arginine biosynthesis, nicotinate and nicotinamide metabolism, pantothenate and CoA biosynthesis, purine metabolism, pyrimidine metabolism, and tryptophan metabolism. Equus asinus L. is rich in amino acids, including aspartic acid, glutamate and serine. In this study, the metabolites L-aspartic acid and N-acetylglutamate were abundant and jointly participated in the biosynthesis of arginine. Arginine, in combination with other substances in the body, enhances the immune system and regulates IL-2 gene expression through methylation [42]. Moreover, L-arginine supplementation can alleviate the side effects of thrombocytopenia and leukopenia and enhance IL-2 immunotherapy [43–46]. Thus, QJSB may enhance the immune system and hematopoietic function through the components of Equus asinus L. by regulating the metabolic pathway of arginine. It has a therapeutic effect on mice suffering from leukopenia. N1-methyl-2-pyridone-5-carboxamide is a downstream metabolite in the tryptophan metabolic pathway. Tryptophan is an essential amino acid in dietary proteins, that can be metabolized into different metabolites in intestinal microbiota and tissue cells, and its metabolites play a key role in maintaining intestinal environmental homeostasis and systemic immunity, and may also influence the development and progression of diseases such as inflammatory bowel disease, tumors, obesity and metabolic syndrome and cardiovascular disease, and liver fibrosis [47]. These findings have important implications for the immunotherapy of tumors and other immune-related diseases [48]. In this study, anthranilic acid, which is also a metabolite of tryptophan, was found at significantly higher levels compared to the Model group [49]. It is hypothesized that QJSB may improve the disturbance of the intestinal environment as well as the treatment of diseases through tryptophan metabolism [50].

In terms of intestinal microbiota, the results of this study found that compared to the Model group mice, the diversity, richness and uniformity of the intestinal microbiota in the QJSB group mice compared to the Model group mice. The abundance of bacteria also differed at the phylum and genus level, followed by the Firmicutes. Firmicutes and Bacteroidota are the two major bacteria in the intestinal microbiota and play an important role in maintaining intestinal homeostasis [51]. The results also revealed that mice in the Model group had a reduced proportion of the Bacteroidota compared to the Normal group. The change in the Bacteroidota and the Firmicutes ratio is considered to be the first sign of intestinal microecological dysregulation. Reduced Firmicutes ratios have also been observed in some chronic autoimmune diseases, such as systemic lupus erythematosus [52]. In the present study, it is hypothesized that QJSB may improve the dysregulation of intestinal microecology to ameliorate leukopenia by regulating the ratio of the Firmicutes and the Bacteroidota. At the genus level, QJSB reverses six major intestinal microorganisms, including the beneficial *Bacteroides*, *Alloprevotella*, *Lactobacillus*, *Alistipes* and the pathogenic *unidentified_Enterobacteriaceae* and *Staphylococcus*. *Bacteroides* is one of the major lineages of bacteria that emerged early in evolution. A recent study showed that the relationship between *Bacteroides* and human hosts is a reciprocal symbiotic process [53] and that the *Bacteroides* are considered beneficial only at the appropriate abundance. The results of this study showed that QJSB could effectively regulate cyclophosphamide-induced disorders of *Bacteroides* and restore normal levels. Thus, it has a positive effect on the intestinal tract. *Alloprevotella* is generally considered to be a bacterium associated with a healthy plant-based diet and acts as a "probiotic" in the human intestine. A positive correlation between L-serine and *Alloprevotella* levels has been reported [54–56]. This microorganism is protective of human health by shaping immune development, immune response and metabolism through the pathways of invading pathogens [57]. Alloprevotella is also recognized as a producer of short-chain fatty acids [58]. *Lactobacillus* make up a relatively small proportion of the intestinal microbiota, but due to their widespread use in intestinal health and disease, they maintain the stability of the intestinal microbiome by producing a variety of tryptophanolytic metabolites that reduce intestinal permeability to glycolytic probiotics [59,60]. Most of them are non-pathogenic and can produce antimicrobial substances, such as hydrogen peroxide and bacteriocins. The probiotic *Lactobacillus* rhamnosus GR-1 has been shown to induce G-CSF production by macrophages [61]. And IL-6 deficiency decreases *Lactobacillus* [62]. Since the early 20th century, it is believed that *Lactobacillus* can compete with pathogenic bacteria, including Streptococcus, Escherichia coli, and Staphylococcus aureus, to play a key inhibitory role and be beneficial to human health [63]. It was also reported that taurine could significantly modulate the intestinal flora and reverse the decrease in the abundance of *Lactobacillus* [64], it was speculated that *Lactobacillus* had a facilitating effect on the increase of taurine content in the intestine. *Lactobacillus* is a potential probiotic and a potent producer of acetic and butyric acids [65]. This coincides with our findings that the abundance of *Enterobacteriaceae* as well as *Staphylococcus* in the QJSB group was significantly lower than in the Model group, confirming the competitive effect of *Lactobacillus* against pathogenic bacteria and the reliability of the experimental results, and it is hypothesized to increase colonic G-CSF and IL-6 levels, and it has a promoting effect on the increase of both acetic and butyric acid. *Alistipes* belongs to a gram-negative bacterium of the phylum Mycobacterium, which is a relatively new genus of bacteria, mainly isolated from medical and clinical samples. Due to the diversity of microbiota in the gastrointestinal tract, there is a strong correlation between dysbiosis and inflammatory bowel disease (IBD). *Alistipes* may be protective against certain diseases, including liver fibrosis, cancer immunotherapy, and cardiovascular disease [66]. From an ecological point of view, *Alistipes* is mainly found in the intestines of healthy individuals [67,68]. And it's also a butyric and valeric acid-producing bacterium [69]. The association between gut microbiota and short-chain fatty acids is closely related.

Therefore, the present study determined the content of SCFAs in the cecum of mice. The results showed that QJSB up-regulated the content of acetic, butyric and valeric acids in the contents of the mouse cecum. SCFAs can have an impact on the integrity of the human intestinal epithelial and mucosal barriers, the immune response, and the diversity of the microbiota [70]. Acetic acid promotes Treg cell differentiation, which stimulates FOXP3 transcription and also positively influences the enhancement of mucosal immunity [71]. Butyrate supports the integrity of the intestinal epithelial barrier by regulating the expression of tight junction proteins and supporting the production of intestinal mucus. It has been shown that butyrate aids intestinal motility by acting as a ligand and activator of the SCFA receptor. The mucosal barrier monolayer of intestinal epithelial cells and their adjacent mucosal layers are the first line of defense for host intestinal immunity, and butyrate has a direct beneficial role in supporting intestinal epithelial barrier integrity. Butyrate supports the integrity of the intestinal epithelial barrier by regulating the expression of tight junction proteins and supporting the production of intestinal mucus. It has been shown that butyrate aids intestinal motility by acting as a ligand and activator of the SCFA receptor. The mucosal barrier monolayer of intestinal epithelial cells and their adjacent mucosal layers are the first line of defense for host intestinal immunity, and butyrate has a direct beneficial role in supporting intestinal epithelial barrier integrity [72]. QJSB modifies the composition of the intestinal flora, increases the levels of SCFA-producing bacteria, and influences the levels of acetic, valeric, and butyric acids, thereby modulating the homeostasis of the intestinal immune system.

When the immune function of the body decreases, it can lead to the proliferation of *E. coli*. bacteria, causing damage to the mucosa of the gastrointestinal tract and inducing an inflammatory response in the intestinal mucosa. Studies have shown that L-serine has a competitive relationship with *Enterobacteriaceae* in the intestinal tract [73,74]. The results of the 16S rRNA genus level showed that the *Enterobacteriaceae* content was significantly increased in the Model group, and the *Enterobacteriaceae* content returned to normal level after taking QJSB. It is possible that through the competitive relationship of L-serine to *Enterobacteriaceae*, the decrease in *Enterobacteriaceae* abundance played a role in repairing the damaged intestinal mucosa. From the correlation results of this study, taurine, cholic acid, ursolic acid, L-aspartate, serine, valylproline, anthranilic acid, N-acetylglutamate, N1-methyl-2-pyridone-5-carboxamide and guanosine) were all negatively correlated with *g_unidentified_Enterobacteriaceae*. It is hypothesized that the administration of QJSB may be followed by a synergistic effect of related intestinal metabolites to jointly fight against pathogenic bacteria and thus maintain intestinal health. Thus, regulation of gut microbes is also essential for the treatment of leukopenia.

QJSB increased the dominant flora in the intestine of leukopenic mice (*Bacteroides*, *Alloprevotella*, *Lactobacillus*, *Alistipes*) while decreasing the associated pathogenic bacteria (*unidentified_Enterobacteriaceae*, *Staphylococcus*). It was confirmed that QJSB is a pathway to treat diseases by regulating the dominant intestinal flora, reducing pathogenic bacteria, and moving the organism in a more beneficial direction.

5. Conclusion

In conclusion, cyclophosphamide-induced leukopenia is triggered by its impairment of the immune system and hematopoietic

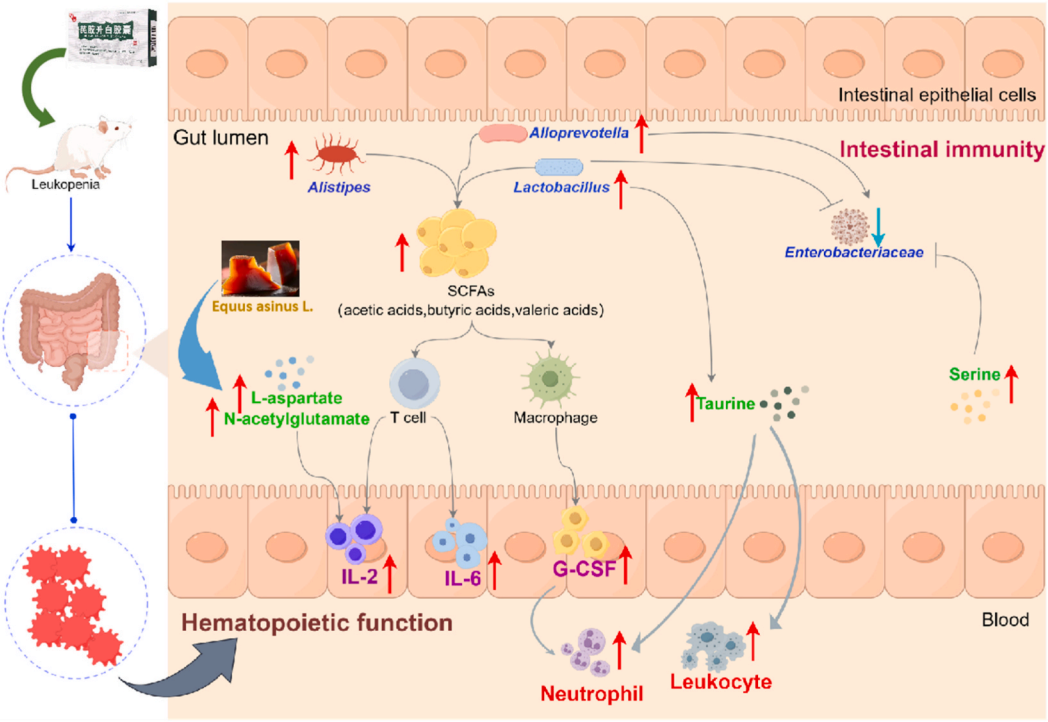

Fig. 11. Possible pathways for QJSB in the treatment of leukopenia.

function, and this study confirms the significant efficacy of QJSB in the treatment of leukopenia. As shown in Fig. 11, administration of QJSB can improve hematopoietic function and enhance the immune system in leukopenic mice by modulating metabolic response pathways, fecal metabolites, and the intestinal microbiota, providing efficacy in the treatment of leukopenia and improving the intestinal tract. It provides effective support for the clinical use of leukopenia patients. It also lays the foundation for further research on leukopenia and the use of intestinal microbes as drug targets for the treatment of leukopenia.

Author contribution statement

Conceptualization, Y.C. (Yu Cao); methodology, Y.C. (Yu Cao) and S.Z.; software, L.T. and S.J.; validation, L.T., S.J. and Y.C. (Yixuan Chen); formal analysis, S.J., Y.C. (Yixuan Chen); investigation, Y.C. (Yu Cao), S.Z.; data curation, Y.C. (Yu Cao), S.Z.; writing—original draft preparation, Y.C. (Yu Cao); writing—review and editing, Y.C. (Yu Cao), S.Z., L.T. and Y.C. (Yixuan Chen) ; supervision, L.L., X.G.; funding acquisition, X.G. All authors have read and agreed to the published version of the manuscript.

Ethics statement

This study was reviewed and approved by [The Animal Care and Use Committee of Guizhou Medical University], with the approval number: [No. 2303319].

Data availability

Data will be made available on request.

Declaration of competing interest

We declare that we have no conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my coauthors that the work described was original research that has no been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed. There is no conflict of interest with "Guizhou Dechangxiang Pharmaceutical Co., Ltd. (China Limited).

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