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Integration of gut microbiota and metabolomics for the hematopoiesis of Siwu paste on anemia rats

Dan Wan [a,b], Xuejuan Liang [a,b], Limei Yang [c], Dan He [a,b], Qing Du [a,b], Wanping Zhang [c], Jianji Huang [c], Linben Xu [a,b], Ping Cai [a,b], Jianhua Huang [a,b], Yiying Xiong [c], Rongrong Zhou [a,d,**], Yongbo Peng [c,*], Shuihan Zhang [a,b,***]

[a] *Hunan Academy of Chinese Medicine, Hunan University of Chinese Medicine, Changsha, 410013, Hunan, PR China*
[b] *Innovative Medicine Institute of Traditional Chinese Medicine, Institute of Chinese Medicine Resources, Hunan Academy of Chinese Medicine, Changsha, 410013, Hunan, PR China*
[c] *Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing Key Laboratory for Pharmaceutical Metabolism Research, College of Pharmacy, International Medical College, The First Affiliated Hospital, Chongqing Medical University, Yuzhong District, 400016, Chongqing, PR China*
[d] *The Affiliated Hospital, Hunan Academy of Chinese Medicine, Changsha, 410013, Hunan, PR China*

A R T I C L E I N F O

Keywords:
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Firmicutes/bacteroidetes

A B S T R A C T

Background: To investigate the regulation mechanism of hematopoiesis of Siwu paste (SWP) in anemia rats, which is a classic Chinese prescription used for nourishing blood or blood deficiency over 1000 years.

Methods: Blood cell and biochemical analysis were used to evaluate the hematopoietic function of SWP in anemia rats. The intestinal microbial composition was analyzed with 16S rRNA gene sequencing, and the metabolites were profiled using UPLC-TripleTOF system nontargeting metabolomics.

Results: SWP can improve the levels of red blood cells, hemoglobin, platelet, hematocrit value, white blood cells, lymphocyte, EPO, TPO, and GM-CSF in anemia rats, and significantly change the microbial community and its metabolites. The correlation analysis of intestinal microbiota-hematopoietic efficacy shows that 13 kinds of different intestinal flora were related to hematopoietic efficacy, in which *Prevotella_1*, *Prevotella_9*, *Lactobacillus,* and *norank_f_Muribaculaceae* were significantly positively correlated with hematopoiesis, nine kinds of intestinal flora are negatively correlated with hematopoietic effect. Compared with anemia rats, 218 potential metabolic biomarkers and 36 metabolites with significant differences were identified in the SWP treatment group, and the key metabolites were mainly amino acids and lipids. An in-depth analysis of metabolic pathways showed that SWP mainly affected 7 metabolic pathways, including aminobenzoic acid degradation and tryptophan metabolism.

Conclusion: The study provides novel insights into the regulation of hematopoiesis of SWP in anemia rats that were correlated with gut microbiota and the metabolites, which through the restoration of the firmicutes/bacteroidetes ratio.

* Corresponding author.
** Corresponding author. Hunan University of Chinese Medicine & Hunan Academy of Chinese Medicine, Changsha, 410013, Hunan, China.
*** Corresponding author. Hunan University of Chinese Medicine & Hunan Academy of Chinese Medicine, Changsha, 410013, Hunan, China.
E-mail addresses: 515306460@qq.com (R. Zhou), pengyongbo2021@cqmu.edu.cn, pengyongbo2000@126.com (Y. Peng), zhangshuihan@hnucm.edu.cn, zhangshuihan0220@126.com (S. Zhang).

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1. Introduction

Traditional Chinese medicine (TCM), which has long been practiced in the clinic in Asian countries, is an important choice for preventing and treating diseases. And *Siwu* paste (SWP) is a classic nutrition prescription from the "Xian Shou Li Shang Xu Duan Secret Recipe", which is usually used for nourishing blood in blood vacuity syndrome (e.g., anemia) of Chinese Medicine [1]. In Chinese medicine, blood deficiency is usually expressed as insufficiency of "stomach" and "spleen", blood loss, and deficiency of "Qi" and blood. SWP has attracted wide attention because of its good blood-enriching effect in different types of anemia, fracture, and abnormal eumenorrhea, especially for its hematopoietic dysfunction caused by bone marrow suppression and fewer side effect [1]. The main ingredients of SWP are steamy *Dihuang* (the prepared root from *Rehmannia glutinosa* (Gaertn.) DC.; as monarch drug), *Baishao* (the root of *Paeonia lactiflora* Pall.; as ministerial drug), *Danggui* (the root of *Angelica sinensis* (Oliv.) Diels; adjuvant drug) and *Chuanxiong* (the root of *Ligusticum chuanxiong* Hort.; adjuvant drug) with the ratio of 1:1:1:1 from the "Xian Shou Li Shang Xu Duan Secret Recipe" [2]. Many modern pharmacological studies have shown that SWP can reduce liver cells apoptosis, improve mitochondrial function, correct fatty acid β oxidation disorder, maintain cells' osmotic pressure, promote energy metabolism, produce estrogen-like effects, and enhance immunity [3,4]. SWP also can promote hemopoiesis, increase white blood cells, red blood cells, and platelets in peripheral blood, and enrich blood by improving the hematopoietic function of bone marrow [5,6]. However, the possible mechanism of SWP in blood supplements for anemia has not been clear.

Recently, some studies have shown that gut microbiota play an important role in the regulation of organism hematopoiesis, such as anemia (e.g., aplastic anemia (AA)) and neutropenia are closely related to changes in intestinal flora [7]. Staffas et al. found that intestinal flora disorder could reduce the number of white blood cells, lymphocytes, and bone marrow cells in bone marrow transplantation (BMT) mice, which affected cell proliferation and differentiation during the hematopoietic process, and proved that intestinal flora is helpful to hematopoietic reconstruction [8]. The co-housing SPF mice with mice born in the same environment could increase flora diversity, restore hematopoietic gene expression, and promote myeloid cell production and T-cell activation [9]. Guo et al. found that intestinal microbiota could promote hematopoietic production and reduce gastrointestinal injury against radiation [10]. Moreover, microbial metabolites enter into the blood circulation and promote hematopoietic production through a variety of key signals. Lactate, a metabolite of the microbiota, promotes hematopoietic stem cells (HSC) in mesenchymal stromal cells stem cell factors required for proliferation, accelerated hematopoiesis, and erythropoiesis [11]. *E. coli* peptidoglycan (NOD1 ligand) can restore HSC and precursors in bone marrow as well as serum concentrations of IL-7, Flt3L, SCF, and ThPO to the levels displayed by specific pathogen-free control animals [12]. Short-chain fatty acids, butyric acid, can activate the differentiation of erythroid progenitor cells, and butyric prodrugs have successfully treated anemia [13]. Therefore, the integration of microbial diversity and metabolomics can further understand the effect of SWP on hematopoietic anemia.

Hence, to explore the interaction between SWP and gut microbiota in hematopoiesis of anemia, 16S rRNA gene sequencing was used to analyze the gut microbiota of SWP in anemia rats, and non-targeted metabolomics was profiled using the UPLC-TripleTOF system. The results show that SWP could significantly change the composition structure of gut microbiota in anemia rats, such as the restoration of the firmicutes/bacteroidetes ratio (e.g., *norank_f_Muribaculaceae, Lactobacillus* and *Prevotella_9),* which was positively correlated with the metabolites (as tryptophan metabolites). Meanwhile, SWP has also affected their related metabolic phenotype.

In all, based on the changed data of gut microbiota and metabolites of SWP on anemia rats, gut microbiota and metabolites may play a key role in the regulation of anemia by SWP, which helps to reveal the possible enrich blood mechanism for SWP in anemia clinical treatment from the perspective of "intestinal flora-metabolites".

2. Materials and methods

2.1. Experimental materials

Siwu Paste (SWP) was obtained from Hunan Times Sunshine Pharmaceutical Co., Ltd. (No. 191005, Changsha, China). According to the national drug standards of China (State Food and Drug Administration: WS-10876 (ZD-0876)-2002-2012Z), the main ingredients of SWP are steamy *Dihuang* (the prepared root from *Rehmannia glutinosa* (Gaertn.) DC.; as monarch drug), *Baishao* (the root of *Paeonia lactiflora* Pall.; as ministerial drug), *Danggui* (the root of *Angelica sinensis* (Oliv.) Diels; adjuvant drug) and *Chuanxiong* (the root of *Ligusticum chuanxiong* Hort.; adjuvant drug) with the ratio of 1:1:1:1 from the "Xian Shou Li Shang Xu Duan Secret Recipe" [1], 178.5 g, respectively. After *Danggui* and *Chuanxiong* were soaked in cold water for 30 min, steam distillation was used to collect 179 mL of distilled liquid and stored it in another pot. The residue, *Baishao,* and steamy *Dihuang* were boiled in water three times, 1 h for the first time and 1.5 h for the second and third times, respectively. The decoction was combined and filtered, and the filtrate was concentrated in the clear paste with a relative density of 1.25–1.30 (80 °C). The appropriate amount of sucrose was added, the filtrate was heated to dissolve, and the concentration was continued until the relative density of 1.25–1.30 (80 °C). And then, cool, and added the above distillate and potassium sorbate by stirring well. The content of paeoniflorin ($C_{23}H_{28}O_{11}$) could be the guideline to control the quality in SWP, the determined content of paeoniflorin is 1.45 mg/g in SWP (the chromatograph as Fig. S1) with HPLC analysis. The content of paeoniflorin in SWP meets the standard of National Medical Products Administration WS-10876 (ZD-0876)-2002-2012Z (the content of paeoniflorin >0.80 mg/g in SWP). The Fu-Fang E'jiao Jiang (No.171102) came from the Shandong Dong-East-E-Jiao Co., Ltd., 60 mL/day in clinic and equivalent to the dosage of 10.8 g/kg in anemia rats. The total amount of nitrogen (N) content was 6.5 mg/mL, which according to the Pharmacopoeia standard that the total nitrogen is not less than 5.5 mg/mL in Chinese Pharmacopoeia (2020 Edition). Cyclophosphamide (CTX, No. 8H259A) was obtained from Baxter International Inc. (Illinois, US). Acetophenylhydrazine

(APH, No. A0310A) was from Meilun Biotechnology Co., Ltd. (Dalian, China). GM-CSF, EPO, and TPO Kitswere from Wuhan Huamei Biological Engineering Co., Ltd. (No. CSB-E04570r, CSB-E07323r, and CSB-E08352r). Chromatographic pure methanol, propanol, formic acid, and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, USA).

2.2. Experimental instruments

7DZ5-WS centrifuge was from Hunan Xiangyi Experimental Instrument Co., Ltd. (Changsha, China); Exion-LC AD liquid chromatography system with BEH C18 chromatographic column (100 mm × 2.1 mm i.d., 1.8 μm made in Waters) and Triple TOF5600 triple quadrupole mass spectrometer was purchased from AB SCIEX (Boston, USA). JXDC-20 nitrogen purging instrument was obtained from Shanghai Jingxin Industrial Development Co., Ltd. LNG-T88 desk-top fast centrifugal concentration dryer was from Taicang Huamei Biochemical Instrument Co., Ltd. Wonbio-96c high-throughput tissue crusher was from Shanghai Wanbai Biotechnology Co., Ltd. SBL-10TD ultrasonic cleaning machine (300W-10L) obtained from Ningbo Xinzhi Biotechnology Co., Ltd. High-speed freezing centrifuge 5430R was obtained from German Eppendorf Co., Ltd. and NewClassic MF MS105DU electronic balance was from the Swiss Mettler Co., Ltd.

2.3. Animal experiment

Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (Regulations on the Administration of Experimental Animals, China, 1988) and were approved by the Animal Ethical Committee of Hunan Academy of Chinese Medicine (No. 20190052). Fifty male SD rats (180–220 g) were purchased from Hunan SJD Experimental Animal Co., LTD. (SCXK 2019-0004; animal certificate number: 1107271911003733), and feed in an SPF animal room with day and night replacement. After one week of acclimatization in the SPF room, SD rats were divided into normal group, model group, low-dose SWP (SWP-L), and high-dose SWP (SWP–H) group (5.67 and 22.68 g/kg body weight, and the based dosage 5.67 g/day/kg body weight is the converted dosage of rats from the clinical dosage is 63 g/day/50 kg body weight, 22.68 g/day/kg is equivalent to four folds of the based dosage.), positive control (10.8 g/kg of Fu-Fang E'jiao Jiang, which is then converted dosage of rats from the clinical dosage is 60 mL/day/50 kg body weight). The anemia model was replicated according to the reference method [1]. Besides the normal group, 2% APH (at doses of 20 mg/kg and 10 mg/kg) or saline was injected subcutaneously on the 1st and 4th days in all other groups, respectively. After subcutaneous injection for 2 h, intraperitoneal injection of CTX (20 mg/kg) on the 4, 6, 8, and 10th days. Except for the normal rats, the anemia rats were given SWP, positive drug, and sterile water for 14 days by gavage, respectively. The thymus, spleen, and liver were taken out, weighed, and the indexes of various organs were calculated. Organ index = organ mass (mg)/body weight (g).

2.4. Sample collection and processing

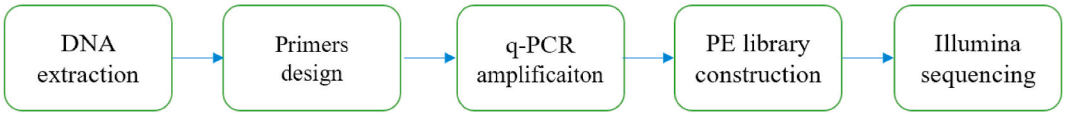
One hour after the last administration, fresh feces were collected and put into sterilized frozen storage tubes at −80 °C for use. Twenty-four hours after the last administration, 2% pentobarbital sodium (2 mL/kg) was used for anesthesia via intraperitoneal injection, and the anticoagulant blood was taken from the abdominal aorta. One part of the blood sample was stayed at room temperature for 30 min, centrifuged at 3500 r/min for 15 min, and the supernatant was stored at −80 °C for use. Thymus and spleen were weighed, the left femur was stored in 4% paraformaldehyde, and the spleen and right femur are stored at −80 °C for later use.

2.5. Blood biochemical analysis

The red blood cell count [RBC ($\times 10^{12}$) cells/L], white blood cell count [WBC ($\times 10^{9}$) cells/L], platelet count [PLT ($\times 10^{9}$) cells/L], hemoglobin concentration (HGB, g/L) and red blood cell volume (HCT) were detected by automated hematology analyzer respectively. The erythropoietin (EPO), thrombopoietin (TPO), and hematopoietic growth factor granulocyte colony-stimulating factor (GM-CSF) in serum were determined according to the instructions of the ELISA kit.

2.6. Gut microbiota diversity analysis

According to experimental Scheme 1, the total DNA of gut microbiota in groups of anemia model and SWP-H was extracted according to the instructions of E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.), the quality of DNA was determined by 1% agarose gel electrophoresis, and their concentration and purity were determined by NanoDrop2000. The 16 S rRNA gene V3–V4 variable region was amplified by PCR with 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTW TCTAAT-3′). The PCR products of the same sample were mixed and recovered with 2% agarose gel. The recovered products were purified by an



Scheme 1. Process of microbial diversity detection.

Axyprep DNA gel extraction kit (Axigen Biosciences, Union City, CA, USA), detected by 2% agarose gel electrophoresis, and quantified by Quantus™ Fluorometer (Promega, USA). NEXTflex™ Rapid DNA-Seq Kit (Bioo Scientific, USA) was used to build the library. The Miseq PE300 platform was sequenced from Illumina company (Shanghai Meiji Biomedical Technology Co., Ltd.).

2.7. Nontargeting metabolomics analysis

According to experimental Scheme 2, about 50 g of the samples (anemia model, normal rats, and SWP-H treatment groups were weighted and thawed at 4 °C, and added with 400 μL extraction solvent (acetonitrile/methanol at the ratio of 1:1, *v/v*) for 30s in vortex apparatus, and sonicated for 30 min in an ice bath (40 KHz), centrifuged at 13,000 rpm for 15 min, and about 400 μL of supernatant was collected. The obtained supernatant was concentrated to dry with nitrogen and reconstituted with 120 μL of 50% acetonitrile, and then sonicated for 5 min in an ice bath, centrifuged at 13,000 rpm for 5 min, the final supernatant was used for UPLC-MS/MS analysis. The UPLC-TripleTOF system was used to detect and analyze all metabolites in the samples of the anemia model, normal rats, and SWP-H groups with a non-targeting strategy. An equal volume of all metabolite samples was mixed to prepare quality control (QC), QC sample is inserted into the other six samples to analyze the difference through UPLC-TripleTOF. And the obtained LC-MS raw data were imported into the Progenesis QI platform, which is a metabolomics processing software (Waters Corporation, Milford, USA). Moreover, the retention time, *m/z,* and MS/MS information are used to automatically match the metabolite information of the human metabolite database (HMDB) and Metlin database to identify the chromatographic peak. The retention time, *m/z,* and MS/MS information are used to automatically match the metabolite information of the human metabolite database (HMDB) and Metlin database (https://metlin.scripps.edu/) to identify the chromatographic peak. The differential metabolites were dug by metabolic pathway annotation in the database KEGG (https://www.kegg.jp/kegg/pathway.html). An equal volume of all sample metabolites mixed.

2.8. UPLC-MS/MS analysis

Chromatographic separation of the metabolites was performed on an ExionLCTMAD system (AB Sciex, USA) equipped with an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters, Milford, USA). The mobile phases consisted of 0.1% formic acid in water with formic acid (0.1%) (solvent A) and 0.1% formic acid in acetonitrile: isopropanol (1:1, *v/v*) (solvent B). The solvent gradient changed according to the following conditions: from 0 to 3 min, 95% (A): 5% (B) to 80% (A): 20% (B); from 3 to 9 min, 80% (A): 20% (B) to 5% (A): 95% (B); from 9 to 13 min, 5% (A): 95% (B) to 5% (A): 95% (B); from 13 to 13.1 min, 5% (A): 95% (B) to 95% (A): 5% (B), from 13.1 to 16 min, 95% (A): 5% (B) to 95% (A): 5% (B) for equilibrating the systems. The sample injection volume was 20 μL and the flow rate was set to 0.4 mL/min. The column temperature was maintained at 40 °C. During the period of analysis, all these samples were stored at 4 °C.

The optimal conditions were set as followed: source temperature, 500 °C; curtain gas (CUR), 30 psi; both Ion Source GS1 and GS2, 50 psi; ion-spray voltage floating (ISVF), −4000V in negative mode and 5000V in positive mode, respectively; declustering potential, 80V; a collision energy (CE), 20–60V rolling for MS/MS. The detection was carried out over a mass range of 50–1000 *m/z*. Internal standard (0.3 mg/mL of L-2-chloro-phenylalanine in acetonitrile) was used during the metabolomics experiments.

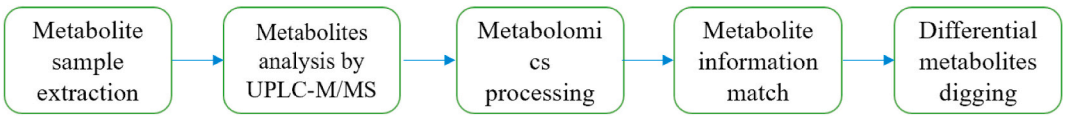
2.9. Statistical analysis

GraphPad Prism 5 software was used to analyze the experimental data, both the mean ± SD value was showing results in three independent measurements. Data analysis was evaluated by one-way ANOVA and Student's test-test respectively with p-value <0.05 as statistically significant. A multivariate statistical analysis was performed using ropls (Version 1.6.2) R package.

3. Results

3.1. Effect of SWP on the physical indexes of anemia model rats

As shown in Fig. 1, the weight and thymus index of the anemia model group decreased significantly than the normal group ($p < 0.01$, Fig. 1A and B), while the spleen index and kidney index increased significantly ($p < 0.01$, Fig. 1C and D). Compared with the anemia group, the kidney index and spleen index of the positive group decreased significantly ($p < 0.05$), the thymus index of the high dose of SWP increased significantly ($p < 0.05$), the kidney index and spleen index decreased significantly ($p < 0.05$). The kidney index of the low dose of SWP was significantly decreased than anemia rats ($p < 0.05$), and there was no significant difference for the thymus index and spleen index.



Scheme 2. Process of Nontargeting metabolomics analysis.

3.2. Effect of SWP on peripheral blood cells and EPO, TPO, and GM-CSF of anemia rats

As shown in Fig. 2A–F, high-dose SWP significantly increased the levels of red blood cells, hemoglobin, platelets, erythrocyte backlog, white blood cells, and lymphocytes of anemia rats as the positive treatment, compared with the anemia rats ($p < 0.05$). Difference from the normal rats in Fig. 2G–I, the levels of EPO, TPO, and GM-CSF in the serum of anemia rats decreased significantly ($p < 0.001$). While compared with anemia rats, the levels of EPO, TPO, and GM-CSF in the serum of the high-dose SWP rats and the positive group increased significantly ($p < 0.01$).

3.3. The operating unit (OTU) of gut microbiota classification and diversity analysis

To study the differences in structural diversity of gut microbiota of rats in different groups, high-throughput sequencing was conducted on three groups of samples, and the richness, uniformity, and diversity of gut microbiota were evaluated by Sob, Ace, and Chao indexes. The results of α diversity analysis showed that the OTU coverage rate of each sample was above 99.5% (Fig. 3A, 3B, and 3C). Compared with the normal group, the abundance index and diversity index of gut microbiota in anemia rats increased significantly. SWP significantly decreased the abundance index and diversity index of gut microbiota with anemia rats. The difference in β diversity of rat gut microbiota was investigated by principal component analysis in Fig. 3D, the results showed that the gut microbiota of anemia rats and the normal rats were obviously separated, which indicated that there are significant differences in the flora structure between the two groups. And the flora of the SWP group tended to the normal rats, which indicated that the flora structure of the SWP group was like that of the normal group and the positive group, so there might be a certain genetic relationship among them (Fig. 3D).

3.4. Composition and structure analysis of gut microbiota

There are 21 samples in this study, both OTU belonged to 26 dominant bacteria genus, such as *norank_f_Muribaculaceae*, *Lactobacillus*, *Prevotella_9*, *Allobaculum*, *Lachnospiraceae_NK4A136_group*, *Treponema_2*, *Ruminococcaceae_UCG-014*, *unclassified_f_Lachnospiraceae*, *Ruminococcaceae_UCG-005*, *Ruminococcus_1* (Fig. 4A). Among them, the dominant genus of bacteria in normal group rats were *norank_f_Muribaculaceae* (25.29%), *Lactobacillus* (25.55%), *Prevotella_9* (11.318%). The dominant bacteria genus in

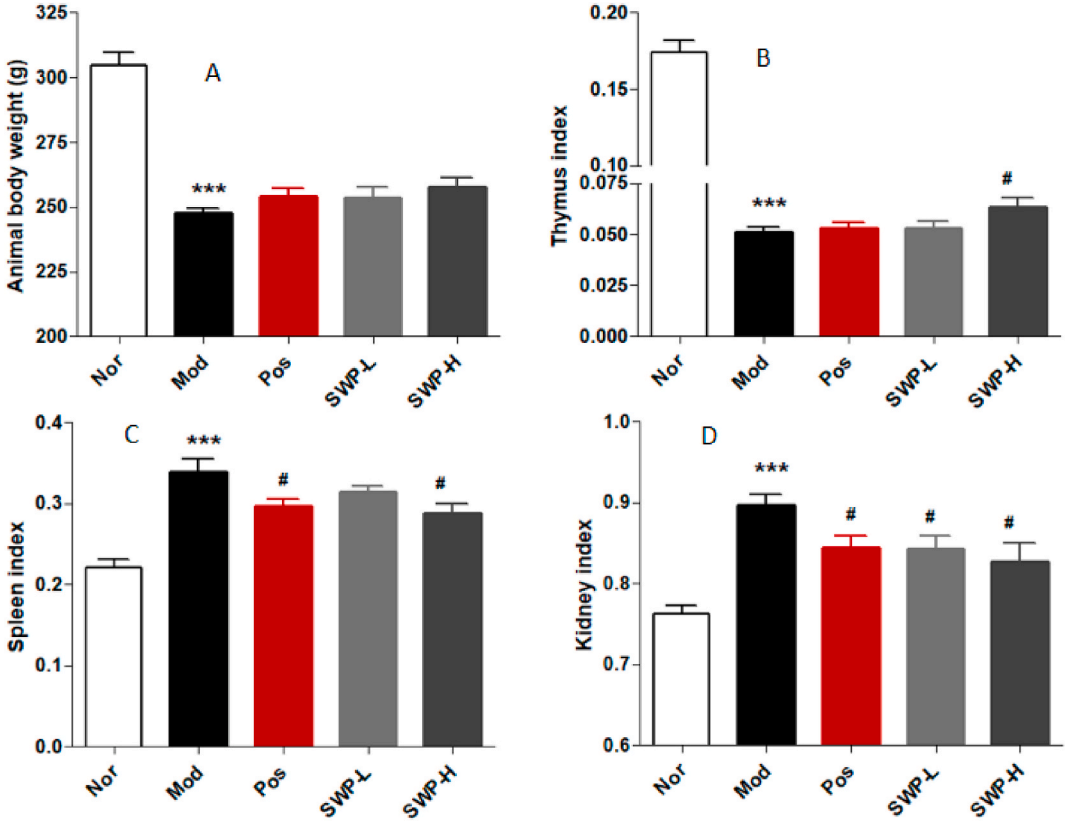


Fig. 1. Effects of SWP on body weight (A), thymus index (B), spleen index (C), and kidney index (D) of anemia model rats (x ± SD, n = 10). ***$p < 0.001$ (*vs* blank group); $^{\#}p < 0.05$ (*vs* model group). Nor, normal rats group; Mod, anemia model rats group; Pos, positive rats group; SWP-L and SWP-H is the low dosage of SWP group and high dosage of SWP group, respectively.

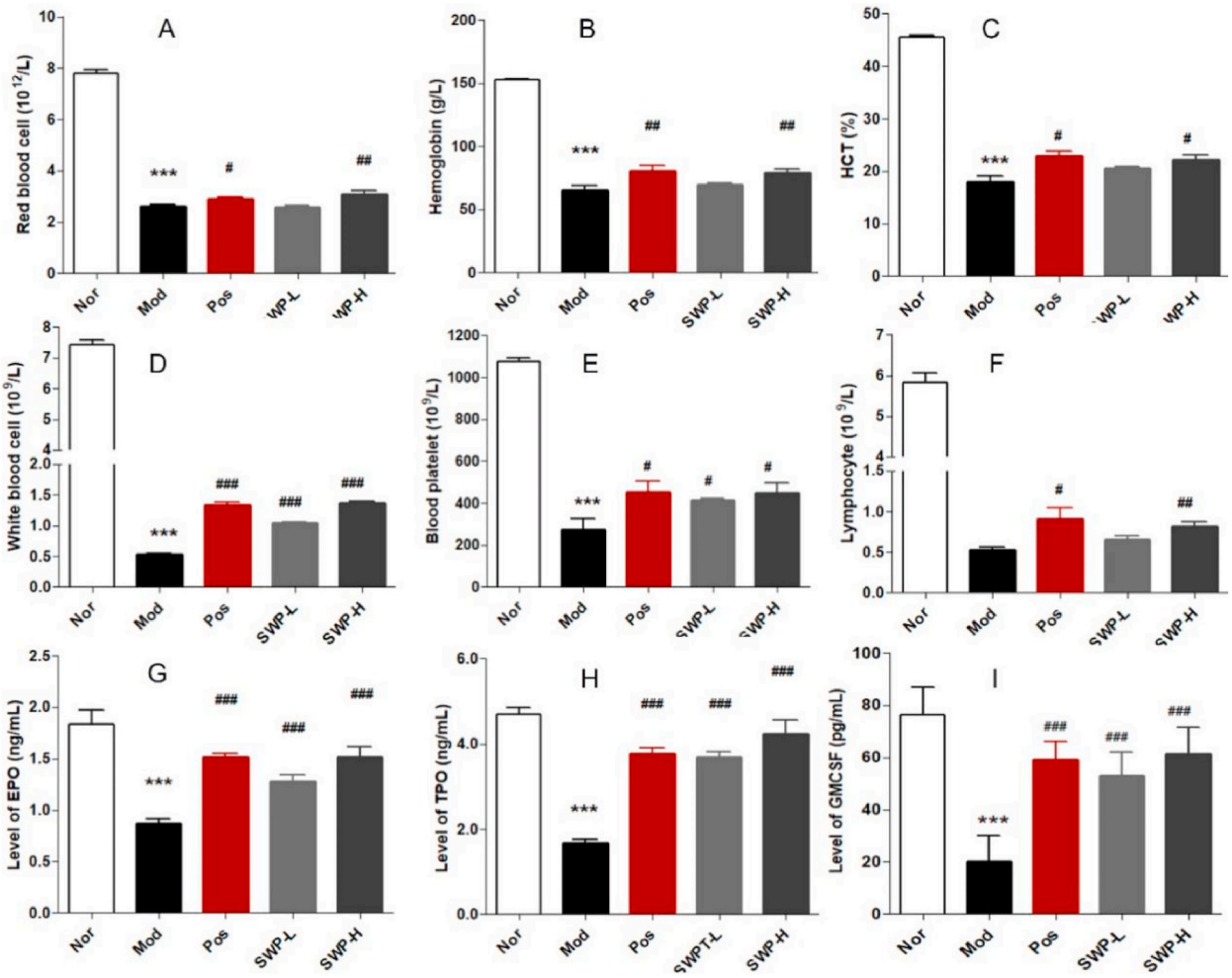


Fig. 2. Effects of SWP on erythrocyte (A), hemoglobin (B), erythrocyte backlog (C), leukocyte (D), platelet (E), lymphocyte level (F), EPO (G) TPO (H) and GM-CSF (I) in serum of anemia model rats. $^{***}p < 0.001$ (*vs* blank group); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ (*vs* model group). Nor, normal rats group; Mod, anemia model rats group; Pos, positive rats group; SWP-L and SWP-H is the low dosage of SWP group and high dosage of SWP group, respectively.

anemia rats were *norank_f_Muribaculaceae* (17.76%), *Lactobacillus* (11.17%), and *Allobaculum* (7.72%). The dominant genus of bacteria in groups of treated rats was *norank _f_ Muribaceae Norank _f _ magnoliaceae* (22.15%), *Prevotella_9* (21.02%), and *Lactobacillus* (14.48%). Furthermore, the Wilcoxon rank sum test was further used to compare the feces of each group in pairs at the genus level. Compared with the normal group, there were 10 bacteria genera with significant differences in the gut microbiota of rats in the model group. Among them, *Lactobacillus* and *Prevotella_9* were down-regulated, and 8 bacteria genera were up-regulated, such as *Allobaculum* and *Roseburia*. Compared with the anemia group, SWP can recall these 10 genera (Fig. 4B and C). LEfSe was used to further determine whether the specific flora of SWP have differential enrichment, compared with anemia rats. And the lower limit of the logarithmic LDA score is 2.0. So, 56 distinguishing genera were identified as the key discriminant factors (Fig. 5).

3.5. Correlation analysis of gut microbiota flora with the hematopoietic index

Spearman correlation coefficient analysis was used to explore the relationship between different gut microbiota and hematopoietic systems during SWP intervention on serum-related indexes in anemia rats. When $|r| > 0.5$ and $p < 0.05$, which indicates that there is a significant correlation between gut microbiota and the hematopoietic system. As shown in Fig. 6, the correlation analysis of intestinal microbiota-hematopoietic efficacy shows that 13 kinds of different intestinal flora related to hematopoietic efficacy, in which *Prevotella_1*, *Prevotella_9*, *Lactobacillus*, *norrank_f_Muribaculae* are significantly positively correlated with the hematopoietic system. While nine kinds of intestinal flora are a significantly negative correlation with the hematopoietic system, such as *Roseburia*, *Allobaculum*, *unclassified_f_ruminococcus, Romboutsia,* and so on (Fig. 6).

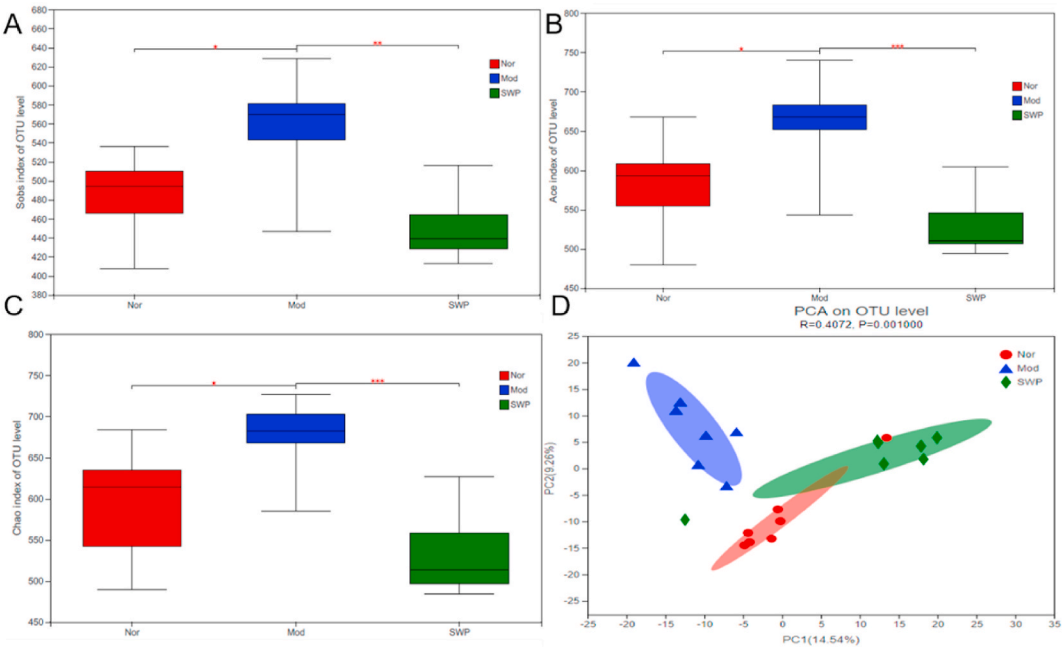


Fig. 3. Gut microbiota analysis in SWP and the other samples (A, sob; B, ace; C, Chao; D, PCA analysis.). Nor, normal rats group; Mod, anemia model rats group; SWP, is the SWP-H treatment group.

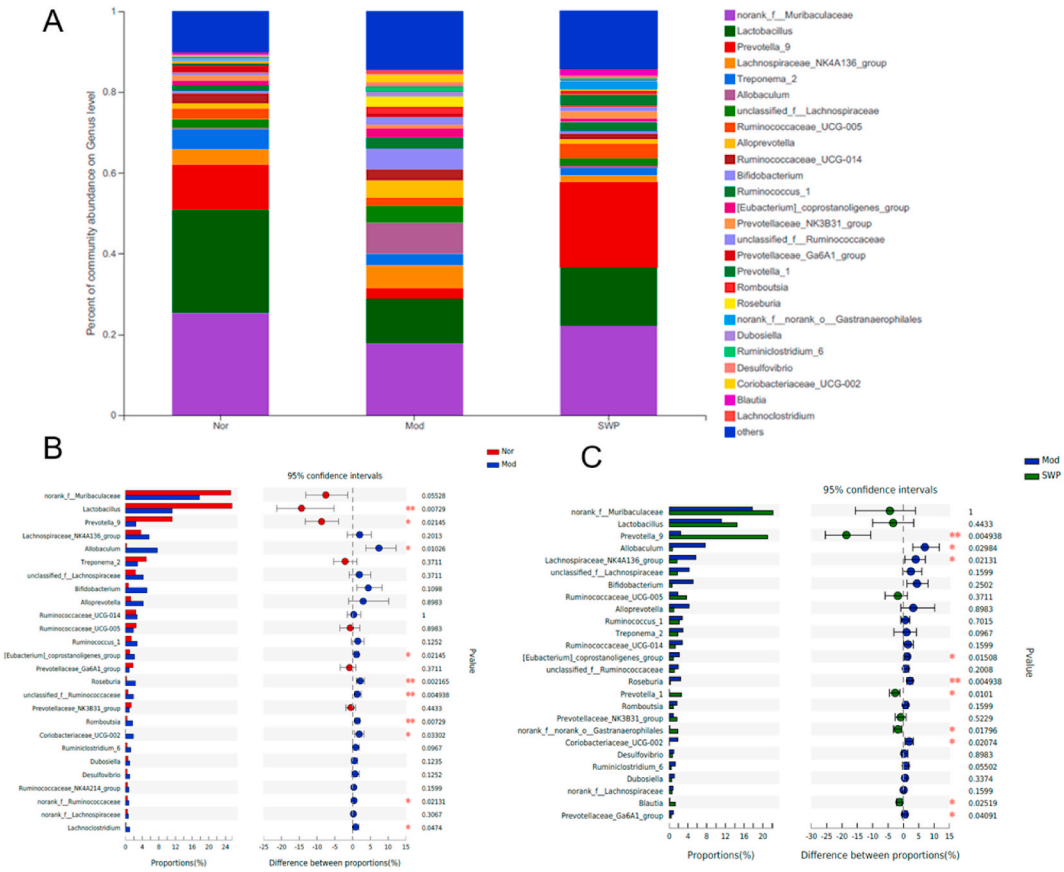


Fig. 4. Gut microbiota composition and difference analysis in anemia rats and SWP treatment. A. Composition of the dominant genus of bacteria in the gut microbiota of rats in anemia rats and SWP group. B and C. Gut microbiota difference analysis of rats in anemia Model (Mod) *vs* Normal rats (Nor) and anemia Model (Mod) *vs* SWP-H treatment (SWP).

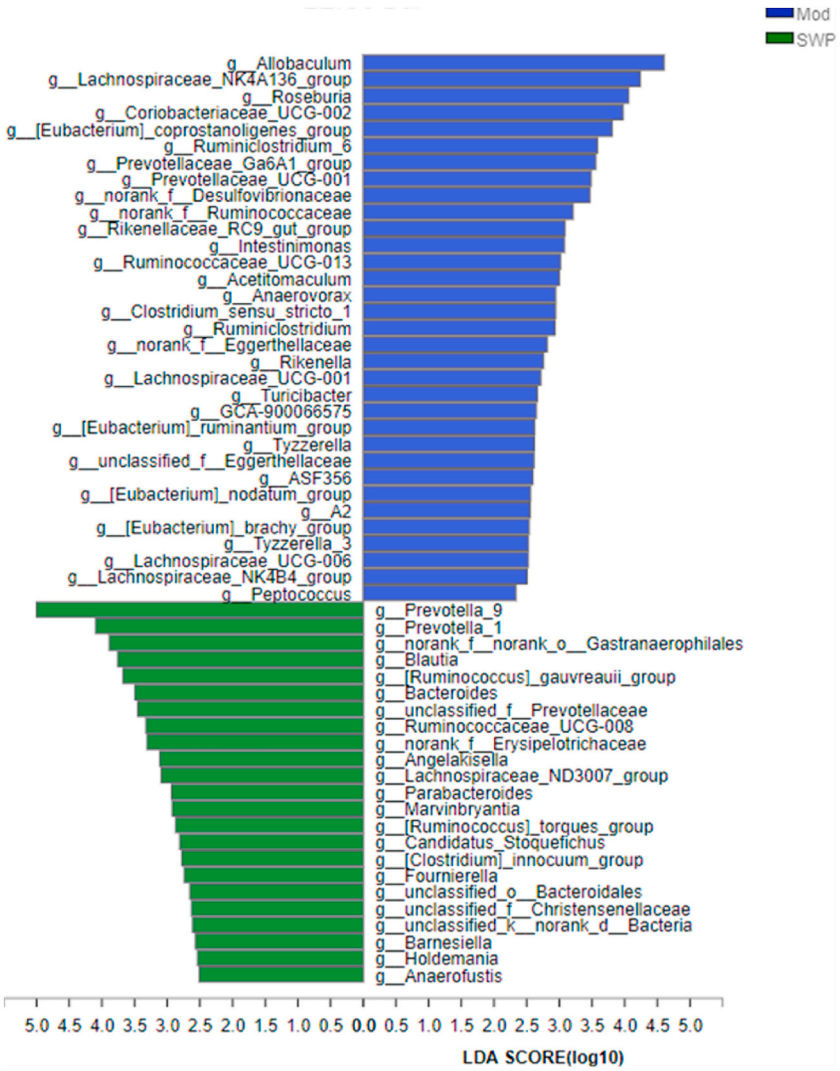


Fig. 5. LEfSe analysis of gut microbiota composition. Mod, anemia model rats; SWP, is the SWP-H treatment group.

3.6. Differential analysis of SWP on fecal metabolic profiles of anemia rats

Multivariate statistical analysis, and principal component analysis (PCA) algorithm were used to distinguish the differences in metabolic data of rats in each group. As shown in Fig. 7A, there are metabolic differences in the three groups. An orthogonal partial least squares discriminant analysis (OPLS-DA) model was established to further identify the metabolites differences of rats in the three groups. The PLS-DA score plot shows that there are significant differences between the two groups [R_2X (cum) = 0.702, R_2Y (cum) = 0.988, Q2 (cum) = 0.956]. It shows that the model is predictive and reliable, and there are significant differences in metabolite abundance among the three groups of rats (Fig. 7B).

3.7. Metabolism biomarkers screening

To obtain the differential metabolic biomarkers between different groups, the important variables of the OPLS-DA model were used. Then, metabolites with VIP score >1 and $p < 0.05$ were considered potential metabolic biomarkers. VIP >2 and $p < 0.05$ in univariate analysis are the metabolites with significant differences. The result showed that a total of 218 potential metabolic biomarkers and 36 metabolites (Table S1) with significant differences were identified in the model group and SWP group. These metabolites are mainly involved in amino acid metabolism, chemical structure transformation map, metabolism of cofactors and vitamins, biosynthesis of other secondary metabolites, lipid metabolism, etc. The thermal map was then constructed to show the 36 metabolites with significant differences (Fig. 7C). Overall, there were 7 metabolites that significantly increased and 29 metabolites significantly decreased in the anemia rats. And rich differences in metabolites are mainly amino acids and lipoids, such as beta-leucine,

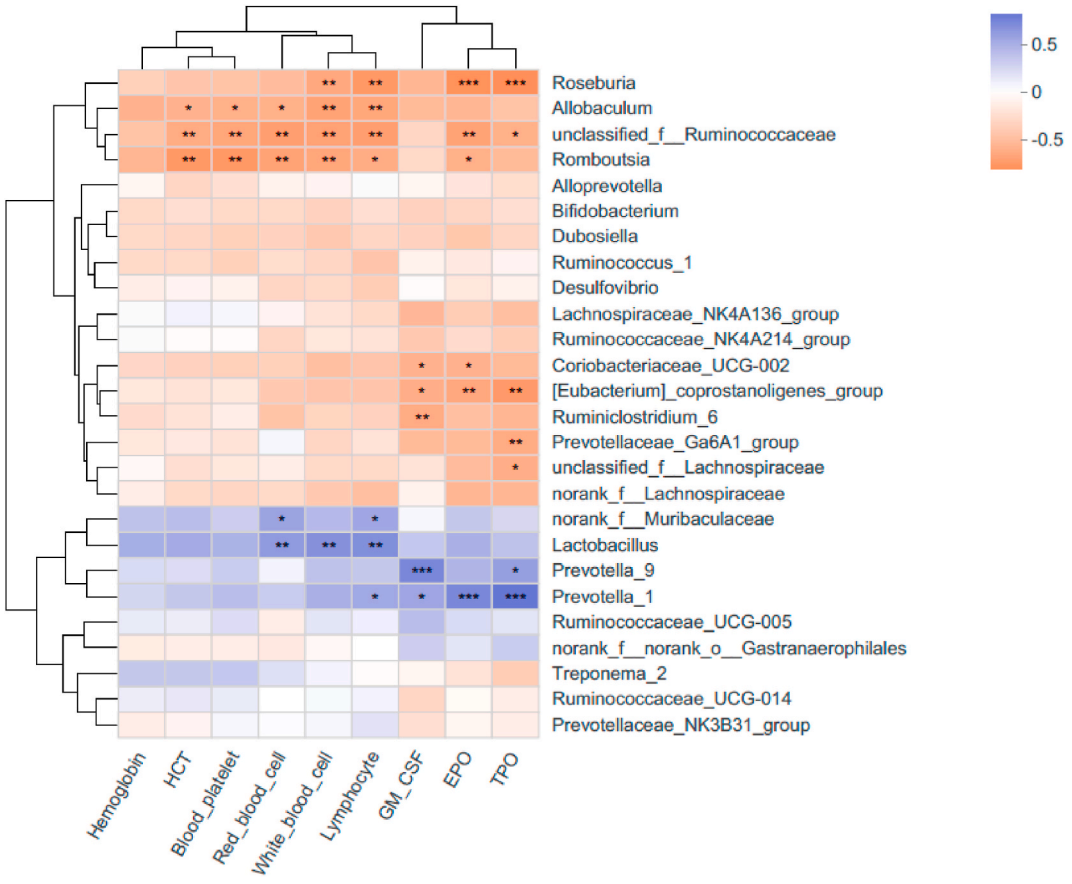


Fig. 6. Thermal analysis of correlation between differential flora and serum indexes.

ricinoleic acid, and 3-hydroxytridecanoic acid.

The metabolic pathways of anemia affected by SWP are shown in Fig. 8A, among which there are 7 metabolic pathways with significant differences ($p < 0.05$). They are aminobenzoate degradation, tryptophan metabolism, biosynthesis of phenylpropanoids, one carbon pool by folate, biosynthesis of plant hormones, biosynthesis of plant secondary, fructose, and mannose metabolism metabolites.

3.8. Correlation analysis between gut microbiota and metabolites

To explore the functional correlation between intestinal dysbacteriosis and change in fecal metabolites, the Spearman correlation coefficient was used to calculate the correlation matrix level between intestinal dysbacteriosis and change in fecal metabolites (26 bacterial groups) and 36 significantly changed metabolites (VIP >2). As shown in Fig. 8B, based on $|r| \geq 0.75$ and $p < 0.01$, 31 significant microbial metabolic correlations were determined. And *prevella_1*, *blautia*, *roseburia,* and *lachnospiraceae_NK4A136_group* were significantly correlated with 16, 14, 13, and 16 fecal metabolites, respectively. In addition, the dominant strain *prevotella_9* was positively correlated with 8-isoquinoline methenamine (hydrochloride), asparaginyl-proline, xanthurenic acid, and tetrahydrofolic acid. And tryptophan metabolite xanthinic acid and 4- (2-Amino-3-hydroxyphenyl)-2,4-dioxobutanoic acid were positively correlated with some superiority strains, such as *Prevotella, norank_f_norank_o_Gastranaerophilales*, and *unclassified_f_Lachnospiraceae*.

4. Discussion

Intestinal microecology is closely related to various physiological functions in humans. The imbalance of the microbial community is related to potential disease, risks, and even the apparent onset of clinical symptoms. Several recent studies have shown that gut microbiota plays a significant role in the regulation of hematopoiesis, such as anemia and neutrophilic granulocytopenia. In this study, the abundance of intestinal flora in the anemia model group was higher than that of normal rats and SWP rats (Fig. S2), which is akin to the reported that the decrease was observed in microbial diversity over time among the hematopoietic stem cell transplantation (HSCT) patients [14]. And β diversity of rat gut microbiota with SWP was similar to that of the normal rats, indicating that the flora structure of SWP treatment was as similar as that of the normal group and the positive group, so there might be a certain relationship

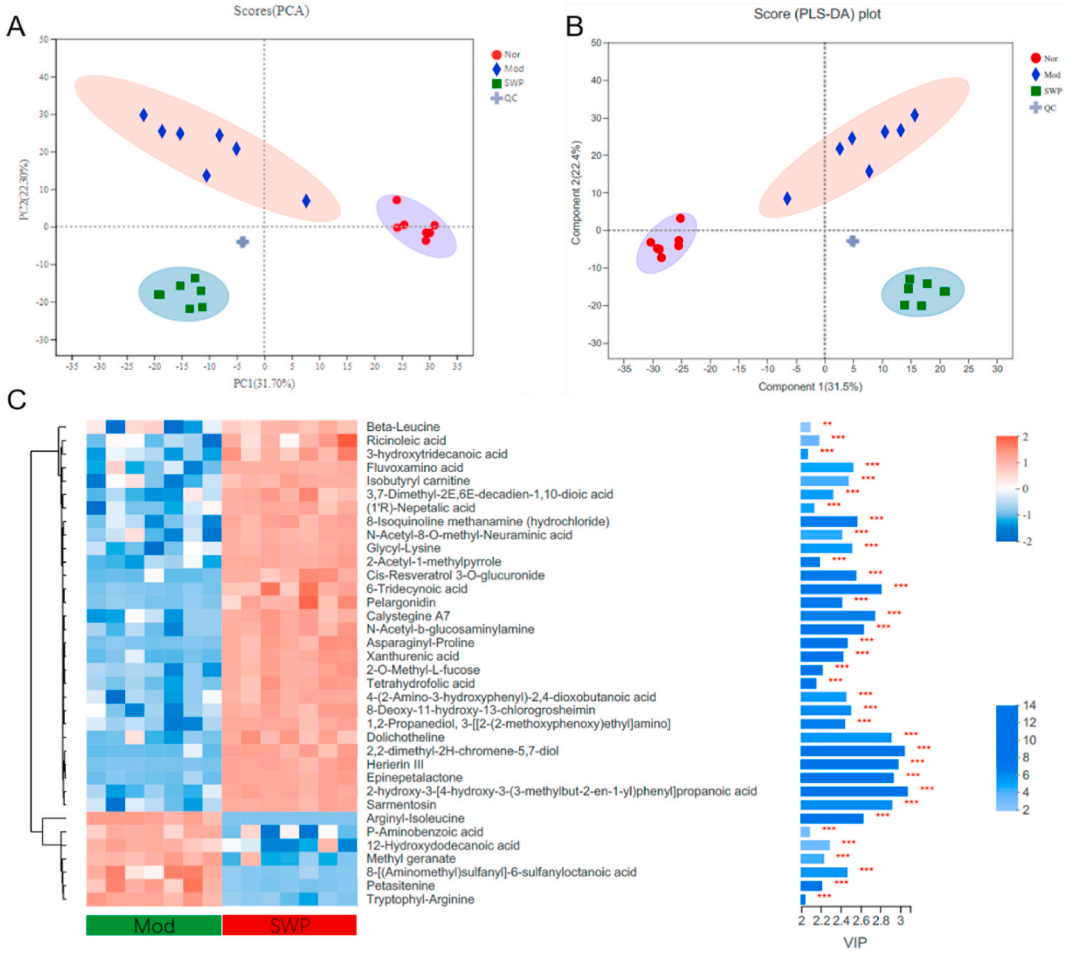


Fig. 7. PCA analysis (A) and PLS-DA score (B) of rats' feces and their metabolites difference (C). Nor, normal rats group; Mod, anemia model rats group; Pos, positive rats group; SWP, is the SWP treatment group; and QC is the quality control sample.

among them. The ratio of firmicutes/bacteroidetes (F/B) may reflect the balance of intestinal microecology and be regarded as a type parameter of organism health. The results of 16S rRNA gene sequencing have shown that the bacteroidetes of rats decreased, firmicutes increased, and the F/B ratio correspondingly increased in anemia rats, which reflected the imbalance of microecology in the anemia intestine. However, the F/B ratio can be restored after treatment with SWP, which shows that SWP could regulate the composition of the gut microbiota of anemia rats at the phylum level. The results of this study showed that SWP could regulate back 10 different genera of bacteria, particularly up-regulate the abundance of three dominant strains *norank_f_muribaculaceae*, *lactobacillus,* and *prevotella_9*. Among which, *muribaculaceae* contributes to the production of propionate [15,16], it can stimulate the proliferation of bone marrow cells and spleen white and red lymphocytes, and reduce the radiation-induced damage of granulocyte-macrophage progenitor cells (GMPs), common myeloid progenitor cells (CMPs) and megakaryocyte erythroid progenitor cells (MEPs) [10]. *Lactobacillus* can secrete gamma-aminobutyric acid (GABA) [17], and the change of GABA signal is related to the regulation of hematopoietic stem cells and megakaryocyte progenitor cells [18]. Moreover, *lactobacillus* can increase immature myeloid progenitor cells in the bone marrow and accelerate the recovery of cyclophosphamide-caused immunosuppression [19]. The results showed that *lactobacillus* had a positive correlation with red blood cells, white blood cells, and lymphocytes in SWP treatment, suggesting that *lactobacillus* could produce bone marrow hematopoietic cells to improve anemia. Prevotella genus belongs to Bacteroides, which does not produce spores, and its main metabolites are acetic acid, succinic acid and a small amount of butyric acid [20], it can promote the production of sphingolipid transmitters and affect the feedback regulation of the immune system and nervous system [21,22]. In patients with myelodysplastic syndrome (MDS), the abundance of prevotella is significantly decreased, and MDS patients are often accompanied by anemia and other symptoms. A high abundance of prevotella can activate intestinal myeloid dendritic cells and activate systemic immunity [23]. Correlation analysis in this study showed that *prevotella_1* and *prevotella_9* were significantly positively correlated with hematopoietic efficacy (e.g., EPO, TPO, and GM-CSF), which indicates that prevotella can improve hematopoietic function through cellular immune regulation.

Gut microbes and microbial derivatives play an important role in regulating host metabolism and immunity, such as short-chain fatty acids (SCFA) and tryptophan metabolites. The characteristics of fecal metabolome can improve the understanding of microbial

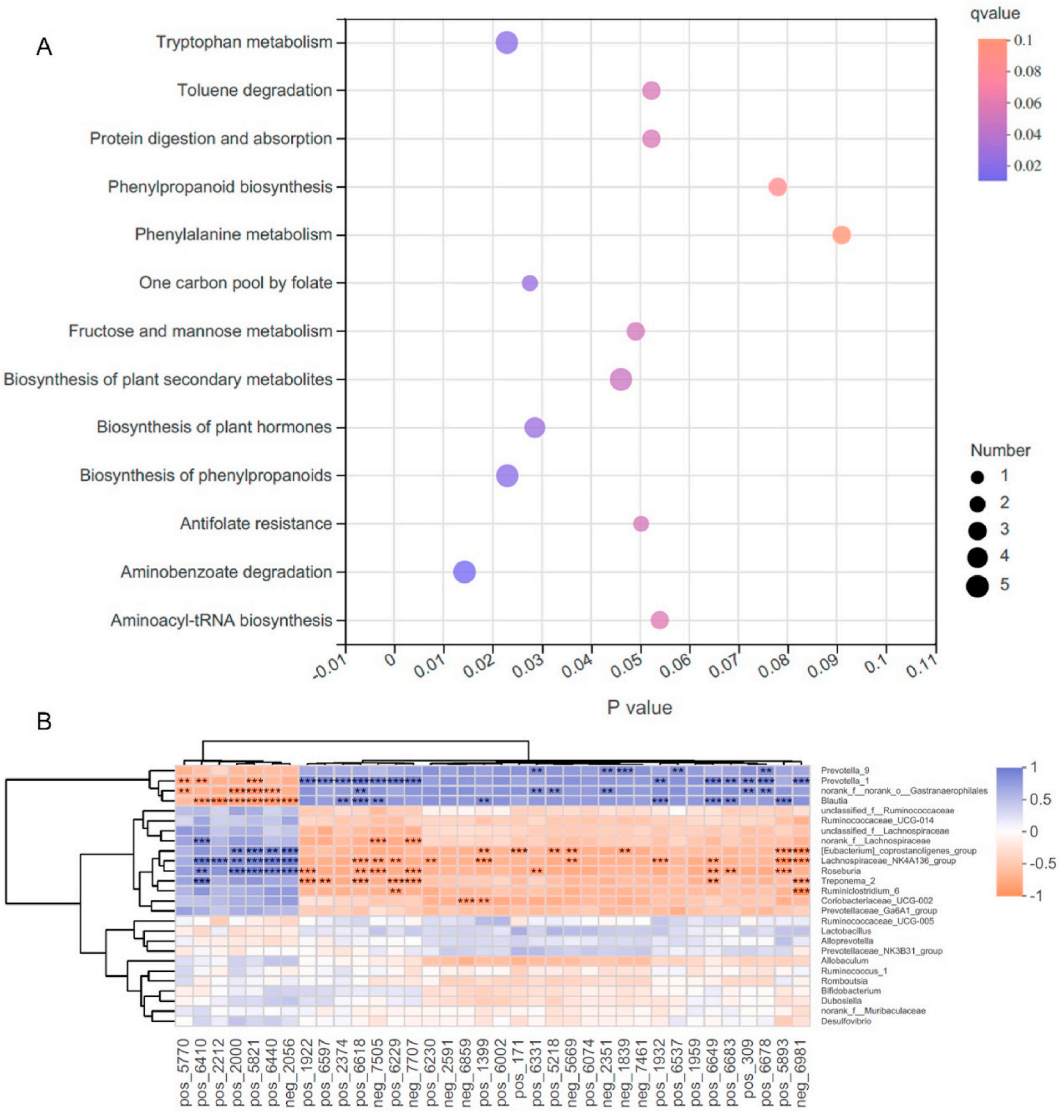


Fig. 8. Bubble diagram of KEGG enriched metabolic pathway (A) and thermal analysis of the correlation between gut microbiota and metabolites (B).

reactions disturbed by gut microbiota. There are significant differences in the fecal metabolic spectrum between anemia model rats and SWP rats. 36 fecal metabolites were identified as biomarkers with VIP >2 and $p < 0.05$ in OPLS-DA, mainly including amino acids and lipids. In addition, seven disturbed metabolic pathways were found in anemia rats ($p < 0.05$), among which the tryptophan metabolic pathway is closely related to the occurrence and development of many clinical diseases, their metabolites can reduce pro-inflammatory cytokines such as TNF-α, interleukin-6, and interferon-γ [24]. Recent studies show that the pathogenesis of immune thrombocytopenia is related to the abnormal metabolism of tryptophan mediated by indoleamine 2, 3-dioxygenase (IDO) [25] and L-tryptophan could reduce radiation-induced thrombocytopenia by inhibiting megakaryocyte differentiation and promoting megakaryocyte apoptosis [26]. In this study, five tryptophan metabolites were observed and it means that SWP can improve platelet abnormalities in anemia rats by regulating the tryptophan metabolic pathway. In our study, *Lachnospiraceae* was positively correlated with tryptophan metabolites, which could promote the recovery of hematopoietic function and reverse the aging of hematopoietic stem cells, this result is consistent with the recent report [27].

These findings have suggested that gut microbiota and metabolites may play a key role in the regulation of anemia by SWP. The effects of identified intestinal bacteria and metabolites on the hematopoietic system will be carried out in the further experiment.

5. Conclusions

In this study, high throughput 16S rRNA gene sequencing and non-targeted metabolomics were used to analyze the effects of SWP

on the gut microbiota and fecal metabolites of anemia rats. By integrating the data of gut microbiota and metabolomics, SWP could regulate back 10 different genera of bacteria, particularly it up-regulates the abundance of three dominant strains *norank_f_muribaculaceae*, *lactobacillus,* and *prevotella_9*. SWP could significantly change the composition structure of gut microbiota and its metabolites of anemia rats, and the changes of gut microbiota were significantly correlated with the changes of metabolites. It is worth noting, *Lachnospiraceae* was positively correlated with tryptophan metabolite, which could promote the recovery of hematopoietic function and reverse the aging of hematopoietic stem cells, these results indicated that SWP not only changed the gut microbiota but also affected its related metabolic phenotype. The data on gut microbiota and metabolites helps to screen the effective metabolites and their corresponding gut microbiota in the treatment of anemia with SWP.

Author contribution statement

Dan Wan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Xuejuan Liang, Limei Yang: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dan He, Qing Du, Linben Xu, Ping Cai, Jianhua Huang, Jianji Huang, Wanping Zhang, Yiying Xiong: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Yongbo Peng, Shuihan Zhang, Rongrong Zhou: Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18024.

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