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RECEIVED 21 January 2025

ACCEPTED 22 May 2025

PUBLISHED 12 June 2025

CITATION

Mo Y, He X, Shi P, Ning Y, Zhou M, Cui H and
Zhang T (2025) Hematopoietic effects of
Fufang E'jiao Jiang revealed by microbiome,
metabolome and transcriptome analyses: a
multi-omics strategy.
Front. Immunol. 16:1561477.
doi: 10.3389/fimmu.2025.1561477

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Hematopoietic effects of Fufang E'jiao Jiang revealed by microbiome, metabolome and transcriptome analyses: a multi-omics strategy

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Introduction: Fufang E'jiao Jiang has been extensively utilized to replenish qi and nourish blood as the homology of medicine and food.

Methods: We analyzed the effects of FEJ on cyclophosphamide and acetylphenylhydrazine-induced anemia mice through gut microbiome analysis, fecal metabolomics, and transcriptome sequencing.

Results: FEJ markedly alleviated the anemia symptoms in the mice. FEJ markedly alleviated the anemia symptoms caused by cyclophosphamide and acetylphenylhydrazine induction. FEJ improved the gut microbiome imbalance by inhibiting the proliferation of harmful bacteria *Turicibacter*, *Akkermansia* and *Tuzzerella*. Fecal metabolomic data showed that FEJ regulated metabolic disorders in anemia mice and was probably associated with L-leucine, L-proline, glycine, phenylalanine, propanoic acid and butanoic acid. Transcriptome analysis indicated the amelioration of anemia was predominantly associated with the hematopoietic cell lineage, osteoclast formation and B cell receptor signaling pathway. According to Spearman's correlation analysis, there was a strong link between gut microbiota and hematopoietic index, metabolites and genes.

Discussion: Our study supports the application of FEJ in anemia treatment.

KEYWORDS

Fufang E'jiao Jiang, anemia, microbiome, metabolome, transcriptome

Introduction

Anemia is a prevalent disease seen in clinical settings that is defined by a reduction in red blood cells (RBC) and hemoglobin concentrations. It impacted up to one-third of the world's population (1). In traditional Chinese medicine theory, anemia is defined by blood deficiency and represents a condition of organ malnutrition and blood dysfunction. Patients or animals suffering from anemia frequently face impaired hematopoietic function, peripheral blood pancytopenia, diminished internal organ function, malnutrition, or myelosuppression during severe illnesses (2). In clinical, two primary strategies are employed to treat anemia: stimulating the production of RBC and providing more iron to the bone marrow. However, these methods are accompanied by negative effects. Severe cases may require blood transfusions, which could potentially result in hemolysis or heart failure (3). Accordingly, the development of safe and efficacious strategies for treating anemia is a concern in the medical community.

Fufang E'jiao Jiang (FEJ) is a syrup made from *Asini Corii Colla* and four food-medicine homology ingredients *Ginseng Radix et Rhizoma Rubra* (*Panax ginseng* C.A.Mey.), *Codonopsis Radix* (*Codonopsis pilosula* Nannf.), *Crataegi Fructus* (*Crataegus pinnatifida* Bunge.) and *Rehmanniae Radix Praeparata* (*Rehmannia glutinosa* (Gaertn.) Libosch. ex DC.) using modern processing and refining technology (4–9). Complex chemical composition of FEJ showed common challenges, including ambiguous bioactive components and unidentified therapeutic targets. A total of 72 chemical ingredients in FEJ were identified by HPLC–MS, including organic acids, flavonoids, phenylethanoid glycosides, notoginsenosides and ginsenosides (10). Oligosaccharides, which have significant immune-boosting effects, were key constituents of FEJ, and 13 oligosaccharides identified via ultra-performance liquid chromatograms combined with triple quadrupole mass spectrometry (UPLC–QQQ–MS) (6). FEJ is produced exclusively by Shandong Dong-E-E-Jiao Co., Ltd in Dong'e, China. Its sales surpassed one billion Yuan in China in 2012. The efficacy of FEJ is replenishing qi and nourishing blood (8) and it has been clinically employed in the adjuvant treatment of anemia by way of blood supplementation (6). In a myelosuppression mouse model, treatment with FEJ promoted the restoration of bone marrow

hemopoietic activity by improving bone marrow hematopoietic niche, preventing bone marrow nucleated cells (BMNCs) from apoptotic cell death and stimulating the expressions of cytokines essential to hematopoiesis (8). The percentage of hematopoietic stem cells and the quantities of burst-forming unit-erythroid (BFU-E) and colony-forming unit granulocyte-monocyte (CFU-GM) in BMNCs of mice with myelosuppressive were significantly increased by FEJ supplementation (11). Nonetheless, a systematic investigation of the underlying molecular mechanism of FEJ's hematopoietic effects has not yet been conducted.

In light of the prominent efficacy of FEJ in the treatment of anemia, this study aimed to elucidate the potential mechanism of FEJ in alleviating anemia in mice induced by cyclophosphamide (CTX) and acetylphenylhydrazine (APH), with an emphasis on the role of gut microbiota, metabolites, and genes. The ability of FEJ to ameliorate hematopoietic dysfunction was assessed, and pathological analyses of bone and spleen tissues were performed. Following the analysis of gut microbiota and fecal metabolites from fecal samples, we proceeded to perform gene expression profiling of the spleen. The findings of this study offered insights into the pharmacological mechanisms of FEJ in regulating hematopoiesis.

Materials and methods

Material preparation

FEJ (No. 2208021) was provided by Shandong Dong-E-E-Jiao Co., Ltd (Dong'e, China). The above plant names have been checked with <http://mpns.kew.org> and <http://www.worldfloraonline.org/taxon/wfo-0000263606>. Accessed on: 10 Jan 2025'. CTX and APH were obtained from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). The ELISA kits for erythropoietin (EPO) were acquired from Enzyme-linked Biotechnology Co., Ltd (Shanghai, China). Kits for Malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and bicinchoninic acid (BCA) were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Twenty-four Kunming (KM) mice, consisting of equal numbers of males and females, and weighing 18–22 g, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were maintained under standard conditions with unlimited access to food and water (22°C ± 1°C temperature and 12 h light/dark cycle) to allow acclimation to the laboratory environment. The study received approval of the Animal Studies Ethics Committee of Shanghai University of Traditional Chinese Medicine (Ethical number: PZSHUTCM2302110002).

Animal experiment

Experimental design (Animal modeling and feeding)

The mice were evenly distributed into three groups: the control group, model group and FEJ group, with 8 mice in each group

Abbreviations: APH, acetylphenylhydrazine; BCA, bicinchoninic acid; BFU-E, burst-forming unit-erythroid; BMNCs, bone marrow nucleated cells; β -TH, β -thalassemia; CFU-GM, colony-forming unit granulocyte-monocyte; CGD, Chronic granulomatosis; Chdh, Choline dehydrogenase; CTX, cyclophosphamide; DEGs, differential expression genes; EPO, erythropoietin; FEJ, Fufang E'jiao Jiang; Gpx4, glutathione peroxidase 4; Gss, glutathione synthetase; Gst, glutathione S-transferase; Gstm5, glutathione S-transferase mu 5; Gys1, glycogen synthase 1; HGB, hemoglobin; Hk2, hexokinase2; H&E, hematoxylin-eosin; HSCs, hematopoietic stem cells; HSPCs, hematopoietic stem and progenitor cells; KM, Kunming; LDA, Linear Discriminant Analysis; MDA, Malondialdehyde; Mgst2, Microsomal glutathione S-transferase 2; Mks, megakaryocytes; NMDS, Non-metric multidimensional scaling analysis; Osm, Oncostatin M; OTUs, operational taxonomic units; PCA, Principal component analysis; PcoA, Principal coordinate analysis; PLT, platelets; RBC, red blood cells; RP, red pulp; SCFAs, short-chain fatty acids; SOD, superoxide dismutase; Thpo, thrombopoietin; TSCC, tongue squamous cell carcinoma; T-AOC, total antioxidant capacity; WBC, white blood cells; WP, white pulp.

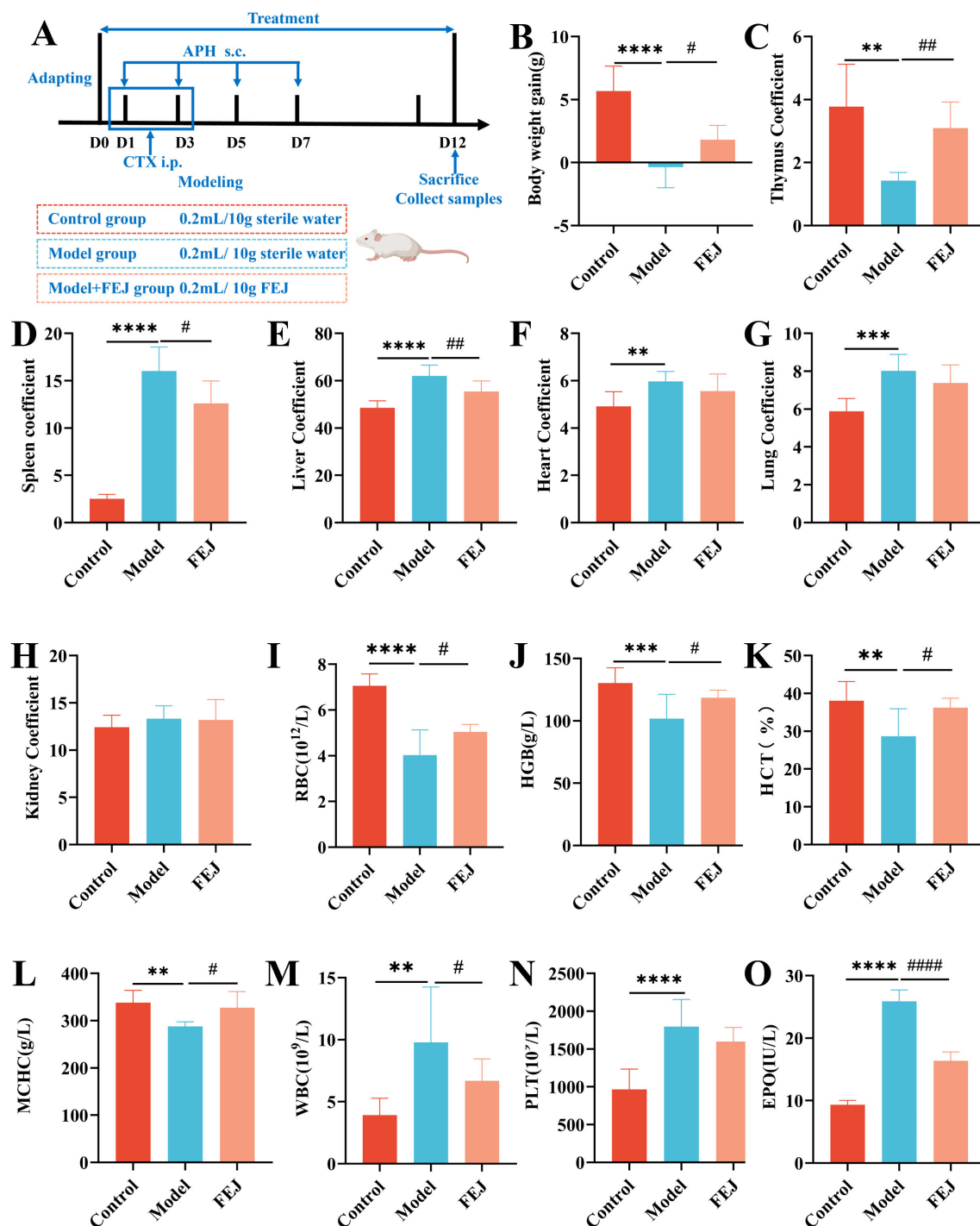


FIGURE 1

Impact of FEJ on weight loss, organ injury, blood routine and hematopoiesis cytokine abnormalities in mice. (A) The process of the experiment. (B) Body weight gain. (C) Thymus coefficient. (D) Spleen coefficient. (E) Liver coefficient. (F) Heart coefficient. (G) Lung coefficient. (H) Kidney coefficient. (I) The level of RBC. (J) The level of HGB. (K) The level of HCT. (L) The level of MCHC. (M) The level of WBC. (N) The level of PLT. (O) The level of EPO. Values are presented as mean $\pm$ SD ($n = 8$). ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus control group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ versus model group.

(Figure 1A). The model and FEJ groups, but not the control group, received subcutaneous injections of APH at a dose of 20 mg/kg on the first, third, fifth and seventh days, and intraperitoneal injections of CTX at a dose of 40 mg/kg on the first to third days to induce anemia (12). Subsequently, the control group and model group

were gavaged with water, whereas the FEJ group was gavaged with FEJ (8 mL/kg) last 12 days. FEJ is particularly popular for its remarkable performance in tonifying blood. In our previous study, we utilized it as a positive control and discovered that an 8 mL/kg dose of FEJ significantly alleviated anemia (13, 14). Consequently,

this dosage was still used in this study. After the final administration, mice were positioned in a sterile frame for fecal collection, and a minimum of three fresh feces particles were gathered for subsequent analysis. The researcher collected blood from the orbital sinus and reserved it for biochemical assays. The mice were anesthetized with ether and euthanized by cervical dislocation. All mice were dissected and thymus, spleen, liver, heart, lung, and kidney tissues were washed in 0.9% normal saline and weighed. The femur and portions of the spleen were preserved in 4% paraformaldehyde. All other samples were quickly transferred in -80°C for later use.

Body weight gain and organ indexes

Body weight gain was calculated using the formula: body weight gain = the final weight (12th day of treatment) (g) - the initial weight (g). The organ coefficient was computed using the formula: *organ coefficient* (mg/g) = *organ wet weight* (mg)/*body weight* (g) (15).

Routine blood test

The blood samples were obtained in tubes containing EDTA- K_2 anticoagulant. The white blood cells (WBC), RBC, hemoglobin (HGB) and platelets (PLT) were quantified in mice using Mindray automatic veterinary blood cell analyzer (BC-30Vet) (16). The remaining blood samples were utilized for additional testing.

Measurements of hematopoiesis related cytokine in serum

To isolate the supernatant, blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C (5415R, Eppendorf, Hamburg, Germany). The serum was then analyzed for EPO levels using enzyme-linked immunoassays at 450 nm, following the manufacturer's instructions (17).

Assessing oxidative stress factors in the liver

The liver tissue was homogenized and the T-AOC, SOD, and MDA levels were analyzed using a commercial kit, following the manufacturer's instructions (18).

Bone histological changes

After decalcification, the femur was processed into paraffin-embedded thin slices. These thin slices were stained with hematoxylin-eosin (H&E) for the examination of the histopathological changes within the bone marrow (15).

Spleen histological changes

The spleen was embedded in paraffin and thin slices of the paraffin blocks were stained with H&E for the histological examination (19). Additionally, paraffin spleen sections were employed for immunohistochemical staining (20). The spleen sections were trans-sectioned through incubation with 3% hydrogen peroxide in methanol for 25 min, incubated with primary antibodies overnight, incubated with a horseradish peroxidase-conjugated secondary antibody, and stained with peroxidase substrate DAB kit. Subsequently, sections were

counterstained with hematoxylin before examination under light microscopy. We analyzed the percentages of positive staining areas using the ImageJ 2.15.1 software (<https://imagej.net/software/fiji/>).

Measurement of short-chain fatty acids levels

The levels of SCFAs in feces were determined using a method adapted from a previous study (21). Briefly, a mixed standard stock solution A was prepared in n-butanol, comprising acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid and isohexanoic acid (Sigma Chemical Co., St. Louis, MO, USA). Stock solution B was prepared with n-butanol and internal standard 2-ethylbutyric acid (Sigma Chemical Co., St. Louis, MO, USA). Standard A and B solutions were mixed and diluted with n-butanol into 7 gradient concentrations of solutions. Then, feces (20 mg) were mixed with 500 μL 0.5% phosphoric acid water, ground and centrifuged at 13000 g for 15 min (Centrifuge 5430R, Eppendorf, Hamburg, Germany). The supernatant (200 μL) was combined with 200 μL of a 10 $\mu\text{g/mL}$ 2-ethylbutyric acid solution in n-butanol, and then centrifuged at 13000 g for 5 min. Samples were then analyzed using a GC system (8890B-5977B GC/MSD, Agilent Technologies, CA, USA) fitted with an HP-FFAP column (30 m $\times$ 0.32 mm $\times$ 0.25 μm , Agilent, CA, USA). The operating conditions were as follows: nitrogen as the carrier gas at a flow rate of 1 mL/min. The temperature was initially set to 80°C , then ramped up to 120°C at a rate of 20°C/min , followed by an increase to 160°C at a rate of 5°C/min , and held at 220°C for 3 minutes. The concentrations of SCFAs were calculated using linear regression standard curve.

Microbiome analysis via 16S rRNA sequencing

The total bacterial DNA was extracted from the fecal samples using the E.Z.N.A.[®] soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The V3-V4 hypervariable region of the 16S rRNA gene was targeted for amplification with primer pairs 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACH VGGGTWCTCTAAT) (22), and performed on an ABI GeneAmp[®] 9700 PCR thermocycler (ABI, CA, USA). Sequencing was utilized on the Illumina MiSeq PE300 platform (Illumina, San Diego, USA). UPARSE 7.1 (23) clustered the optimized sequences into operational taxonomic units (OTUs) at a 97% similarity level. Bioinformatic analysis was conducted on the Majorbio Cloud platform (<https://cloud.majorbio.com>). Non-metric multidimensional scaling analysis (NMDS) was conducted to analyze differences in bacterial community composition at the OTU level. A Linear Discriminant Analysis (LDA) score greater than 3.5 was considered the distinguishing feature. Each group comprised six samples.

Metabolomics analysis

The procedure for extracting fecal metabolites involved the following steps: Weighing 100 mg of feces, mixing with 400 μL of a 1:1 methanol/water solution, homogenizing using a Tissue Grinder (Tiss-24, Jingxin Industrial Development Co., Shanghai, China) and subsequently centrifuging for 10 min at 4°C and 13000 rpm. 300 μL mixture was added 10 μL of heptadecanoic acid methanol solution,

dried under nitrogen and combined with 50 μ L of methoxyl amine hydrochloride pyridine solution for 90 minutes (24). After the reaction, 50 μ L BSTFA was added and the mixture was subjected to a silicon alkylation reaction at 70°C for 1 hour. Finally, the supernatant was collected for analysis.

Metabolites analysis was performed using an Agilent GC-MS system equipped with an Agilent J&W DB-5ms Ultra Inert column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m). Nitrogen served as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 μ L. The temperature program began at 80°C, then ramped up to 120°C at a rate of 20°C/min, followed by a further increase to 160°C at a rate of 5°C/min, and held at 220°C for 3 minutes.

Experimental data were analyzed using the metaboanalyst 6.0 online platform. Statistical analyses, including Student's t-test and multivariate analysis of variance, were conducted. Metabolites with a VIP score > 1 and a *p*-value < 0.05 were considered significantly differentially expressed.

Gene expression profiling analysis

Spleen tissue RNA was extracted using the MJZol total RNA extraction kit (Majorbio, Shanghai, China) following the provided guidelines. The messenger RNA was then isolated through polyA selection with oligo(dT) beads and fragmented. Next, double-stranded cDNA was synthesized with the SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) employing random hexamer primers. The cDNA was processed for end-repair, phosphorylation and adapter addition in line with library construction protocol. Libraries were selected for 300 bp fragments on 2% Low Range Ultra Agarose and then PCR amplified with Phusion DNA polymerase (NEB) for 15 cycles. Libraries were quantified with Qubit 4.0 and sequenced on the NovaSeq X Plus platform (PE150). Reads underwent trimming and quality control using fastp (25) and were aligned to reference genome HISAT2 software (26). StringTie was employed to assemble reads in a reference-guided manner (27). DEGs (differential expression genes) were identified by the transcripts per million reads (TPM) expression analysis, with RSEM for gene quantification (28). DESeq2 was applied for differential expression analysis (29), identifying DEGs with $|\log_2\text{FC}| \geq 1$ and $\text{FDR} < 0.05$ as significantly differentially expressed. KEGG pathway analysis was performed using Python scipy software at Bonferroni-corrected *p*-value < 0.05.

Statistical analysis

We used GraphPad prism 8.3.0 software for statistical analysis. Results were expressed as mean $\pm$ SD, and statistical significance was set at *p* < 0.05.

Results

Impact of FEJ on body weight, organ indexes, peripheral blood cells and EPO

The model group exhibited a significant reduction in body weight gain and thymus coefficient compared to the control group

(*p* < 0.01, Figures 1B, C), and a significant increase in the coefficients of spleen, liver, heart and lung (*p* < 0.01, Figures 1D–G, Supplementary Figure S1). FEJ treatment showed a trend towards enhancing body weight gain and thymus coefficient and improving the swelling of spleen and liver (*p* < 0.05). However, FEJ had no significant effects on the heart and lung coefficient (*p* < 0.05, Figure 1H). No significant difference in the kidney coefficient was observed among the control, model and FEJ groups.

After 12 days treatment with FEJ, we determined the levels of blood routine indicators (Figures 1I–N). The model group showed significantly decreased levels of RBC, HGB, HCT and MCH (*p* < 0.01), and significantly increased levels of WBC and PLT (*p* < 0.01) compared to the control group, confirming the successful establishment of the anemic mouse model. Treatment with FEJ significantly elevated the RBC, HGB, HCT and MCHC (*p* < 0.05) and reduced the WBC (*p* < 0.01). Similarly, ELISA kits were used to detect the level of EPO in serum. FEJ significantly ameliorated the increase of EPO level in the model group (*p* < 0.0001, Figure 1O).

Impact of FEJ on liver oxidative stress markers

T-AOC, SOD and MDA levels in the liver serve as oxidative stress indicators. The control and FEJ groups demonstrated significantly higher levels of T-AOC and SOD compared to the model group (Figures 2A, B). On the other hand, the MDA content was significantly elevated in the model group compared to the control and FEJ groups (Figure 2C).

Impact of FEJ on bone histological features

The bone marrow is crucial for hematopoiesis in mice, serving as active hematopoietic tissue (30), and the impact of FEJ on bone marrow histopathology was assessed using H&E staining. Compared with the control group, the model group exhibited a significant abnormal proliferation and severe fatty degeneration of the bone marrow cells, leading to increased adipocyte formation and a subsequent decrease in erythroid, granulocytic and megakaryocytic cell counts. However, the FEJ group displayed an elevated bone marrow cellular density with plenty of hematopoietic cells, and there was a significant decrease in the number of adipocytes (Figure 2D).

Impact of FEJ on spleen histological features

The spleen is also an active hematopoietic tissue (30). H&E staining revealed distinct red pulp (RP) and white pulp (WP) in the spleen of the control group, with clear and obvious boundaries, and an apparent splenic trabecular structure. In contrast, the RP and WP were not clearly defined, and the WP was atrophied in the

model group. FEJ treatment led to a clearer boundary between the RP and WP and an expansion of the WP's marginal area (Figure 2E). Bcl-2 and Bax are important proteins in regulating cell apoptosis. We observed that the expression of Bcl-2 decreased

significantly ($p < 0.01$), the expression of Bax increased significantly ($p < 0.05$) and the ratio of Bax to Bcl-2 increased ($p < 0.05$) in the model group. Although FEJ administration was not able to restore the ratio of Bax to Bcl-2 ($p > 0.05$), it significantly enhanced the

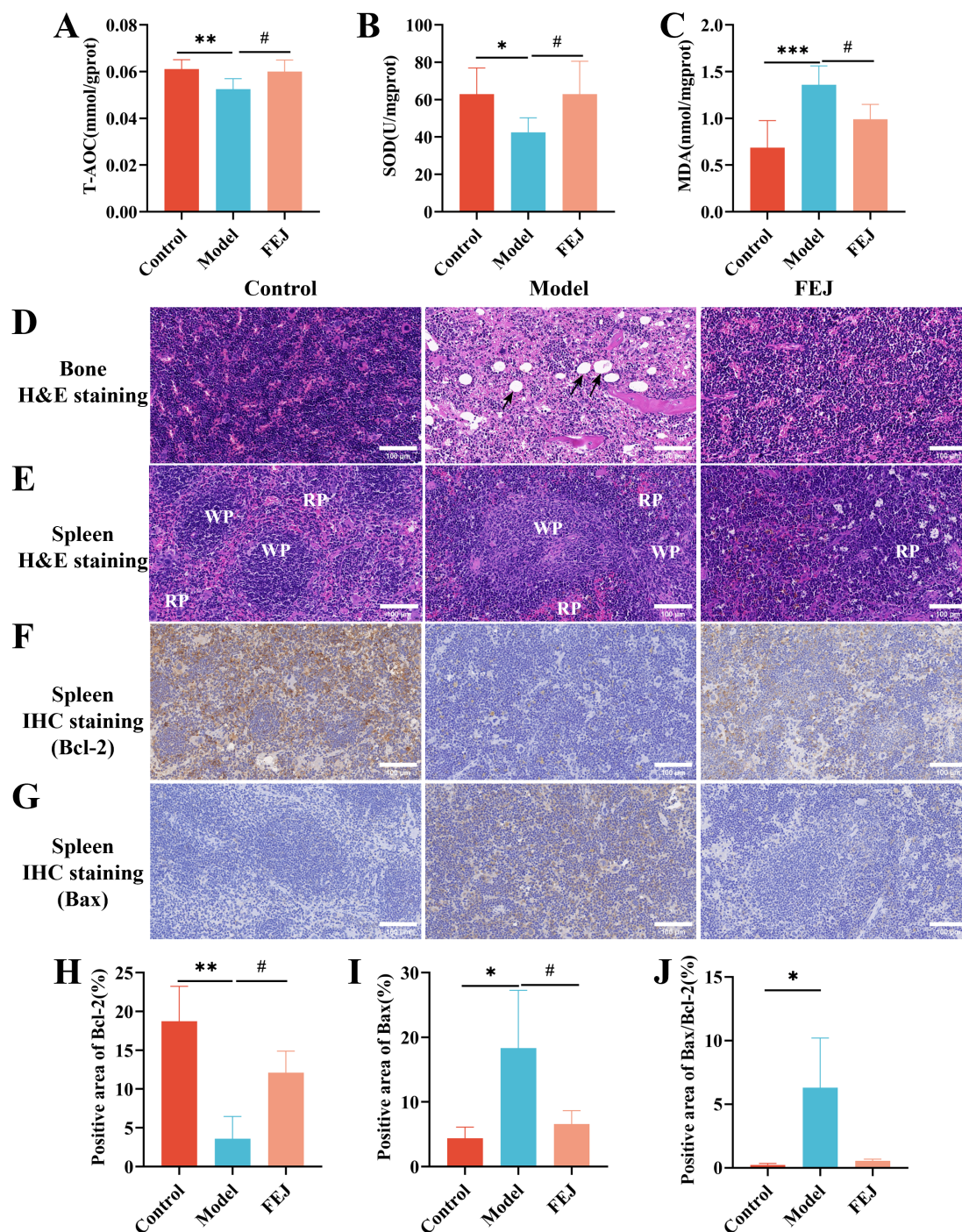


FIGURE 2

FEJ alleviated liver oxidative stress and promoted the recovery of bone and spleen tissue damage. The level of T-AOC (A), SOD (B) and MDA (C) in liver. Values are presented as mean $\pm$ SD ($n = 6$). Pathological sections of bone marrow (D) and spleen (E). (400 $\times$, scale bar: 100 μ m). Immunohistochemical staining for Bcl-2 (F) and Bax (G) of spleen. (400 $\times$, scale bar: 100 μ m). The percentages of positive staining area of immunohistochemical staining for Bcl-2 (H), Bax (I) and Bax/Bcl-2 (J). The black arrow indicates myeloid cell adipocytosis in (D). In (E) red pulp is denoted by RP, white pulp is denoted by WP. Values are presented as mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control group. # $p < 0.05$ versus model group.

expression of Bcl-2 ($p < 0.05$) and significantly reduced the expression of Bax significantly ($p < 0.05$) compared to the model group (Figures 2F–J).

Impact of FEJ on the SCFAs in the feces

Acetic acid, propionic acid, isobutyric acid, butanoic acid, isovaleric acid, valeric acid, isohexanoic acid, hexanoic acid and total SCFAs levels were significantly decreased in the model groups compared to the

control group ($p < 0.05$) (Figures 3A–I). Additionally, the FEJ group exhibited higher production of SCFAs compared to the model group. The most significantly abundant SCFAs were found to be propanoic acid, butanoic acids and total SCFAs ($p < 0.05$).

Impact of FEJ on gut microbiome

The rank-abundance curves and the rarefaction curves (Sobs index on OTU level) exhibited a flat profile (Figures 4A, B),

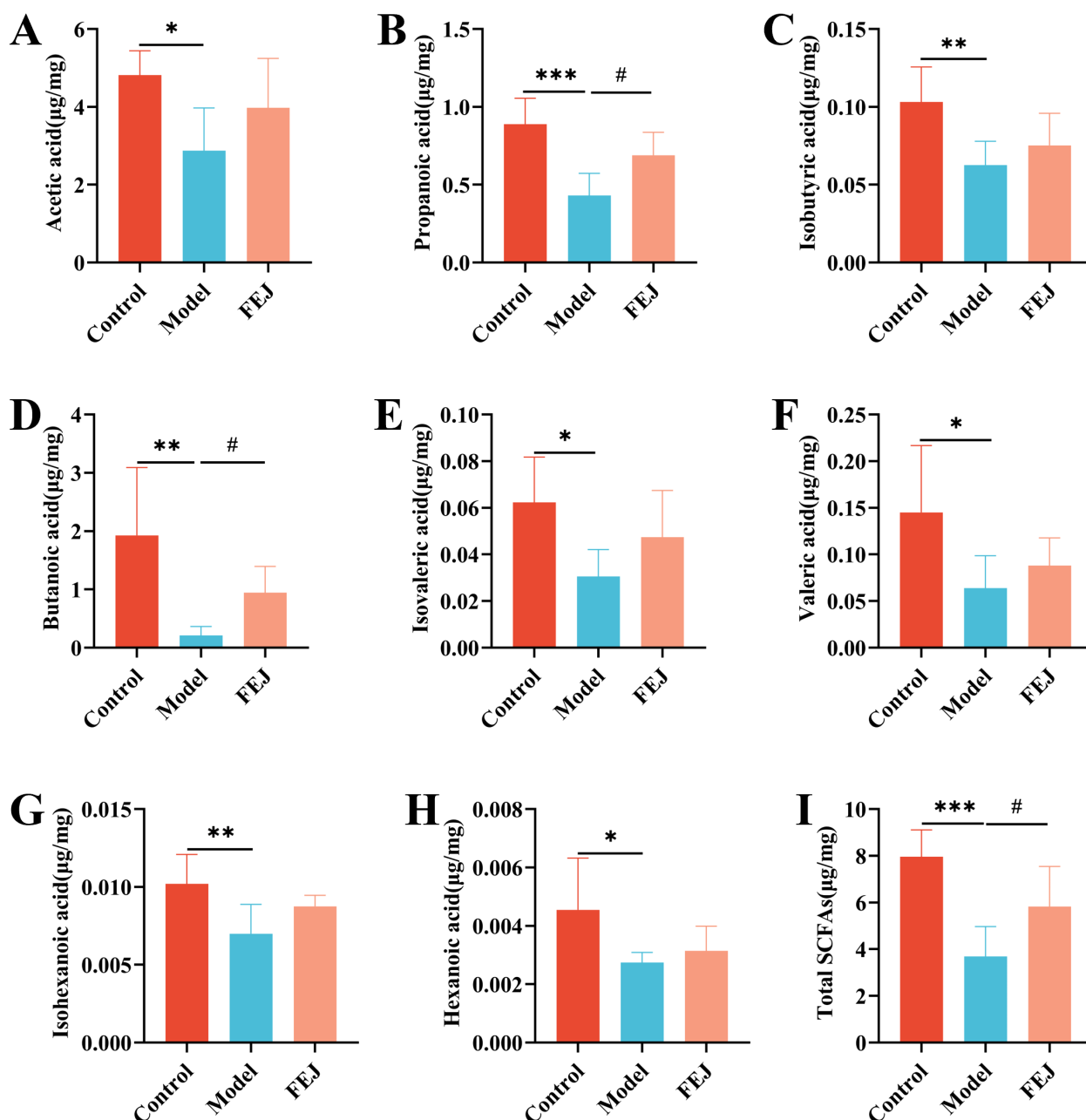
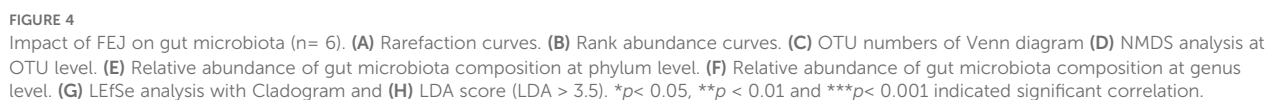


FIGURE 3

FEJ promoted the production of SCFAs in feces contents. (A) Acetic acid. (B) Propionic acid. (C) Isobutyric acid. (D) Butanoic acid. (E) Isovaleric acid. (F) Valeric acid. (G) Isohexanoic acid. (H) Hexanoic acid and (I) Total SCFAs. Values were expressed as means $\pm$ SD ($n=6$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control group. # $p < 0.05$ versus model group.

Figure 4E illustrated a histogram of species abundance, highlighting that the predominant intestinal bacteria at the phylum level consisted mainly of *Bacteroidota*, *Firmicutes*,



Patescibacteria, *Verrucomicrobiota*, *Proteobacteria* and *Desulfobacterota*, with *Bacteroidota* being the most dominant bacteria. Among them, the model group significantly down-regulated *Bacteroidota*, while up-regulated *Firmicutes* and *Verrucomicrobiota*. Conversely, FEJ treatment counteracted these changes, up-regulating *Bacteroidota* and down-regulating *Firmicutes* and *Verrucomicrobiota*.

As shown in Figure 4F, the distribution of genus level of intestinal bacteria was made up primarily of *norank_f_Muribaculaceae*, *Lactobacillus*, *Bacteroides*, *Lachnospiraceae_NK4A136_group*, *Alloprevotella*, *norank_f_norank_o_Clostridia_UCG-014*, *Candidatus_Saccharimonas*, *Faecalibaculum*, *Turicibacter* and *Akkermansia*. However, the abundance of *Turicibacter*, *Akkermansia*, *Romboutsia*, *Family_XIII_UCG-001*, *UCG-009*, *Negativibacillus*, *Tuzzerella*, *unclassified_f_Peptostreptococcaceae*, *norank_f_Erysipelatoclostridiaceae* and *unclassified_p_Firmicutes* significantly increased in the model group compared to the control group ($p < 0.05$). Following FEJ treatment, there was a notable decrease in the abundance of *Family_XIII_UCG-001*, *UCG-009*, *Negativibacillus* and *Tuzzerella* ($p < 0.05$).

We used LEfSe analysis to determine the classified bacterial taxa that were altered. The LDA scores and taxonomic diagram are displayed in Figures 4G, H. Twenty-seven distinct classes of taxa were identified, exhibiting varying degrees of richness among three groups. *C_Bacteroidia* were the dominant microbiota in the control group, *p_Firmicutes* and *g_Turicibacter* were the dominant microbiota in the model group and *g_Faecalibaculum* was the primary microbiota in the FEJ group. Overall, these findings indicate that FEJ treatment reversed the composition and structure of the gut microbiota of CTX and APH-induced mice.

Impact of FEJ on fecal metabolome

Metabolites in fecal samples were analyzed from the control, model group and FEJ groups in order to overall assess the metabolomics regulation of FEJ intake in anemic mice. Principal component analysis (PCA) clearly indicated a trend of separation between the control group and the model group, suggesting differences in metabolites between the two groups. Additionally, the spatial distances of the FEJ group were closer to the control compared to the model group (Figure 5A). OPLS-DA based S-plot visualization is often used to explore different metabolites between groups and assess integrating VIP (>1), p (<0.05). The results demonstrated that the groups were well grouped as shown in Figures 5B–E. A permutation test was conducted on the model to prevent overfitting and the results indicated that the model was applicable (Figures 5F, G). Following a comparison with the database, a total of 30 and 16 differential metabolites were identified between the model and FEJ groups, respectively (Table 1). The variation of all differential metabolites in the two comparison groups (the control group compared with the model group and the model group compared with the FEJ group) was displayed in Figure 5H. The two comparison groups shared 15 differential metabolites. After FEJ treatment of anemic mice, five

metabolites were upregulated, such as ethanedioic acid, D-glucopyranoside, octadecanoic acid, sebacic acid and isoquinoline, whereas ten metabolites were downregulated, such as L-valine, L-leucine, L-proline, glycine, threonine, 2-piperidone, ornithine, phenylalanine, pyrimidinetrione and L-tyrosine.

Subsequently, the significant metabolites were conducted for metabolic pathway analysis via metaboanalyst. Seven primary altered pathways were identified in the model vs. control/FEJ groups (Figure 5I, impact value > 0.1) as follows: phenylalanine, tyrosine and tryptophan biosynthesis, glycine, serine and threonine metabolism, phenylalanine metabolism, starch and sucrose metabolism, arginine and proline metabolism, glyoxylate and dicarboxylate metabolism and tyrosine metabolism.

Impact of FEJ on spleen transcriptome

We performed transcriptome analysis on spleen tissues from the control, model and FEJ groups to interpret the effects of FEJ on anemia mice. This analysis revealed a total of 8679 DEGs between the model group and the control group, and 2059 DEGs in the FEJ group compared to the model group (Figures 6A, B). PCA revealed distinct clusters among the groups. The model group was distinguished from the control group, and the FEJ group showed intermediate variability between the control and model groups in Figure 6C. The Venn diagram and cluster analysis heat map revealed the restoration of 1297 DEGs after FEJ treatment, which were previously skewed by CTX/APH (Figures 6D, E). Based on the KEGG pathways classification, six, six, three, two, two and one pathways were associated with human diseases, organismal systems, cellular processes, environmental information processing, genetic information processing and metabolism pathways, respectively (Figure 6F). The KEGG enrichment analysis revealed that the DEGs reversing by FEJ were found significantly enriched in Fc gamma R-mediated phagocytosis, NF- κ B signaling pathway, hematopoietic cell lineage, GnRH secretion, RNA polymerase, osteoclast differentiation and multiple immune-related signaling pathways (B cell receptor signaling pathway, leukocyte transendothelial migration, natural killer cell mediated cytotoxicity, chemokine signaling pathway, primary immunodeficiency and T cell receptor signaling pathway), etc ($p < 0.05$, Figure 6G).

Analyses of the microbiome, metabolome and transcriptome correlations

Analysis of correlations between hematopoietic index and microbiome

Spearman's correlation analysis was conducted to explore the connection between the hematopoietic-related traits and the genus level of the intestinal flora (Figure 7A). The richness of above mentioned ten kinds of different gut microbiota exhibited positive correlations with WBC, PLT, EPO and MDA while negative correlation with SOD, HGB, T-AOC, RBC, HCT and MCHC.

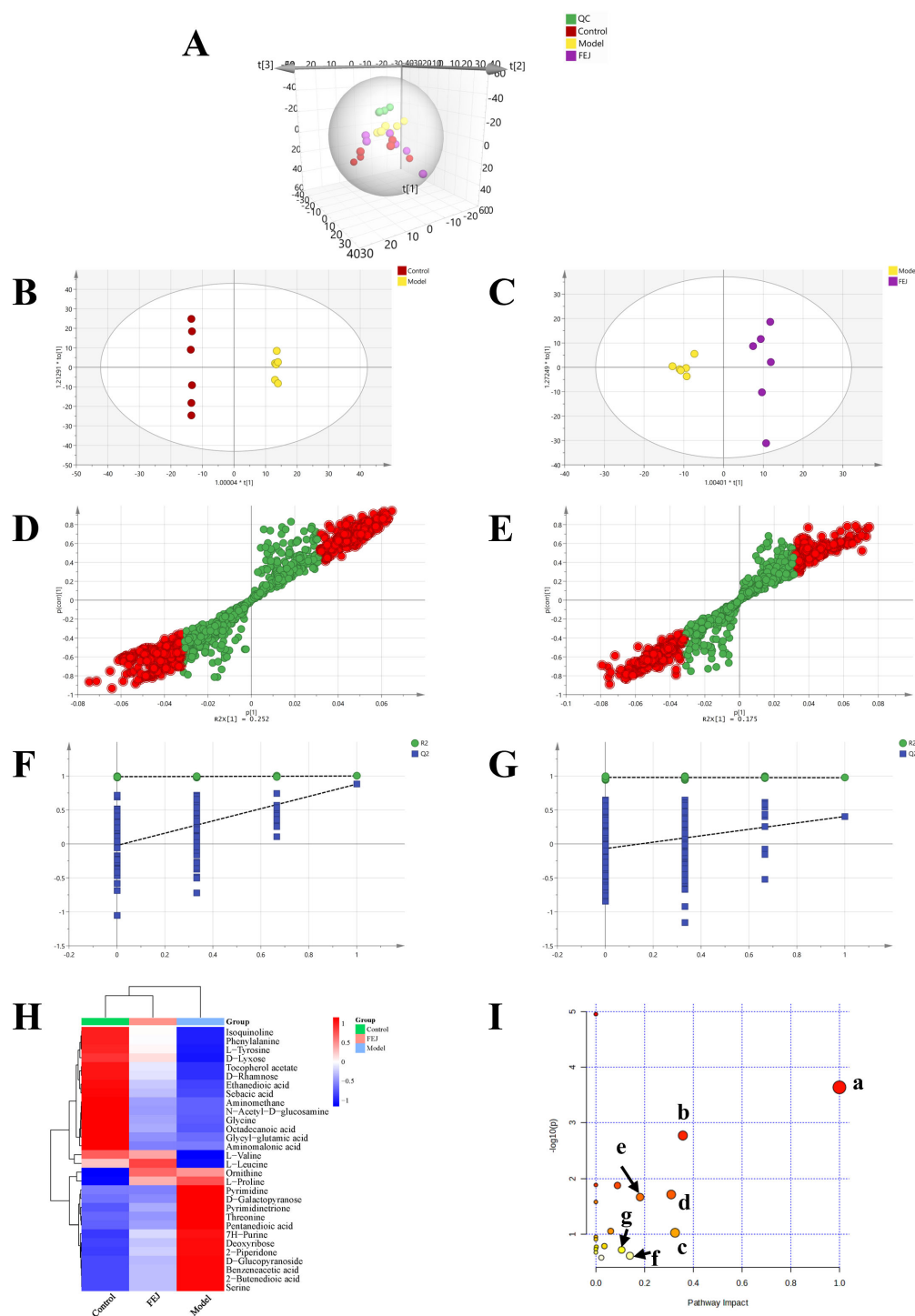


FIGURE 5

Impact of FEJ on fecal metabolic profile in anemia mice ($n = 6$). **(A)** The PCA score plots among all groups ($R^2X = 0.657$, $Q^2 = 0.381$). The OPLS-DA score plots between **(B)** the control and model groups ($R^2Y = 1$, $Q^2 = 0.879$), **(C)** the model and FEJ groups ($R^2Y = 0.974$, $Q^2 = 0.403$). The permutation test analysis between **(D)** the control and model groups, **(E)** the model and FEJ groups. Score plot from OPLS-DA model classifying **(F)** the control and model groups, **(G)** the model and FEJ groups. **(H)** The heatmap of all the differential metabolites. **(I)** Summary of pathway analysis in the fecal. a-phenylalanine, tyrosine and tryptophan biosynthesis; b-phenylalanine metabolism; c-starch and sucrose metabolism; d-glycine, serine and threonine metabolism; e-arginine and proline metabolism; f-tyrosine metabolism; g-glyoxylate and dicarboxylate metabolism; KEGG, Kyoto Encyclopedia of Genes and Genomes.

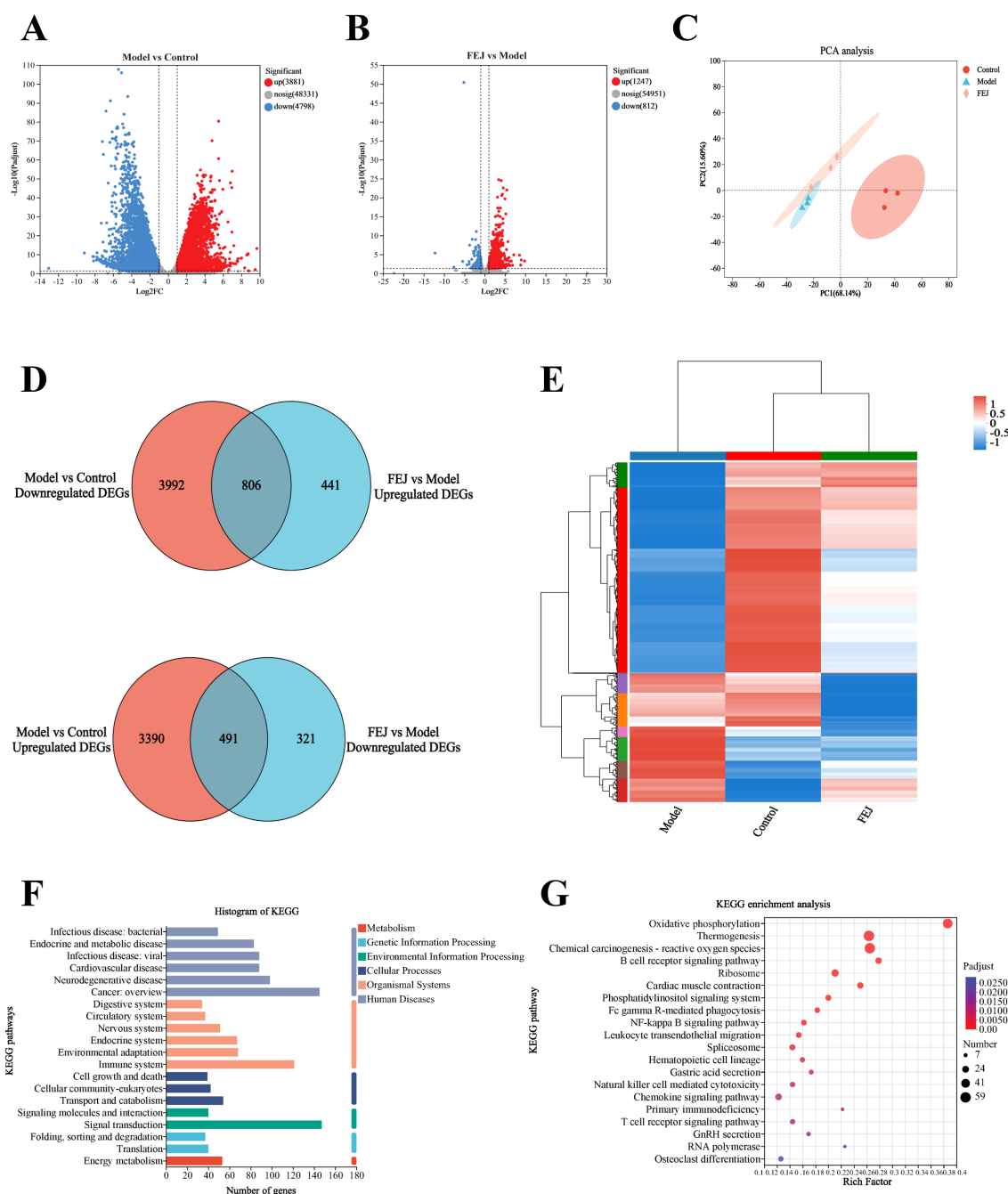


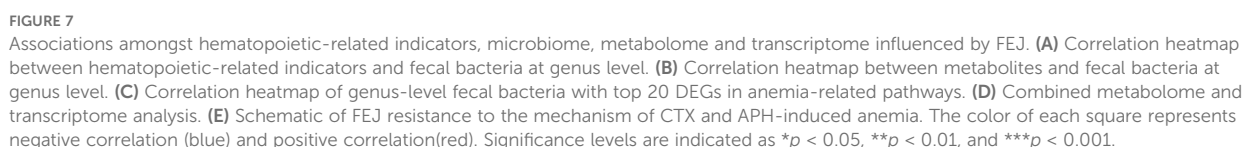
FIGURE 6

Impact of FEJ on spleen transcriptome profile in anemia mice. Volcano plots of differentially expressed genes in (A) the control group vs model group, (B) the model group vs FEJ group (DEGs, $n = 3$ mice per group). (C) PCA among the three groups. (D) Venn diagram of up-regulated and down-regulated DEGs among the three groups. (E) Heatmap showing the cluster analysis of DEGs. (F) Functional annotation of DEGs among the three groups. (G) KEGG pathway enrichment analysis of DEGs among the three groups. KEGG, Kyoto Encyclopedia of Genes and Genomes.

Analysis of correlations between microbiome and metabolome

FEJ influenced the gut microbiota composition and metabolic profiles, with Spearman's correlation analysis revealing correlations between 15 significantly changed fecal metabolites and 10 gut microbiota at the genus level (Figure 7B). L-valine, L-leucine,

phenylalanine, pyrimidinetrione, ornithine, 2-piperidone, threonine, L-tyrosine, L-proline and glycine were significantly positively correlated with 7, 8, 8, 5, 5, 5, 5, 7, 5 and 6 gut microbiota, respectively. Ethanedioic acid, octadecanoic acid, isoquinoline, D-glucopyranoside and sebacic acid were negatively correlated with 1, 8, 7, 2 and 5 gut microbiota.



correlation with at least three representatives of fecal *Akkermansia*, *Unclassified_p_Firmicutes*, *Family_XIII_UCG-001*, *Romboutsia*, *Unclassified_f_Peptostreptococcaceae*, *Norank_f_Erysipelatoclostridiaceae*, *Tuzzerella*, *UCG-009*, *Negativibacillus* and *Turicibacter*.

TABLE 1 Differentia metabolites among the control group, model group, and FEJ group.

NO	Metabolites	Formula	RT (min)	m/z	p(Con vs Mod)	VIP(Con vs Mod)	Trend(Con vs Mod)	Trend (Mod vs FEJ)
1	ethanedioic acid	C ₂ H ₂ O ₄	11.16	73.1	0.029	1.2300	↓*	↑*
2	L-valine	C ₅ H ₁₁ NO ₂	12.86	145.1	0.002	1.8429	↑**	↓**
3	L-leucine	C ₆ H ₁₃ NO ₂	13.98	159.1	0.002	1.6967	↑**	↓*
4	L-proline	C ₅ H ₉ NO ₂	14.50	142.0	0.011	1.4305	↑*	↓*
5	glycine	C ₂ H ₅ NO ₂	14.64	174.0	0.000	1.9300	↑****	↓**
6	threonine	C ₄ H ₉ NO ₃	15.92	117.0	0.002	1.5396	↑**	↓*
7	2-piperidone	C ₅ H ₉ NO	17.02	128.0	0.000	1.4375	↑****	↓*
8	ornithine	C ₅ H ₁₂ N ₂ O ₂	19.09	142.0	0.004	1.5736	↑**	↓*
9	phenylalanine	C ₉ H ₁₁ NO ₂	19.28	219.0	0.000	1.9824	↑****	↓**
10	pyrimidinetrione	C ₄ H ₄ N ₂ O ₃	21.46	256.1	0.005	1.8164	↑**	↓*
11	D-glucopyranoside	C ₆ H ₁₂ O ₆	21.70	204.0	0.014	1.1096	↓**	↑*
12	L-tyrosine	C ₉ H ₁₁ NO ₃	22.68	219.0	0.003	1.5188	↑**	↓*
13	octadecanoic acid	C ₁₈ H ₃₆ O ₂	25.91	73.1	0.000	1.8535	↓****	↑*
14	sebacic acid	C ₁₀ H ₁₈ O ₄	28.05	331.1	0.022	1.5156	↓*	↑*
15	isoquinoline	C ₉ H ₇ N	30.39	192.0	0.000	2.3461	↓****	↑**

*p < 0.05, **p < 0.01 and ****p < 0.0001 indicated significant correlation. VIP value was obtained from the OPLS-DA model. The symbol ↑ (FC > 1) up or ↓ (FC < 1) down arrows represent the relatively increased or decreased levels of the metabolites, respectively.

Analysis of correlations between microbiome and transcriptome

According to padjust value < 0.05, we screened out hematopoietic-related functional pathways, including B cell receptor signaling pathway, NF-κB signaling pathway, leukocyte transendothelial migration, hematopoietic cell lineage, T cell receptor signaling pathway and osteoclast differentiation. The top 20 DEGs were related to hematopoiesis pathways based on the Log2 FC (the FEJ group vs. the model group). Then, Spearman’s correlation analysis was conducted to examine the gut microbiota and the top 20 DEGs involved in hematopoietic pathways. As depicted in Figure 7C, Akkermansia, Romboutsia, Turicibacter, Family_XIII_UCG-001 and Tuzzerella were notably associated with KEGG pathways implicated in anemia. For instance, Romboutsia, Turicibacter and Tuzzerella showed a significantly negative correlation with Edaradd, Ncf2, Cd19, Cd22, I15ra, Fcer2a, Ncf1, and Plcg2. Additionally, Akkermansia, Romboutsia and Turicibacter exhibited significantly negative correlations with Pirb and Vav3. Moreover, Romboutsia and Turicibacter were significantly negatively correlated with Atm, and Family_XIII_UCG-001 was negatively correlated with Edaradd, Ncf2, and Plcg2.

Integrative analysis of metabolome and transcriptome

An integrative analysis of the transcriptome and metabolome was carried out to investigate the links between DEGs and metabolites. Figure 7D illustrated that 304 KEGG pathways were altered at the transcriptional level and 19 at the metabolome level. It was worth mentioning that 14 KEGG pathways were affected in both, among which nine pathways were associated with anemia,

namely, starch and sucrose metabolism; valine, leucine and isoleucine biosynthesis; glutathione metabolism; arginine and proline metabolism; porphyrin metabolism; glycine, serine and threonine metabolism; phenylalanine metabolism; pantothenate and CoA biosynthesis; and tyrosine metabolism. Starch and sucrose metabolism belongs to carbohydrate metabolism. Valine, leucine and isoleucine biosynthesis, glutathione metabolism, arginine and proline metabolism, glycine, serine and threonine metabolism, phenylalanine metabolism and tyrosine metabolism belong to amino acid metabolism. Porphyrin metabolism and pantothenate and CoA biosynthesis belong to the metabolism of cofactors and vitamins.

Discussion

The global burden of anemia is considerable, accounting for 8.8% of disability from all conditions (31). Anemia increases in prevalence with increasing age and frailty (32). Even without underlying health conditions, mild anemia is associated with impaired functional capacity, reduced physical performance and a diminished quality of life (33). People with anemia exhibit different traits from normal individuals, including body weight, spleen, liver, hematopoietic cytokines, fecal microbes, host genes and metabolites (34, 35). We observed that FEJ had beneficial effects on anemia induced by CTX and APH in mice and investigated its antianemia mechanism through microbiome, metabolome, and transcriptome analyses.

After modeling, the anemia mice exhibited a poor appearance, including easily shedding fluffy hairs, pale ears, noses, faces and feet,

sleepiness, mobility retardation and emaciation. FEJ could markedly reverse the weight loss and appearance caused by anemia modeling. The blood routine data revealed that the peripheral RBC, HGB, HCT and MCHC of the model mice were decreased significantly, which are hallmark indicators of anemia. Meanwhile, WBC counts in the model group were higher than in the control group, indicating a compensatory leukocytosis triggered by CTX and APH (36). After treatment with FEJ, the peripheral blood cell count also showed a significant return to normal values. EPO is a hematopoietic growth factor which regulates blood cell production (37), mainly stimulating RBC production. EPO secretion generally helps to maintain normal levels of RBC and HGB for effective oxygen delivery to body (38). In regenerative anemia, elevated EPO levels respond to decreased HGB levels, reflecting a loss of erythrocytes due to hemolysis or bleeding (39). In our investigation, the EPO concentrations of serum samples in the model group were higher than in the control group, but decreased significantly with FEJ intervention, suggesting that FEJ may ameliorate hematopoietic damage by regulating EPO production. These findings implied that FEJ may facilitate hematopoietic rehabilitation in the model mice, which confirmed its traditional efficacy of tonifying blood.

It has been demonstrated that APH and CTX can impair the body's antioxidant system, reducing its capacity for oxidative stress (40). T-AOC and SOD serve as pivotal indicators of antioxidant capacity. A dysregulation of oxidative stress response results in inadequate antioxidant capacity and lipid peroxidation, ultimately increasing the lipid peroxidation marker MDA, further aggravating bodily injury (41). FEJ increased the T-AOC and SOD levels in the liver while reducing MDA levels, indicating that FEJ enhanced antioxidant capacity in anemia mice.

The bone marrow has hematopoietic and immune defense functions, containing hematopoietic cells at various maturation stages (42). CTX can disrupt the blood-forming microenvironment, leading to inhibition of bone marrow hematopoiesis (15). FEJ could alleviate the severe damage of bone marrow in the histopathological lesions of the anemia model by decreasing adipocyte numbers and increasing hematopoietic cell counts in the bone marrow.

The thymus serves as a pivotal lymphoid organ responsible for the generation of T cells, which are essential for regulating both cellular and humoral immunity (43). The spleen is a critical organ for hematopoiesis, immunity and clearance of particulate matter from the blood, and its size and constituents are altered by anemia. The degrees of spleen enlargement are diagnostic features for anemia (44–47). Notably, FEJ successfully reversed the abnormalities in the size of the spleen and thymus. Moreover, FEJ was capable of restoring the spleen's structural integrity to normal and facilitating the recovery of splenic function. Bax and Bcl-2 are apoptosis regulatory proteins. Bcl-2 can protect cells from mitochondrial damage and inhibit apoptosis, while Bax can increase mitochondrial membrane permeability, thereby promoting cell apoptosis (48). In the present investigation, apoptotic spleen cells and Bax expression were significantly increased and Bcl-2 expression was decreased in the model group. Treatment with FEJ notably normalized Bcl-2 and Bax expression, curtailing

spleen cell apoptosis. Collectively, these observations imply that FEJ may promote the recovery of hematopoietic and immune function.

SCFAs, which are predominantly produced by the gut microbiome through the fermentation of complex carbohydrates, play a crucial role in maintaining intestinal homeostasis and barrier function (49). Acetic acid, propionic acid and butyric acids are the most abundant SCFAs. Anemic patients exhibit reduced SCFAs concentrations in feces or serum due to the impaired synthesis of SCFAs. Butyrate and supplementation with butyrate can restore iron metabolism to ameliorate anemia (50). Additionally, propionate can modulate the differentiation of bone marrow hematopoietic cells and effectively combat iron deficiency anemia (51). In this research, the concentrations of propanoic acid, butanoic acid and total SCFAs in fecal samples treated with FEJ were markedly elevated compared to those in the model group, suggesting that FEJ improved anemia by increasing the concentration of SCFAs.

Compared with the control group, some pathogenic bacteria exhibited significant changes in the model group, such as *Turicibacter*, *Akkermansia*, *Romboutsia*, *Desulfobacterota*, *Negativibacillus* and *Erysipelotrichaceae*. FEJ modulated the composition of the gut microbiota in anemia mice at the genus level, significantly decreasing the abundance of genera such as *Family_XIII_UCG-001*, *UCG-009*, *Negativibacillus* and *Tuzzerella*. *Turicibacter*, related to inflammation, has been linked to intestinal barrier disruption upon its elevation (52). *Akkermansia* was previously identified as a bacterium with probiotic properties and a health marker in healthy individuals. Multiple studies *in vivo* revealed its critical role in modulating inflammation, enhancing immune function, maintaining energy homeostasis, inhibiting pathogenic microbes, improving metabolic disorders, suppressing tumors, regulating autoimmune responses and alleviating psychological disorders. Clinical observations indicated a reduced abundance of *Akkermansia* in patients with diabetes, obesity, intestinal disorders and metabolic diseases compared to healthy individuals (53). However, other studies showed adverse effects associated with elevated abundance of *A. muciniphila*, including on the induction of Parkinson's disease and ulcerative colitis (54). Additionally, the pathobionts *Akkermansia* expanded in the mice with severe anemia and monocytopenia (55), and overcolonization of *Akkermansia* was found to exacerbate intestinal diseases through disrupting the intestinal barrier by consuming excessive mucin (56).

The abundance of *A. muciniphila* significantly increased in 4-day fasted Syrian hamsters and 30-day fasted Burmese pythons, associated with dietary iron inadequate (57, 58). This mucin-degrading bacteria gained a competitive advantage during nutrient deprivation due to its ability to utilize mucin as a constant nutrient source. Host mucus production and secretion were correlated with dietary iron content. Furthermore, *A. muciniphila* exhibited advantage for growth in vehicle-treated mice with iron-deficiency anemia (IDA) induced by an iron-deficient diet. The study hypothesized that IV iron treatment elevated plasma iron levels, providing luminal iron source to other gut microbes and thereby reducing *A. muciniphila* abundance in iron-supplemented groups (59). These contradictory findings highlight the need for comprehensive

investigations to validate the characteristics of *Akkermansia*. Additionally, *Romboutsia* proliferates in gastrointestinal disorders, including irritable bowel syndrome, and its levels is positively correlated with pro-inflammatory cytokines, such as TNF (60). The strain *Desulfovibrio desulfuricans* in the phylum *Desulfobacterota* can exacerbate intestinal permeability and boost systemic inflammation when administered orally (61, 62). Mice with obesity-related disorders have a high content of *Negativibacillus* in cecal (63). *Erysipelotrichaceae* strains are associated with colitogenic effects (64). The abundance of *Family_XIII_UCG-001* increases when inflammation occurs (65). The increased risk of disease is positively correlated with *UCG-009* abundance (66). *Tuzzerella* is associated with the development of IBD, as its upregulation can promote an inflammatory response and increase intestinal permeability (67). Consuming polystyrene microplastics can cause liver damage, leading to an increase in pathogenic *Tuzzerella* (67, 68). DHA-enriched phosphatidylcholine alleviate NAFLD by improving hepatic oxidative stress (MDA, SOD) and reducing the abundance of *Tuzzerella* (69).

In this study, Spearman's correlation analysis was conducted to examine the relationship between gut microbiota and hematopoietic indicators. *Akkermansia* and *Tuzzerella* showed significant positive correlations with WBC, PLT, EPO and significant negative correlations with HGB, RBC, HCT and MCHC. In addition, a positive correlation was found between *Tuzzerella* and MDA, but a negative correlation was found between *Tuzzerella* and other oxidative stress indicators (T-AOC and SOD). The results suggested that *Akkermansia* and *Tuzzerella* may be potential markers for improving hematopoietic function. Therefore, FEJ may ameliorate anemia by inhibiting the proliferation of harmful bacteria and by modulating the structure of gut microbiota, thereby controlling inflammation, intestinal barrier and liver oxidative stress in mice.

Fecal metabolites include own metabolites of the host and the metabolites of gut microbiota, which can reveal the activity of microbial communities and their symbiotic relationship with the host. Fecal metabolomics based on GC-MS technology has been applied on the therapeutic effect of FEJ in CTX and APH-induced anemic mice for the first time in our study. The fecal metabolomics profile revealed a disorder in amino acid metabolism in the model group, predominantly affecting neutral and acidic amino acids with a polyanionic group structure. The presence of the negative ion group exerts a profound influence on the oxygen-binding capacity of Hb, facilitating its linkage with dextran (70). The elevated valine level in anemia rats reflects liver failure due to the usage of APH (70). Generally, valine and leucine collaborate to support normal body growth, assist in tissue repair, regulate blood sugar, and supply energy to the body (71). Moreover, the liver antioxidant activity is disrupted in anemia mice induced with CTX and APH, which could be related to the elevated glycine level (72). In addition, glycine plays a role in regulating serum iron and accelerating serum protein synthesis. Threonine has the function of enhancing immunity. It is reported that *Angelica sinensis* exerted blood enrichment by decreasing abnormally elevated glycine and threonine levels (73). FEJ could significantly decrease glycine and threonine, speculating that the FEJ nourished blood by regulating glycine, serine and

threonine metabolism. Phenylalanine is an essential aromatic amino acid, and abnormally high levels of plasma phenylalanine in patients with severe aplastic anemia may cause bone marrow and hematopoietic stem cell injury (74). A damaged liver can't catabolize phenylalanine and tyrosine, leading to elevated concentrations of them in individuals with liver disease (75). FEJ influenced the metabolic pathways of phenylalanine, tyrosine and tryptophan biosynthesis, in line with previous reports (76). By regulating the concentrations of these amino acids, FEJ potentially facilitated hemoglobin's oxygen affinity, enhanced liver antioxidant activity, and improved the cellular microenvironment involved in anemia, suggesting hepatoprotective effects and hematopoietic support. Moreover, proline and ornithine were significantly different metabolites between the model group and the FEJ group, with their enrichment noted in the arginine and proline metabolism pathway. Arginine is capable of biosynthesizing both proline and ornithine (77, 78). Proline promotes the formation of osteoblastogenesis. It is speculated that FEJ regulated the formation and function of osteoblasts by participating in arginine and proline metabolism pathway. Methyl α -D-glucopyranoside, which has demonstrated a strong binding capacity to hemoglobin E in molecular docking studies, is a potential drug candidate for deficient hemoglobin synthesis in β -thalassemia (β -TH) (79). Octadecanoic acid, a beneficial saturated fatty acid, helps to maintain membrane dynamics. Low levels of octadecanoic acid can weaken the RBC membrane. Starch and sucrose metabolism along with dicarboxylate metabolism altered in β -TH patients (80), which is consistent with our findings.

In the correlation analysis, *UCG-009*, *Family_XIII_UCG-001*, *Tuzzerella* and *Negativibacillus* was found to be positively correlated with the levels of L-leucine, phenylalanine, L-proline and glycine and negatively correlated with the levels of octadecanoic acid. Anemia and blood deficiency are linked to a spectrum of metabolic disturbances including amino acid metabolism (16, 81, 82). These results suggested that FEJ may regulate phenylalanine metabolism, glycine, serine, and threonine metabolism, arginine and proline metabolism, as well as octadecanoic acid in anemic mice by influencing these gut microbiota. *Turicibacter*, *Tuzzerella*, *Negativibacillus* and *Romboutsia* were found to be significantly negatively correlated with propanoic acid and butanoic acid. Gut dysbiosis inhibited the production of SCFAs in thalassemia mice (83). The increase in the harmful bacteria *Turicibacter*, *Tuzzerella*, *Negativibacillus* and *Romboutsia* disrupted a balance of gut flora, resulting in decreased SCFAs content (84–88). SCFAs and anemia-related metabolic pathways interact closely with intestinal flora (89). The relative abundance of *Tuzzerella* and *Negativibacillus* were upregulated in anemia mice and FEJ treatment could significantly restore their abundance to normal levels. FEJ may alleviate anemia by down-regulating *Tuzzerella* and *Negativibacillus*, thereby promoting the production of SCFAs. The changes in gut microbiota induced by FEJ were likely to cause corresponding variations in metabolism, which emphasized the role of FEJ in modulating intestinal metabolism and maintaining gut microbiota homeostasis in anemic mice. Especially, targeted metabolomics of

SCFAs based on GC-MS and 16S rRNA sequencing combining with Spearman's correlation analysis of hematopoietic indices were firstly employed to find potential targets.

A previous study showed that the nourishing blood mechanisms of E'jiao in bone marrow cells, as revealed via RNA sequencing in a 5-fluorouracil-induced myelosuppression mouse model, were associated with hematopoietic cell lineage, osteoclast differentiation, T cell receptor signaling pathways and NF- κ B signaling pathways, etc (90). Spleen is one of the primary organs and is most susceptible to anemia. Spleen dysfunction is closely linked to blood deficiency and immune dysregulation. The accumulated bone marrow progenitor cells and RBC can induce splenic enlargement, exacerbating anemic symptoms (91). However, no RNA-seq-based study has been used in the therapeutic effect of FEJ on spleen function in CTX and APH-induced anemic mice. Among the in spleen pathways modulated by FEJ treatment, the B cell receptor signaling pathway stood out, involving *Plcg2* and *Vav3*. The knockdown *Plcg2* in megakaryocytes (Mks) reduces thrombopoietin (*Thpo*) expression, leading to decreased bone marrow hematopoietic stem cells (HSCs) quiescence and repopulation potential, as well as extramedullary hematopoiesis (92). *Vav3* is critical for B and T cells development and osteoclast function (93). FEJ also regulated the NF- κ B signaling pathway, including *Edaradd*, *Card10* and *Atm*. *Edaradd* knockdown in tongue squamous cell carcinoma (TSCC) cells reduces proliferation and induces apoptosis (94), while *Atm* deficiency disrupts hematopoiesis and reduces myeloid and lymphoid hematopoietic cells in zebrafish (95, 96). Transcriptomic data showed FEJ significantly modulated hematopoietic cell lineage biomarkers (*Cd19*, *Cd22*, *Il5ra*, *Fcer2a*, *Cd33* and *Csf2ra*), which are pivotal for hematopoietic cells maturation and specialization (97, 98). *Cd19* promotes B lymphocyte maturation and activation (99), while *Cd22* negatively regulates B-cell signaling pathways (100, 101). *Il5ra* and *Fcer2a* promote eosinophil development and neutrophil apoptosis, respectively (102, 103). FEJ also regulated the osteoclast formation including *Lilrb4a*, *Ncf2*, *Ncf1*, *Pira12*, *Pira1*, *Lilra6* and *Pirb*. Endochondral ossification is tightly associated with the generation of the hematopoietic stem cell in bone marrow (104). *Ncf2* indirectly promotes osteoclast differentiation (105), and mutations in *Ncf2* or *Ncf1* are correlated with chronic granulomatosis (CGD), a genetic disorder often accompanied by anemia (106). *Pirb* supports the stemness of hematopoietic stem and progenitor cells (HSPCs) in hematopoietic organs (107). The FEJ group restored EPO levels and *OsM* expression, potentially through modulation of hematopoietic cell lines and osteoclastic bone marrow. Elevated EPO levels represent the physiological response to anemia (108). Animals lacking the Oncostatin M (OsM) receptor suffer from anemia (109, 110) and EPO stimulation activates OsM in erythroblasts, thereby stimulating osteoclast differentiation (108, 111, 112).

The Spearman's correlation analysis revealed significant negative correlations between intestinal flora including *Akkermansia*, *Romboutsia*, *Turicibacter*, *Family_XIII_UCG-001* and *Tuzzerella*, and biomarker genes in B cell receptor signaling pathway, NF- κ B

signaling pathway, hematopoietic cell lineage and osteoclast differentiation. Benzene exposure deregulates the B cell receptor signaling pathway by down-regulating *CD22*, inhibiting NF- κ B activation, causing an oxidative stress imbalance, thereby ultimately inhibiting spleen cell proliferation and promoting spleen cell apoptosis (113). It was reported that decreased *Tuzzerella* abundance is associated with relieved hepatic oxidative stress (MDA, SOD) (69), consistent with our findings. We identified that *Tuzzerella* has a significant correlation in regulating hematopoietic function and oxidative stress using Spearman's correlation analysis. Therefore, *Tuzzerella* may regulate oxidative stress and splenic injury to reduce anemia through various signaling pathways.

The correlation analysis between transcriptome and metabolome profiles indicated that twenty-one DEGs and ten differential metabolites were enriched in fourteen anemia-related pathways. Microsomal glutathione S-transferase 2 (*Mgst2*), glutathione synthetase (*Gss*), glutathione peroxidase 4 (*Gpx4*) and glutathione S-transferase (*Gst*), mu 5 (*Gstm5*) are differential genes in glutathione metabolism. *Mgst2* can mitigate ROS-mediated lipid peroxidation. The direct binding of coniferyl ferulate to *Mgst2* alleviates the toxic effect of xylene on HSPCs induced by oxidative stress (114). Variants in *Gss* can reduce *Gss* activity, leading to lower GSH levels, which in turn cause red blood cell rupture and anemia (115). Mice exhibit anemia and ineffective erythropoiesis when *Gpx4* is deletion (116). *Gstm5* is associated with refractory anemia with excess blast progression (117). Starch and sucrose metabolism requires the participation of glycogen synthase 1 (*Gys1*) and hexokinase2 (*Hk2*). Enarodustat protects against renal anemia by increasing the expression of *Gys1*, leading to upregulated glycogen synthesis (118). *Angelica sinensis* polysaccharides facilitate the expression of the glycolytic gene *Hk2*, which promoted splenic glycolysis and extramedullary stress erythropoiesis, thereby ameliorating anemia (119). Choline dehydrogenase (*Chdh*) is a differential gene in glycine, serine and threonine metabolism. In the hemopoietic systems, *Chdh* exhibits significant enzyme activity in granulopoietic cells, while erythroblasts, megakaryocytes and platelets show only weak positive activity. These results are related to cellular lipid metabolism and blood cell functions (120). Integrative analysis of transcriptomic and metabolomic data indicated that FEJ prevented anemia by interfering with amino acid metabolism and carbohydrate metabolism.

Conclusion

This study comprehensively investigated the hematopoietic characteristics of FEJ for the first time by integrating multi-omics analysis of fecal metabolomics, intestinal microbiota and spleen transcriptome profiles in CTX and APH-induced anemic mice. FEJ exhibited beneficial effects on hematological parameters, organ indexes, peripheral blood routine, hepatic oxidative stress and tissue damage in the bone and spleen. FEJ effectively ameliorated gut microbiome dysbiosis by reducing the abundance of pathogenic genera, including *Family_XIII_UCG-001*, *UCG-009*, *Negativibacillus* and *Tuzzerella* in anemia mice, while establishing *Akkermansia* and *Tuzzerella* as potential microbial markers for

hematopoietic enhancement. Distinct metabolic profile variations revealed key fecal biomarkers including propanoic acid, butanoic acid, L-leucine, phenylalanine, L-proline, glycine and octadecanoic acid, which potentially regulated phenylalanine metabolism, glycine, serine and threonine metabolism, arginine and proline metabolism. Transcriptome analysis identified spleen hematopoietic regulators, including B cell receptor signaling pathway (*Plcg2*, *Vav3*), NF- κ B signaling pathway (*Edaradd*), hematopoietic cell lineage biomarkers (*Cd19*, *Cd22*, *Il5ra*, *Fcer2a*) and osteoclast formation (*Ncf2*, *Ncf1*, *Lilra6*, *Pirb*), as potential diagnostic indicators (Figure 7E). The Spearman's correlation analysis revealed significant associations between hematopoietic indicators and the gut microbiota, with functional connections between microbial communities and hematopoietic related genes and amino acid metabolism. An integrated metabolome and transcriptome analysis demonstrated FEJ's regulatory impact on metabolic disturbances induced by anemia, particularly amino acid metabolism and carbohydrate metabolism. The results indicated FEJ ameliorated anemia through regulating the amino acid metabolism, inhibiting the proliferation of harmful bacteria, controlling inflammation and liver oxidative stress and reflecting the multi-target therapeutic effect of traditional Chinese medicine. These findings offer valuable insights into application of FEJ as a healthy food and Chinese patent medicines for anemia treatment.

Data availability statement

The data presented in the study are deposited in the NCBI repository (<https://www.ncbi.nlm.nih.gov/>), accession number PRJNA1203393 and PRJNA1203422.

Ethics statement

The experimental protocol was approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine (Ethical number: PZSHUTCM2302110002). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YM: Conceptualization, Software, Writing – original draft, Writing – review & editing. HC: Conceptualization, Supervision,

Writing – review & editing. TZ: Conceptualization, Writing – original draft, Writing – review & editing. XH: Writing – original draft. PS: Visualization, Writing – original draft. YN: Software, Writing – review & editing. MZ: Conceptualization, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was financially supported by the project of Laboratory of Sustainable Utilization of Panax Notoginseng Resources, State Administration of Traditional Chinese Medicine (Grant NO.2022030001).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1561477/full#supplementary-material>

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