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Pseudostellaria heterophylla improves intestinal microecology through modulating gut microbiota and metabolites in mice

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Pseudostellaria heterophylla improves intestinal microecology through modulating gut microbiota and metabolites in mice

Running title: Effect of *P. heterophylla* on mice gut health

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Abstract

BACKGROUND: *Pseudostellaria heterophylla* is a Chinese medicine and healthy edible that is widely used to for its immunomodulatory, antioxidant, antidiabetic, and antitussive properties. However, the potential function of *P. heterophylla* in intestinal microecology remains unclear. In this study, we investigated the impact of *P. heterophylla* on immune functions and evaluated its potential to regulate the gut microbiota and metabolome.

RESULTS: The results showed that *P. heterophylla* significantly increased the content of red blood cells, total antioxidant capacity, and expression of immune factors, and decreased platelet counts when compared to the control under cyclophosphamide injury. In addition, *P. heterophylla* altered the diversity and composition of the gut bacterial community; increased the abundance of potentially beneficial *Akkermansia*, *Roseburia*, unclassified *Clostridiaceae*, *Mucispirillum*, *Anaeroplasma* and *Parabacteroides*; and decreased the relative abundance of pathogenic *Cupriavidus* and *Staphylococcus* in healthy mice. Metabolomic analyses showed that *P. heterophylla* significantly

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increased the content of functional oligosaccharides, common oligosaccharides, vitamins, and functional substances. Probiotics and pathogens were regulated by metabolites across 11 pathways in the bacterial–host co-metabolism network.

CONCLUSION: We demonstrated that *P. heterophylla* increased the abundance of probiotics and decreased pathogens, and further stimulated host microbes to produce beneficial secondary metabolites for host health. Our studies highlight the role of *P. heterophylla* on gut health, and provided the new insights for the development of traditional Chinese medicine in the diet.

Keywords: *Radix pseudostellariae*; Gut microbiome; Untargeted metabolomics; Host Immunity

INTRODUCTION

Pseudostellaria heterophylla is a plant mainly cultivated and produced in a geo-authentic production zone in Fujian, Guizhou and Anhui Provinces of China. The average annual production of fresh *P. heterophylla* roots is approximately 38,000 tons, with an estimated value higher than 120 million USD. The roots of this traditional Chinese medicinal herb contain amino acids, polysaccharides, cyclic peptides, and sapogenins, which are thought to have beneficial effects against anorexia, fatigue, spleen asthenia, asthenia after severe illness, and palpitations [1, 2]. *Pseudostellaria heterophylla* has been widely used in Chinese medicine and diet for 100 years and has recently received increasing attention. Previous studies have supported the beneficial

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pharmacological activities of the main components of *P. heterophylla*, such as the function of polysaccharides, saponins, amino acids, volatile oily substances, and cyclic peptides [2]. However, the potential function of *P. heterophylla* on the intestinal microbiome of target organisms remains unclear.

Extensive studies have shown that natural products and their bioactive components in medicinal plants possess high biological activity and potential pharmacological value [3, 4]. *P. heterophylla* and its extract can reduce the incidence of spleen deficiency in mice, improve the physical ability of mice with spleen deficiency, and significantly improve the health of immune organs [5, 6]. Previous study indicate the tonifying spleen Chinese medicine can improve the composition of intestinal microecology and cure the disease of spleen deficiency, and improve the imbalance of gut microbiota composition by inhibiting the reproduction of harmful bacteria, enhancing body immunity and regulating gastrointestinal motility function [7, 8]. Gut microorganisms are known as host “second genome” [9], and can create natural protective barriers and perform proper immune functions for the body [8, 10, 11]. Mammalian guts possess a remarkable abundance of microorganisms and have an important role in maintaining overall organismal health [12]. Interactions between the intestinal microbiota and host systems are known to play a major role in various diseases, providing further proof of the importance of the gut microbiota to host health [12, 13]. Therefore, exploring the effects of *P. heterophylla* on changes in the gut microbial community can provide a mechanistic explanation for the beneficial health effects of *P. heterophylla*.

The gut microenvironment provides a barrier to prevent the translocation of

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pathogens or harmful agents across the gut epithelial cells into the surrounding lymphoid system and blood [14, 15]. The gut microbiota plays an essential role in the conversion of proteins, lipids, carbohydrates, and non-nutritive small chemical compounds from traditional Chinese Medicine herbs into chemical metabolites that may have beneficial or adverse effects on host health [15, 16]. Recent advances have revealed that the gut microbiota and their metabolites are necessary for immune homeostasis and affect host susceptibility to many immune-mediated disorders [17]. Previous studies have indicated that gut microbiota can influence the host immune system via specific metabolites [18]. The gut microbiota is indeed able to produce diverse metabolites (e.g., short-chain fatty acids, polyamines, and aryl hydrocarbon receptor ligands), which can affect the host immune response [17]. The “healthy” microbiome of the intestine is crucial to the immune response and immune tissue through regulating the metabolites and microbial surface antigens [17]. However, the existence of a link between the consumption of *P. heterophylla*, changes in the microbiome, and the potential influence of immune responses needs to be experimentally proven.

To this end, we analyzed the main composition of *P. heterophylla* and studied the effects of *P. heterophylla* on the immune system of mice under cyclophosphamide injury. In addition, we studied the effects of *P. heterophylla* on the gut microbiome and metabolism of mice. We investigated the effect of *P. heterophylla* on immune function and evaluated the potential ability of *P. heterophylla* to regulate the gut microbiome and metabolome.

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MATERIAL AND METHODS

Main components of P. heterophylla

Amino acids in *P. heterophylla* were extracted following a previously described method [19]. Briefly, *P. heterophylla* samples were hydrolyzed in phenol and hydrochloric acid for 24 h and then evaporated until dry. The residue was then dissolved in distilled water. The above solution was then derivatized in triethylamine acetonitrile, phenyl isothiocyanate acetonitrile, and n-hexane. Amino acid standards were also derivatized and used for detection. An HPLC system (ACE Excel SuperC18, 5 μm, 250 × 4.6 mm) was used to analyze the amino acid content. Mobile phase A was 0.1 mol /L Sodium acetate solution (pH 6.5): acetonitrile = 93:7; mobile phase B was acetonitrile:water=4:1. The column temperature was maintained at 36 °C and the detection wavelength was 254 nm. The analysis was carried out using the elution gradient as follows: 0-15 min (0%-15% B), 15-18 min (15%-24% B), 18-25 min (24%-40% B), 25-30 min (40% B), 30-30.1 min (40%-100% B), 30.1-50 min (100% B), 50-50.1 min (100%-0% B), 50.1-70 min (0% B).

The total polysaccharide content was extracted from water extraction and alcohol analysis, determined by the phenol-sulfuric acid method, and calculated based on the regression equation [20]. The extraction of heterophyllin B was performed as previously described [20]. HPLC-MS (Thermo LTQ XL model) was used to detect the heterophyllin B content. The mobile phase comprised water (A) and acetonitrile (B). The HPLC-MS parameters were as follows. Scan Mode: negative ion mode; Sheath Gas Flow Rate: 50arb; Vaporizer temperature, 400 °C; auxiliary gas flow rate, 20 arb; capillary

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temperature, 350 °C; Capillary Voltage, -3 V. The elution conditions of HPLC-MS are listed in Table S1.

Preparation of P. heterophylla suspension

Sodium carboxymethyl cellulose was dissolved in sterilized water to prepare a dispersant with a concentration of 0.5%. Because sodium carboxymethyl cellulose is insoluble in water, it must be dissolved quickly in a magnetic stirrer at 50-70 □ within 1.5-2 hours. To avoid contamination, the prepared dispersant was stored at 4 °C and used when ready. *Pseudostellaria heterophylla* suspension was prepared from a mixture of superfine powder of *P. heterophylla* and sodium carboxymethyl cellulose dispersant and then stored at -20 °C.

Pharmacological activities of P. heterophylla

Male BALB/c mice (20 ± 2 g, 6-7 weeks old) were purchased from Wushi Laboratory Animal Co., Ltd (Fuzhou). According to Xu Wensheng's formula [$S\ (m^2) = 0.0061 \cdot H\ (cm) + 0.0128 \cdot W\ (kg) - 0.1529$, where H is the average human height and W is the average human weight] and the recommended oral dose of *P. heterophylla* in the Pharmacopoeia of the People's Republic of China, we chose a daily gavage dose of 4,505 mg/kg. The mice were fed adaptively for 7 days and randomly divided into three groups with five individuals in each group, including the control group (CK), cyclophosphamide group (CTX), and *P. heterophylla* group (CTX+B). Cyclophosphamide is able to suppresses both cellular and humoral immunity in animals[21]. In immunopharmacology, cyclophosphamide is widely used to generate immunosuppression models. The CTX+B group was administered 4,505 mg/kg/day of

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P. heterophylla by intragastric gavage for 40 days. The CK and CTX groups were administered the same amount of sodium carboxymethyl cellulose.

After 40 days of intragastric administration, 150 mg/kg cyclophosphamide was injected into each group, except for the control group, which was intraperitoneally injected with normal saline. The mice were dissected within 48 h after modeling, and the food was removed without stopping water at night before dissection. All research procedures were based on the laboratory animal care and use guidelines (China).

After blood collection from the mice, 40 μL of whole blood was immediately absorbed into the pre-prepared blood routine dilution solution and sent to the Fuzhou Air Force Hospital for routine blood testing within 1 h. The remaining whole blood was left at room temperature for 2 h, centrifuged at 3500 rpm, and the supernatant was used to measure the serum antioxidant activity. Serum antioxidant activity was determined using a serum antioxidant activity kit (Nanjing Jiancheng bioengineering, China).

Mouse liver tissue was placed in liquid nitrogen and ground into a powder, and the tissue powder (0.5 g) was accurately weighed to extract the total RNA using a total RNA extraction kit (TransGen Biotech, Beijing, China), and then the extracted RNA was reverse transcribed. The expression levels of IL-2, IFN-γ, and TNF-α were determined using RT-PCR, and the conditions for primers and PCR amplification are listed in Table S2. Five independent quantitative PCR analyses were performed for each treatment.

Gut microbiota and metabolomics analysis

Male BALB/c mice (20 ± 2 g, 6–7 weeks old) were fed adaptively for 7 days and

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randomly divided into two groups with six individuals in each group, including the control group (K) and *P. heterophylla* treatment group (B). Group B received 4,505 mg/kg/day of *P. heterophylla* by intragastric gavage for 40 days. The same amount of sodium carboxymethyl cellulose was administered to the K group. The feces of the mice in the two groups were collected at 9:00 a.m. every day for four days.

Gut microbial community

The total DNA of mouse feces was extracted using a genomic DNA Extraction kit (MP Biomedicals). The concentration of the extracted DNA was measured using a Nanodrop spectrophotometer (Thermo Scientific). We amplified the V3–V4 region of the 16S rRNA gene to characterize the bacterial community using primers 341F and 806R [22]. The PCR products were purified, pooled, and sequenced on an Illumina MiSeq platform using PE250 chemistry.

Extraction and analysis of fecal metabolites

We weighed 50 mg mice feces, added 1000 μL extract (methanol: acetonitrile: water = 2:2:1, containing L-2-chlorophenylalanine 2 μg/ml internal standard); Violent shock for 30 s, added steel balls, ground at 35 Hz for 4 min, and conducted ultrasonic treatment in ice water bath for 5 min; Placed the sample at -80 □ for 1 h, centrifuged to get 400 μL supernatant (4 □, 1000 rpm, 15 min) with vacuum freeze-drying; Re-dissolving with 200 μL 50% acetonitrile, swirling for 30 s, ultrasonic in ice water bath for 10 min; Centrifuged 75 μL supernatant and tested it in the upper part of the injection bottle (4 □, 13000 rpm, 15 min); All samples were mixed with 10 μL supernatant into QC samples for computer detection.

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The extracts were detected using non-targeted metabolomic ultra-performance liquid chromatography (Agilent 1290, Agilent Technologies). The detection conditions were as follows. Mobile phase A: 25 mmol/L ammonium acetate and 25 mmol/L ammonia water; mobile phase B: acetonitrile; flow rate: 0.5 ml/min; column temperature: 25 °C; sample tray temperature: 4 °C; injection volume: positive ion, 2 μL; negative ion, 2 μL. The gradient elution procedure is presented in Table S3. All mouse feces samples from the two groups were extracted and analyzed in six replicates.

Statistical analysis

All statistical analyses were performed using R software unless otherwise specified. The Shannon and Chao1 values of the bacteria were calculated at the OTU level. The Bray-Curtis distance was used to perform PERMANOVA and visualize the structure of microbial communities through PCoA. The differential abundances of microbial taxa and pharmacological activities were eliminated using variance analysis (ANOVA) and Tukey's test (significance level of P value < 0.05), and then visualized using GraphPad Prism software (7.00). Correlations between the metabolites and bacterial abundance were evaluated using Spearman's correlation tests. Gut metabolites were analyzed with PLS-DA, and multivariate statistical models were constructed using SIMCA14.0. Using MetOrigin, we discriminated the origins of gut metabolites, analyzed the enrichment of metabolic pathways, evaluated the interactions of gut microbial metabolites, and integrated statistical correlations with the visualization of the Sankey network [23].

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Results

Pharmacological activities of P. heterophylla

We detected 13 different amino acids in *P. heterophylla* extract. Glutamic acid and arginine contents were greater than 10 mg/g (Figure 1A). The histidine, serine, threonine, leucine, phenylalanine, lysine, and tyrosine residues were also abundant. *P. heterophylla* contains polysaccharides and heterophyllin B. The pharmacological results showed that cyclophosphamide had a significant effect on the physiological properties of the mice, indicating that the model was successfully established by intraperitoneal injection of 150 mg/kg cyclophosphamide at one time (Figure 1C-G). Moreover, we found that the CTX+B group had slightly increased white blood cells, red blood cells, and total antioxidant capacity when compared to the CTX group. The platelet count was lower in the CTX+B group than in the CTX group. The CTX+B group had a significant effect on the content of superoxide dismutase (SOD) compared to the CTX group. CTX+B treatment significantly increased the expression of interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) in the liver compared with the CTX group (Figure 1B).

Effects of P. heterophylla on the change of gut microbiota

Our results suggest that *P. heterophylla* treatment did not influence the mouse weight (Figure S1). High-throughput sequencing generated 512,634 reads across the 12 samples, with an average of 42,720 reads/sample. The sequences of all samples were grouped into 35,111 bacterial OTUs, with each OTU number varying from 2663 to 3534

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(Figure S2). The results showed that *P. heterophylla* significantly decreased Chao 1 and had no significant effect on the Shannon index of the gut microbiota of the mice (Figure 2A and 2B). The results of the Bray-Curtis distance and PCoA analysis showed that the composition of the bacterial community was generally separate when compared with the control treatment (Figure 2C and S3). The bacterial communities were classified into nine phyla, dominated by Bacteroidetes, Firmicutes, Proteobacteria, Deferribacteres, and Verrucomicrobia (Figure 2D). *P. heterophylla* treatment increased the relative abundance of Verrucomicrobiaceae, Eubacteriaceae, Deferribacteraceae, Christensenellaceae, and Anaeroplasmataceae families, and decreased the abundance of Alcaligenaceae, F16, Oxalobacteraceae, and Staphylococcaceae in the gut microbiota (Figure 3A). Meanwhile, *P. heterophylla* treatment had a positive effect on the abundance of the genera *Akkermansia*, *Roseburia*, *Anaerofustis*, unclassified*Clostridiaceae*, *Mucispirillum*, *Christensenellaceae*, *Anaeroplasma*, *Anaerostipes*, *Coprobacillus*, *Flexispira* and *Parabacteroides*, and decreased the abundance of *Dorea*, *Sutterella*, *Cupriavidus* and *Staphylococcus* (Figure 2E and 3B). PICRUSt analysis showed that the detected microbiota mainly perform functions within the carbohydrate metabolism, amino acid metabolism, energy metabolism, cell motility, membrane transport, replication and repair, infectious diseases, and endocrine system pathways (Figure 3C and S4-S8).

Effects of P. heterophylla on gut metabolomics

The results of PCA and OPLS-DA suggested that the gut metabolomic

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composition was generally different between *P. heterophylla* and the control treatment (Figure 4A and B). A total of 1,690 metabolites were detected by the non-targeted metabolomic method using the positive ion mode, with 677 discernible metabolites and 92 differential metabolites (65 upregulated metabolites and 27 downregulated metabolites under *P. heterophylla* treatment) (Figure 3C and D). Combining the positive and negative ion modes, 29 potential biomarkers (VIP> 1, $p < 0.05$) were annotated to the detailed names (Table S4). The levels of alfuzosin, stachyose, pioglitazone, urea, raffinose, hippuric acid, maltopentaose, 1-aminocyclohexanecarboxylic acid, creatine, maltotriose, honokiol, His-Val, creatinine, homoveratric acid, pantothenate, nicotinamide (vitamin B3), L-leucine, nicotinamide ribotide, anthranilic acid (vitamin L1), D-maltose, ethyl hydrogen malonate, and butabarbital were significantly upregulated in *P. heterophylla* treatment. *P. heterophylla* treatment significantly decreased the contents of 2'-deoxy-d-ribose, 4-imidazoleacetic acid, Phe-Thr, cimetidine, and carbamazepine compared to the control group. The differential metabolites were aligned to 20 KEGG pathways in the positive ion mode. Among them, arginine and proline metabolism and ABC transporters constituted the majority, followed by vitamin digestion and absorption (Figure 4E). Carbamazepine, creatine, creatinine, isomaltose, L-leucine, maltotriose, nicotinamide (vitamin B3), pantothenate, stachyose, and urea are involved in the regulation of these pathways (Table S5). In addition, all the pathways were upregulated except for the drug metabolism cytochrome P450.

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Correlation analysis between gut microbiome and metabolomics

Our Spearman's correlation results showed that the abundances of *Rikenellaceae*, *Ruminococcus*, *Parabacteroides* and *Helicobacter* were positively correlated with the most upregulated metabolites under *P. heterophylla* treatment, while they were significantly negatively correlated with downregulated metabolites (Figure S9). Metabolite origin analysis showed that the total number of 677 detected metabolites were initially classified into four groups: 11 host-specific metabolites, 53 bacterial metabolites, 159 bacteria-host co-metabolites, and 454 other metabolites (drug- and food-related) (Figure 5A). In addition, 50, 3, and 7 related metabolic pathways matched the co-metabolism, host, and bacterial pathway databases (Figure 5B). Among these pathways, 15, 2, and 3 metabolic pathways were significantly associated with *P. heterophylla* treatment (Table S6). Origin-based functional analysis showed that lysine degradation, arginine and proline metabolism, and biotin metabolism were unique to the microbiota. Biotin metabolism and phenylalanine metabolism were unique to the host, and 15 metabolic pathways related to amino acid metabolism, purine metabolism, biotin metabolism, and pantothenate and CoA biosynthesis were shared by the host microbiota.

Based on functional analysis, phenylalanine metabolism was exclusively specific to the host. Toluene degradation and aminobenzoate degradation were unique to the microbiota, and five metabolic pathways related to biotin metabolism, arginine and proline metabolism, lysine degradation, ascorbate and aldarate metabolism, and pyrimidine metabolism were shared by both the microbiota and co-metabolism. The

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"bacteria–host" metabolic network indicated that 30 metabolites were associated with 17 bacteria in the 11 pathways, and most of them were positively correlated under *P. heterophylla* treatment (Figure 5C). The microbiota network of biotin metabolism, lysine degradation, and arginine and proline metabolism showed that 5 metabolites were associated with 16 bacteria.

Based on the bacterial metabolites, we identified three significant metabolic pathways that were related to *P. heterophylla* treatment using Bio-Sankey networks: arginine and proline metabolism, biotin metabolism, and lysine degradation (Figure 6 and 7). Eleven metabolites were involved in the arginine and proline metabolism pathways, which participated in three different metabolic reactions (R01884, R02825, and R02922). Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Bacilli, Bacteroidia, and Clostridia were identified as the main classes closely related to the arginine and proline metabolism pathways (Figure 6A-C). *Staphylococcus*, *Clostridium* and *Desulfovibrio* were identified as the main genera closely related to the biotin metabolism pathway (Figure 6D). Fifteen metabolites were involved in the lysine degradation pathway, which participated in five different metabolic reactions and were associated with Burkholderiaceae and Alcaligenaceae families (Figure 7).

Discussion

Pseudostellaria heterophylla regulates the immune function of mice

P. heterophylla is popularly used in the medicine and in traditional Chinese diet. As a diet, it is often used to make soups, which usually takes the form of water

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decoctions. In this study, we found that *P. heterophylla* contains abundant amino acids, polysaccharides, and heterophyllin B. Modern pharmacological studies showed that *P. heterophylla* polysaccharide enhanced cell-mediated immunity, lowered blood lipid and blood pressure, increased hemolysin content in the blood after immunization, and treatment of diabetes and its complications [6, 24]. Heterophyllin B of *P. heterophylla* can improve mouse memory via neurite regeneration and immunomodulation [25]. Our results showed that *P. heterophylla* increased the content of white and red blood cells and decreased the content of platelets following cyclophosphamide injury. This indicated that total *P. heterophylla* was able to improve cell immunity in mice. In addition, *P. heterophylla* treatment significantly increased the expression of IL-2, IFN-γ, and TNF-α in the mouse liver. IL-2 is a crucial T-cell growth factor that supports self-tolerance through its action on Regulatory T cells [26]. TNF-α is an important cytokine that is involved in the immune response. IFN-γ promotes activation of antigen-presenting cells. These factors play an important role in the development of immune cells. Previous studies have indicated that a decrease in the content of these factors may weaken immune function and lead to disorders of lymphokines [27, 28]. Therefore, our study suggests that *P. heterophylla* effectively inhibits cyclophosphamide-induced injury and enhances immune function in mice.

Pseudostellaria heterophylla mediates the gut microbiota and increase probiotic bacteria

The gut microbiota has co-speciated with the host over millions of years, likely

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shaping the host's metabolism and immune system and becoming intertwined with the host's physiology [29]. With increasing research on the gut microbiota, substantial studies have shown that Chinese medicinal herbs improve diseases by mediating the gut microbiota [30-32]. Our results suggest that *P. heterophylla* alters the diversity and composition of the gut bacterial community, which is consistent with a previous study suggesting that the crude polysaccharides of *P. heterophylla* effectively regulate gut microbiota [6]. The gut microbiota mainly consists of Bacteroidetes and Firmicutes phyla, which contain a large number of bacteria producing different types of polysaccharide-degrading enzymes and then degrading polysaccharide carbohydrates to various short-chain fatty acids [33, 34]. These short-chain fatty acids can modulate immune responses of the host, regulate epithelial barrier function, and provide an energy source for the gut microbiota [35].

Probiotics can effectively improve and regulate the gut microbiome, especially by affecting the health of the gut [36]. The abundance of beneficial *Akkermansia*, *Roseburia*, unclassified *Clostridiaceae*, *Mucispirillum*, *Anaeroplasma* and *Parabacteroides* increased under *P. heterophylla* treatment. *Akkermansia* is a common strict anaerobic bacteria in the intestinal tract and new probiotics, which can produce short-chain fatty acids and play an important role in immune responses of gut adaptive [37], and is effective in relieving obesity, treating type 2 diabetes, and reducing epilepsy susceptibility [38]. *Roseburia* spp. are commensal bacteria that produce short-chain fatty acids, are associated with several diseases (including obesity and allergies), and can also serve as probiotics for the restoration of beneficial microbiota [39]. *Clostridiaceae* are able to

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decompose carbohydrates to short-chain fatty acids in vitro [40], and play a role in maintaining gut barrier integrity to decrease pathogens colonization [41]. *Anaeroplasma* can protect against atherosclerosis [42]. *Mucispirillum* is the only known representative of Aferobacter in mammals and can competitively utilize electron receptors in the intestinal mucosa to alleviate *Salmonella* infection [43]. *Parabacetoides* is negatively correlated with obesity and can alleviate metabolic dysfunction [44]. In addition, *P. heterophylla* treatment decreased the abundances of *Staphylococcus* and *Cupriavidus*. *Staphylococcus* spp. are opportunistic human pathogens that are infamous for infections worldwide [45]. *Cupriavidus* is considered an opportunistic pathogen associated with human infections with compromised immune systems [46, 47]. In our study, the changing microbes were mainly involved in regulating obesity and immune response. The gut microbiota may directly or indirectly alter the weight and immunological function of mice. Previous studies have shown that polysaccharides and heterophyllin B of *P. heterophylla* can effectively regulate the gut microbiota community [6, 48]. Our current results further suggest that *P. heterophylla* can remodel the gut biome structure, increase the relative abundance of probiotics, and decrease that of pathogens. Although we cannot provide direct evidence for the improvement of immune function and gut microbiome by the detailed components of *P. heterophylla*, the ecological function of various microbes and their interactions with the host immune system under polysaccharide and heterophyllin B treatment should be explored in future studies.

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Pseudostellaria heterophylla influences the metabolism process of mice gut

The metabolomic results showed that *P. heterophylla* significantly altered the composition of gut metabolome. *P. heterophylla* treatment significantly increased the contents of functional oligosaccharides (stachyose and raffinose), common oligosaccharides (maltopentaose, isomaltose, maltotriose, and D-maltose), vitamin B3 variants (nicotinamide ribotide and nicotinamide), and functional substances (alfuzosin, pioglitazone, honokiol, creatine, and creatinine). Among them, alfuzosin is mainly used to treat functional symptoms of hypertension or benign prostatic hyperplasia [49]. Pioglitazone is a common type 2 diabetes drug that can inhibit the proliferation of prostate cancer cells [50]. Honokiol exhibits striking antibacterial, antiviral, and antitumor effects [51]. Stachyose and Raffinose were praised as super bifidus factors and provided favorable conditions for the proliferation of gut *Bifidobacteria* in mice, which contributed to improving defecation function, preventing dental caries, and promoting trace element absorption and nutrient element synthesis [52]. Nicotinamide ribotide and nicotinamide are the amide forms of vitamin B3 and are able to cure respiratory diseases, cardiovascular and cerebrovascular diseases, immune inflammatory diseases, and type I diabetes [53]. These upregulated metabolites were mainly assigned to arginine and proline metabolism, ABC transporters, and vitamin digestion and absorption pathways. Arginine is absorbed by cells, metabolized into proline, and used as a precursor to collagen [54]. A previous study showed that the impaired bacterial production of B vitamins altered host metabolism and gut microbial community functions and affected host immunity [55, 56]. This indicated that the gut metabolizes *P. heterophylla* and produces beneficial secondary metabolites for host health.

Metabolite origin analysis suggested that most of the metabolites belonged to bacteria and bacteria–host co-metabolites. Our results also showed that biotin metabolism, arginine and proline metabolism, lysine degradation, ascorbate and aldarate metabolism, and pyrimidine metabolism were shared by both the microbiota and co-metabolism. *P. heterophylla* treatment significantly affected biotin metabolism, lysine degradation, arginine and proline metabolism, phenylalanine metabolism, pyrimidine metabolism, and other amino acid metabolism pathways (Table S6). Homoeostasis of biotin metabolism is crucial for healthy growth and function of bacteria [56]. Previous study suggested that the shrimp fed with seaweeds was able to utilize the phenylalanine metabolism pathways to increase the immunomodulatory responses and disease resistance [57]. Pyrimidine metabolism participates in the biological processes of nucleic acid synthesis and the formation of CDP-activated precursors for membrane phospholipids, and pyrimidine derivatives possess antimicrobial, antiviral, and antiprotozoal activities [58]. Previous research suggested that the gut microbiota utilizes the amino acids produced from the host or food to synthesize proteins, and synthesizes the essential amino acids de novo, which regulates the homeostasis of amino acids [59]. Clinical applications of amino acids contribute to improving the gut microbiota and mucosal immunity [60]. The amino acid-derived molecules produced by the gut microbiota affect host health by regulating gut microbiota composition, metabolism, or host immunity [61]. PICRUSt and Bio-Sankey network analyses showed that the detected microbiota and metabolites were mainly

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involved in amino acid metabolism and carbohydrate metabolism pathways (Figure 3, 6, and 7). This indicated that the abundant amino acids and heterophyllin B in *P. heterophylla* might effectively regulate the gut bacterial community and further improve host immunity. Microbiome and metabolome cross-talk showed that probiotics (*Akkermansia*, *Roseburia* and *Parabacteroides*) and pathogens (*Cupriavidus* and *Staphylococcus*) were regulated and associated with *P. heterophylla* treatment in the co-metabolism network (Figure 5C). In the microbiota network, more bacteria were negatively affected by metabolites. The metabolites of these pathways are involved in mediating bacterial composition. The gut microbiota and its metabolites play a crucial role in the onset of many metabolic diseases [62]. Our results indicate that the interaction between the host and microbiota participates in regulating the health of intestinal microecology.

Conclusions

In conclusion, our results indicate that *P. heterophylla* can influence the structure of the gut bacterial microbiome, increase the abundance of probiotics and decrease pathogens, and further stimulate host microbes to produce beneficial secondary metabolites for host health (Figure 8). Thus, we believe that *P. heterophylla* has a beneficial effect on host health by influencing both host immunity and gut health, suggesting its wider use for *P. heterophylla* to be used as a probiotic medicinal plant for *P. heterophylla*. However, the existence of a link between the consumption of *P. heterophylla*, changes in the microbiome, and the influence of immune responses still needs to be experimentally proven.

Author contributions

Shengkai Zhang: Data curation, formal analysis, and methodology. Tao Kang: Formal analysis and methodology. Antonino Malacrinò: Review and editing. Zhen Zhang: Formal analysis, review, and editing. Zhongyi Zhang: Review and editing. Wenxiong Lin: Review and editing, supervision. Hongmiao Wu: Data curation, formal analysis, Methodology, Writing-original draft, funding acquisition, and supervision.

Conflicts of interest

There are no conflicts of interest to declare.

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Figure Legends

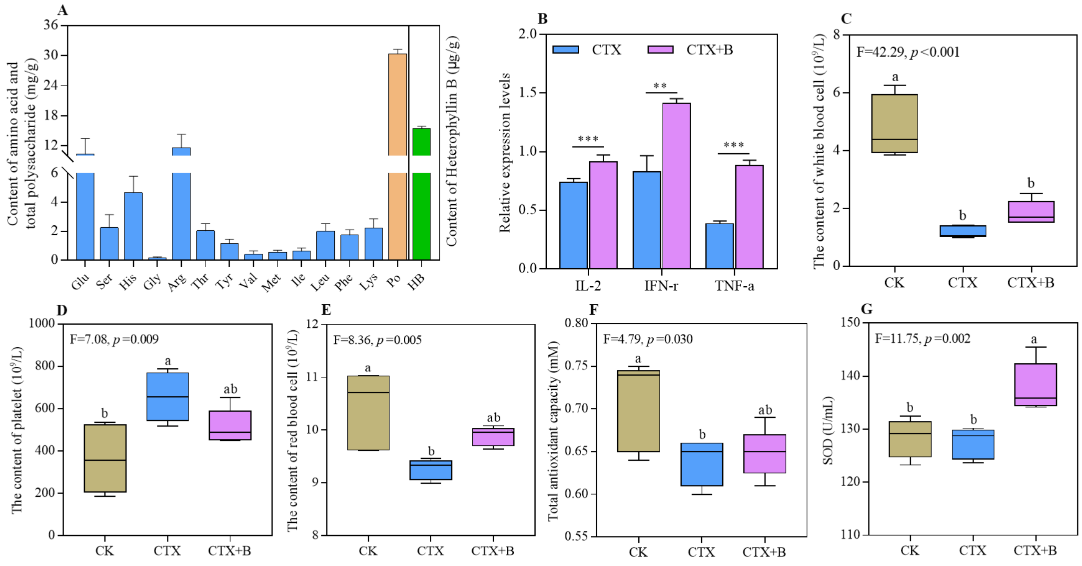

Figure 1 Contents of amino acid, total polysaccharide and heterophyllin B in the *P. heterophylla* **(A)**. Effect on the expression of liver immune factors in mice under *P. heterophylla* treatments **(B)**. Effect of *P. heterophylla* on physiological and biochemical indexes of mice **(C-G)**.Po and HB represent the polysaccharide and heterophyllin B.

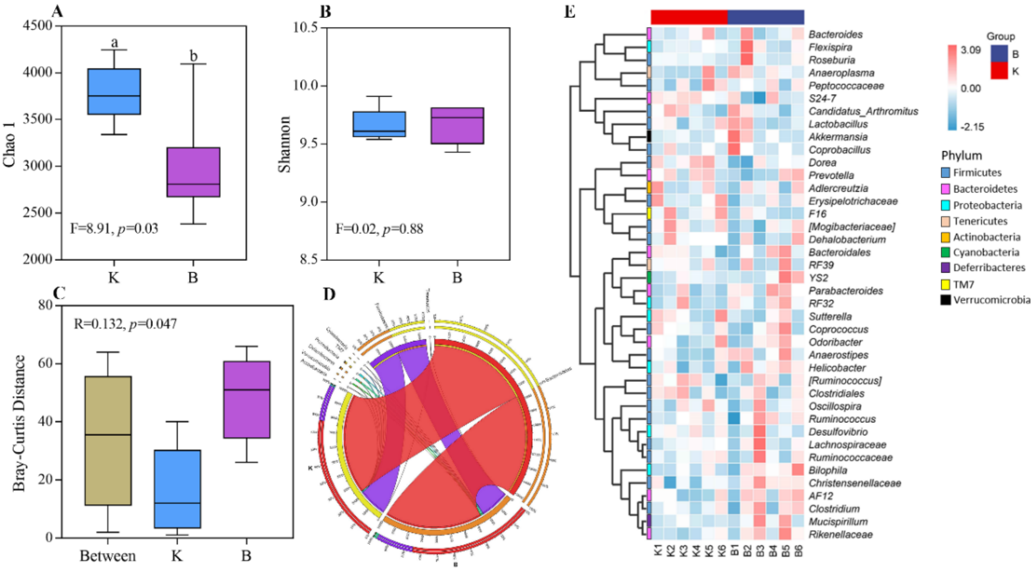

Figure 2 Effect of *P. heterophylla* on Chao 1 **(A)**, Shannon **(B)** diversity and bray-curtis distance **(C)** of gut bacteria. CIRCOS plots showing the distribution of links among gut bacterial phylum **(D)**. Heat map analysis of the most abundant gut bacterial genus detected in *P. heterophylla* treatment **(E)**. K represents control group; B represents *P. heterophylla* treatment group.

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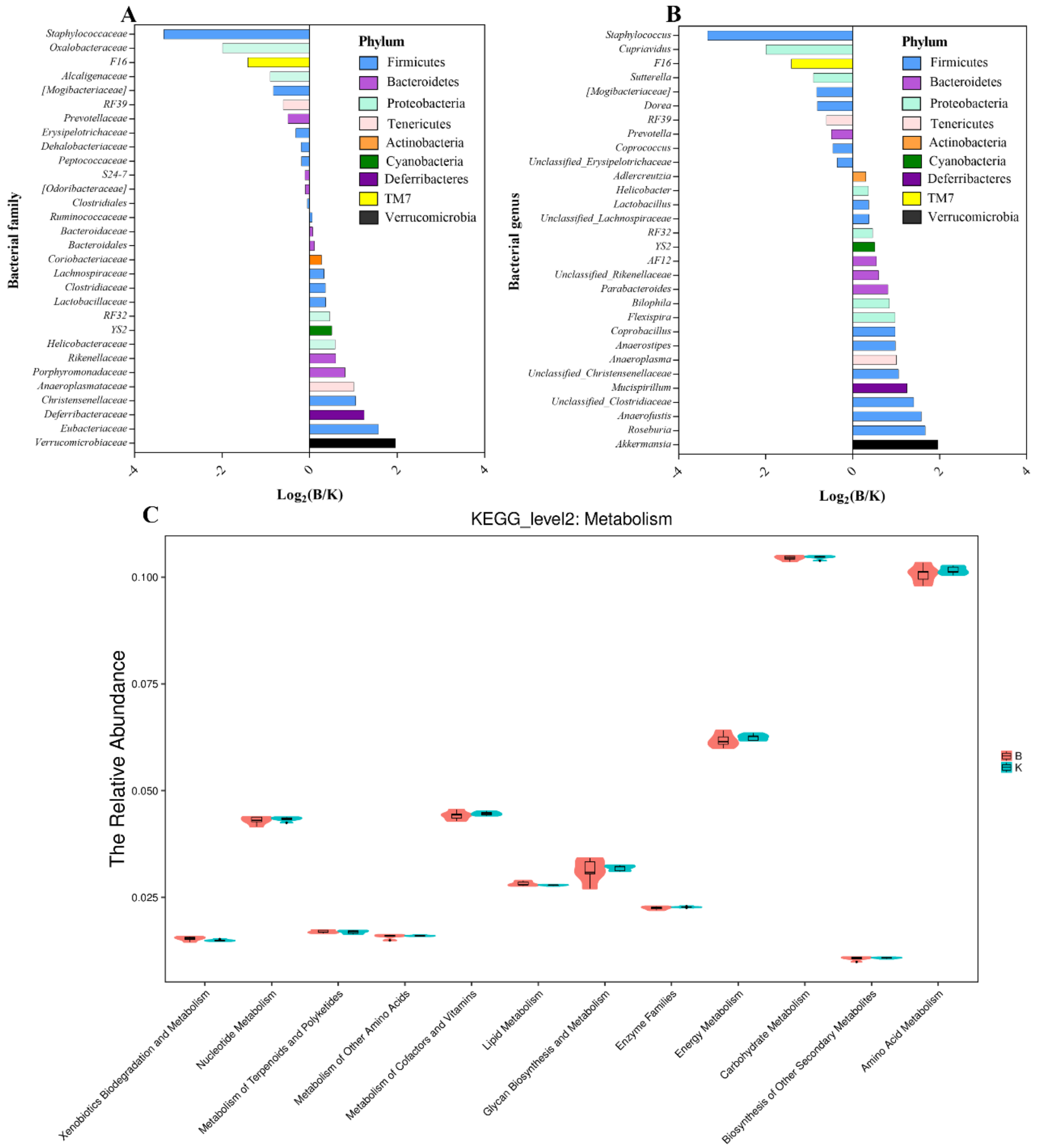

Figure 3 Changes in the relative abundances of bacterial family **(A)** and genus **(B)** under *P. heterophylla* treatment. PICRUSt distribution map of the predicted metabolism pathway **(C)**. The abscissa in the figure is the KEGG second grade functional group, and the ordinate is the relative abundance of each functional group in each sample.

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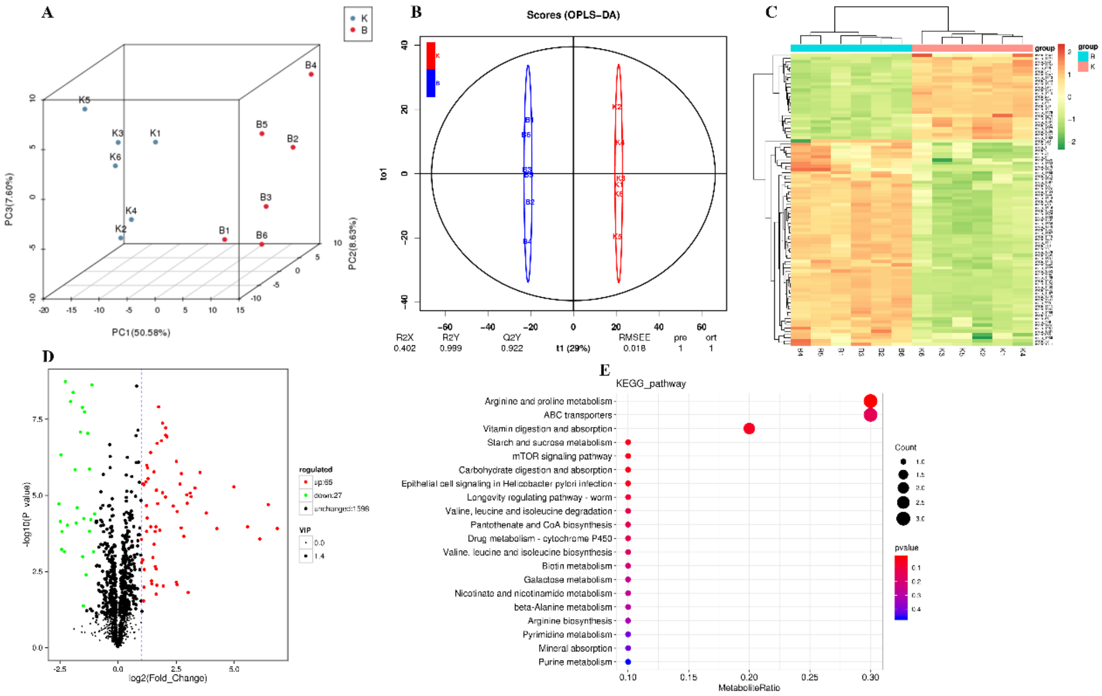

Figure 4 Principal component analysis (A) and orthogonal partial least-squares discriminant analysis (B) of gut metabolites. Heat map analysis of the differential metabolites (C). Volcano map of differential metabolites under positive ion mode (D). Up and down regulated represent higher and lower abundance of metabolites under *P. heterophylla* treatment. KEGG enrichment of differential metabolites under positive ion mode (E).

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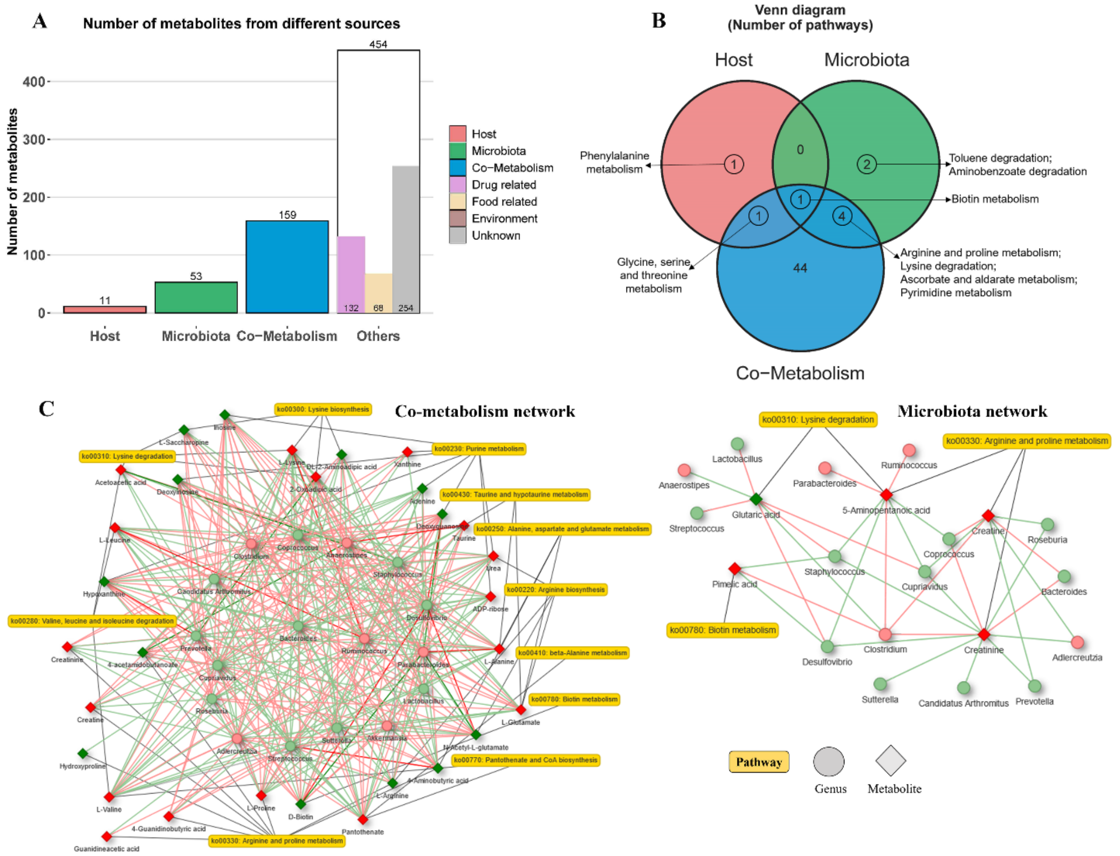

Figure 5 Number of metabolites in mice, microbiota, co-metabolism and others categories (A). The venn diagram showed the number of enriched metabolic pathways using origin-based metabolic pathway enrichment analysis (B). Correlation network analysis of *P. heterophylla* treatment on host-microbiota and microbiota (C). Dot and diamond shapes represent the indicate microbes and metabolites, respectively. Red round/diamond represents a higher abundance/content under *P. heterophylla* treatment, grenade round/diamond represents a lower abundance/content. Green/Red lines represent negative/positive correlations.

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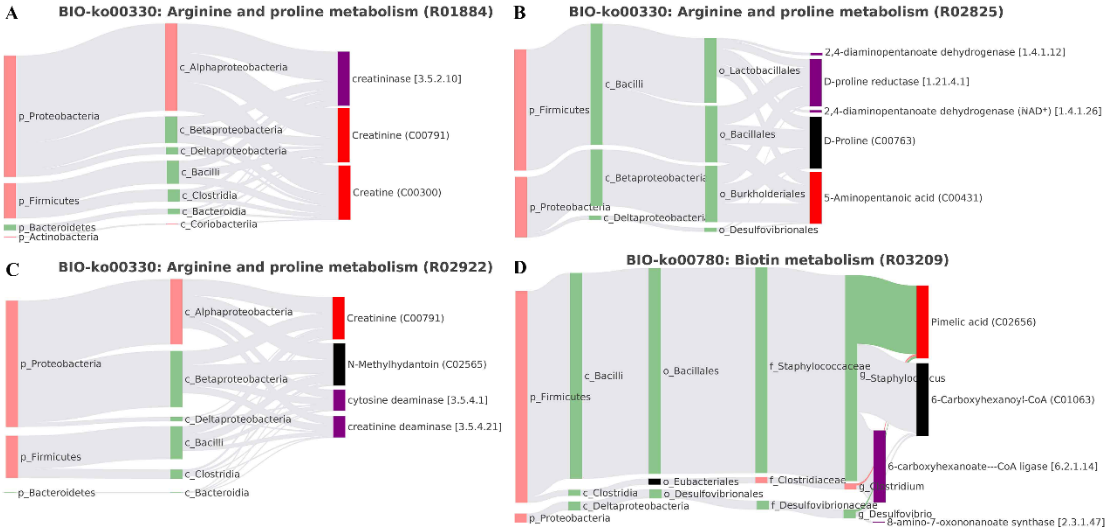

Figure 6 The BIO-Sankey Network for metabolic reaction of microbiota in arginine and proline metabolism, and biotin metabolism.

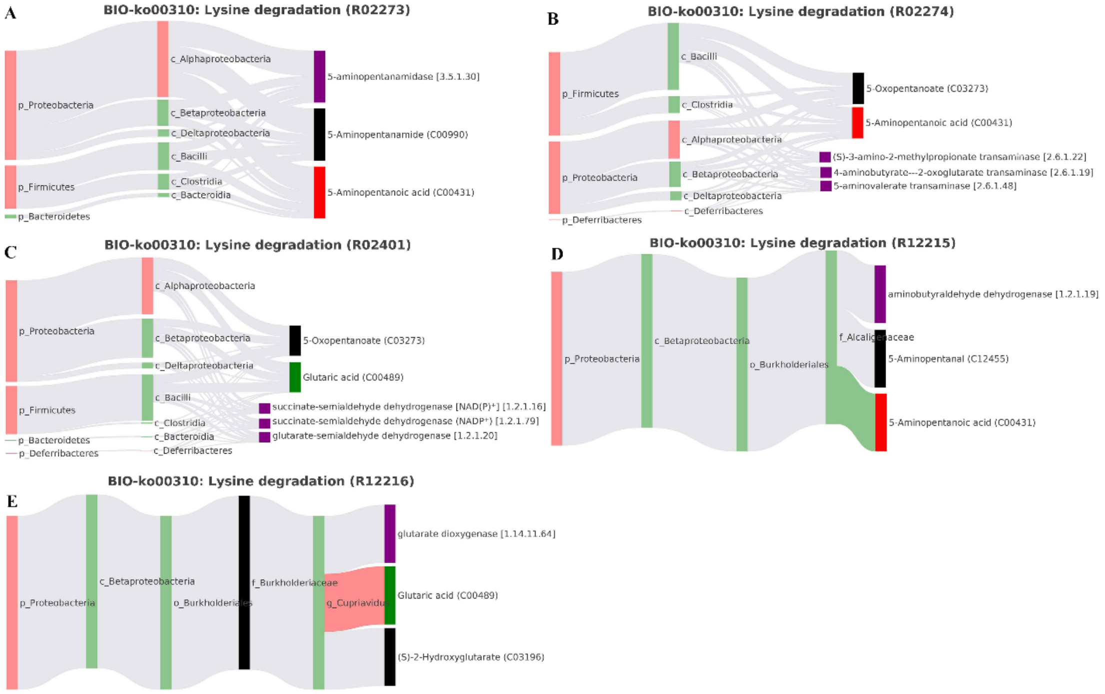

Figure 7 The BIO-Sankey Network for metabolic reaction of microbiota in lysine degradation.

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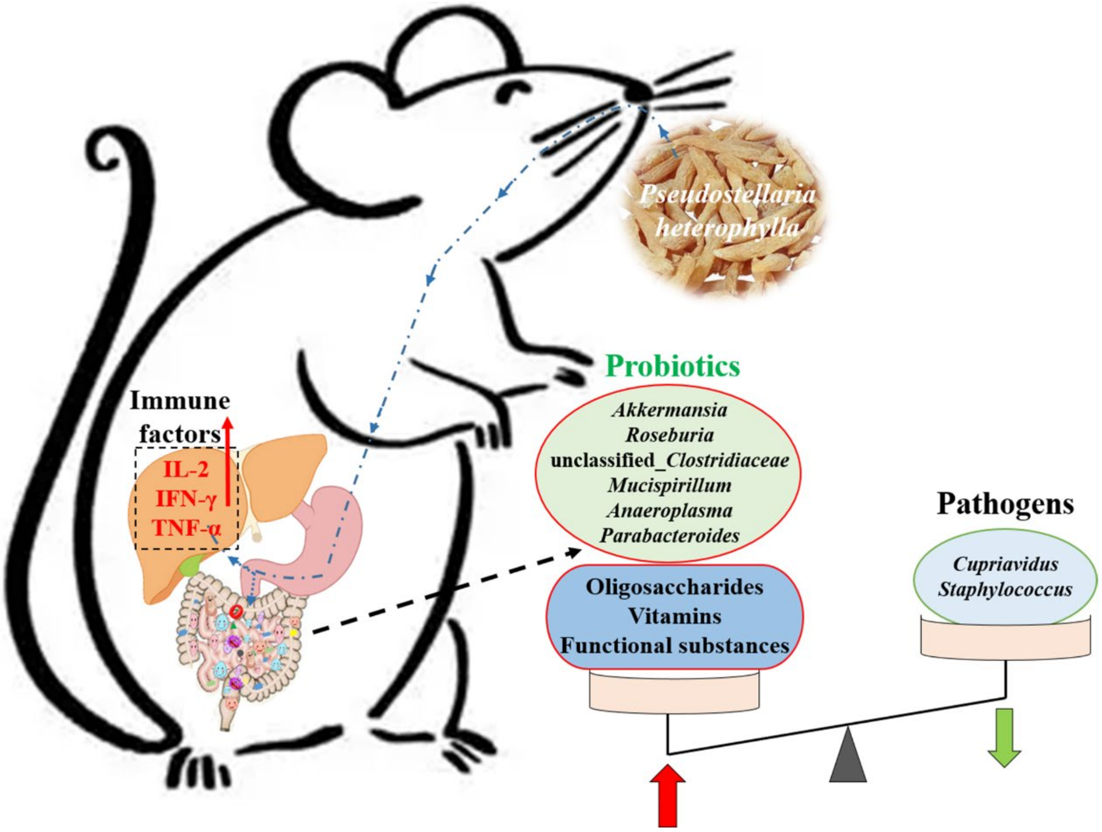

Figure 8 Schematic representation of gut microbiome and metabolites changes under *P. heterophylla* treatment.

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