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Prophylactic Effects of *Astragalus* Polysaccharides on Depression-Like Behaviors in Rats With Chronic Unpredictable Mild Stress: A Role of Gut-Microbiota–Brain Axis

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Keywords: *Astragalus* polysaccharides | Depression | "Gut microbiome–immunity–metabolome" axis | Gut immunity | Metabolomics | Microbiomes

ABSTRACT

Radix Astragalus (RA) has been used in the treatment of depression. *Astragalus* polysaccharide (APS) is one of the effective components of RA. However, the antidepressant mechanism of APS is still unclear. This research utilized 16S rRNA analysis, microbial analysis, and metabolomics analysis to elucidate the antidepressant mechanism of APS at both micro and macro levels. Correlation analysis was performed on the perspective of "behavioral indicators-intestinal bacteria-immune factors-differential metabolites" to show the relationships among various indicators. The results showed that APS could significantly regulate the depressive behaviors of depressed rats, alleviate the imbalance of Th17/Treg, increase the expression level of anti-inflammatory factor IL-10, while reduce the expression level of pro-inflammatory factor IL-22. APS significantly reduced the expression levels of lysine, alanine, and arginine of depressed rats, as well as the abundance of *Aerococcus*. Lysine and arginine were the most closely related to behaviors, immune factors, and gut bacteria. The current findings revealed the antidepressant mechanism of APS from the perspective of the "gut microbiome–immunity–metabolome" axis. This study provides a new strategy for proving the antidepressant effects of plant polysaccharides and lays a solid foundation for the discovery of new drugs from botanical drugs and the improvement of patients' quality of life.

Abbreviations: APS, *Astragalus* polysaccharides; CUMS, chronic unpredictable mild stress; FST, forced swimming test; LDBT, light-dark box test; MDD, major depressive disorder; MW, molecular weight; MWM, Morris water maze; NC, negative control; OFT, open-field test; PX, Paroxetine; RA, *Radix Astragalus*; RT-qPCR, quantitative real-time PCR; SPT, sucrose preference test.

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1 | Introduction

Depression is causing excessive disability and mortality, bringing huge global economic and social burdens. World Health Organization (WHO) estimates that more than 350 million people worldwide are suffering from depression, and worse, the number of cases is increasing each year (Ortiz et al. 2022). At present, serotonin reuptake inhibitors (SSRIs) are one of the mostly used antidepressant drugs. Yet, efficiencies of these drugs are far from satisfactory due to various side effects, for example, gastrointestinal reactions (nausea, vomiting, and diarrhea) (Coupland et al. 2018; Gerhard, Wohleb, and Duman 2016; Huang et al. 2023). Therefore, treatments with notable the efficiencies but low or none side effects are urgently needed. In this regard, natural products have demonstrated huge potential and benefits.

Natural polysaccharides have potent biological activities, including antitumor, antidiabetic, antiviral, and immunomodulatory effects (Guo et al. 2021; Liu, Li, et al. 2022b). After entering the intestine, polysaccharides interact with the intestinal flora and degrade into monosaccharides or oligosaccharides. In the meantime, gut homeostasis, for example, gut microbiome and immunity, has been reported to be crucial in depression (Han et al. 2022; Mazza et al. 2020; Peirce and Alviña 2019). Nowadays, gut microbes have become a hot topic in depression, one of the most prevalent psychiatric disorders with complex pathogenesis (Ménard, Hodes, and Russo 2016; Weersing et al. 2017). Intestinal microorganisms can affect the occurrence and development of depression through the gut-brain axis (Liu, Wang, et al. 2023a, b). The abundance of pro-inflammatory species, for example, *Enterobacteriaceae* and *Desulfovibrio* in patients with depression is higher, whereas the abundance of short-chain fatty acid producing-bacteria, for example, *Faecalibacterium* is lower (Simpson et al. 2021). *Roseburia intestinalis* ameliorate CRS-induced mouse depressive behaviors via promoting tryptophan hydroxylase-2 (TPH_2) or -1 (TPH_1) expression (Zhou et al. 2023). *Bifidobacterium breve* CCFM1025 can alleviate depression by regulating tryptophan metabolism (Tian et al. 2022). Besides, gut is also the largest immune and endocrine organ. Immune factors and balances, for example, interleukin 10 (IL-10), interleukin 17 (IL-170s., interleukin 22 (IL-22), transforming growth factor-β (TGF-β), and Th17/Treg balance also participate in the occurrence and development of depression (Köhler et al. 2017; Huang et al. 2022a, 2022b; Westfall et al. 2021). Of note, we also should notice that intestine is also the largest organ for digestion and absorption. Therefore, intestinal homeostasis provides a new perspective for exploring the pathogenesis of depression and the antidepressant mechanism of APS. On the basis of the fact that intestinal microorganisms affect the host through metabolites, metabolomics will be an effective approach to reflect the changes of intestinal homeostasis in patients with depression, as have been demonstrated by a number of studies (Han et al. 2022; Li et al. 2022). Consequently, we start from the "gut microbiome–immunity–metabolome" axis to reveal the antidepressant mechanism of APS.

Radix Astragalus (RA), Huang qi in Chinese, belongs to Leguminosae family. As a homologous medicine and food, RA has been used for thousands of years in China. According to the theory of traditional Chinese medicines (TCMs), RA invigorates spleen's functions and replenishes *Qi*. Of note, RA has also been used in treating depression for long time (Liu et al. 2021a). *Astragalus* polysaccharides (APSs) are one of major active ingredients of *Astragalus*, which exhibits a variety of pharmacological activities, such as regulating immunity (Tong and Hou 2006), anti-diabetes, anti-oxidation, anti-tumor, anti-inflammatory, and neuroprotection (Dong et al. 2020; Jin et al. 2014; Liu et al. 2018). However, the underlying mechanisms of the anti-depression effects of APS have not been fully elucidated, which is the reason why APS has not been widely used.

In this study, we aimed to investigate the multi-factor mechanisms of depression and the anti-depression mechanisms of APS from the perspective of "gut microbiome–immunity–metabolome" axis by analyzing gut microbiome, immunity, and metabolome. We employed a classical depression rat model by subjecting rat in chronic unpredictable mild stress (CUMS) condition. Subsequently, we assessed the effects of APS on abnormal behaviors of depressed rats. The immune factors, including IL-10, IL-17, IL-22, and TGF-β, were measured. At the same time, gut microbiota of depressed rats was analyzed by 16S rRNA gene sequencing. ^{1}H NMR-based metabolomics coupling with multivariate data (MVD) analysis were conducted on feces that collected from these rats. Fecal metabolic profiles, differential metabolites, and corresponding metabolic pathways that related to CUMS-induced depression were screened. On top of this, the regulatory effects of APS were demonstrated. Finally, both intra- and inter-layer networks were constructed in terms of integrating both macroscopic and microscopic parameters. Our current findings are of significance in terms of revealing depression from the perspective of "gut microbiome–immunity–metabolome" axis, and the antidepression effects of polysaccharides from plants, providing ideas for clinical researches and development of new antidepressants, and ultimately serving depressed patients in clinic.

2 | Materials and Methods

2.1 | Reagents

Paroxetines (PX) (molecular formula: $C_{19}H_{20}NO_3F \cdot HCl$) were purchased from the Beijing Wansheng Pharmaceutical Co. Ltd. (Beijing, China). The sucrose was obtained from the Sangon Biotech Co. Ltd. (Shanghai, China). Deuterated water (D_2O) and sodium 3-trimethylsilyl [2,2,3,3-2d4]—propionate (TSP) were bought from the Norell (Landisville, USA) and the Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), respectively. Phosphate was obtained from the Shanxi Baiao Biotechnology Co. Ltd. (Taiyuan, China).

Reverse transcription kits and RT-qPCR kits were purchased from TaKaRa Biotechnology Co. Ltd. (Beijing, China) (639505). Rat IL-10 enzyme-linked immunosorbent assay (ELISA) kit (D731011-0096), rat IL-17 ELISA kit (D731078-0096), rat IL-22 ELISA kit (D731248-0096), and rat TGF-β ELISA kit (D731125-0096), chloroform and ethanol were bought from the Sangon Biotech Co. Ltd. (Shanghai, China).

2.2 | Preparation of APS

APS was prepared as follows (Cao, Li, and Qin 2019). *Astragalus* powder (15 g) was extracted with 300 mL deionized water for 4 h at 90°C. The supernatant was then obtained by centrifugation (4000 r/min, 30 min) and followed by freeze-drying. APS solution was prepared by enzymatic digestion (papain 200 U) and deproteinized with trichloroacetic acid. The reaction was carried out in a water bath at 45°C for 6 h, and then 10% trichloro acetic acid was added to the reaction system to a total volume of 200 mL, stirred in an ice bath for 15 min, and left to stand for 30 min. The supernatant was mixed with ethanol to 90% ethanol content and left overnight, and followed by freeze-drying. The molecular weight (MW) of APS was determined by high-performance gel permeation chromatography.

2.3 | Animals Experiment

Specific pathogen-free (SPF) male Sprague-Dawley rats (8 weeks old, 220–240 g) were purchased from the Experimental Animal Center of the Chinese Military Medical Sciences Academy (No. SCXK2021-0011). All rats were housed under standard experimental conditions with room temperature at 23°C ± 2°C, relative humidity 50% ± 10%, and 12 h light–dark cycle. Rats were allowed to be acclimated to the environment for 7 days prior to experimentation. Food and water were available to rats at all times during the acclimatization process, as well as during the experimental process. All the experimental protocols that used in the present study were approved by the Committee on the Ethics of Animal Experiments of Shanxi University (Approval No. SXULL 2022022).

We conducted the CUMS procedure in accordance with the previous description (Liu, Wang, Wei, et al. 2021b). After 7-day adaptation, all rats were randomly assigned to four groups ($n = 8$): the negative control group (NC), the CUMS model group (MS), PX group, and APS group (200 mg/kg/day), referring to the previous researches (Awad et al. 2020; Yang et al. 2014; Zhou et al. 2021). The dose of PX was 5 mg/kg/day in this study, referring to the study of Amodeo et al. (2015). One week after modeling, intragastric administration was performed until the end of the experiment. Except for rats in NC group, rats in the other three groups were repeatedly exposed to a series of chronic unpredictable mild stressors, including 24 h food deprivation, 24 h water deprivation, exposure to an experimental room at 50°C for 10 min, swimming in cold (4°C) water for 5 min, tail clamping for 2 min, foot shock every 10 s for 10 times, restraint for 3 h, disruption of circadian rhythm, and exposure to ultrasonic (40 MHz) electromagnetic radiation for 3 h. One stressor was applied daily. The entire stress procedure lasted for 4 weeks, with stressors applied in a completely random order.

2.4 | Behavioral Tests

2.4.1 | Body Weight

During the experimental period, the body weight of each rat was recorded every week.

2.4.2 | Sucrose Preference Test (SPT)

Prior to SPT, rats were accustomed to 1% sucrose solution for 72 h. All rats could drink water or sucrose freely after 24 h of CUMS and deprivation of water and food. The following formula was used to calculate the sucrose preference of each rat:

$$\text{Sucrose preference}\,(\%) = (\text{volume of sucrose intake}/\text{total volume of intake}) \times 100\%$$

2.4.3 | Open-Field Test (OFT)

An OFT was conducted to assess the exploratory behaviors of rats as well as the tension within the rats, which may be used as an indirect indicator of spontaneous locomotor activities and anxiety behavior levels. The open-field apparatus consisted of a bare box (150 × 150 cm^2), which was equally divided into 5 × 5 cells. There was a 5-min observation period for each rat after being placed in the central square of the apparatus. After 1-min acclimatization, the following parameters were recorded in the following 4 min including the duration time in the center cell, the grooming time, the total distance travelled, and the rearing time. After the completion of one rat, the entire area was cleaned to eliminate the influences of odors on the next rat.

2.4.4 | Light-Dark Box Test

The typical light/dark box has two compartments connected to each other with an accessing between the compartments. The rat was usually placed in the light chamber first and the latency of the first entry into the dark compartment, the percentage of time spent in the light and dark compartments, and the numbers of dark-to-light transitions were recorded. The chamber was cleaned with 70% ethanol between testing each rat.

2.4.5 | Forced Swimming Test

The forced swimming (FS) experiment is a behavior of desperation experiment, in which rats are placed in a cylindrical transparent glass container (50 cm high) with a water depth of 30 cm and a water temperature of (23 ± 2)°C. After 2 min of acclimatization, the immobility time of each rat was recorded with a stopwatch for the next 4 min. The immobility time of each rat was determined by floating on the water surface and stopping struggling.

2.4.6 | Morris Water Maze (MWM) Test

MWM was used to test the ability of spatial memory of rats. The maze was a circular pool with 120 cm diameter and 40 cm height, filling with (25 ± 2)°C water. We made the water opaque by adding ink into it, which was then separated into four equal quadrants. A hidden square platform (10 cm in diameter) was located 1 cm below the water surface in the center of quadrant two. In the acquisition trails, a rat was put into the tank to allow to find a hidden platform within 120 s (one trial). In addition, at the end of

every trial, each rat was allowed to stand on the platform for 30 s. Two trials per day were performed for 4 days in total. The hidden platform was placed in the same position. On the 5th day, after removing the platform, rats were placed in the pool at the opposite quadrant of platform position, and then were given 60 s to find the platform out. A video-tracking system was used to record the body tracks of each rat, as well as the number of platforms that it found.

2.5 | Sample Collection

At the end of experiment, all rats were put into metabolic cages to collect feces. Fecal samples were immediately stored at −80°C for metabolomics analysis prior to sacrificing. After 12 h of fasting, all rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g). The whole small intestine from the pylorus to the ileocecal junction of each rat was removed, and its length was measured. Meanwhile, blood samples were collected from the abdominal aorta of rats. Plasma was isolated from blood, which was then collected into cryopreservation tubes. Plasma samples were frozen at −80°C. The cecal contents of rats were collected into cryopreservation tubes, which were frozen at −80°C for following analysis.

2.6 | RT-qPCR Analysis

2.6.1 | Extraction of RNA From Colon Samples of Rats

First, 200 mL TriZol reagent and 50–100 mg colon tissue sample was added to aN RNase-free Eppendorf tube. Then, they were homogenized thoroughly for 2–3 min with a homogenizer on ice. The homogenate was centrifuged at 12,000 g for 15 min at 4°C.

A volume of 400 µL of upper colorless water phase was transferred into 1.5 mL Eppendorf tube. Afterward, the same amount of precooled anhydrous ethanol was added and mixed for 15 s. After discarding the supernatant, the remains were washed with 1.5 mL 75% ethanol for 1–2 times. Later, the mixture was centrifuged at 7500 g for 15 min at 4°C.

Finally, 30–50 µL of RNase-free water was added into the mixture, resulting in RNA.

2.6.2 | RNA Concentration and Purity Determination

Nucleic acid RNA was selected by a Nano-100 microspectrophotometer for detection. The concentration of 1 µL RNA and the ratios of A260/A280 and A260/A230 were determined. The A260/A280 ratio of pure RNA was about 2.0, and the A260/A230 ratio was at least 2.0.

2.6.3 | Agarose Gel Electrophoresis

Agarose solid powder (1 g) was mixed with 100 mL 1 × TBE working solution and added into a conical flask. It was heated to a boil in a microwave oven, and then cooled to about 60°C at room temperature. Overall, 1% agarose gel solution was then poured into gel making plate to set the gel. Finally, 5 µL of RNA and 1 µL of 6 × loading buffer were added to the spot wells. After about 15 min of RNA electrophoresis at 80 V and 80 A, the gel was observed and photographed.

2.6.4 | Quantitative Reverse Transcription PCR (RT-qPCR)

The primers were designed with Primer 5 software and synthesized by the Sangon Biotech Co. Ltd. (Shanghai, China). The primers and primer sequences were shown in Table 1. We performed qPCR in three steps with 94°C pre-denaturation for 30 s, 94°C denaturation for 5 s, 58°C fade for 15 s, 72°C extension for 10 s, and 50 cycle collection of fluorescence signals. After the reaction, the solubility curves of amplification product were plotted. The solubility curve of amplification product was plotted. The availability of primers or the specificity of amplification product was judged by observing whether the peak of solubility curve was single. The relative quantification of target gene mRNA expression was performed by 2-CT method in combination with the corresponding CT values (see details in the Table S1).

2.7 | Enzyme Linked Immunosorbent Assay

Samples of colon tissues were rinsed in a precooled PBS solution, weighed, and cut up. With adding nine times volume of PBS, the clipped tissues were grounded by a homogenizer. After homogenization, the homogenate was centrifuged for 10 min at 5000 *g*. Finally, the supernatant was collected for analysis.

2.8 | Gut Microbiota Analysis

Gut microbiota sequencing of cecal contents was performed by Personal Biotechnology Co. Ltd (Shanghai, China). The relative abundances of microbial community at phylum level, genus level, and species level were calculated by original sequence, using DADA2 analysis, and Vsearch analysis in QIIME2. In order to assess alpha diversity within samples, Shannon, Simpson, Plelou_e, Observed_species indices, and rarefaction curves were used. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) were used to compare differences in species diversity among samples in beta diversity analysis. Linear discriminant analysis effect size (LEfSe) analysis was used to find and screen potential microbial markers that are responsible for the differences among groups. Moreover, taxonomic tree in packed circles were used to visualize distribution of the top 10 gut bacteria in the 4 groups at the genus level.

2.9 | Microbial Co-Occurrence Pattern Analysis

The microbial co-occurrence pattern analysis was performed by iNAP (http://mem.rcees.ac.cn:8081), an integrated network analysis pipeline for microbiome studies (Feng et al.2022). First, the majority selection was carried out on the basis of the operational taxonomic units (OTUs) abundance data to filter the OTUs that were less detected among all samples. The standard of filter value is recommended as the 50% of the sample numbers.

TABLE 1 | Primer sequences applied in qPCR experiments.

Genes	5′–3′ forward	5′–3′ reverse
RORγt	CAGTACGTGGTGGAGTTCGC	TAGGGGCAGAAACCAGTCTTAG
Foxp3	CTGGACAACCCAGCGATGAT	GGGTAGGATCCTTGTGGTGC
β-Actin	TCAGGTCATCACTATCGGCA	GGCATAGAGGTCTTTACGGAT

Spearman correlation coefficient was next calculated among the filtered OTUs to obtain similarity matrix (Spearman's $r > 0.6$). To determine an appropriate cutoff to construct network, the adjacent correlation matrix was used according to the random matrix theory. Correlation matrix and *p*-value matrix were then used to generate network on the basis of a cutoff to gain associated node and edge attributes ($p < 0.01$). The topological properties of network and modularization and Z_i–P_i result for module hubs were obtained subsequently. Finally, Gephi software 0.9.5 was used to visualize the network. Microbiome analysis of intestinal microbes was conducted on the genes cloud platform (https://www.genescloud.cn/), provided by the Shanghai Personal Biological Technology Co. LTD (Shanghai, China).

2.10 | Metabolomics Analysis

2.10.1 | Preparation of Fecal Samples

An amount of 100 mg of fecal sample was added to 1000 μL of PBS (0.1 mol/L, pH = 7.4). After being vortexed sufficiently, freeze–thaw cycles were performed for three times. Finally, the mixture was placed in an ice bath for sonication for 20 s, and then stood for 40 s. The procedure was repeated for 20 times. All samples were centrifuged at 15,493 g for 15 min at 4°C. Afterward, 100 μL of D_2O containing 0.0025% (W/V) TSP was added into 500 μL supernatant. A total of 450 μL supernatant was pipetted into a 5 mm NMR tube for NMR analysis.

2.10.2 | ^{1}H NMR-Based Metabolomics Analysis

^{1}H NMR spectra of fecal samples were acquired on a Bruker 600-MHz AVANCE III NMR spectrometer (Karlsruhe, Germany) at 25°C. A one-dimensional Carr–Purcell–Meiboom–Gill pulse sequence with water suppression was used. A total spin relaxation delay (RD) of 320 ms was used. Each sample spectrum consisted of 64 scans requiring 5 min of acquisition time with the following parameters: spectral width = 12,019.2 Hz, spectral size = 65,536 points, pulse width = 30° (12.6 μs), and RD = 1.0 s.

By using MestReNova 6.0 (Santiago, Spain), all acquired NMR spectra were manually phased, baseline-adjusted. In addition, the spectra were segmented into consecutive regions of 0.01 ppm chemical shift bins across the region δ 0.63–8.98. As containing the residual water signals, the regions of δ 4.57–5.85 were removed from spectra. Finally, the remaining regions of spectra were normalized to the total integrated area of spectra in order to compensate concentration differences among samples.

At the same time, both Human Metabolome Database (http://www.hmdb.ca), Massbank (https://www.massbank.jp), KEGG (https://www.kegg.jp), and PubChem (https://pubchem.ncbi.nlm.nih.gov/), and related literatures were referred to identify metabolites (Chen et al. 2019a; Zhou et al. 2023).

2.10.3 | MVD Analysis and Visualization of Metabolic Networks

Normalized data were analyzed by MVD analysis using SIMCA 14.0 (Umetrics, Sweden). Principal component analysis (PCA), partial least-square-discrimination analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed to identify depression and APS-related variations.

Meanwhile, simultaneous permutation tests were used to evaluate the quality of each MVD model constructed. Differential metabolites were identified on the basis of the following criteria: VIP (variable importance in the projection) values ≥ 1.0 that obtained from OPLS-DA models and p values < 0.05 that calculated from Students' *t*-test.

Furthermore, to confirm the targeted metabolic pathways and to visualize the metabolic networks, metabolic pathways were constructed and analyzed by Metabolomic Pathway Analysis (MetPA) on the MetaboAnalyst 5.0 platform (http://www.metaboanalyst.ca).

Finally, to visualize the improvement effects of PX and APS on depressed rats in terms of metabolic pathways, $p < 0.05$ was used as a screening condition.

2.11 | Correlation Analysis

Spearman correlation analysis was used to calculate the correlation coefficients between behaviors and gut microbes, behaviors and differential metabolites, andgut microbes and differential metabolites. The data were screened and imported into Excel.

Heat-maps of correlations were drawn by using the Sangerbox 3.0 platform (http://sangerbox.com/home.html). To understand the correlations among behavioral index, gut microbes, and differential metabolites, Cytoscape 3.9.0 (NRNB, USA) was used to draw the relevant network diagrams.

2.12 | Statistical Analysis

All behavioral data were expressed as mean ± standard error of mean (SEM). Behavioral data analysis was performed by the IBM SPSS Statistics 26.0 (Chicago, USA). Mean ± SEM were

analyzed with one-way ANOVA. Data that do not conform to the normal distribution were analyzed by Kruskal–Wallis H. All column bars were drawn by GraphPad Prism software 8.0 (San Diego, CA, USA). Figures 2 and 4 were drawn by Sangerbox 3.0 platform. Figure 3 was drawn by genescloud platform (https://www.genescloud.cn/home). A value of $p < 0.05$ was considered to be statistically significant.

3 | Results

3.1 | Characteristics of APS

In this study, APS used for the treatment of depression was isolated from *Astragalus* by water extraction and alcohol precipitation, which yielded 8.5%, that is, 8.5 g APS from 100 g *Astragalus*. The MW of APS was determined to be 1699,100, by high-performance gel permeation chromatography. APS was composed of glucose (Glc), galactose (Gal), arabinose (Ara), rhamnose (Rha), and galacturonic acid (Gal A) at a molar ratio of 1.5:1:5.4:0.08:0.1.

3.2 | APS Significantly Ameliorated the Abnormal Behaviors of Depressed Rats

Experimental period and grouping information were shown in Figure 1A. The initial body weight of rats did not differ among four groups at week 0. After 4-week CUMS experiment, the body-weight of depressed rats (MS) was significantly lower than that of NC group [$F_{(3,20)} = 39.071$, $p < 0.001$, Figure 1B]. Noticeably, PX group [$F_{(3,20)} = 39.071$, $p < 0.05$] and APS group [$F_{(3,20)} = 39.071$, $p < 0.001$] significantly increased the body weight of rats as compared to the MS group.

In SPT, OFT, and light-dark box test (LDBT), sucrose preference rates [$F_{(3,20)} = 69.136$, $p < 0.01$], crossing grid number [$F_{(3,20)} = 17.237$, $p < 0.001$], and light box activity time rates [$F_{(3,20)} = 19.482$, $p < 0.05$] of depressed rats were significantly lower than that of negative control rats. In contrast, PX and APS significantly increased the sucrose preference rates [PX: $F_{(3,20)} = 69.136$, $p < 0.001$; APS: $F_{(3,20)} = 69.136$, $p < 0.001$], the number of crossings [PX: $F_{(3,20)} = 17.237$, $p < 0.001$; APS: $F_{(3,20)} = 17.237$, $p < 0.001$], and light box activity time rates [PX:$F_{(3,20)} = 19.482$, $p < 0.01$; APS:$F_{(3,20)} = 19.482$, $p < 0.001$] of depressed rats. Of note, the effects of APS on sucrose preference rates [$F_{(3,20)} = 69.136$, $p < 0.001$] and light box activity time rates [$F_{(3,20)} = 19.482$, $p < 0.01$] of depressed rats were stronger than that of rats in PX group (Figure 1C–G).

In FS test (FST), the immobility time of depressed rats was significantly increased as compared with negative control rats [$F_{(3,20)} = 11.823$, $p < 0.001$]. PX [$F_{(3,20)} = 11.823$, $p < 0.001$] and APS [$F_{(3,20)} = 11.823$, $p < 0.001$] significantly decreased the immobility time of depressed rats (Figure 1H, I).

In MWM test, compared with negative control rats, the time of depressed rats finding the platform significantly increased [$F_{(3,20)} = 16.208$, $p < 0.001$]. In contrast, PX [$F_{(3,20)} = 16.208$, $p < 0.001$] and APS [$F_{(3,20)} = 16.208$, $p < 0.001$] significantly decreased the time of depressed rats finding the platform (Figure 1J,K), recovering to normal levels.

3.3 | APS Recovered the Th17/Treg Imbalance and Inflammatory Response of Depressed Rats

Compared with negative control rats, the expression level of RORC gene in Th17 cells of depressed rats significantly increased [$F_{(3,16)} = 14.310$, $p < 0.05$]. However, the expression level of FOXP3 gene in Treg cells of depressed rats significantly decreased when compared to negative control rats [$F_{(3,16)} = 13.166$, $p < 0.05$]. This observation suggested that CUMS induced an imbalance of Th17/Treg in rats. In contrast, PX and APS increased the expression level of RORC gene in Th17 cells. PX [$F_{(3,16)} = 13.166$, $p < 0.05$] and APS [$F_{(3,16)} = 13.166$, $p < 0.05$] decreased the expression level of FOXP3 gene in Treg cells of depressed rats. Both PX and APS alleviated the imbalance of Th17/Treg. Of note, APS exhibited stronger effects than PX (Figure 2A).

The expression levels of IL-10, IL-17, IL-22, and TGF-β of rats in different groups were shown in Figure 2B–E. The expression levels of IL-10, IL-22, and TGF-β of depressed rats were lower than that of negative control rats, whereas the expression level of IL-17 was significant higher [$F_{(3,20)} = 10.349$, $p < 0.05$].

Compared with depressed rats, both PX and APS significantly increased the expression levels of IL-10 [PX: $F_{(3,20)} = 4.433$, $p < 0.05$; APS: $F_{(3,20)} = 4.433$, $p < 0.05$] and IL-22 [PX: $F_{(3,20)} = 9.738$, $p < 0.05$; APS: $F_{(3,20)} = 9.738$, $p < 0.05$]. PX and APS also increased the expression level of TGF. In contrast, PX [$F_{(3,20)} = 10.349$, $p < 0.05$] and APS [$F_{(3,20)} = 10.349$, $p < 0.05$] reduced the expression level of IL-17 of depressed rats. The effect of APS was comparable to that of PX.

3.4 | APS Significantly Ameliorated the Abnormality of Gut Microbiota of Depressed Rats

The rarefaction curves were close to the saturation plateau, indicating that sequencing results adequately reflected the diversity that contained in the current samples (Figure S1). There were significant differences in species composition, α-diversity, and β-diversity of gut microbiota of rats between MS group and other groups. PCoA analysis (PCo1 = 28.7%, PCo2 = 13.7%) based on Bray–Curtis distance of four groups (Figure 3A) showed that the MS group clustered far away from the NC group, suggesting depression changes the microbiota compositions. PX and APS were clustered with NC group except for one rat, pointing that PX and APS could improve the abnormality of intestinal bacteria that interrupted by depression. The results of NMDS analysis were similar to that of PCoA (Figure 3B). There were significant differences in the abundance, diversity, and the evenness of gut microbiota of rats. Shannon, Simpson, Plelou_e, and Observed_species indices of rats in MS group were higher than that of NC group. In contrast, PX and APS treatments significantly decreased the Shannon, Simpson, and Plelou_e indices of depressed rats. Of note, the effects of APS treatment on Shannon, Simpson, Plelou_e, and Observed_species indices of depressed rats were stronger than that of PX (Figure 3C). Meanwhile,

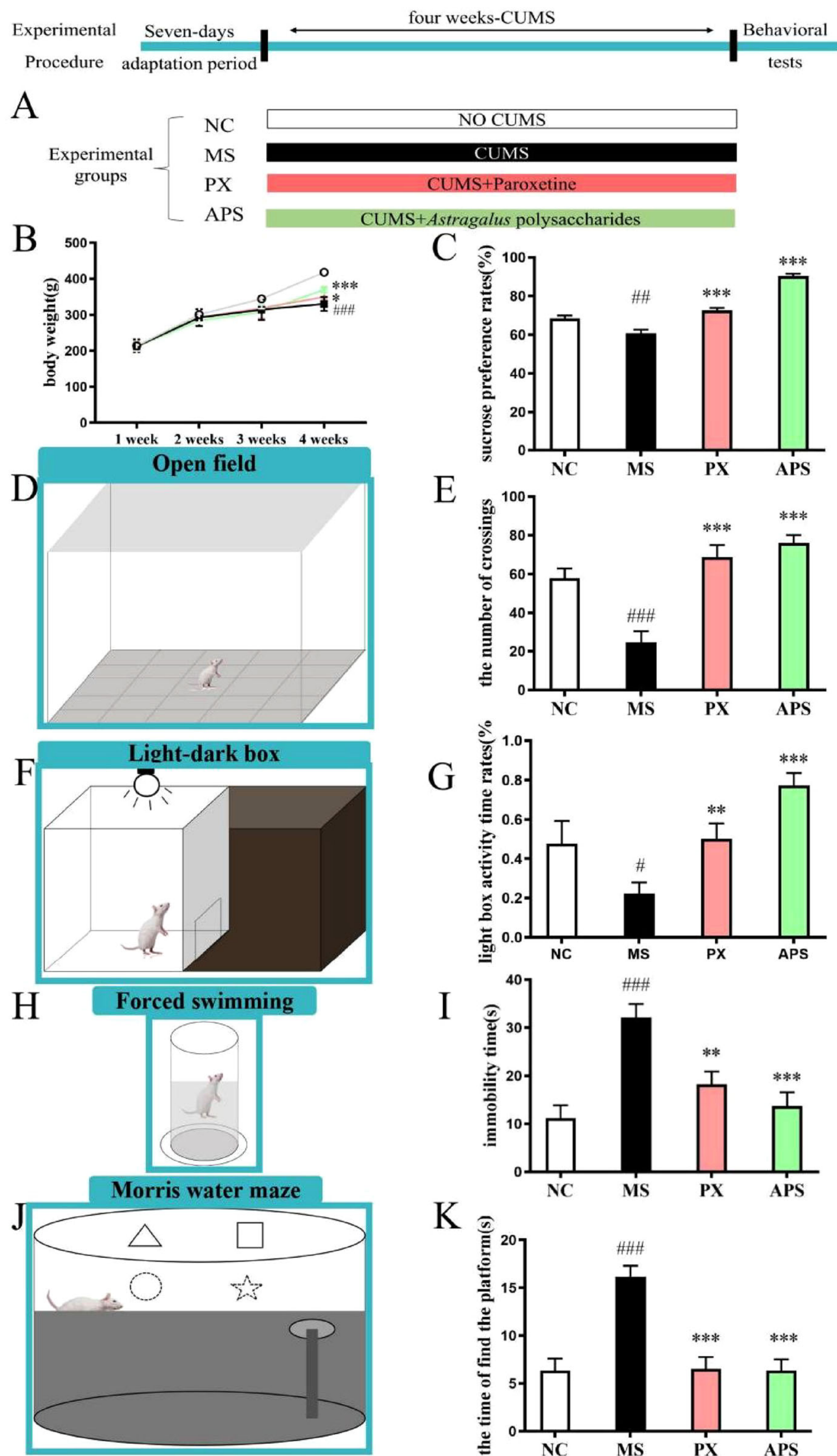

FIGURE 1 | The behavioral performances of rats in the negative control group (NC), the chronic unpredictable mild stress (CUMS) model group (MS), the paroxetine group (PX), and the *Astragalus* polysaccharides group (APS), including the experimental procedure and groups (A), body weight trends of rats (B), sucrose preference rates (SPT) (C), the number of crossings of rats in the open-field test (OFT) (D, E), light box activity time rates in the light-dark box test (LDBT) (F, G), immobility time in forced swimming test (FST) (H, I), and the time of find the platform in Morris water maze test (MWM) (J, K). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, significant differences as compared to the NC group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significant differences as compared to the MS group ($n = 6$). Values were expressed as means ± SEM. Body weight trends of rats: $F_{(3,20)} = 39.071$, Sucrose preference rates: $F_{(3,20)} = 69.136$. The number of crossings of rats in the open-field test: $F_{(3,20)} = 17.237$. Light box activity time rates in the light-dark box test: $F_{(3,20)} = 19.482$. Immobility time in forced swimming test: $F_{(3,20)} = 11.823$. The time of find the platform in Morris water maze test: $F_{(3,20)} = 16.208$.

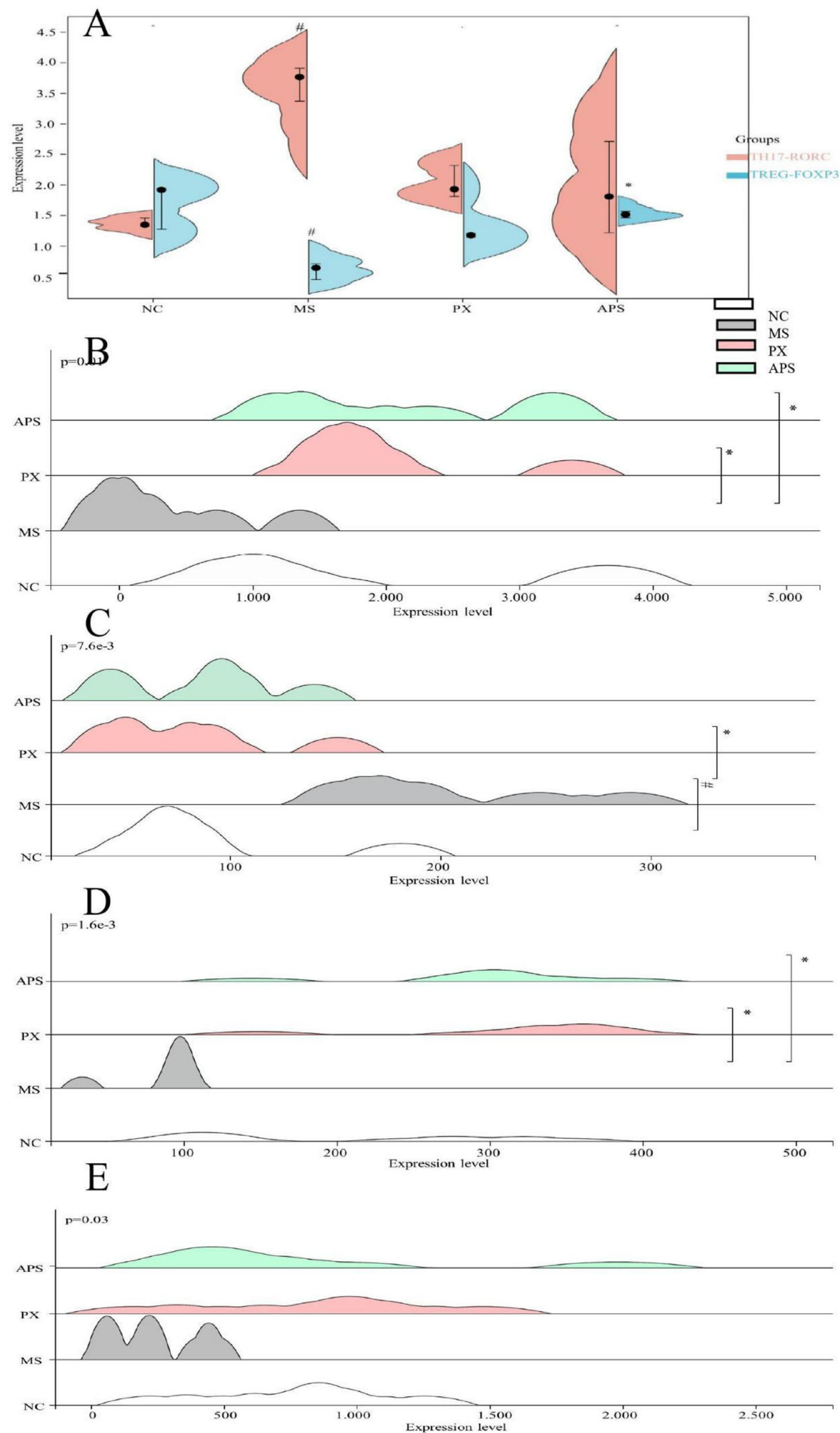

FIGURE 2 | The levels of gene expression in Th17 cell and Treg cell (A), the levels of IL-10 cytokines (B), IL-17 cytokines (C), IL-22 cytokines (D), and TGF cytokines (E) of rats in the negative control group (NC), the chronic unpredictable mild stress (CUMS) model group (MS), the paroxetine group (PX), and the *Astragalus* polysaccharides group (APS). Values were expressed as means ± SEM (The levels of gene expression in Th17 cell and Treg cell, $n = 5$; the levels of IL-10 cytokines, IL-17 cytokines, IL-22 cytokines, and TGF cytokines, $n = 6$). $^{\#}p < 0.05$, significant differences as compared to the NC group, $^{*}p < 0.05$, significant differences as compared to the MS group. The levels of gene expression in Th17 cell: $F_{(3,16)} = 14.310$. The levels of gene expression in Treg cell: $F_{(3,16)} = 13.166$. The levels of IL-10 cytokines: $F_{(3,20)} = 4.433$. The levels of IL-17 cytokines: $F_{(3,20)} = 10.349$. The levels of IL-22 cytokines: $F_{(3,20)} = 9.738$. The levels of TGF cytokines: $F_{(3,20)} = 2.463$.

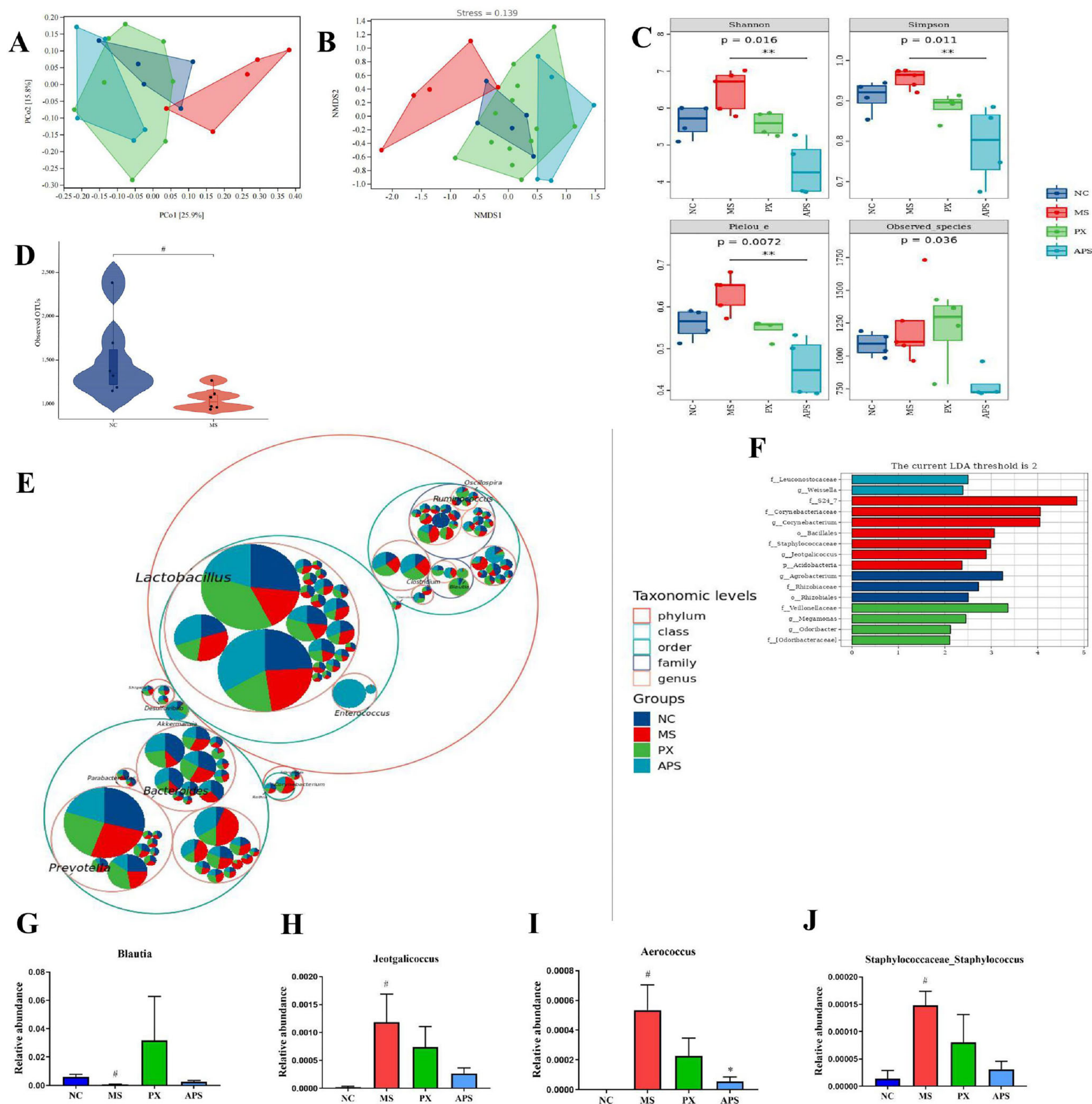

FIGURE 3 | Difference in the fecal microbiota of rats, including principal coordinates analysis of microbial unweighted UniFrac compositional differences (A), nonmetric multidimensional scaling (B), alpha diversity analysis based on Shannon, Simpson, Pielou, and Observed (C) ($n = 4$), the community richness calculated by observed OTUs (D), taxonomic histogram obtained using LEfSe analysis (E), taxonomic tree in packed circles (F), relative abundances of *Blautia*, *Jeotgalicoccus*, *Aerococcus*, and *Staphylococcaceae_Staphylococcus* in rats of the negative control (NC) group, the chronic unpredictable mild stress (CUMS) (MS) group, the paroxetine (PX) group, and the *Astragalus* polysaccharides (APS) group. $^{\#}p < 0.05$, significant differences as compared to the NC group, $^{*}p < 0.05$, significant differences as compared to the MS group. The community richness calculated by observed OTUs: $F_{(1,10)} = 5.059$, the relative abundances of *Blautia:* $F_{(3,20)} = 9.495$, the relative abundances of *Jeotgalicoccus:* $F_{(3,20)} = 2.742$, the relative abundances of *Aerococcus:* $F_{(3,20)} = 5.312$, the relative abundances of *Staphylococcaceae_Staphylococcus:* $F_{(3,20)} = 3.883$. MS, model group; NMDS, nonmetric multidimensional scaling.

depression led to a reduction in community richness (α diversity), as shown by OTUs [$F_{(1,10)} = 5.059$, $p < 0.05$, Figure 3D]. The taxonomic tree in packed circles results showed that *Lactobacillus, Prevotella, Bacteroides, Ruminococcaceae_Ruminococcus, Oscillospira, Enterococcaceae_Enterococcus, Parabacteroides, Blautia, Coprococcus*, and *Corynebacterium* were the top 10 gut bacteria in abundance at the genus level (Figure 3E). CUMS significantly changed the composition of intestinal microbiome in rats.

To identify specific bacterial taxa of gut microbiota of rats in different groups, the compositions of gut microbiota of four groups were compared by LEfSe (Figure 3F). LDA scores showed that *corynebacterium* was the key bacterial type that led to microflora imbalance of depressed rats. The LDA score of family *Leuconostocaceae* was the highest in APS group. At the genus level, four gut microbes were found to be most significantly differed between depressed rats and negative control rats, including *Blautia* [$F_{(1,10)} = 5.059$, $p < 0.05$, Figure 3D], *Jeotgalicoccus* [$F_{(3,20)} = 2.742$, $p < 0.05$], *Aerococcus* [$F_{(3,20)} = 5.312$, $p < 0.05$], and *Staphylococcaceae_Staphylococcus* [$F_{(3,20)} = 3.883$, $p < 0.05$]. Compared with the MS group, *Aerococcus* was significantly lower in APS group [$F_{(3,20)} = 3.883$, $p < 0.05$, Figure 3G–J].

3.5 | APS Ameliorated the Intestinal Disorders of Depressed Rats

Studying the interactions among members of microecology (i.e., the co-occurrence pattern) may help further explore the role of APS in depression. Therefore, we performed a network analysis to visualize the co-occurrence pattern in gut microbial communities. After quality filtering and OTU clustering, we performed Spearman's correlation analysis and constructed a co-occurrence network. We found the network of MS group consisted of 236 nodes and 323 edges, whereas that of APS group consisted of 302 nodes and 447 edges (Figure 4A,B), indicating that more members engaged in significant interactions by APS. These results indicated that the network structure significantly differed between the two groups. Moreover, the intestinal microecology of APS group had a more connected and complex co-occurrence pattern than that of MS group.

To identify the key members (i.e., keystone species) in the co-occurrence pattern, we assessed the topological role of each node on the basis of two properties, that is, within-module connectivity (Z_i) and among module connectivity (P_i) (Jiang et al. 2015b). We classified all species into four subcategories, that is, peripherals, connectors, module hubs, and network hubs. Module hubs represented close connections within a module, and connectors represent high connections among modules. Therefore, module hubs and connectors helped identify keystones species. According to the Z_i–P_i plot, in the MS group, the node derived from *Ruminococcus* (OTU 223) was categorized as a module hub, and two nodes derived from *unclassified_Prevotellaceae* (OTU 723) and *unidentified_S24-7* (OTU 1518) were categorized as connectors (Figure 4C). This result suggested that they play a leading role in the interaction between community members. By contrast, in the APS group, seven nodes, including *Prevotella* (OTU 36), *Lactobacillus* (OTU 58), which belong to beneficial bacteria, *unidentified_Peptostreptococcaceae* (OTU 232), *Adlercreutzia* (OTU 431), *Oscillospira* (OTU 623), *unidentified_RF39* (OTU 805) and *Adlercreutzia* (OTU 893), were classified as module hubs, and the node, namely, *unidentified_Ruminococcaceae* (OTU 704), was classified as a connector (Figure 4D). This result indicated that APS prompted beneficial bacteria to guide a positive co-occurrence pattern in the intestinal microecology, thereby promoting gut homeostasis of depressed rats.

3.6 | Differential Metabolites and Pathways Involved in CUMS-Induced Depression and the Antidepression Effects of APS

The representative ^{1}H NMR spectra of fecal samples of rats that collected from NC, MS, PX, and APS groups were shown with major metabolites being labeled. In total, 33 fecal metabolites were identified (Table S2, Figure S2).

PCA score plot showed separation between the NC group and the MS group, indicating that CUMS significantly disturbed the fecal metabolic profiles of rats. Second, APS and PX groups were clustered with the NC group, suggesting that the two groups were able to reverse the abnormalities of fecal metabolic profiles of depressed rats. APS showed an equivalent effect with PX (Figure 5A). Furthermore, PLS-DA was performed to maximize the differences of metabolic profiles of rats from different groups and to show the intergroup metabolic differences. $R^2X = 0.331$ and $Q^2Y = 0.708$ showed that PLS-DA model was valid and without over-fitting (Figure 5B,C).

Both VIP > 1.0 and $p < 0.05$ were used to screen differential metabolites involving in CUMS-induced depression, as well as to assess the antidepression effects of APS (Figure 5D). Compared with negative control rats, the concentrations of lysine [$F_{(3,20)} = 11.832$, $p < 0.01$], alanine [$F_{(3,20)} = 7.714$, $p < 0.01$], and arginine [$F_{(3,20)} = 11.942$, $p < 0.01$] in depressed rats were significantly increased, whereas the content of Glc decreased. APS significantly reduced the levels of lysine [$F_{(3,20)} = 11.832$, $p < 0.001$], alanine [$F_{(3,20)} = 7.714$, $p < 0.001$], and arginine [$F_{(3,20)} = 11.942$, $p < 0.001$] of depressed rats. PX could significantly regulate the abnormal levels of four fecal metabolites, in terms of reducing the levels of lysine [$F_{(3,20)} = 11.832$, $p < 0.001$], alanine [$F_{(3,20)} = 7.714$, $p < 0.001$], and arginine [$F_{(3,20)} = 11.942$, $p < 0.001$], while increasing the level of Glc (Figure 5F).

As shown in Figure 5E, five metabolic pathways were found to be involved in pathophysiologic mechanisms of CUMS-induced depression, including arginine biosynthesis, arginine and proline metabolism, aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, and biotin metabolism. APS significantly regulated three metabolic pathways, that is, aminoacyl-tRNA biosynthesis, biotin metabolism, and arginine biosynthesis.

3.7 | The Correlations Among Behavioral Indicators of Rats, Immune Factors, Gut Bacteria, and Differential Metabolites

In behavioral indicators, body weight had the greatest influence, which was positively correlated with the expressed levels of IL-10 and Treg in immune factors, whereas it negatively correlated with the expressed level of Th17 in immune factors (Figure 6B,D,F). Among differential metabolites that involved in CUMS-induced depression, lysine had the greatest influence, which positively correlated with the contents of alanine and arginine in differential metabolites, the abundance of *Staphylococcaceae_Staphylococcus* in gut bacteria, the behavioral indicators of FST, and the expressed level of IL-17 in immune factors, respectively. On the contrary, it negatively correlated with the expressed level of IL-22 in immune factors (Figure 6C–E). Among immune

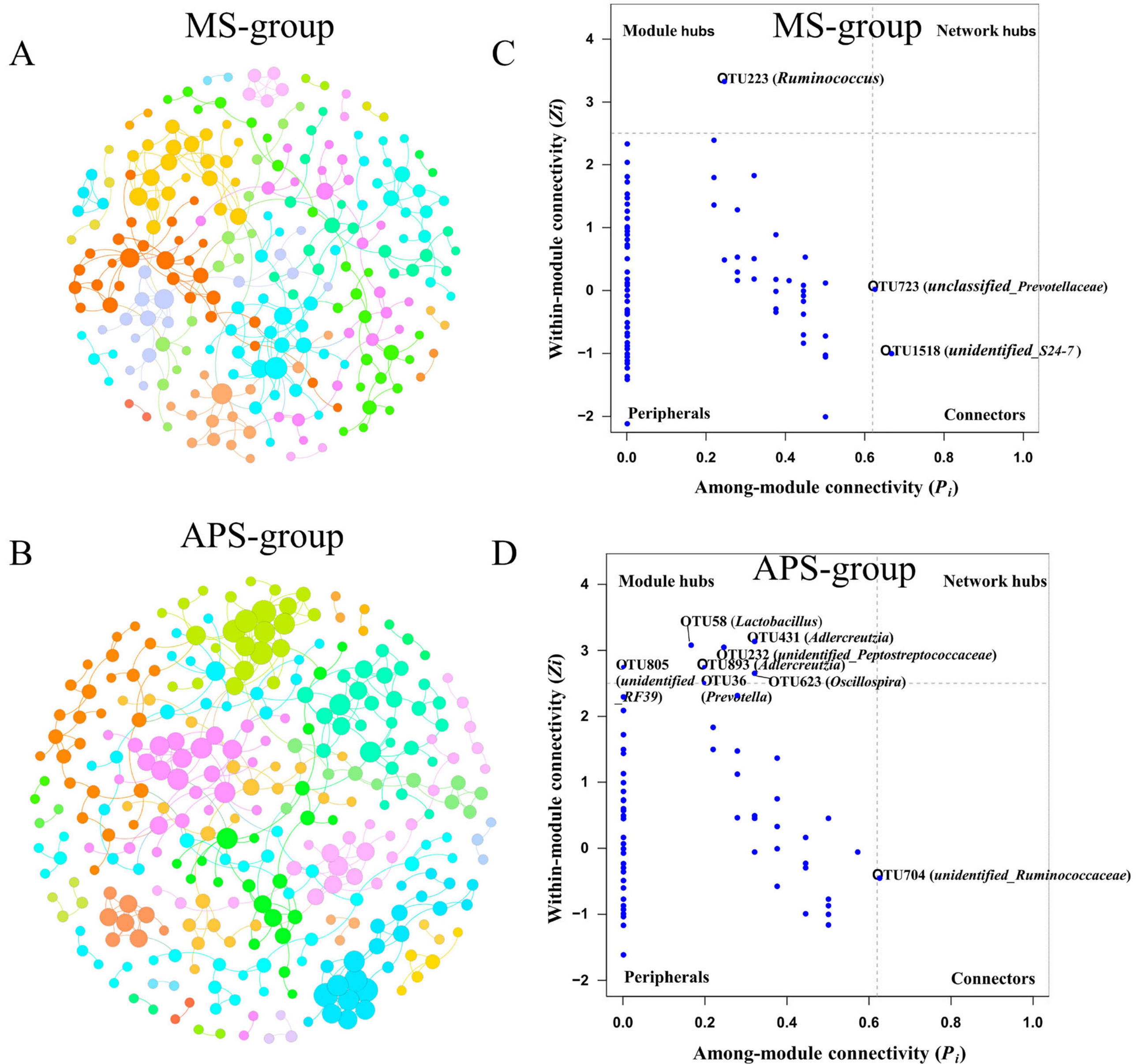

FIGURE 4 | APS improved co-occurrence patterns in the intestinal microecology to promote gut homeostasis. Co-occurrence network of the MS group (A) and the APS group (B) based on Spearman's correlation analysis. Each node represented an OUT and the size of each node was proportional to the number of edge (that is, degree). Different colors represented different modules. A connection (that is, edge) stood for a strong (Spearman's r > 0.6) and significant ($p < 0.01$) correlation. Z_i–P_i plot for the MS group (C) and the APS group (D). Z_i–P_i plot showed the distribution of nodes on the basis of their topological roles in aggregate-related networks. The module hubs and connectors were labeled with OTU numbers and corresponding bacteria. APS, *Astragalus* polysaccharides; MS, model group.

factors, IL-17 was observed to have the greatest influence, which was positively correlated with the expressed level of Th17 in immune factors, the contents of lysine, alanine, and arginine in differential metabolites, and the abundance of *Aerococcus* in gut bacteria, respectively. On the contrary, it was negatively correlated with the expressed levels of IL-22 and Treg in immune factors (Figure 6A,E,F). Among gut bacteria, we observed the greatest influence of *Staphylococcaceae_Staphylococcus*, which positively correlated with the abundance of *Aerococcus* in gut bacteria, the contents of lysine, alanine, and arginine in differential metabolites, respectively. Conversely, it negatively correlated with the expressed levels of IL-22 and Treg in immune factors, the abundance of *Blautia* in gut bacteria, respectively (Figure 6A–C). In addition, we found that the levels of lysine and arginine were the most closely related to behavioral indicators, immune factors, and gut bacteria (Figure 6G).

4 | Discussion

In this study, the antidepression effects and the underlying mechanisms of APS on depression were, for the first time, demonstrated from the perspective of "gut microbiome–immunity–metabolome" axis by integrally applying 16S rRNA gene sequencing and ^{1}H NMR-based metabolomics. We concluded that APS significantly improved depressive behaviors of depressed rats, as

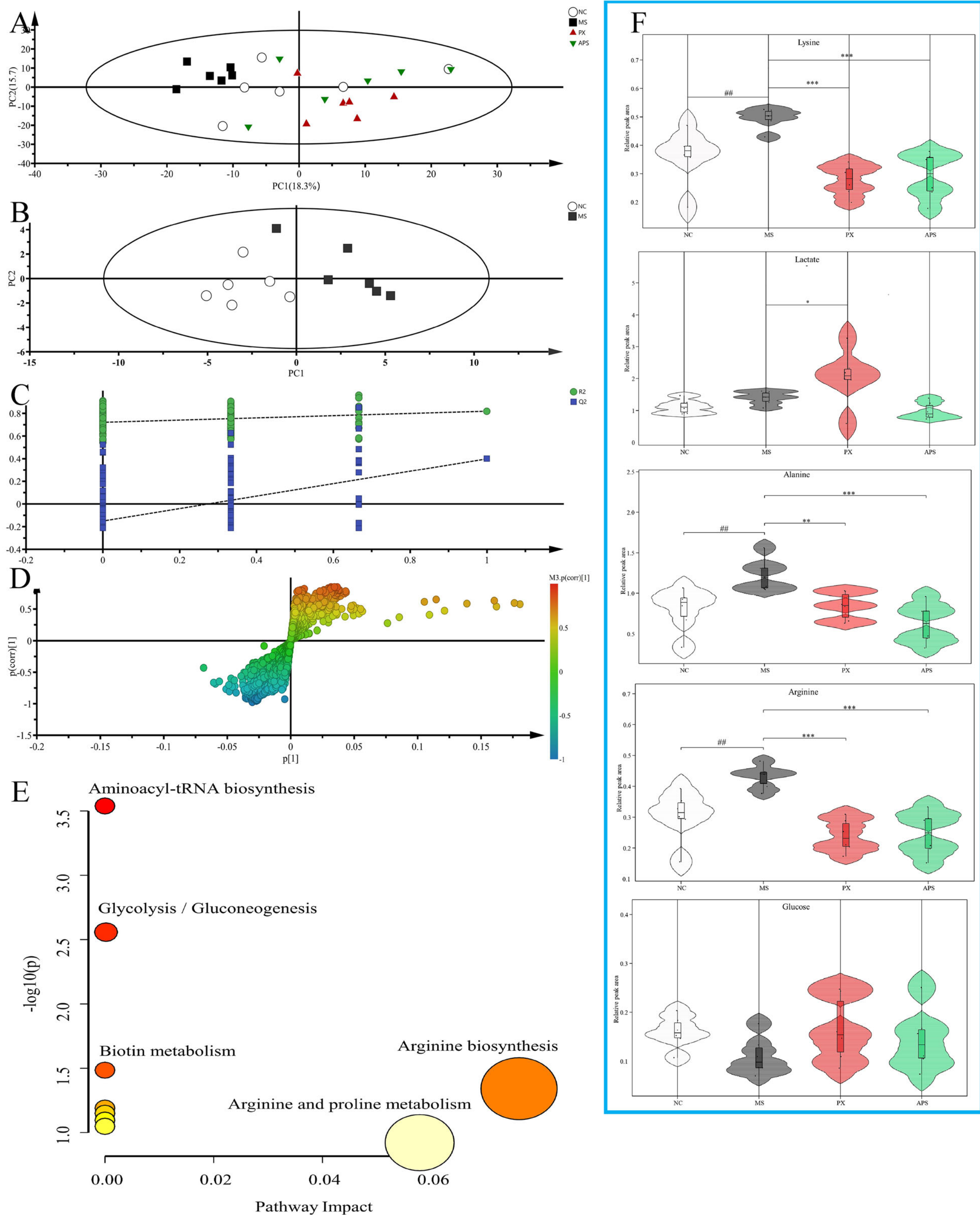

FIGURE 5 | Score plot of principal component analysis (PCA) (A) of the negative control (NC) group, the chronic unpredictable mild stress (CUMS) (MS) group, the paroxetine (PX) group, and the *Astragalus* polysaccharides (APS) group, and score plot of partial least square-discrimination analysis (PLS-DA) (B), the model permutation (C), the corresponding S-Plot (D) of orthogonal partial least squares discriminant analysis (OPLA-DA) of the NC group and the MS group ($n = 6$), as well as the metabolic pathways (E) involving in CUMS-induced depression (NC vs. MS). Relative peak areas of the differential metabolites (F) of the NC, the MS, the PX, the APS group. In (C), the PLS-DA model was validated by 200 random permutations. In (E), the sizes and the colors were built on pathway count and p values, respectively. All data were expressed as mean ± SEM ($n = 6$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the NC group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the MS group. Relative peak areas of the differential metabolites: lysine: $F_{(3,20)} = 11.832$; lactate: $F_{(3,20)} = 5.990$; alanine: $F_{(3,20)} = 7.714$; arginine: $F_{(3,20)} = 11.942$; glucose: $F_{(3,20)} = 1.315$. MS, model group.

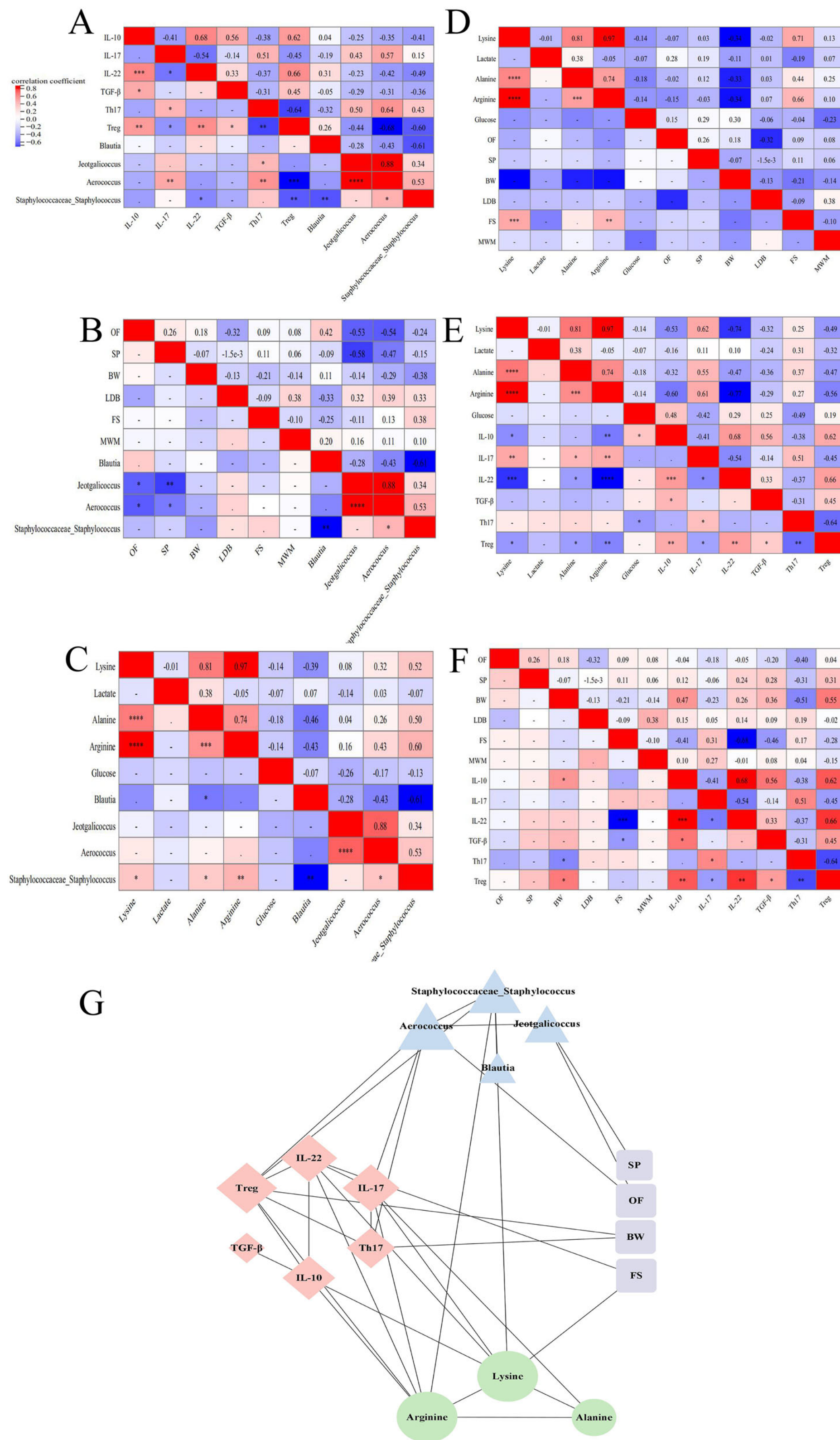

FIGURE 6 | Correlation analysis between immune factors and gut bacteria (A), between behavioral indicators and gut bacteria (B), between differential metabolites and gut bacteria (C), between differential metabolites and behavioral indicators (D), between differential metabolites and immune factors (E), between behavioral indicators and immune factors (F), as well as among behavioral indicators, gut bacteria, immune factors, and differential metabolites (G). In (A–F), red and blue indicated positive correlations and negative correlations, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. In (G), the green, the orange, the blue, and the purple indicated differential metabolites, immune factors, gut bacteria, and behavioral indicators, respectively. FST, forced swimming test; IL-10, interleukin 10; IL-17, interleukin 17; IL-22, interleukin 22; MWM, Morris water maze; TGF-β, transforming growth factor-β.

well as the disorders of gut microbiome, immune factors, and fecal metabolome. As such, the current findings broaden and lay solid foundations for the clinical applications of APS, which for instance could be potentially served as a candidate of treating depression.

As a complicated mental disorder, depression has been reported to be attributed to a combination of genetic, environmental, and even lifestyle risk factors (Gao et al. 2018a). Such an illness is usually characterized with unhappiness and low mood, accompanied with the lack of heed, fatigue, insomnia, and so forth (Zhou et al. 2012). Other than that, depressed patients are more often frail and have substantially higher frailty scores than nondepressed counterparts (Oude Voshaar et al., 2021). The present results on depressed rats showed consistent results with depressed patients. The reduced activity of depressed rats in the open field test indicates that CUMS exposure has significant influences on anxiety, exercise, and exploration levels (Wang et al. 2018a). The observed reduction in sucrose consumption in SPT reflects the suppression of brain reward system associated with CUMS, which serves as a proxy for depression in rodents (Strekalova et al. 2004). CUMS has also been associated with significant changes in body weight, which aligns with previous studies, and likely reflects combined effects of stress, food deprivation, and sucrose consumption paradigms used during CUMS protocol (Dong et al. 2019; Gáll et al. 2020; He et al. 2020). Following our discriminate analysis, we observed diminished the number of crossings of rats in OFT, reduced light box activity time rates in LDBT, decreased sucrose preference rates in SPT, increased immobility time in FST, increased the time of finding the platform in MWM, and lower body weights in rats that exposed to CUMS for four continuous weeks as compared with negative control rats.

By contrast, these changes could be significantly ameliorated by both APS and PX. This finding agrees with the results of Wang et al. (Wang et al. 2018b). The above results not only indicate the reliability and the stability of CUMS model that we used in this study but also suggest that APS and PX significantly returned the abnormal behaviors of depressed rats to normal status. *Astragalus* is a TCM that has been used for thousands of years to improve the function of body, especially through tonifying *Qi*. *Astragalus* alleviates physical fatigue in mouse under simulated plateau environment. Concerning active components in herbs, a variety of plant polysaccharides has been shown to own anti-fatigue effects (Gao et al. 2018b; Zhang et al. 2020; Zhang et al. 2022). On the basis of the present results, we speculate that APS, the main active ingredient of *Astragalus*, also plays an anti-fatigue role, consequently improving the depressive symptoms of rats that induced by CUMS.

Gut microbiome represents a complex ecosystem in which members rarely live independently from each other; instead, the interaction between the members is responsible for the stability of gut homeostasis to influence host health and diseases (Bear et al. 2020; Simpson et al. 2021; Wu et al. 2021). For instance, we previously found that CUMS significantly disturbed gut microbiome of depressed rats (Liu, Wang, et al. 2023a). By using 16S rDNA sequencing technology, the structures of gut microbiota of depressed rats were significantly altered by CUMS. At genus level, four gut microbiota of depressed rats induced by CUMS were screened to be significantly differed, namely, *Blautia*, *Jeotgalicoccus*, *Aerococcus*, and *Staphylococcaceae_Staphylococcus*. We found that the abundance of *Blautia* of depressed rats was lower. APS significantly decreased the levels of *Jeotgalicoccus, Aerococcus*, and *Staphylococcaceae_Staphylococcus*. It has been showed that *Blautia*, *Lactobacillus*, *Bradyrhizobium*, and *Faecalibaculum* are all well-known probiotics (Deng et al. 2020). *Blautia* is one of the bacteria that produce acetate in the intestine. Supplementation of *Blautia* can alleviate depression (Ye et al. 2023). Tao et al. (2024) found that after fecal microbiota transplantation in TRG5 knockout mice, the mice showed anxiety and depression, the content of 5-HT in serum and hippocampus decreased, while the abundance of *Jeotgalicoccus* increased. *Aerococcus* is an α-hemolytic and microaerophilic or facultatively anaerobic Gram-positive coccus that has been shown to be associated with anxiety or depression in women (Wu et al. 2017). *Staphylococcaceae _ Staphylococcus* can activate the NF-κB pathway and cause inflammatory response (Bao et al. 2023). We speculate that *Staphylococcaceae _ Staphylococcus* may mediate the occurrence of depression through inflammatory response. Therefore, APS improved the depressive symptoms of depressed rats in terms of increasing abundances of beneficial bacteria. The current results highlight the important roles of gut microbiome in CUMS-induced depression, as well as the antidepression effects of APS.

The immune system also plays an important role in the occurrence and development of depression (Lin et al. 2018). Gastrointestinal tract is the largest immune organ for systemic immunity in animals, composing various types of cells (Zheng et al. 2020). Among others, Th17 and Treg cells represent two CD4+ T cell subsets but with opposing principal functions, playing important functions in cancer immunity and infectious, as well as autoimmune diseases (Guéry and Hugues 2015; Lochner, Wang, and Sparwasser 2015). Th17 cells regulate immune and pro-inflammatory functions mainly through secreting IL-17, IL-22, and other cytokines, thus participating in the occurrence and development of immune diseases, inflammatory diseases, and tumors (Korn et al. 2009). At the same time, Treg cells exert immune-regulatory effects by releasing inhibitory cytokines, for example, IL-10 and TGF-β to suppress inflammatory immune responses (Hatzioannou et al. 2021). As such, the balance of Th17 and Treg cells is critical for developing autoimmunity (Lee 2018). Of particular interests, gut microbiota alters Th17 to Treg ratios of depressed rats. The imbalance of Th17 to Treg cells has been implicated in the development of chronic stress-induced depression in mice (Hong et al. 2013). In this study, we found that not only the levels of pro-inflammatory factors increased, whereas anti-inflammatory factors in depressed rats decreased, but also the Th17/Treg ratio was significantly increased in depressed rats. However, it was a very interesting phenomenon observed in this study that unlike the trend of expression at the cellular level, IL-22 of depressed rats secreted by Th17 cells was significantly decreased. Generally, IL-22 is considered as a pro-inflammatory factor and plays a critical role in various inflammatory diseases, that is, allergic airway inflammation (Leyva-Castillo, Yoon, and Geha 2019). In recent years, increasing evidence suggests that the role of IL-22 can be both pathogenic and protective, depending on inflammatory environment (Zenewicz 2021). After reviewing literatures, we found that IL-22 could play a protective role in chronic colitis by enhancing the integrity of intestinal mucosa (Huang et al. 2022b).

Studies have shown that probiotic supplements alleviate depressive behaviors in rats by reducing pro-inflammatory cytokines (Aygun et al. 2022). Similarity, anti-inflammatory factors are also involved in neuroprotective efficiency of depressed rats (Albrakati et al. 2021). APS significantly regulated the abnormal levels of pro- and anti-inflammatory factors of depressed rats. Meanwhile, the abnormality of Th17/Treg balance was significantly restored by APS. All in all, APS could exert antidepression effects in terms of significantly regulating intestinal immune balance.

Feces, the final metabolites of organism, are produced by interactions between host and gut microbiota. Therefore, fecal metabolome and related information can remarkably reflect the status and functions of gastrointestinal function. For instance, variations in fecal amino acids (Rauf et al. 2022) and metabolites (Feng et al. 2018) can characterize the changes in gut microbiota associated with CUMS-induced depression. In this regard, changes and functions of fecal metabolites could reveal dynamic changes of depression, as well as mechanisms underlying the antidepression effects of APS.

We found that CUMS significantly altered the metabolic profiles of rats and further abnormalized the levels of fecal metabolites, for example, the significant increases of lysine, lactate, alanine, and arginine, whereas the significant decrease of Glc. Lysine is related to lysyl-tRNA synthetases. In vivo metabolism of depressed rats has also been reported to involve in aminoacyl-tRNA biosynthesis (Enea et al. 2019; Geng et al. 2020). Meanwhile, lysine is the most significant excitatory neurotransmitter in central nervous system (CNS) (Battur et al. 2009). Wang et al. (2023) found that the content of lactic acid in Hspa12 a mouse with anti-anxiety behavior increased. Lactate usually forms under anaerobic condition that shifts pyruvate in the direction of lactate via lactate dehydrogenase (LDH). It has been reported that a decrease of LDH level in serum is an independent risk factor for suicide attempt in major depressive disorder (MDD) patients (Ghrewati et al. 2018; Yao, Liu, and Li 2022). In addition, the modulation of lactate may be useful in treating sleep onset problems that associated with depression (Murack and Messier, 2019). Mitani et al. (2006) found that the level of alanine in patients with depression was positively correlated with the degree of depression. Alanine, also produced from pyruvate via alanine transaminase, is considered to be a potential biomarker than lactate of hypoxia (Sanni et al. 2001). Alanine transaminase is related to depressive-like behaviors that caused by chronic restraint stress-induced depressed mice (Lin et al. 2021). Beyond this, alanine can be synthesized from pyruvate by Glc-alanine cycle (Chen et al. 2019b). An increase of alanine may be related to Glc-alanine cycle, which converts pyruvate to alanine by glutamate-pyruvate transaminase (Douzi et al. 2020). The significant increase of arginine suggests that oxidative stress may be involved in CUMS-induced depression (Bakir et al. 2019). Detka et al. (2014) found that prenatal stress increased the content of Glc in the midbrain of depressed rats, which was inconsistent with our results. It may be due to the different modeling methods. APS significantly improved amino acids metabolism and Glc metabolism, thus improving and alleviating depressed symptoms of rats.

Meanwhile, five metabolic pathways were found to be significantly involved in CUMS-induced depression, including arginine biosynthesis, arginine and proline metabolism, aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, and biotin metabolism. It has been reported that depression tightly connected with host energy metabolism concerning arginine and proline metabolism (Ma et al. 2019). Arginine and proline metabolism, a primary pathway for the biosynthesis of arginine and proline, was also reported significantly disturbed in the prefrontal cortex of learned helplessness rat model in a metabolomics study (Zhou et al. 2017). It has shown that polyamines could enhance phosphorylation processes, and that phosphorylation of functional proteins in neurons might involve in the therapeutic mechanisms of antidepressants (Benelli et al. 1999). The results of poststroke depression analysis showed that aminoacyl-tRNA biosynthesis was the pathway containing the most associated metabolites (Liu, Li, et al. 2022b). Simultaneously, Jia et al. found that the disorder of “aminoacyl-tRNA biosynthesis” could be related to poststroke depression by affecting biological processes, such as energy failure, oxidative stress, apoptosis, and glutamate toxicity (Jia et al. 2021). In addition, “aminoacyl-tRNA biosynthesis” is an important metabolic pathway in human psychiatric disorders, such as MDD and attention-deficit/hyperactivity disorder (Yang et al. 2020). A combined analysis of feces, serum, liver, and hippocampal metabolites from mice that underwent fecal microbiota transplantation from MDD patients showed significant changes in “aminoacyl-tRNA biosynthesis” (Li et al. 2018). Recently, a metabolomics study on urine samples of rats also found that “aminoacyl-tRNA biosynthesis” was associated with depression in rats with metabolic disorder induced by CUMS (Zhang et al. 2021). APS regulated the aminoacyl-tRNA biosynthesis, biotin metabolism, and arginine biosynthesis, thus improving and alleviating the abnormal symptoms of depressed rats.

Last but not least, we constructed both inter-layer and inner-layer networks to display correlations among behavioral indicators, gut microbiota, immune factors, and differential metabolites involving in CUMS-induced depression, as well as the mechanisms underlying the antidepression effects of APS. As for behavioral indicators, immobility time in FST was most positively correlated with the levels of lysine and arginine. FST model provide a rapid and reliable behavior screening test for antidepression study (Gupta et al. 2011). It has been reported that L-arginine can be a markers of depression severity in both males and females with first depressive episode (Ogłodek et al. 2017). IL-17, a pro-inflammatory factor, was positively correlated with *Aerococcus*. It has been reported that kidneys infected with *Aerococcus urinae* showed signs of histological inflammation and elevated levels of pro-inflammatory cytokines (Gilbert et al. 2021). *Staphylococcaceae_Staphylococcus* was negatively correlated with IL-22 and Treg cells that secrete anti-inflammatory factors. *Staphylococcus aureus* has been found to be involved in the anti-inflammatory response of mastitis (Chen et al. 2022). In addition, lysine and arginine were the most closely related to behavioral indicators, immune factors, and gut bacteria between APS and depressed group. Yet, the mechanisms that we have demonstrated herein still need to be further revealed and analyzed by integrally applying various approaches, including but not limiting, proteomics, genomics, and molecular biology.

In this study, for the first time, from the perspective of “gut microbiome–immunity–metabolome” axis, we integrally applied microbiome, immunology, and metabolomics to investigate the

antidepression effects of APS, as well as the underlying mechanisms. The results showed that APS significantly improved depressive symptoms of rats in terms of regulating and improving multiple pathways with multi-targets. From the perspective of gut microbiota, APS significantly increased the abundances of beneficial bacteria, for example, *Blautia*. From the perspective of gut immunity, APS significantly restored the abnormal levels of immune factors and regulated gut immune balance. From the perspective of fecal metabolism, APS significantly recovered the abnormal levels of fecal metabolites and corresponding metabolic pathways. Finally, of note, inter- and inner-layer relationship networks were constructed on the basis of correlations among behaviors, gut microbiome, immunity, and fecal metabolome of depressed rats, as well as the regulatory effects of APS. This study will not only help us understand the pathogenesis of depression from a novel perspective of "gut microbiome–immunity–metabolome" axis, but will also provide substantial experimental evidence for applying APS on treating depression, as well as novel research strategies and perspectives for discovering new antidepressants from natural products, especially polysaccharides. It lays a foundation for our later clinical trials to explore the antidepressant mechanism of APS from a clinical perspective. At the same time, it also provides a new idea for our future researches, suggesting that we can start from natural products and combine antidepressant drugs to treat depression, improving patients' mood and their quality of life.

Author Contributions

Xiaojie Liu: conceptualization, methodology, supervision, project administration, writing–original & -review and editing. **Wenlu Xu** and **Ziyu Zhao**: experimental operation, data analysis, data visualization, writing–original draft. **Xiaoling Wu** and **Senyan Wang**: experimental operation, data analysis. **Chen Jian** and **Mengyu Li**: experimental operation, data visualization. **Yulan Wang** and **Xuemei Qin**: conceptualization, project administration, writing–review and editing. All authors have read, improved, and approved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

ETHICS STATEMENT

Our article did not carry out human experiments, only animal experiments. Outside of the experimental procedure, we did our best to reduce the stimulation and pain of animals outside of the experiment from the aspects of diet, living environment and hygiene.

①Diet: In order to ensure that animals are not plagued by hunger and thirst, we provide sufficient food and water to ensure their health and vitality while avoiding malnutrition or other health problems that caused by the lack of food.

②Living: We did our best to provide a suitable living environment for animals. All rats were raised at room temperature 23 ± 2°C, relative humidity 50 ± 10 %, light and dark cycle 12 h, so that they could can get comfortable space and rest time.

③Hygiene: In order to prevent unnecessary suffering and injury to animals, we reduce the suffering of animals due to injuries through effective disease prevention measures and treatment.

④Activity: In order to ensure that animals can move freely, we give animals sufficient space to move outside the experiments.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.