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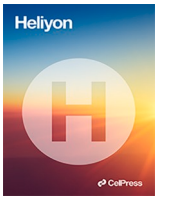

Research article

Polysaccharides from *Polygonatum cyrtonema* Hua prevent depression-like behaviors in mice with chronic unpredictable mild stress through refining gut microbiota-lipopolysaccharide-paraventricular nucleus signal axis

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A R T I C L E I N F O

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A B S T R A C T

As natural polysaccharide cannot directly pass the blood-brain barrier, it is of potential importance to investigate the systemic anti-depression mechanisms of polysaccharides (PSP) from *Polygonatum cyrtonema* Hua, a well-known herbal medicine with the anti-depressant activity. Here, we explored the underlying mechanisms of effects of PSP on chronic unpredictable mild stress (CUMS)-induced depression-like behavior in mice from the perspective of the microbiota-gut-brain axis. The results demonstrated that PSP intervention for 14 days significantly improved CUMS-induced depressive-like behaviors. Interestingly, PSP treatment increased the relative abundance of *Muribaculaceae*, *Dubosiella* and *Lactobacillus* and decreased the relative abundance of *Akkermansia*, *Helicobacter* and *Clostridium_methylpentosum* in the colon of CUMS mice. Meanwhile, PSP blocked CUMS-induced impairment of intestinal barrier function, inhibited the levels of corticosterone and lipopolysaccharide (LPS), and increased the level of 5-hydroxytryptamine in the serum. Importantly, PSP treatment prevented abnormal neuronal activation and altered local field potential (LFP) in the paraventricular nucleus of CUMS mice, especially the decrease of power spectral density in delta and theta frequency bands. Finally, the results of LFP and c-fos staining after multiple repetitions of LPS injection showed consistencies with CUMS. Taken together, our study indicates that PSP ameliorates depression-like behavior likely via modulating the gut microbiota-lipopolysaccharide-paraventricular nucleus signal axis.

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1. Introduction

Depression is a severe neuropsychiatric disorder characterized by persistent depressed mood, slowed thinking, and diminished will [1], affecting more than 300 million people worldwide. As a major contributor to the burden of mental health-related illness nowadays [2], its pathogenesis mainly includes dysregulation of neurotransmitter levels, inflammation, impaired synaptic plasticity, and disruption of intestinal flora [3,4]. The current pharmacological treatment of depression has the disadvantages of a low cure rate, high adverse effects and relapse rate [5]. Therefore, it is urgent to further explore the pathogenesis of depression and search for novel preventive drugs.

The gut-brain axis is an important bidirectional communication pathway between the gut and brain [3,6]. An increasing number of clinical studies have pointed out that changes in the composition and function (*e.g.*, metabolites) of the gut microbiota are closely related to the onset and progression of depression [7,8]. Transplanting the fecal microbiota of depressed patients induced depressive-like behaviors in rodents, suggesting that microbial ecological dysregulation may precede the onset of depressive illness [9,10]. The hypothalamic paraventricular nucleus (PVN) is a crucial brain region receiving peripheral signals to regulate emotions, especially the development of depressive behaviors [11,12]. However, whether gut microbiota dysfunction is associated with the PVN and the exact mechanism during the development of depression are still not well verified.

Polygonatum sibiricum F. Delaroche (PS) is a traditional Chinese medicine with anti-aging, immunity boosting, liver protection, and cardiovascular improvement effects [13]. The theory of traditional Chinese medicine (TCM) theory proposes that the deficiencies of five Zang, Qi and blood are the main causes of depression, while PS has the ability to antagonize those pathological changes. *Polygonatum sibiricum* polysaccharide (PSP) is one of the main active components of PS, which has antioxidant, anti-inflammatory, and anti-tumor effects [14]. Our previous studies revealed that PSP could ameliorate lipopolysaccharide (LPS)- and chronic unpredictable mild stress (CUMS)-induced depression-like behaviors in mice by mediating cerebral calpain-1-NOD-like receptor protein 3 (NLRP3) signal, repairing hypothalamic-pituitary-adrenal (HPA) axis hyperactivity, and inhibiting inflammatory responses [15,16]. Whereas polysaccharides, as macromolecular substances, are usually unable to enter the brain directly through the tightly connected vascular endothelial cell gap [17], the systemic mechanism of their antidepressant action is still not fully elucidated. The present study aims to elucidate the intrinsic mechanism of PSP in the treatment of depression from the microbiota-gut-brain axis, and to provide a theoretical basis for the development of novel antidepressant drugs.

2. Materials and methods

2.1. Animals and treatments

C57BL/6J mice (male, 20–23 g, 2 months old) were purchased from the Hangzhou Ziyuan Laboratory Animal Technology Co., LTD (License number: SCXK (Zhejiang) 2019-0004). The mice were housed at 22–24 °C and a humidity of 45–65 % with free access to water and food.

2.2. Preparation of PSP

100 g of dried rhizomes of PS were taken, pulverized and added to a solution of petroleum ether as previously described [15]. The mixture was refluxed in a Soxhlet extractor for 3 h to remove the low polar fraction. The filtered mixture was soaked in water for 2 h and then refluxed twice. The polysaccharide solution was collected and the precipitate was obtained by evaporating the solvent. After that, the precipitate was dissolved in ultrapure water and extracted at least three times with sevag reagent [chloroform: n-butanol = 4:1 (V/V)] to remove the protein, which was confirmed by UV spectrophotometer. The polysaccharide solution was dialyzed in distilled water for 2 days to obtain the PSP at the molecular weight from 6 kDa to 14 kDa. After concentration, the polysaccharide sample was obtained by vacuum freeze-drying, and used for subsequent analysis and pharmacological experiments. The structure and monosaccharide components have been shown in our previous publication [15].

2.3. CUMS model and experimental groups

The mice were established as a CUMS model by referring previous publication [18] and 14 kinds of stimuli were selected (fasting for 24 h; water fasting for 24 h; tilting the rat cage for 12 h; shaking the cage for 5 min; restraining for 2 h; tail pinching for 5 min; swimming in cold water at 4 °C; swimming in hot water at 45 °C; wetting the bedding for 24 h; removing the bedding for 24 h; noise for 3 h; circadian reversal, odour stimulation for 3 h, and electroshock stimulation, 1 mA, 2 s, three times), and two stimuli were randomly selected each day for 28 days. From the 3rd week of modeling, the CUMS + PSP group and the CUMS + Flu group were given PSP (400 mg/kg) and Fluoxetine (Flu, 10 mg/kg) by gavage for 14 days, respectively. The body weights were measured once a week during the modeling period. The behavioral and electrophysiological experiments were conducted sequentially after the end of modeling.

During the CUMS modeling process, two animals died. Finally, 8 mice in each group were allocated: the control group, the CUMS group, the CUMS + PSP group, and the CUMS + Flu group. In our previous study, we have conducted the dose-dependent effect of PSP on depression and suggested 400 mg/kg as the optimal dose of PSP [15]. Therefore, the mice in the CUMS + PSP group were given 400 mg/kg PSP dissolved in distilled water via intragastric administration for 14 days, while the mice in the other groups were given equal amounts of water. All experimental protocols were approved by the Animal Ethics Committee of Anhui University of Chinese Medicine (Approval number: AHUCM-mouse-20210205).

2.4. LPS treatments

After one week of adaptation, the mice were randomly divided into two groups (N = 6 per group): the control group and LPS group. The mice were injected intraperitoneally with LPS (2 mg/kg) once a day for 3 days, as previously described [15]. The local field potential was measured at 1 h before LPS injection and on day 1, day 2 and day 3 after LPS injection.

2.5. Forced swimming test (FST)

The animals were placed in an inescapable water tank and forced to swim in order to observe their behavioral responses, and the immobile time of animals was recorded to reflect their degree of depression. Mice were placed individually in a 2000 mL glass container for 6 min at 22 ± 3 °C and a water surface height of 10 cm. The mice were allowed to move for 6 min and the immobility time of last 4 min was recorded using SuperMaze software.

2.6. Tail suspension test (TST)

After the forced swimming experiment, the mice were rested for 4 h, and then the TST was performed. The depression of the mice was reflected by recording the immobility time during the experiment. The mice were fixed 2 cm from the end of their tails and hung upside down, with their heads about 50 cm from the tabletop. The mice were allowed to move for 6 min, and the immobility time was recorded with SuperMaze software for the last 4 min.

2.7. Open field test (OFT)

OFT was performed using an open field box, as previously described [15]. The total distance and time explored in the open field was recorded within 5 min. In this study, the time and distance spent in the central area of the open field to represent the anxiety-like behaviors. To minimize the effect on the next test, the box was wiped with 75 % alcohol after each test.

2.8. 16s rRNA gene sequencing

Fresh faecal samples from mice were collected and immediately frozen in liquid nitrogen, and the genomic DNA of the samples was extracted, after which the purity and concentration of the extracted DNA were detected using agarose gel electrophoresis; the genomic DNA were amplified using bacterial 16s rRNA gene-specific primers, and the PCR products were purified and sequenced. After that, the sequences are further clustered into operational taxon or system type (OTUs) at 100 % recognition rate using QIIME2. The degree of community differentiation was analyzed using Unifrac distance-based non-metric multidimensional scaling (NMDS), and Linear discriminant analysis Effect Size (LEfSe) was analyzed using linear discriminant analysis (LDA) estimation to identify high-dimensional gut microbes. Sequencing results were also compared with Greengene data for metabolic function prediction of intestinal microbiota.

2.9. Hematoxylin and eosin (HE) and alcian blue/periodic acid Schiffs (AB/PAS) staining

The fixed colon tissues of mice were processed by paraffin embedding, and the slices were stained with HE and AB/PAS according to the previous publications [19,20]. The colon tissues were observed and imaged under a light microscope.

2.10. Enzyme linked immunosorbent assay (ELISA)

To explore the potential mechanism of metabolites produced by differential microbiota on the development of depression, ELISA was used to detect the levels of 5-hydroxytryptamine (5-HT), corticosterone (CORT), and LPS in the serum of mice. The experiments were performed according to the instructions of ELISA kits of 5-HT (JL12087, Jianglai Biologicals), CORT (JL11918, Jianglai Biologicals), and LPS (JL20691, Jianglai Biologicals), respectively. After that, the absorbance was detected at 450 nm, and the levels of 5-HT, CORT, and LPS were calculated from the standard curve.

2.11. Western blot

Colon tissues were homogenized on ice and lysed for 30 min. Protein concentration was then determined using a BCA assay kit (Thermo Fisher Scientific). The protein samples were separated by SDS-PAGE gel and transferred to a nitrocellulose (NC) membrane. The NC membrane was blocked in PBST containing 5 % fat-free milk for 2 h. After that, NC membranes were washed 3 times with PBST and incubated overnight at 4 °C with the primary antibodies: β-actin (1:1000, CST, #3700), Occludin (1:1000, CST, #91131), Claudin 5 (1:1000, CST, #49564), ZO-1 (1:1000, CST, #13663). After washing 3 times with PBST, the membranes were incubated with the peroxidase-conjugated secondary antibodies (1:10,000, ZEN BIO) for 2 h at room temperature. The protein bands were visualized by ECL reagent (Tanon, shanghai) and analyzed using Image J software.

2.12. Immunofluorescence

The mice were placed in water 1 h before tissue collection to swim freely for 5 min. The heart of the mouse was then perfused with saline until the liver and limbs were whitened, and there was no blood flow from the right atrial appendage, and then perfused with 4 % paraformaldehyde (PFA). Then the brain tissue was quickly removed and fixed in 4%PFA for 24 h. Subsequently, the tissues were then dehydrated in a solution of 20 % and 30 % sucrose, subsequently. The tissue were then embedded in optimal cutting temperature compound (OCT) and sliced continuously (thickness of 35 μm) on a frozen slicer (Leica, CM1950). After that, the slices were incubated with c-fos antibody (1:1000, Santa Cruz) at 4 °C overnight. Then the slices were washed three times with PBS and incubated with goat anti-mouse Alexa Fluor 488 (1:100) for 2 h at room temperature. After sealing, the expression of c-fos in the PVN was observed by laser confocal microscope (Olympus, Japan) and analyzed using Image J software.

2.13. Measurement of local field potential (LFP)

The mice were anaesthetized with 2 % isoflurane and fixed on the stereotactic instrument. The skull was exposed after routine sterilization, and the coordinates of the PVN were localized (AP: 0.82 mm, ML: ±0.25 mm, DV: 4.6 mm). Four stainless steel cranial

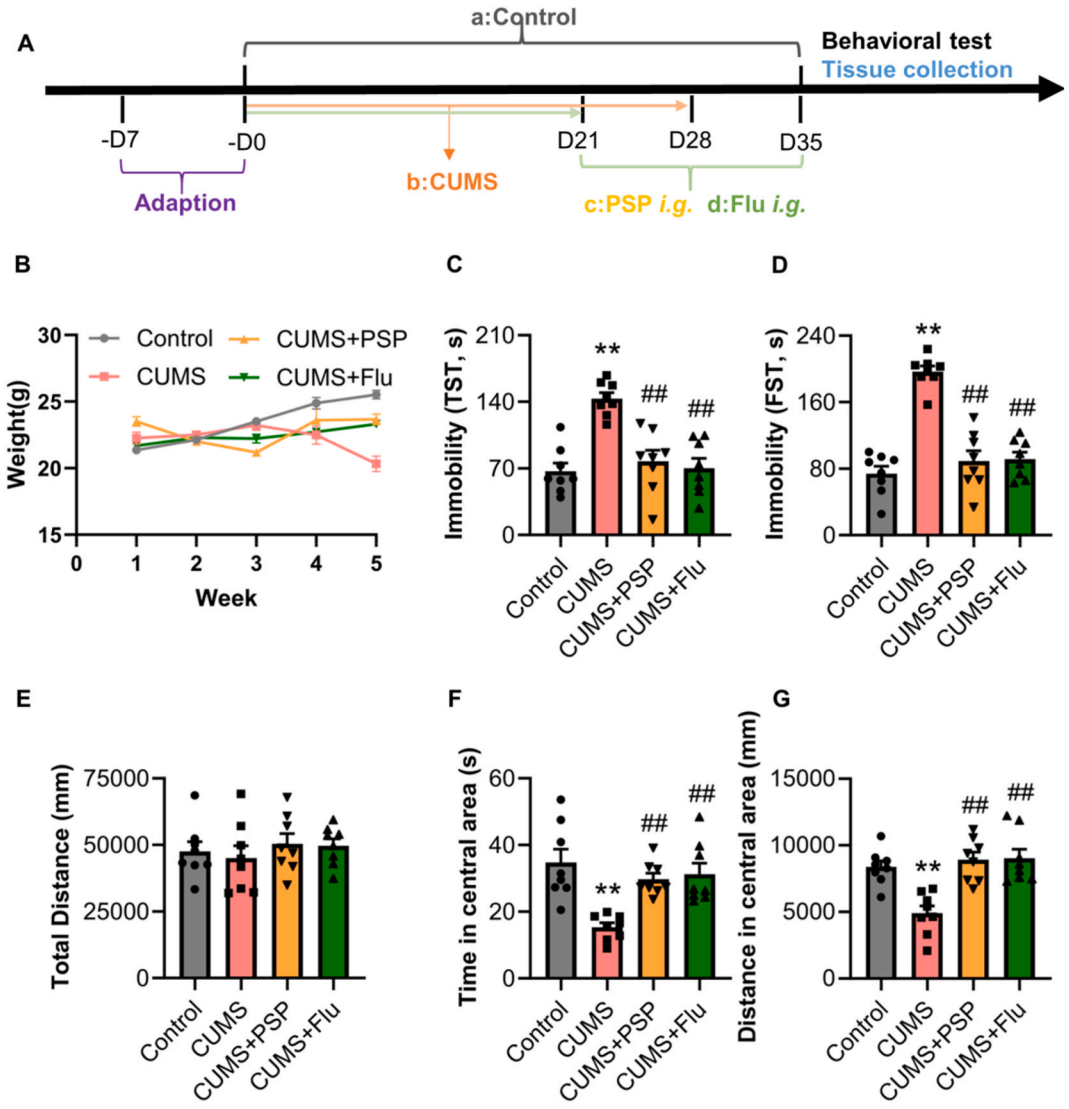

Fig. 1. PSP improves depression-like behavior in CUMS mice. (A) Schematic illustration of the study design. (B) Body weight. (C) Immobility time of mice in the TST. (D) Immobility time of mice in the FST. (E) Total distance traveled in the OFT. (F) Time spent in the center area in the OFT. (G) Distance in the center area in the OFT. Data are expressed as mean ± SEM (N = 8 per group) (**$p < 0.01$, vs. Control group, $^{\#\#}p < 0.01$, vs. CUMS group).

nails were inserted into the skull, and the four silver wires of the C-type electrodes were wrapped around each of the cranial nails. Two myoelectric electrodes were inserted into the neck muscle tissue. LFP electrodes were then implanted into the target brain region and fixed to the skull with dental cement after the electrodes reached the PVN region. After the mice recovered for 3 d, the LFP were recorded accompanied with the induction of depression-like behaviors using tail suspension for 5 min. The changes in the raw electroencephalogram (EEG) of the mice were monitored by a small animal EEG recording system (Medusa), and the raw EEG was filtered from 0.5 to 100 Hz to obtain the LFP. The power values were obtained by preprocessing the EEG data using Neuro explorer and MATLAB. The power spectral densities were calculated for the frequency bands δ (0.5–4 Hz), θ (4–8 Hz), α (8–13 Hz), β (13–30 Hz), and γ (30–80 Hz).

2.14. Statistical analysis

All data from the experiments were expressed as mean ± standard error (SEM) and analyzed using GraphPad Prism 8.0 software by independent samples *t*-test, one-way or two-way ANOVA followed by the Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PSP improved depression-like behavior in CUMS mice

In our previous study, we have characterized the structure of PSP and quantified the components of monosaccharides using 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization method [15]. In this present study, we further clarified the mechanism underlying the protection of PSP on depression from a systemic perspective. Experimental procedure was shown in Fig. 1A and the body weight of CUMS mice showed a decreasing trend, while the body weight of PSP-treated mice increased, but no significant difference was observed among different groups (Fig. 1B, $p > 0.05$). The results of FST and TST showed that the immobility time of CUMS mice was significantly higher than that of the control group, and PSP administration resulted in a significant decrease in the immobility time of CUMS mice (Fig. 1C and D, $p < 0.05$). The results of OFT showed that there was no significant difference in the total movement distance among different groups (Fig. 1E, $p > 0.05$), which indicated that the motor function of mice was normal. Whereas compared to the control group, CUMS group moved significantly less time and distance in the central area within the estimation of 5 min, PSP administration resulted in significantly more time and distance in the central area (Fig. 1F and G, $p < 0.05$).

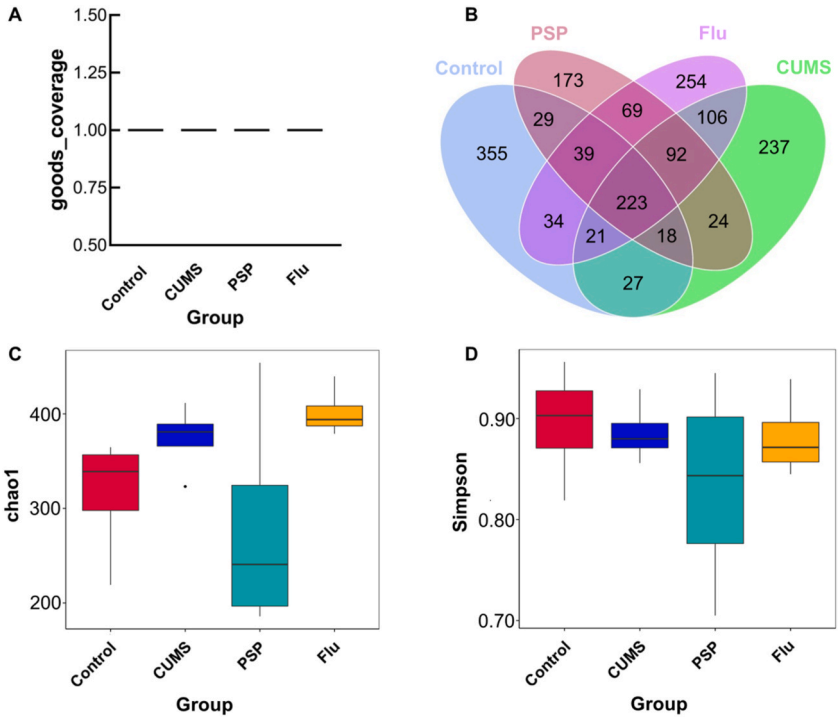

Fig. 2. PSP improves the composition and abundance of intestinal microbiota in CUMS mice. (A) Good's coverage value. (B) Venn diagram based on OUTs. (C) Chao1 index. (D) Simpson index.

3.2. PSP improved the composition and abundance of intestinal microbiota in CUMS mice

The Good's coverage value reached 100 % in each group (Fig. 2A), indicating that the sequencing depth of the samples was reasonable and the coverage was high, which was in line with the actual microbial situation. Compared to the control group, there were 237 and 173 OTUs unique to the CUMS and PSP groups, respectively (Fig. 2B), implying that the number of OTUs in the intestinal microbiota of the mice was altered by CUMS modeling, while PSP administration partially ameliorated those abnormal changes. The chao1 index is used to estimate the total number of species in each community. As shown, the CUMS group produced more unobserved species compared to the control group, suggesting that CUMS modeling caused more unknown microbiota to be present in the intestines and that PSP administration ameliorated this abnormality (Fig. 2C). Simpson index was used to reflect microbial diversity, with larger values indicating lower community diversity. The results showed that the CUMS group had a lower species richness compared to the control group, and the PSP group had a lower species richness compared to the CUMS group. In species diversity, the values of the CUMS and PSP groups showed a constant decreasing trend, but without significant difference (Fig. 2D, $p > 0.05$).

Beta diversity was used to evaluate the composition and differences of microbial communities in the intestines. The Principal Coordinates Analysis (PCoA) method is a technique used to analyze multivariate data, which converts high-dimensional data into low-dimensional data to better demonstrate the relationship between the data. PCoA analysis based on unweighted Unifrac distance showed that the CUMS group significantly deviated from the control group, while the PSP group was more convergent to the control group, which implied that PSP administration improved the intestinal microbiota of CUMS mice (Fig. 3A). In addition, PCoA analysis based on weighted Unifrac distance showed that after PSP administration, the intra-group differences were reduced, and the overlap between the PSP and control groups was higher than that of the CUMS group (Fig. 3B). These data suggest that administration of PSP improves the composition of intestinal microbiota in CUMS mice.

At the phylum level, the relative abundance of *Firmicutes* and *Bacteroidota* in the intestines of CUMS mice decreased, while the relative abundance of *Verrucomicrobiota*, *Campilobacterota*, and *Proteobacteria* increased, compared with that in the control mice. Those

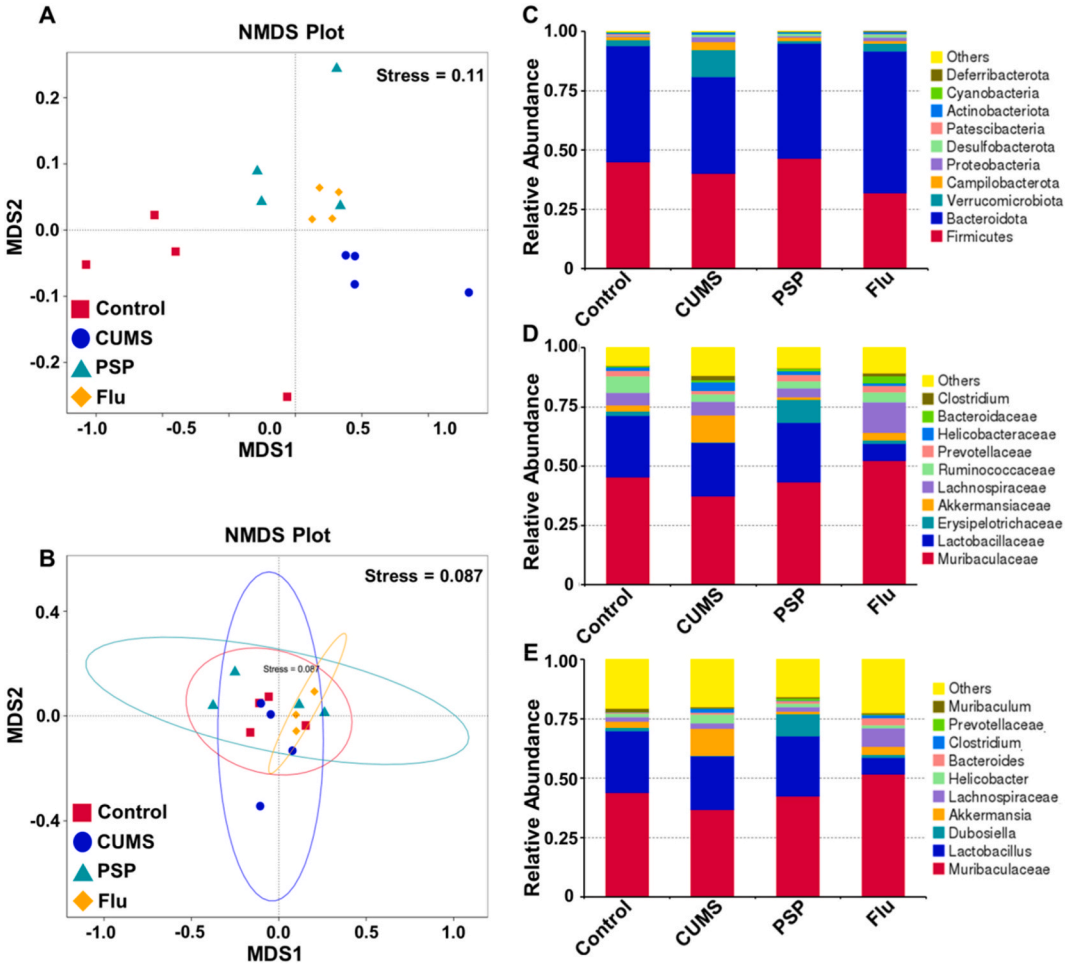

Fig. 3. β-diversity analysis and different levels of colony composition. (A) PCoA analysis of unweighted Unifrac distances. (B) PCoA analysis of weighted Unifrac distances. (C) Top10 microbiota at the family level. (D) Top10 colonies at the family level. (E) Top10 colonies at the genus level.

changes caused by CUMS were improved after PSP administration (Fig. 3C). In addition, at the family level, the relative abundance of *Muribaculaceae*, *Lactobacillaceae*, *Ruminococcaceae* and *Erysipelotrichaceae* decreased in the CUMS group, while the relative abundance of *Akkermansiaceae* and *Helicobacter* increased in relative abundance compared to the control group. However, the changes in the intestinal microbiota of mice were ameliorated by PSP administration (Fig. 3D).

At the genus level, compared to the control group, the CUMS group showed a decrease in the relative abundance of *Muribaculaceae*,

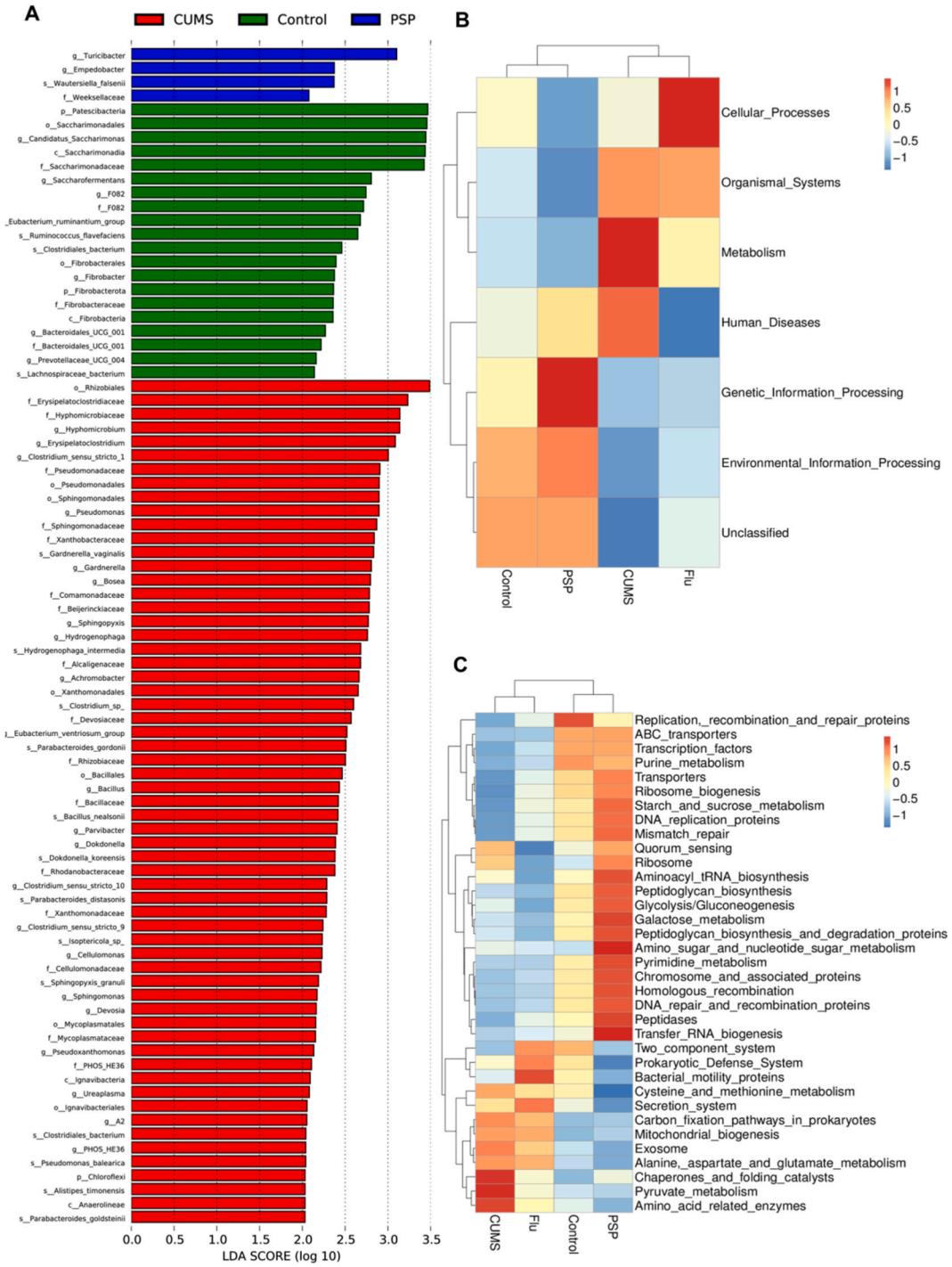

Fig. 4. LEfSe analyses of intestinal microbiota and prediction of gene function of mouse microbiota. (A) LDA scores among Control, CUMS and PSP groups. (B) Heatmap of gene function prediction of intestinal microbiota in mice at the phylum level using KEGG analysis. (C) KEGG pathway enrichment analysis at the order level.

Dubosiella, and *Lactobacillus*, and an increase in the relative abundance of *Akkermansiaceae*, *Helicobacter*, and *Clostridium_methylpentosum*. Those changes in the intestinal microbiota of mice were ameliorated by PSP administration (Fig. 3E).

We used LEfSe to analyze the biomarkers with significant differences among control, CUMS and PSP groups, and the results showed that a total of 20 biomarkers were detected in the control group, 61 biomarkers in the CUMS group, and 4 biomarkers in the PSP group (Fig. 4A). At the outline level, the results of KEGG enrichment analysis showed that there was inhibition of biology such as genetic and environmental information processing and hyperactivity in organismal systems and metabolic processes in the CUMS group compared to the control group, whereas the above biological processes were significantly ameliorated by PSP administration (Fig. 4B). At the order level, compared with control group, CUMS inhibited biological processes such as replication, recombination and repair proteins, ABC transporters, transcription factors, purine and pyrimidine metabolism, ribosome synthesis, starch and sucrose metabolism, mismatch repair, peptidoglycan biosynthesis and degradation proteins, glycolysis, galactose metabolism, chromosomes and related proteins, homologous recombination, two-component system, and bacterial movement proteins. In addition, CUMS mice showed hyperactivity in cysteine and methionine metabolism, glutamic acid, alanine and aspartic acid metabolism, secretory system, carbon sequestration pathway of prokaryotes, mitochondrial biosynthesis, exosomes, pyruvate metabolism, amino acid related enzymes and other biological processes, while PSP administration could improve the above biological processes (Fig. 4C).

3.3. PSP improved impairment of intestinal barrier function in CUMS mice

In order to investigate the intrinsic mechanism of PSP regulating intestinal microbiota, we carried out the pathological observations on colon tissues of mice. The results showed that the colon structure of control mice was intact and the glands were closely arranged; while the epithelial cells of the colon tissues of CUMS mice were broken, the structure of the mucosal epithelial layer and the lamina propria was destroyed, the number of glands was reduced, the cup cells were lost, the mucus secretion was reduced, and the surface of the crypts could be seen to be irregular, and crypt branches appeared. While the number of cup cells on the colon tissues increased, the structure was significantly improved, the number size and arrangement of glands tended to be normal, and mucus secretion was significantly increased after PSP administration (Fig. 5A and B).

Intestinal barrier function is regulated by tight junction proteins. We examined the intestinal tight junctions, including occludin, claudin 1, and zona occludens 1 (ZO-1), and the results showed that compared to the control group, CUMS induced a significant decrease in the expression of occludin, claudin 1, and ZO-1 in the colon. By contrast, the expression levels of occludin, claudin 1, and ZO-1 in the colon were significantly increased after treatment with PSP (Fig. 5C, D, E, and F, $p < 0.05$). This suggests that PSP ameliorates the impairment of intestinal barrier function in CUMS mice by upregulating the expression of relevant tight junction proteins.

The results of ELISA showed that LPS level was significantly increased in the serum of CUMS group compared to the control group, which was significantly reduced after PSP administration (Fig. 5G, $p < 0.05$). In addition, the 5-HT level was significantly decreased, and the CORT level was significantly increased in the serum of mice induced by CUMS. By contrast, those two indicators of depression returned to the normal level after PSP administration (Fig. 6H and I, $p < 0.05$).

3.4. PSP inhibited c-fos$^+$ expression and suppressed the changes of oscillations in the PVN of CUMS mice

The results showed that the number of c-fos$^+$ neurons was significantly increased in the PVN of CUMS mice. Consistent with the effect of Flu, PSP administration significantly reduced the number of c-fos$^+$ neurons in the PVN (Fig. 6, $p < 0.01$). Fig. 7A shows the signals of LFPs in the PVN of mice. The results showed that compared with the control group, the energy value of 100 s LFPs in the PVN of CUMS group was significantly reduced. Consistent with the effect of Flu, PSP administration significantly increased the energy of LFPs in the PVN of CUMS mice. In addition, the power spectral density (PSD) of δ, θ, and α bands in the PVN of CUMS mice was significantly reduced. Consistent with the effect of Flu, PSP administration significantly reduced the PSD of δ, θ, and α bands in the PVN of CUMS mice (Fig. 7B, C, $p < 0.01$). In addition, there was no significant difference in the PSD of β and γ bands among the groups (Fig. 7B, C, $p > 0.05$).

3.5. Exogenous LPS injection mimicked the effect of CUMS on c-fos$^+$ expression and changes of oscillations in control mice

We also did the c-fos staining and measured the hippocampal oscillations after LPS administration. The results showed that the number of c-fos$^+$ neurons was also significantly increased in the PVN of mice induced by LPS (Fig. 8A, $p < 0.01$). Furthermore, the results showed that the PSD in δ and θ bands were significantly increased after the first day of LPS injection in mice. However, the PSD of δ band decreased significantly on the second and third day after continuous injection of LPS, and the PSD of θ band also decreased significantly on the second day after LPS injection (Fig. 8B, $p < 0.05$).

4. Discussion

Our study found that CUMS altered the composition and abundance of the intestinal microbiota and induced impairment of the intestinal barrier function and aberrant activation of the neurons in the PVN. By contrast, PSP administration ameliorated the physiological stress responses, including intestinal microbiota dysbiosis and impairment of the barrier function, as well as hyperactivity of neurons in the PVN. In addition, PSP suppressed the altered EEG oscillations in the PVN caused by CUMS, especially the decrease of δ and θ bands. This study provided a systemic mechanism underlying the preventive effect of PSP against depression.

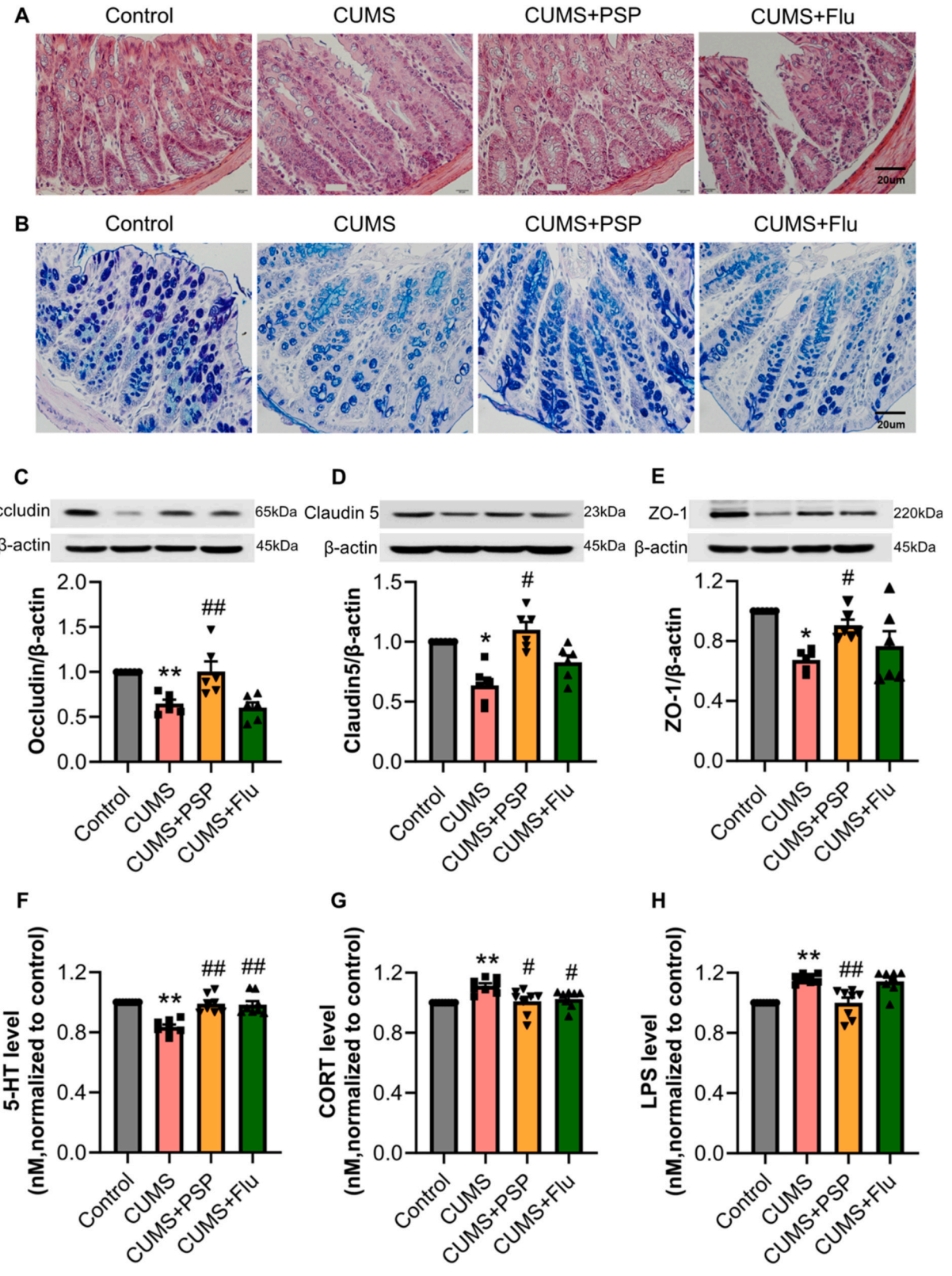

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Fig. 5. PSP improves impairment of intestinal barrier function in CUMS mice. (A) HE staining (× 20). (B) AB-PAS staining (× 20). (C) Representative bands of Occludin, Claudin 1, ZO-1 and β-actin. (D) Expression levels of Occludin (N = 6 per group). (E) Expression levels of Claudin 1(N = 6 per group). (F) Expression level of ZO-1(N = 6 per group). (G) Expression level of 5-HT in the serum (N = 8 per group). (H) Expression level of CORT in the serum (N = 8 per group). (I) Expression level of LPS in the serum (N = 8 per group). Data are expressed as mean ± SEM (*$p < 0.05$, vs. Control group, $^{\#}p < 0.05$, vs. CUMS group).

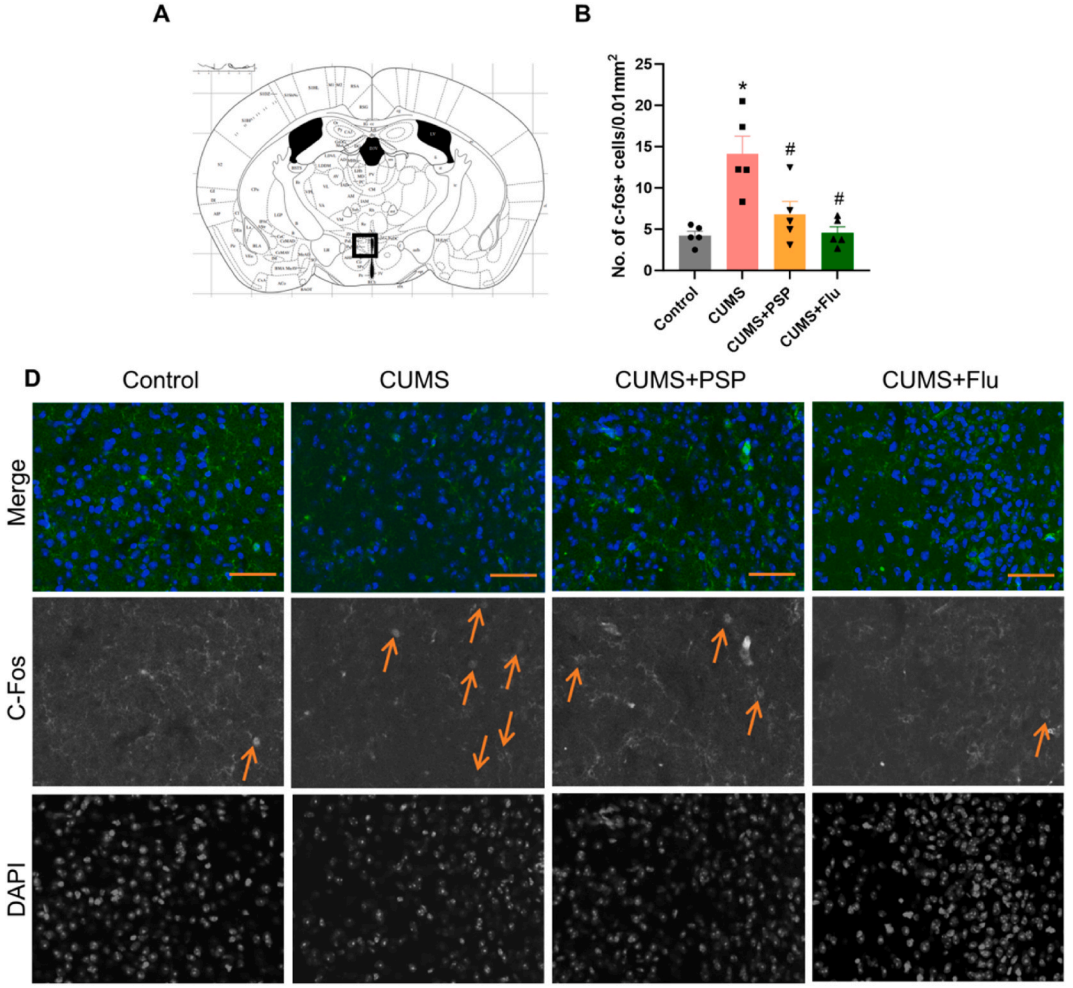

Fig. 6. PSP inhibits c-fos^{+} expression in the PVN of CUMS mice. (A) The PVN region in brain atlas. (B) Quantitative data of c-fos^{+} cells in the PVN of mice induced by CUMS. (C) Representative images of c-FOS staining in the PVN of mice induced by CUMS. Scale bar: 50 μm. Data are expressed as means ± SEM (N = 5 per group) (*$p < 0.05$, vs. Control group, $^{\#}p < 0.05$, vs. CUMS group).

4.1. PSP modifies CUMS-induced dysfunction of gut microbiota and production of LPS

Chronic stress can influence the stability and diversity of the intestinal microbiota [21]. It has been found that the phylum Firmicutes, the phylum *Actinobacteria* and the phylum *Bacteroidota* have the most significant changes in depression [9,22]. In addition, a study enumerated the microbiota species that increased and decreased during the development of depression [23], which corresponded to the fact that at the phylum level, the intestinal microbiota of CUMS mice showed a decrease in *Firmicutes* and an increase in the abundance of *Proteobacteria*. It has been shown that *Firmicutes* prevent intestinal barrier dysfunction by fermenting carbohydrates into various short-chain fatty acids [24]. In addition, a higher abundance of *Proteobacteria* in the gut of mice is considered to be more susceptible to bacterial translocation [25]. Whereas increased bacterial translocation is associated with activation of the immune system and the HPA axis, its translocation products may lead to increased production of inflammatory biomarkers [26]. In this study, the abundance of *Bacteroidota* in the intestine of CUMS mice decreased, which was consistent with the results of fecal samples from patients with clinical depression [27,28]. *Bacteroidota* has a role in regulating the dynamic balance of microbiota and in enhancing the immunity of the host [29,30] and may be involved in the metabolism of carbohydrates, bile acids, and steroids, and in the promotion of intestinal mucosa formation [31]. The administration of PSP was able to normalize the abundance of those microbiota.

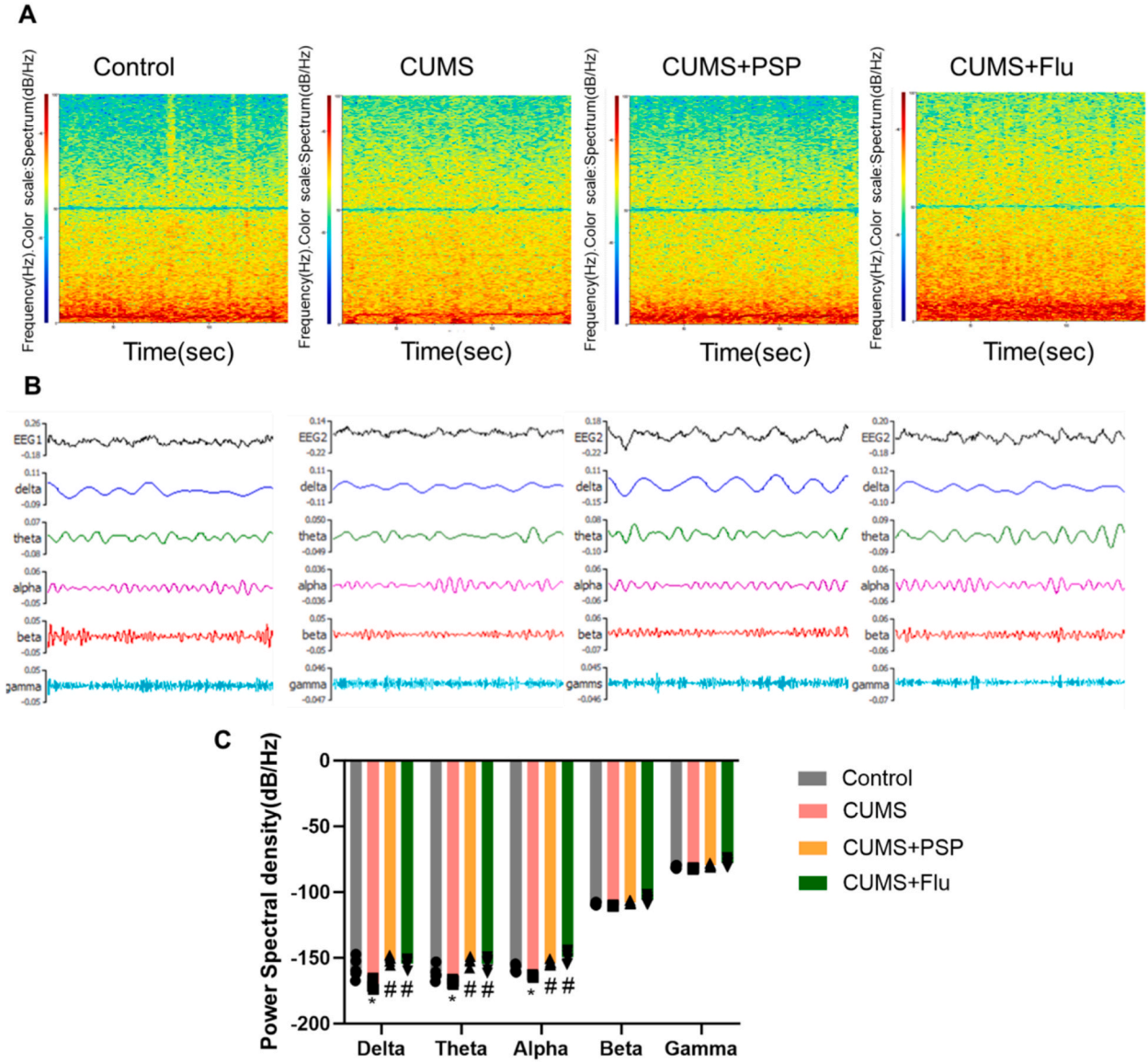

Fig. 7. PSP suppresses the changes of LFP oscillations in the PVN of CUMS mice. (A) Sample LFP recordings in the PVN of mice induced by CUMS. (B) LFPs of each frequency band in the PVN of mice induced by CUMS. (C) Power spectral density of each frequency band in the PVN of mice induced by CUMS. Data are expressed as means ± SEM (N = 5 per group) ($^{*}p < 0.05$, vs. Control group, $^{\#}p < 0.05$, vs. CUMS group).

At the family level, our results showed a lower abundance of families *Lactobacillaceae*, *Ruminococcaceae* and *Muribaculaceae* in the intestinal tract of CUMS mice and a higher abundance of *Helicobacter* and *Akkermansiaceae*, which were consistent with previous publications [21,32]. It has been found that stress can affect the intestinal microbiota resulting in a decrease in *Lactobacillus*, which is considered to be an important factor in depression [33]. *Lactobacillus* has also been shown to play an important role in regulating immune and inflammatory pathways by affecting the kynurenine/tryptophan ratio [34]. *Helicobacter*, as a Gram-negative and microaerobic bacterium, usually colonizes the gastrointestinal tract and may contribute to chronic inflammation, ulcers and even cancer [35]. *Akkermansiaceae* can disrupt the intestinal mucosal barrier, and make the gut susceptible to pathogens, leading to local inflammation [36]. *Ruminococcaceae*, the main butyrate producer in the gut, are sensitive to the presence of oxygen, and when large quantities of reactive oxygen species are produced in the gut, it will die rapidly [37]. The disappearance of this bacterium in the intestine will make inflammation more likely and promote damage to the intestinal mucosal barrier, leading to the entry of harmful molecules into the blood system, such as LPS, tumor necrosis factor (TNF), etc. [38,39]. *Muribaculaceae* and *Prevotellaceae* are considered to be significantly and positively correlated with 5-HT, DA and NE [40]. *Prevotellaceae*, as a beneficial bacterium, protects the intestinal mucosa and regulates immune function, and it is also closely associated with transforming growth factor beta 3, a cytokine that regulates intestinal barrier function [41]. Interestingly, PSP administration improved the abundance of those microbiota.

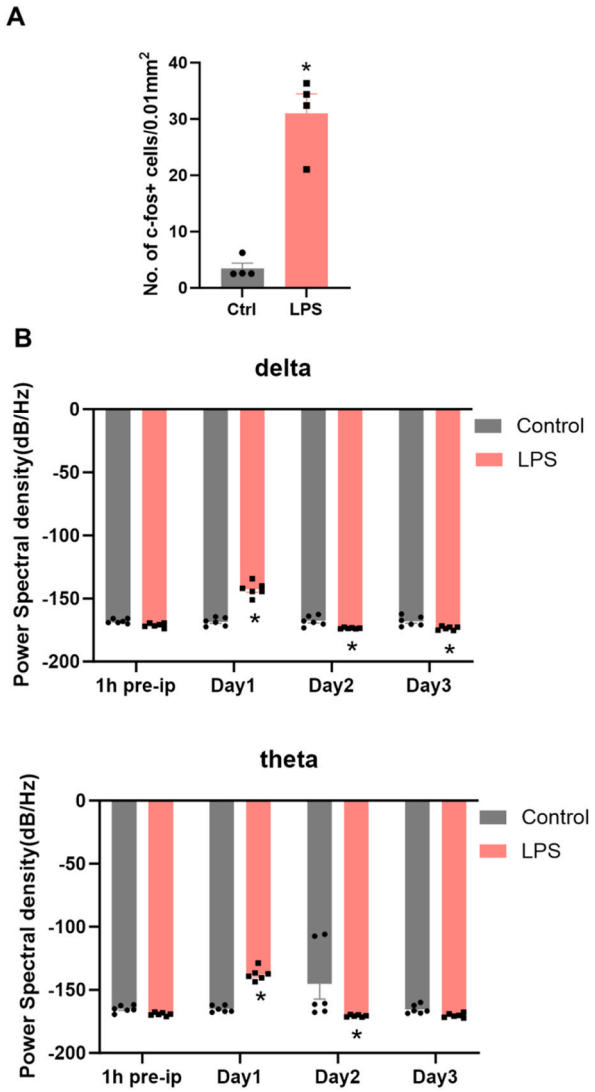

Fig. 8. Exogenous LPS injection mimics the effect of CUMS on c-fos^{+} expression and changes of oscillations in control mice. (A) Quantitative data of c-fos^{+} cells in the PVN of mice induced by LPS (N = 4 per group). (B) Power spectral density of delta and theta frequency band in the PVN of mice induced by LPS (N = 6 per group). Data are expressed as means ± SEM (*$p < 0.05$, vs. Control group).

The maintenance of intestinal barrier function is dependent on the role of intestinal microbiota [42]. Claudin 1, Occludin and ZO-1 are important adhesion proteins for tight junctions. Claudin 1 is expressed in a wide range of tissues and cell types and plays an important role in the formation and function of intercellular junctions [43]. Occludin stabilizes intercellular junctions by interacting with other tight junction proteins and is also involved in regulating intercellular permeability. ZO-1, a cytoskeletal protein, belongs to the accessory proteins and is involved in the formation and maintenance of intercellular junctions together with Claudins and Occludin [44]. In the present study, we observed impaired intestinal mucosal integrity and decreased expression of tight junction proteins in CUMS mice, which were ameliorated by PSP administration. Intestinal microbiota metabolites regulate the assembly of tight junction proteins, influence the expression of tight junction-associated proteins Claudins, Occludin, and ZO-1 and are involved in the maintenance of intestinal barrier function [45,46]. This suggests that our disordered intestinal microbiota impairs intestinal barrier function, abnormally elevates intestinal permeability, and increases LPS production, eliciting depression. PSP, con the contrary, reduces the damage to the intestinal barrier and inhibits the inflammatory response by regulating the intestinal microbiota.

4.2. PSP modulates the neuronal activation in the PVN of CUMS mice

In the present study, we further showed that neurons were abnormally activated in the PVN of mice induced by CUMS. In contrast, PSP inhibited neuronal activation in the PVN of CUMS-induced mice, suggesting that PSP may improve depression-like behavior in mice by increasing the abundance of beneficial bacteria and decreasing the abundance of harmful bacteria in the intestines of CUMS

mice, and by preventing abnormal activation of the neurons in the PVN and hyperactivation of the HPA axis. The PVN is a crucial brain region for regulating emotions and stress responses and plays a pivotal role in the development of anxiety and depression. CRH neurons in the PVN are closely related to the development of depression [11,12], and it may be an effective target for novel antidepressant drugs. It is well known that hyperactivity of the HPA axis due to prolonged and sustained high stress is a key mechanism in the pathogenesis of depression [47]. The present study further confirmed that CUMS mice had significantly higher CORT level and lower 5-HT level. *Lactobacillus* alleviates stress-related symptoms by modulating corticosterone levels and the expression of c-fos and corticotropin releasing factor (CRF) in the PVN in both human and animal models [48]. Feeding a mouse model with *Lactobacillus* asepticus lysate increased oxytocin levels in the PVN and was consistent with a decrease in corticosterone levels [49]. In addition, peripheral *Helicobacter* Vacuolating Cytotoxin A (VacA) administration crosses the blood-brain barrier and activates the Ucn1-CRF receptor axis in the hypothalamus, causing anxiety [50]. These results suggest that intestinal microbiota may affect specific brain regions such as the PVN to trigger the onset of depression and anxiety and that PSP may improve depressive-like behaviors in CUMS mice by modulating intestinal microbiota to affect the PVN neurons.

4.3. Intestinal microbiota-LPS-PVN neuronal activation might underlie the pathogenesis and treatment of depression

In this study, we examined the LPS level in mice induced by CUMS and found that damage to the intestinal mucosa in CUMS mice was associated with higher levels of LPS, which was significantly reduced by PSP administration. This suggests that the antidepressant effect of PSP may be closely related to the level of LPS [15]. It has been reported that bioactive molecules produced by the gut microbiota such as LPS are associated with the development of major depression [51], and it has been suggested that the expression of pro- and anti-inflammatory cytokines can be modulated by inhibiting gut bacterial LPS, thereby alleviating stress-induced mental states such as depression [52,53]. PS has been reported to ameliorate hypertension in rats by inhibiting LPS-induced vascular endothelial dysfunction [54]. In addition, numerous studies have indicated that *Helicobacter* can secrete LPS [55,56]. Combined with those reports, the abundance of some intestinal bacteria such as *Helicobacter* induced by CUMS in mice increased, which may lead to the increase of intestinal LPS level, and the abnormal composition and abundance of intestinal microbiota caused by CUMS may lead to the destruction of intestinal microenvironment homeostasis, resulting in the decrease of intestinal barrier function and inflammation [57].

Interestingly, we found that neurons in the PVN were also abnormally activated in LPS-treated mice. The results showed that LPS levels were abnormally elevated in CUMS mice with disturbed intestinal microbiota, and mice that were persistently stimulated by LPS exhibited similar oscillatory wave changes as observed in the CUMS mice, which suggest that LPS may be a major mediator in the crosstalk between the intestinal microbiota and the brain region of PVN, and affects the depression-like behaviors of the mice. In the present study, we found that PSD in theδ, θ, and α bands showed decreases in CUMS mice. We further examined the characteristics of changes of oscillations in mice using multiple injections of LPS, and the results showed that after single LPS stimulation, mice exhibited a significant increase in PSD in the delta and theta frequency bands. Interestingly, consistent with the results of CUMS mice, the PSD of δ and θ bands decreased after repeated LPS stimulation. That is to say, both CUMS and LPS-induced mice showed reduced levels in the low frequency bands (δ, θ), suggesting that those low frequency bands may play a dominant role in the development of depression. Importantly, our results further confirm the link between δ and θ activity and mood states in animals and humans [58,59]. This could provide a new perspective for the diagnosis of depression in its early stages.

4.4. The strengths and limitations of the current study

The antidepressant effects of PSP have been widely reported. Unlike previous studies [15,16], this research systematically elucidates the antidepressant mechanisms of PSP, ranging from gut microbiota and inflammatory responses to central regulation, providing an experimental foundation for the application and transformation of PSP. However, this study also has notable limitations. Firstly, the systemic antidepressant mechanisms of PSP still require further in-depth investigation. Although we observed changes in specific gut flora, in this study, we were unable to supplement those specific gut flora to confirm their role in PSP's antidepressant effects. Secondly, the gut flora themselves may directly cross the blood-brain barrier and affect brain function. Additionally, changes in the gut flora can regulate alterations in other metabolites, which may serve as intermediate factors in the regulation of depression. Although our research suggests that LPS may be one mechanism, other mechanisms remain to be uncovered. Lastly, there are numerous mechanisms of depression within the brain, and we have only explored the mechanism from the perspective of PVN neuron activation. In future research, we will further investigate which specific neurons are involved in PSP's antidepressant effects.

5. Conclusion

Our data indicate that PSP ameliorate depression-like behavior likely via modulating the gut microbiota-lipopolysaccharide-paraventricular nucleus signal axis, which would provide new explanation for the anti-depressant action of PS.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Ethics Committee of Anhui University of Chinese Medicine (Approval number: AHUCM-mouse-20210205).

Consent for publication

Not applicable.

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Data availability statement

Data will be made available based on reasonable request.

CRediT authorship contribution statement

Xinya Wang: Data curation. **Xueqing Wang:** Data curation. **Feng Gao:** Formal analysis, Data curation. **Shaojie Yang:** Formal analysis, Data curation. **Yilan Zhen:** Conceptualization. **Xuncui Wang:** Conceptualization. **Guoqi Zhu:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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No.

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