



# Effects of Astragaloside IV on Depressive Behaviour and Intestinal Flora in Post-Stroke Depressed Rat Models

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## ABSTRACT

The objective of this study was to investigate whether there is a correlation between post-stroke depression (PSD) and intestinal microflora imbalance, and to explore the regulatory effects of Astragaloside IV (AS-IV) on intestinal microflora in PSD rats. A brain ischemia-reperfusion model was replicated using the Longa suture method. At the same time, the behavioural changes and intestinal flora changes in rats were used as indexes to compare the antidepressant effects of a blank (CON) group, a PSD model group, an AS-IV group, a fluoxetine (FLU) group and a combined administration group (AS-IV+FLU group). Compared with the CON group, the body weight of rats in the PSD, AS-IV, FLU and AS-IV + FLU groups were significantly reduced. The number of rats crossing the platform in the water maze experiment was significantly reduced, and the number of rats standing vertically in the open field experiment was significantly reduced, and the differences were statistically significant ( $P < 0.05$ ). Four weeks after AS-IV or AS-IV + FLU was injected, compared with the PSD group, the body weight of rats in the AS-IV, FLU and AS-IV + FLU groups were significantly increased; the number of rats crossing the platform in the water maze experiment was significantly increased, and the number of rats standing vertically in the open field experiment was significantly increased, with statistical significance ( $P < 0.05$ ). Compared with the CON group, Firmicutes' abundance in the PSD group was significantly increased; Bacteroidetes abundance was significantly decreased, and the differences were statistically significant ( $P < 0.05$ ). To conclude the intestinal microflora of poststroke depressed rats is significantly different from that of CON rats. The present study demonstrated that AS-IV can improve the abundance of *Prevotella\_9* and improve the depressive behaviour of rats to some extent by regulating intestinal microflora.

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Conception and design of the research: KJ, JL, CH, LF, LQ.

Acquisition of data: CH, LF. Analysis and interpretation of the data: CH, LF. Statistical analysis: LF, LQ.

Obtaining financing: KJ. Writing of the manuscript: KJ, JL, CH.

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## Key words

Post-stroke depression, Intestinal microflora, PSD rat model, Astragaloside IV, *Prevotella\_9*

## INTRODUCTION

Post-stroke depression (PSD) is a common ailment after stroke and is related to higher mortality, poorer recovery ability, more obvious cognitive impairment and lower quality of life compared with stroke without depression (Medeiros *et al.*, 2020). In severe cases, there will be a higher rate of disability (Blöchl *et al.*, 2019) and a higher

rate of suicidal ideation (Bartoli *et al.*, 2017). Based on a large number of studies, we found that the incidence of PSD within 2 years was between 11%–41%. Present knowledge indicates that the pathophysiological mechanism of PSD mainly involves hypothalamus-pituitary-adrenal axis dysfunction, increased inflammation, intestinal flora imbalance, neurotransmitter transfer, neurotrophic hypothesis and neuroplasticity (Guo *et al.*, 2022).

PSD can occur at any stage after stroke, which will not only delay the recovery of damaged nerves but also greatly increase the difficulty of nursing and family burden (Liu and Lu, 2019). The specific pathophysiology of PSD remains unknown. At present, its first-line treatment is mainly antidepressant drugs and psychotherapy (Starkstein and Hayhow, 2019; Villa *et al.*, 2018). Most of the drugs used in these treatments have the limitation of slow efficacy, typically taking 2–4 weeks to take effect, and approximately 40% of patients with a depressive disorder

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do not qualify for this type of treatment. The problems of slow drug onset time, significant side effects and low efficiency greatly reduce the compliance of depressed patients with taking drugs, leading to aggravation of the condition and increasing the risk of suicide (Kalbouneh *et al.*, 2022).

Representative among these treatment drugs are tricyclic antidepressive agents and selective serotonin reuptake inhibitors (Li *et al.*, 2022). Drugs have inherent side effects, however, such as the development of drug resistance, and adverse effects are often reported including sexual dysfunction and gastrointestinal symptoms, neuropsychiatric symptoms, and other systemic symptoms (Anagha *et al.*, 2021), rendering their overall efficacy flawed (Villa *et al.*, 2018).

The gut microbiota is the main microbial community that resides in the human body. It comprises approximately 1,800 distinct phyla and 40,000 species of bacteria that are involved in numerous aspects of human health and disease and engage in two-way communication with the central nervous system (CNS) via the microbial gut brain axis (Cryan *et al.*, 2019). Lee *et al.* (2020) showed that stroke patients had Bifidobacteriaceae and *Clostridium* disorders, which initially suggested a relationship between changes in the intestinal flora and depression and stroke. Kang *et al.* (2021) showed that the number of harmful bacteria, such as *Enterococcus faecalis* and *Escherichia coli*, was higher in PSD patients than in non-depressed patients after stroke, and the content of beneficial bacteria, such as *Bifidobacterium*, was lower than in non-depressed patients after stroke, which suggested the presence of microecological disturbances in the intestinal flora of PSD patients.

Intestinal flora is closely related to the occurrence and development of various human diseases (He *et al.*, 2020), and an imbalance in intestinal flora is related to anxiety, depression, Parkinson's disease and other neurological diseases (Suganya and Koo, 2020). According to the intestinal flora mechanism theory, based on the intestinal flora gut brain axis, the intestinal flora can affect the distribution of neurotransmitters through changes occurring in the flora, thereby effectively participating in the regulation of the CNS (Forsythe *et al.*, 2010). Due to limited technical conditions, long-term dynamic monitoring of patients' intestinal flora microecology is not possible, and further research is needed on the interaction between diseases and intestinal flora (Chen *et al.*, 2023).

It is well documented that stress may disrupt the balance of the gut microbiome in patients suffering from PSD and that this disruption is closely related to the severity of the condition in depressed patients. Therefore, maintaining the balance of intestinal microbiota can be

adopted as a focus of research in the treatment of PSD.

Astragalus has a long medicinal history in traditional Chinese medicine (Liu *et al.*, 2017; Qiu *et al.*, 2017). Studies confirmed that Astragalus has a variety of pharmacological effects including CNS protection, anti-oxidation and antibacterial and virus inhibition, among which calming, analgesic, anti-depression and anti-anxiety behaviours have become the focus of current research (Sun *et al.*, 2015). The extract is commonly used in the clinical treatment of various human diseases, and its therapeutic efficacy is mainly attributed to one of the main bioactive components, Astragaloside IV (AS-IV) (Zhang *et al.*, 2020; Murata *et al.*, 2017). AS-IV (chemical formula, 3-O- $\beta$ -D-pyranxylosyl-6-O- $\beta$ -D-glucopyranosyl-cycloastragalool) is the most abundant saponin purified from Astragalus (Zu *et al.*, 2009; Chu *et al.*, 2010; Kwon and Park, 2012). Studies have shown that AS-IV exerts a variety of biological activities, such as anti-oxidation (Luo *et al.*, 2021) and anti-inflammation (Wan *et al.*, 2018). It has a positive effect on regulating the structure and diversity of intestinal flora (Xu *et al.*, 2018) and gut-derived metabolites and promoting intestinal transport (He *et al.*, 2020; Gong *et al.*, 2021).

The human genome cannot encode enough carbohydrate-active enzymes and, as a result, the human body relies on the metabolism of intestinal microorganisms to help it absorb polysaccharides. Different types of intestinal microorganisms selectively digest different polysaccharides, which affects the reproduction and colonisation of microorganisms in the digestive tract. In addition, AS-IV can also regulate the intestinal environment through its beneficial effect on the intestinal mucosa.

Through changes in the body's internal environment, AS-IV can also play an indirect role in regulating intestinal flora. Fluoxetine (FLU) is a clinically and widely used selective 5-hydroxytryptamine (5-HT) reuptake inhibitor that selectively inhibits the 5-HT transporter, blocks the reuptake of 5-HT by the presynaptic membrane and prolongs and increases the effects of 5-HT to produce antidepressant effects. The treatment of PSD rats with AS-IV as a comparison with a FLU group in the current study enabled observing whether there was any reduction in depressive symptoms among rats and whether the results could provide new therapeutic ideas for the treatment of PSD through the regulation of intestinal flora. Accordingly, this study explored the correlation between PSD and an intestinal flora imbalance through experiments and verified its regulatory effect on intestinal flora using AS-IV as a carrier, providing new ideas for the treatment of PSD.

## MATERIALS AND METHODS

### *Animals*

Adult healthy male Sprague–Dawley rats, SPF grade, weighing 260g±10g, were provided by the Animal Experimental Center of Xinxiang Medical University. The rats were housed adaptively at a room temperature of 25°C and relative humidity of 55% for 1 week, and 30 eligible rats were selected for inclusion.

### *Medicine, reagents and instruments*

For this study, AS-IV: Item no. A7970 was purchased from Solarbio Corporation. FLU hydrochloride capsules (20mg/tablet) produced by Shanghai Shangyao Traditional Chinese and Western Medicine Pharmaceutical Co., Ltd., were also purchased (traditional Chinese medicine approval number: H19980114).

All reagents and instruments were operated according to the manufacturer's instructions and included the following. Day root extraction kit: magnetic beads method Qiagen company (Qiagen, Valencia, California, USA); CTAB extraction method: CTAB, Nobleryder, China; Buffer: Phusion® High-Fidelity PCR Master Mix with GC Buffer (Product number M0531S), New England Biolabs, USA; Enzyme: Phusion® High-Fidelity DNA polymerase (product number M0530S, New England Biolabs, USA); PCR instrument: Bio-Rad T100 gradient PCR instrument, Manufacturer: BIO-RAD, model: T100, USA; Universal DNA purification and recovery kit (TianGen, China, catalogue No. DP214) for product purification; Library construction kit: Library construction was performed using the NEB Next® Ultra DNA Library Prep Kit from NEB, Illumina Inc. (Illumina, San Diego, CA, USA, catalogue No.E7370L); Sequencing instrument: The Illumina Novaseq6000 was used for sequencing (Illumina, San Diego, CA, USA).

### *Grouping and model preparation*

The rats were adaptively housed at a room temperature of approximately 25°C and 55% relative humidity for 1 week, with food and water *ad libitum* and a natural circadian rhythm light. The baseline values of an open-field test (OFT) and a water maze test (WMT) were measured, and 30 rats with similar scores were selected for enrolment (Yu *et al.*, 2019).

The rats were randomly divided into 5 groups: The CON, PSD, AS-IV, FLU and AS-IV + FLU groups, with 6 rats in each group. The PSD, AS-IV, FLU, APS + FLU and CON groups were compared using the Longa suture method to create a middle cerebral artery occlusion (MCAO) model (Tang *et al.*, 2020). Sutures were inserted through the right common carotid artery bifurcation, and

the embolisation time was 1.5 h. The right middle cerebral artery was blocked and the cerebral ischemia/reperfusion (I/R) model was established.

Neurological function of I/R model rats was measured using the Longa method 24 h after surgery, and the scores was recorded as 1~4 (scores: 0–no neurological symptoms; (1) the contralateral front paw cannot be fully extended; (2) rotation to the left; (3) leaning to the left; (4) cannot walk automatically, there is a loss of consciousness). On the second day after surgery (Longa *et al.*, 1989) the PSD rat model was established using the chronic unpredictable mild stress (CUMS) preparation method: (1) fasting for 20 h; (2) fasting for 17 h; (3) the mouse cage was placed at 45°C for 17 h; (4) continuous lighting for 17 h; (5) day and night cycles reversed; (6) electroshock to the sole for 10 min; (7) behaviour constraint for 2 h; (8) tail clamping for 1 min (Leonard and Maes, 2012; Heim *et al.*, 1997; Yang *et al.*, 2019). To avoid the development of tolerance to single or regular stress stimulation, the above groups were alternately treated with multiple unpredictable stimulation methods and were housed in a single cage. One of the above 8 types of stressors was selected randomly every day for 4 weeks.

### *Behavioural experiments*

At the start of the experiment, the rats were monitored for body weight and PSD behaviour scores using the OFT and WMT at a fixed time every week.

#### *Open-field test (OFT)*

The OFT reflects how the rats behave in an unfamiliar environment, and when the rats exhibit depressive behaviour, the number of upright positions decreases. An hour before the start of the experiment, the rats were placed in a test laboratory to acclimate to the environment. The opening test device is an open column box with a length of 100 cm and a height of 40 cm. The camera is fixed on the bracket above the open field box, and the camera is connected to a computer through a USB adapter or video cable. Before running the test, the box is cleaned by spraying and wiping it with 70% ethanol and a paper towel. The experiment rats were removed from the cage and placed on the edge of the open field box. The number of times the rats stood vertically and crossed the centre within 1 min was observed as the score of the experimental activity.

#### *Water maze test (WMT)*

The water maze experiment detected the learning and memory ability of the rats, and its learning and memory impairment was also a basic feature of clinical PSD. Data acquisition and analysis software was used to record

relevant data and images, and the cognitive levels of spatial learning and memory among the rats were evaluated. The experimental device is a cylindrical heated swimming pool with a diameter of approximately 2 m, a water depth of 25 cm and a water temperature of  $24 \pm 1^\circ\text{C}$ , which is divided into zones A, B, C and D and a platform.

Water maze training was also carried out during the rats' adaptive feeding period for 1 week. The specific training aspects included the following. (1) Adaptive training: The hidden platform was removed, and the rats swam in the pool at the same time every day to adapt to the environment; this stage lasted three days. (2) Positioning navigation experiment: The rats were put into the water facing the pool wall at a fixed time every morning, and the time required for the rats to find and climb onto the platform in the pool was recorded, i.e. the latency time of escaping from the wall. If the animal did not find the platform within 120 s, its escape latency was recorded as 120 s. The experimenter guided the animals that had not found the platform within 120 s to the platform and let them stay on the platform for 10 s. The positioning navigation experiment lasted four days, and the space exploration experiment was carried out with a rest for one day after the experiment. (3) Space exploration experiment: The hidden platform was removed and the quadrant where the platform was located was taken as the target quadrant. The rats were put into the pool from this quadrant, and their movements were tracked and recorded within 120 s. Record the time and distance the animal reached the target quadrant, and the number of times they crossed the area where the platform was located.

#### *Intervention technique*

The following intervention techniques were effected for the different groups. (i) CON group animals did not receive any stimulation, had free access to food and water, were given the same amount of normal saline gavage without any treatment. (ii) PSD model group animals were subjected to chronic stress stimulation every day and kept alone without injection. (iii) AS-IV group animals were subjected to chronic stress stimulation and isolated daily and were given AS-IV 30 mg/kg by gavage once a day from day 28 following surgery for 4 weeks. (iv) FLU group animals were subjected to chronic stress stimulation and kept alone every day; on day 28 after surgery, fluoxetine 12.5 mg/kg-1 was given by gavage once a day for 4 weeks. (v) AS-IV + FLU group animals were subjected to chronic stress stimulation and isolated daily and were given AS-IV 30 mg/kg-1 + FLU 12.5 mg/kg-1 by gavage once a day for 4 weeks from day 28 following surgery.

The administration of dose applied was sourced from the fourth edition of *Methodology of Pharmacological*

*Experiment* (2010).

#### *High-throughput 16S rDNA sequencing of rat gut microbiota*

Sample collection and preservation were conducted as follows. The faeces of rats in the 5 groups were collected at fixed time points, placed in sterile EP tubes and stored in a refrigerator at  $-80^\circ\text{C}$  for later use.

For total DNA extraction and PCR amplification of samples, the genomic DNA was extracted total DNA extraction. Its quality was assessed by agarose gel electrophoresis and quantified by an ultraviolet spectrophotometer (Feng *et al.*, 2019). The hypervariable region of 16S rDNA in the total DNA was amplified as the target fragment, and the diluted genomic DNA was used as a template for PCR. And the sequencing library was constructed. The final effective tags were obtained; the effective tags of all samples were clustered and the sequence with the highest frequency was selected as representative of operational taxonomic units (OTUs). The community composition of each sample was calculated at each taxonomic level, i.e. kingdom, phylum, class, order, family, genus and species.

#### *Data processing*

OTU clustering, diversity analysis and taxonomic analysis were performed. The dilution curve, rank abundance curve, the intestinal flora species accumulation curve, alpha diversity index, PCA, PCoA and NMDS were conducted and plotted. The SPSS Statistics 25.0 software was used to analyse the experimental data. The experimental measurement data were expressed as mean $\pm$ standard deviation, and the least significant difference in one-way analysis of variance was used for comparison between groups, and a result of  $P < 0.05$  was considered statistically significant.

## RESULTS

#### *Body weight of the PSD rats*

The results showed that after four weeks of modelling, compared with the CON group, the PSD model, AS-IV, FLU and AS-IV + FLU groups had a significant reduction in body weight ( $P < 0.05$ ; Table I).

#### *Behavioural responses*

The WMT results reflected the learning and memory ability of rats. Compared with the CON group, the PSD model, AS-IV, FLU and AS-IV + FLU groups had a significant reduction in the number of platform quadrant crossings ( $P < 0.05$ ).

The OFT results reflected the desire among the rats



to explore an unfamiliar environment. The number of vertical standing times per minute in the PSD model, AS-IV, FLU and AS-IV + FLU groups decreased significantly, and the difference was statistically significant ( $P < 0.05$ ). Compared with the PSD model group, there was no significant difference in the AS-IV, FLU and AS-IV + FLU groups ( $P > 0.05$ ), indicating that the PSD rat model had been successfully prepared (Table I).

After 4 weeks of drug injection, as shown in Table II, compared with the CON group, the body weight of the PSD model group was significantly reduced ( $P < 0.01$ ), while the body weight of the AS-IV, FLU and AS-IV + FLU groups were significantly increased compared with the PSD model group following drug intervention ( $P < 0.01$ ). The therapeutic effects of the AS-IV, FLU and AS-IV + FLU groups were similar ( $P > 0.05$ ). Compared with the PSD model group, the AS-IV, FLU and AS-IV + FLU groups showed a significant increase in the number of platform crossings during the WMT (all  $P < 0.01$ ), indicating that the learning and memory ability of the three groups were significantly improved. In the OFT, compared with the PSD model group, the rats in the AS-IV, FLU and AS-IV + FLU groups also had a significant improvement in the frequency of vertical standing ( $P < 0.01$ ), indicating

that the desire to explore in a strange environment also improved within the three groups, and there was no significant difference among the three groups ( $P > 0.05$ ). In conclusion, traditional AS-IV Chinese medicine improved the depressive behaviour of PSD rats to an extent, and there was no significant difference compared with the FLU group ( $P > 0.05$ ; Table II).

#### Analysis of gut microbiota diversity

##### Box plot of species accumulation

As shown in Figure 1A, in this experiment, with an increase in sample size, the position of the box plot tends to remain flat, indicating that the microbiota species in this environment will not increase significantly with an increase in sample size. This indicated that the sample size of the experiment was sufficient for the subsequent data analysis.

##### Species diversity curves

In this experiment, in the CON group, PSD model group, AS-IV, FLU and AS-IV + FLU groups, under the premise of no more than the existing sample sequencing amount, the dilution curve gradually flattened with an increase in sequencing data in each group, indicating that

**Table I. Effect of Astragaloside (AS) and Fluoxetine (FLU), administered alone and in combination on the body weight, number of crossing platform in water maze and number of vertical standings in open field test of PSD rats (mean  $\pm$  standard deviation).**

Group	n	weight(g)	Number of platform quadrant crossings/min	Number of vertical standing/min
CON group	6	420.8 $\pm$ 8.6	2.17 $\pm$ 0.31	2.67 $\pm$ 0.67
PSD model group	6	299.5 $\pm$ 9.0**	0.83 $\pm$ 0.31**	0.67 $\pm$ 0.67*
AS-IV group	6	325.5 $\pm$ 5.6**	0.83 $\pm$ 0.40**	1.00 $\pm$ 0.63*
FLU group	6	319.7 $\pm$ 8.1**	0.67 $\pm$ 0.33**	1.00 $\pm$ 0.44*
AS-IV+FLU group	6	328.0 $\pm$ 8.8**	0.50 $\pm$ 0.22**	0.50 $\pm$ 0.34*

\* indicates that compared with the blank group, \* $P < 0.05$ , \*\* $P < 0.01$ .

**Table II. Effect of astragaloside and fluoxetine, administered alone and in combination on body weight, number of platform crossings in WMT, and number of vertical standings in OFT in PSD rats after 4 weeks of bolus injection (mean  $\pm$  standard deviation).**

Group	n	Weight (g)	Number of platform quadrant crossings/min	Number of vertical standing/min
CON group	6	514.00 $\pm$ 29.60	2.33 $\pm$ 0.82	2.67 $\pm$ 1.37
PSD model group	6	380.67 $\pm$ 32.36	0.83 $\pm$ 0.75	0.83 $\pm$ 0.75
AS-IV group	6	440.00 $\pm$ 22.69**	1.83 $\pm$ 0.98*	2.17 $\pm$ 0.98*
FLU group	6	418.67 $\pm$ 18.73**	2.00 $\pm$ 0.63*	2.17 $\pm$ 0.98*
AS-IV+FLU group	6	431.83 $\pm$ 23.62**	1.83 $\pm$ 0.41*	2.33 $\pm$ 0.82*

\* indicates \* $P < 0.05$  and \*\* $P < 0.01$  compared with PSD model group.

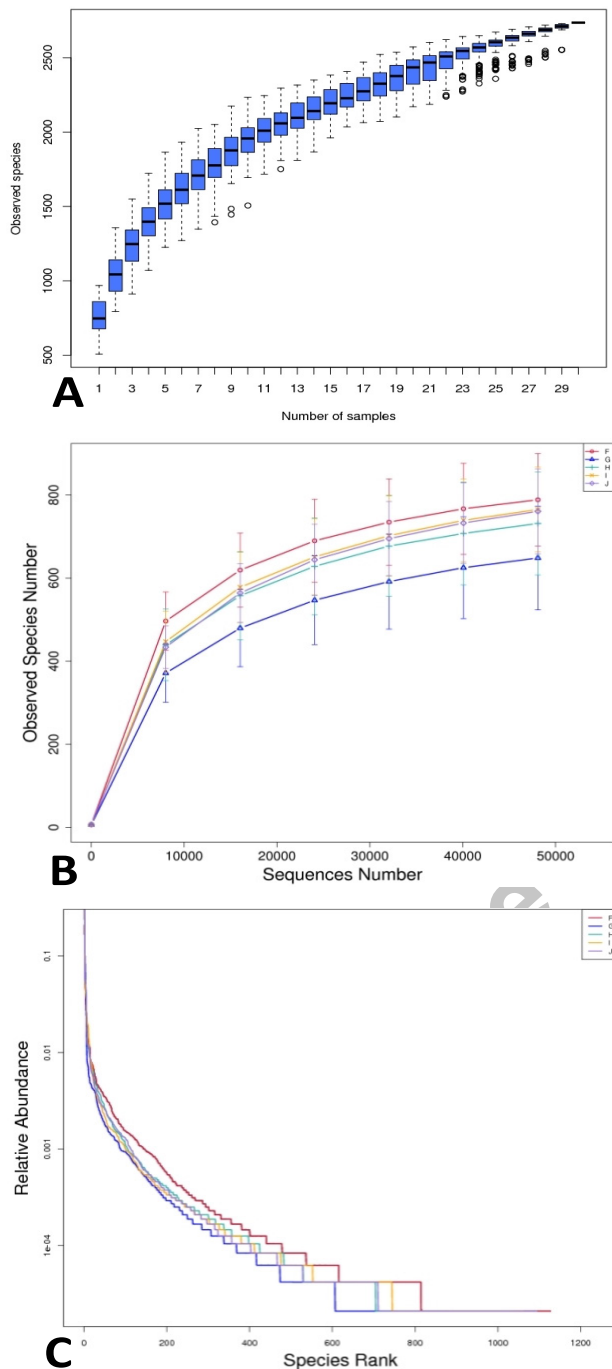


Fig. 1. Effect of astragaloside and fluoxetine, administered alone and in combination on box plot of species accumulation (A), species diversity curves (B) and hierarchical clustering curves (C) PSD rats after 4 weeks of injection. Groups F, G, H, I, and J were CON group, PSD model group, AS-IV group, FLU group, and AS-IV+FLU group, respectively, after 4 weeks of injection, with 6 samples in each group.

the sequencing data quantity was reasonable. The horizontal axis of the Figure 1B is the number of randomly sampled sequencing sequences from a particular sample, while the vertical axis is the number of constructed observed species, based on this number. Figure 2A shows that species abundance in the CON group was greater than in all of the other groups; the PSD model group had the lowest abundance, while the AS-IV, FLU and AS-IV + FLU groups all had similar abundance.

#### Hierarchical clustering curves

In this experiment, as shown in Figure 1C, the CON group had the widest curve width and the largest span in the horizontal direction, indicating the highest species richness. In the vertical direction, the CON group had the flatter curve, indicating the most uniform species distribution. In the horizontal direction, the curve width of the PSD model group was the narrowest, and its span was the shortest, indicating the lowest species richness. In the vertical direction, the curve of the PSD model group was more tortuous, indicating the most uneven species distribution. The results of the AS-IV, FLU and AS-IV + FLU groups were between those of the CON and PSD model groups.

#### Species annotation abundance analysis

Based on the annotation results, column charts of relative abundance at the phylum and genus levels were created for each sample, which was convenient for visually observing the species of bacteria in the gut microbiota with a high relative abundance and their proportion at the taxonomic level in each sample.

At the phylum level, the top three dominant species (Fig. 2A) mainly included *Firmicutes*, *Bacteroidota* and *Verrucomicrobiota*. Compared with the CON group, the PSD model group had a significant increase in the species abundance of firmicutes and a significant reduction in *Bacteroidetes* ( $P < 0.05$ ). Following intervention using AS-IV, FLU, and integrated AS-IV + FLU medication, the abundance of *Firmicutes* in these three groups decreased to varying degrees; in the FLU group, these were significantly decreased ( $P < 0.05$ ). The *Bacteroidetes* species abundance increased to different degrees, and the FLU group was significantly improved ( $P < 0.05$ ).

At the genus level (Fig. 2B), the dominant gut microbiota species mainly included *Ligilactobacillus*, *Lactobacillus*, and *Prevotella\_9*. The abundance of *Ligilactobacillus* in the PSD model group was significantly higher than in the CON group, and the difference was statistically significant ( $P < 0.05$ ).

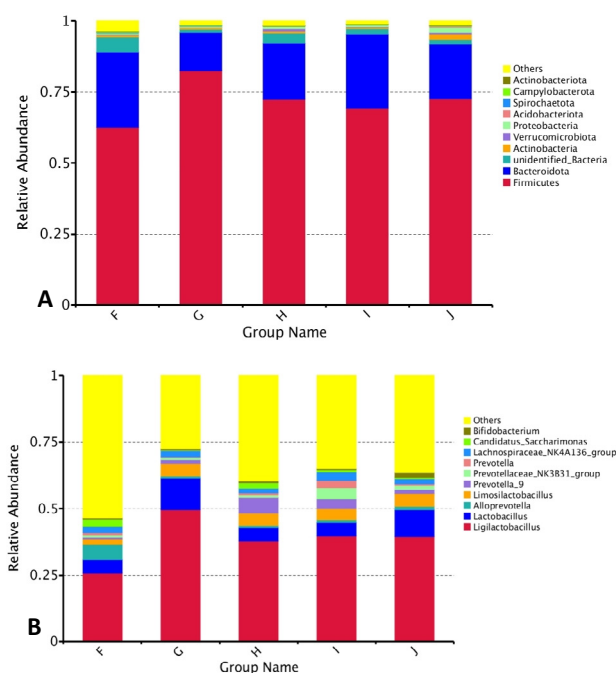


Fig. 2. Annotated abundance analysis of *Firmicutes*, *Bacteroidota*, and *Verrucomicrobiota* species (A) and *Ligilactobacillus*, *Lactobacillus*, and *Prevotella\_9* species (B). Groups F, G, H, I, and J were CON group, PSD model group, AS-IV group, FLU group, and AS-IV+FLU group, respectively, after 4 weeks of injection, with 6 samples in each group.

After medical intervention, the abundance of *Ligilactobacillus* in the AS-IV, FLU and AS-IV + FLU groups were lower than in the PSD model group, but the difference was not statistically significant ( $P > 0.05$ ). The abundance of *Lactobacillus* in the PSD model group was higher than in other groups, but the difference was not statistically significant ( $P > 0.05$ ). The AS-IV group had a significant increase in the abundance of *Prevotella\_9* compared with the CON and PSD model groups ( $P < 0.05$ ). The results indicated that AS-IV could significantly increase the abundance of *Prevotella\_9* (The purple band). At the generic level, *Prevotella\_9*, as one of the dominant species, is widely recognised as a probiotic with the ability to promote the production of short-chain fatty acids (SCFA). SCFA can play a significant role in the production of 5-HT, which, in turn, is involved in the regulation of the nervous system of PSD rats and can alleviate depressive symptoms.

#### Sample complexity analysis

##### Alpha diversity analysis

As shown in Table III, the Shannon index of group

F (CON group) was significantly higher compared with group G ( $P < 0.01$ ), and the Simpson index of group F was significantly higher compared with group G ( $P < 0.05$ ). Compared with group G (PSD model group), the alpha Diversity index in groups H (AS-IV group), I (FLU group) and J (AS-IV+FLU group) was also increased, but the difference did not reach a significant level ( $P > 0.05$ ). The results showed that group F had the highest community diversity and the most uniform species distribution, while the other three groups had improved diversity and species distribution after different interventions. The ACE, Chaol, Shannon and Simpson indexes of groups H, I and J were higher compared with group G, and the Shannon index of group H was higher compared with groups I and J, but there was no statistical significance ( $P > 0.05$ ). These results indicated that the community diversity and species distribution of the other three groups were improved under different interventions, but the effects were not significant.

Table III. Effect of astragaloside and fluoxetine, administered alone and in combination on the Alpha diversity index for each group of PSD rats (mean  $\pm$  standard deviation).

Item	ACE	Chaol	Shannon	Simpson
F	853.15 $\pm$ 126.80	838.55 $\pm$ 122.90	5.99 $\pm$ 0.86	0.91 $\pm$ 0.06
J	740.83 $\pm$ 151.26	717.01 $\pm$ 147.64	4.28 $\pm$ 0.72**	0.78 $\pm$ 0.09*
H	809.07 $\pm$ 127.86	795.89 $\pm$ 130.22	5.11 $\pm$ 1.24	0.85 $\pm$ 0.12
I	859.58 $\pm$ 118.53	847.61 $\pm$ 118.60	4.94 $\pm$ 0.95	0.84 $\pm$ 0.09
J	860.75 $\pm$ 131.73	843.52 $\pm$ 128.71	5.11 $\pm$ 0.56	0.87 $\pm$ 0.04

Groups F, G, H, I, and J were the CON group, PSD model group, AS-IV group, FLU group, and AS-IV+FLU group, respectively, with 6 samples in each group. ACE, The number of otus in the community was estimated. Shannon, the total number of categories in the sample and their proportion. Simpson, to characterize the diversity and evenness of species distribution within a community. Chaol, Estimate the total number of species included in the community sample. \*Indicates that compared with the blank group,  $*P < 0.05$ ,  $**P < 0.01$ .

##### Beta diversity analysis

In this experiment, the PCoA and NMDS methods were used to evaluate the beta diversity of rat gut microbiota, and the closer the distance between replicates, the more similar the species composition was for this sample. As shown in Figure 3, the proximity between groups F and H suggests that the species composition within the groups is similar. The distance between groups G, I, and J increased, suggesting that the structure of the microbiota was significantly separated among the three groups. The results indicated that PSD seriously affected the beta diversity of intestinal flora in rats and that AS-IV traditional Chinese medicine could improve this phenomenon to some extent.

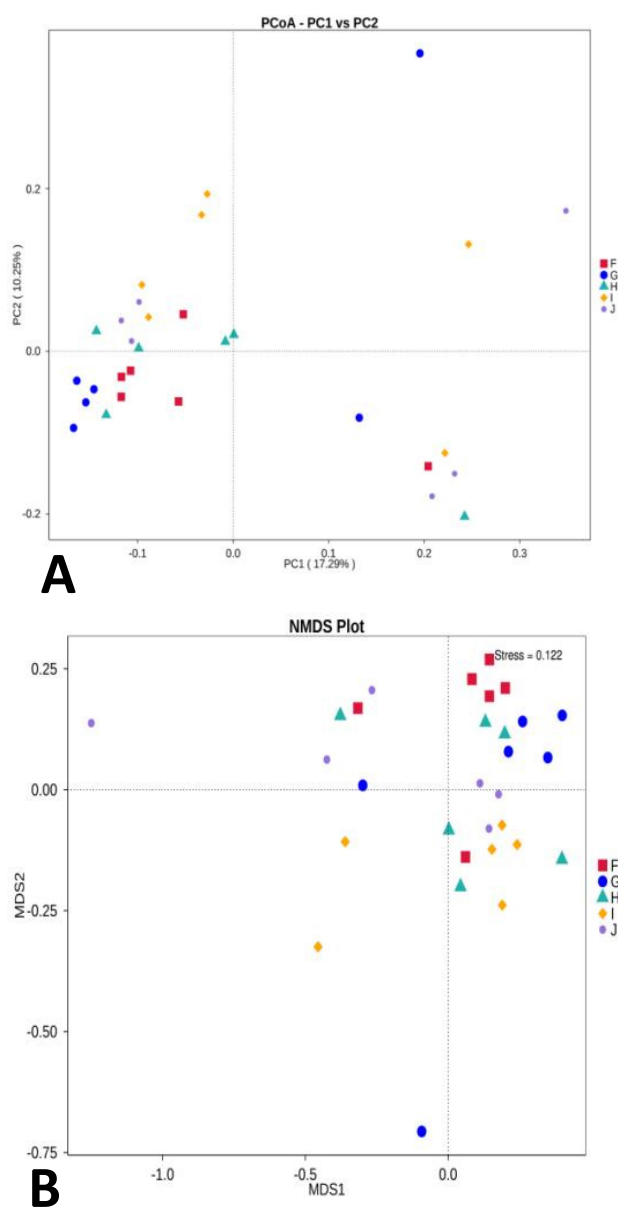


Fig. 3. Assessment of Beta diversity of rat gut microbiota by PCoA (A) and NMDS method (B).

Groups F, G, H, I, and J were CON group, PSD model group, AS-IV group, FLU group, and AS-IV+FLU group, respectively, after 4 weeks of injection, with 6 samples in each group.

#### Multi-level species difference analysis

In this study, species differences and significance analysis from the phylum to species levels of intestinal flora in groups F, G, H, I and J were compared. As shown in Figure 4, the relative abundance of the Saccharimonadia class, Saccharimonadales order, Saccharimonadaceae

and *Candidatus saccharimonas* in group F was higher. However, the relative abundance of *Firmicutes*, bacilli, Lactobacillales and Lactobacillaceae in group G was higher.

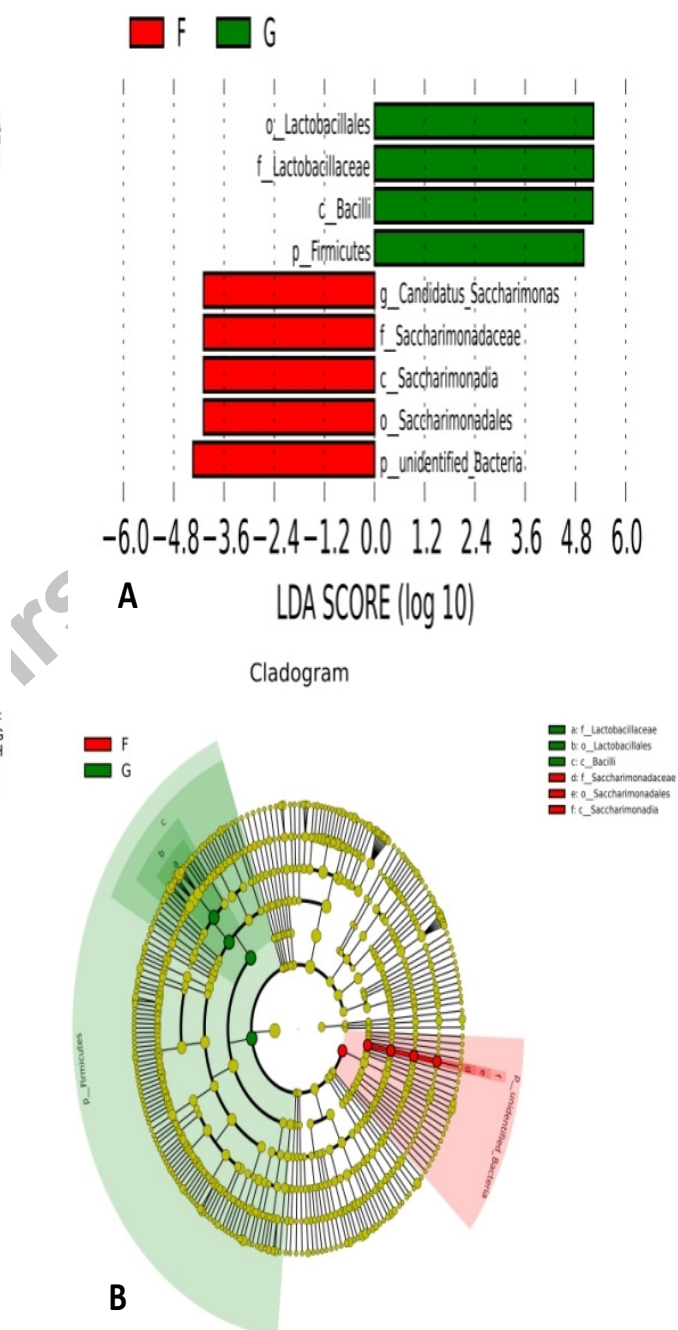


Fig. 4. LDA discriminant (A) and evolutionary branch map (B) of intestinal microorganisms in each group after 4 weeks of drug treatment.



## DISCUSSION

In terms of behaviour, compared with the CON group, the weight of the rats in the PSD model, AS-IV, FLU and the AS-IV + FLU groups decreased significantly after the model was established. In the open field test, the number of vertical standing instances among the rats and the number of platform crossings by rats in the WMT was significantly reduced, indicating that the PSD model had been successfully established. After four weeks of AS-IV injection, the rats in the AS-IV group had a significant increase in body weight compared with the PSD model group. In the WMT, reflecting the learning and memory ability of rats, compared with the PSD model group, the number of platform crossings made was significantly increased in the AS-IV group. Concurrently, the number of vertical standing instances among the rats in the AS-IV group was also significantly improved in the open field test, reflecting that the rats' desire to explore the unfamiliar environment was also improved. Compared with the FLU and AS-IV + FLU groups, there was no significant difference among the remaining three groups, and AS-IV and FLU could improve the depressive behaviour of PSD rats to a certain extent.

16S rDNA amplicon sequencing technology is an important means for studying the composition and structure of microbial communities in environmental samples (Cariou *et al.*, 2018; An *et al.*, 2022; Lieberman *et al.*, 2021). In this study, 16S rDNA high-throughput sequencing technology was used to sequence the intestinal microbial genes of rat faecal samples to further determine the composition and relative abundance of intestinal microbial communities in PSD rats, as well as the differences in intestinal microbial community and relative abundance between PSD and CON rats. Concurrently, AS-IV was used as a carrier to further verify the relationship between PSD and gut microbiota.

Previous studies have found that gut microbial disorders are closely related to depressive symptoms. The majority of 5-HT in the human body is produced and stored in the gastrointestinal tract (Lin and Yang, 2008). Intestinal microorganisms and their metabolic activities can affect the production of 5-HT, and sporulation bacteria and SCFA can play a significant role in the production of 5-HT (Yano *et al.*, 2015; Reigstad *et al.*, 2015). In addition, tryptophan, a precursor of 5-HT, is also affected by intestinal microorganisms, which can penetrate the blood-brain barrier and increase 5-HT content in the brain, thus directly participating in the regulation of the nervous system (Marcobal *et al.*, 2013). In this experiment, there were significant differences in intestinal flora between PSD rats and the CON group, specifically, the species

diversity and uniformity of intestinal flora in PSD rats were significantly lower than those in the CON group. At the same time, the species differences between the PSD and the CON groups were large, and the structure of bacterial flora was more obvious. The AS-IV, FLU and AS-IV + FLU groups showed significant increases in the diversity and uniformity of intestinal flora, indicating that all of them could improve the abundance and diversity of intestinal flora in PSD rats to a certain extent. Compared with the CON group, at the phylum level, the abundance of Firmicutes was significantly increased, and the abundance of Bacteroidetes was significantly decreased in the PSD model group. At the genus level of intestinal flora, the dominant species included *Ligilactobacillus*, *Lactobacillus* and *Prevotella\_9*. The abundance of *Ligilactobacillus* in the PSD model group was significantly higher than in the CON group. The AS-IV group had a significant increase in the abundance of *Prevotella\_9* compared with the CON and PSD groups. *Prevotella*, a Gram-negative anaerobic bacterium, are common symbiotic bacteria (Shah and Collins, 1990) and dominant in the intestines (Shah *et al.*, 2009; Wu *et al.*, 2011). *Prevotella* can synthesise SCFA in vivo under specific conditions (Louis *et al.*, 2014; Neves *et al.*, 2020). An increase in *Prevotella* abundance is beneficial to the synthesis of 5-HT and plays a role in the improvement of PSD. The experiment results showed that the AS-IV group had a more significant increase in the abundance of *Prevotella\_9* in the intestines than the FLU and AS-IV + FLU groups. Combined with the comprehensive analysis of behavioural results, AS-IV alone was found to have a degree of significance for maintaining the balance of intestinal flora, regulating the abundance and diversity of microbial species and improving depressive behaviour after a stroke in PSD rats. These findings indicate AS-IV may be a potential regulator for PSD. It was demonstrated that AS-IV could reverse abnormal intestinal microbial levels. The safe use of AS-IV is also demonstrated through the rich history of AS therapeutics in China.

This study also found that the proportion of beneficial intestinal bacteria in the PSD model group was higher than in the CON group. This phenomenon is considered to be related to the negative feedback regulation of the gut-brain axis. Stroke directly causes neurotransmitter disorders at the lesion and indicates primarily a decrease in 5-HT, which causes and aggravates depressive symptoms. During this period, intestinal flora that can produce 5-HT and other neurotransmitters increase in a compensatory manner, secreting more neurotransmitters to participate in the nervous system regulation. Intestinal flora participates in CNS activities such as brain emotion, stress response and cognition through various pathways, such as the

neuro-endocrine-immune and metabolic systems (Diaz Heijtz *et al.*, 2011; Hsiao *et al.*, 2013; Cryan *et al.*, 2012; Tian and Nie, 2016). Therefore, the phenomenon of increased intestinal beneficial bacteria can be combined with changes in 5-HT content in the body and brain of rats to uncover whether there is a correlation between them.

Few studies exist to date on the relationship between gut microbiota and PSD treatment, and there is no consensus on treatment due to the diversity of gut microbiota and differences between individuals. The heterogeneity and sample size of existing studies are limited, and some key questions remain unanswered. This experiment conducted in this study also has limitations. The small sample size only establishes a correlation between PSD and intestinal flora from a macro perspective, but the specific mechanism of action and accurate target of action are not precise. Furthermore, intestinal flora are complex and changeable, and many factors will affect them. As a new research direction, the complex relationship between intestinal flora and the body is worth further study, and additional research into the specific pathways and neural circuits involved in the influence of intestinal flora on the body will be of great benefit to improving the treatment of PSD and may even provide new ideas and methods for the treatment of various other diseases. Additional future research is needed to explore maintaining the balance of intestinal microbes using the depression model to improve this condition. Moreover, how AS-IV could be used in clinical practice and its potential side effects or interactions with other treatments should also be studied. It is believed that with additional research, the pathogenesis of PSD can be further clarified in the future.

## CONCLUSION

In conclusion, this study suggests that PSD is closely related to the regulation of intestinal flora, which can affect or be negatively regulated by intestinal flora. Furthermore, AS-IV could improve the depressive behaviour of PSD rats and showed obvious advantages for improving intestinal flora regulation and this field of study that is likely to deliver breakthroughs in anti-PSD treatment.

## DECLARATIONS

### *Funding*

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### *Ethics approval and consent to participate*

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics

Committee on Animal Welfare of First Affiliated Hospital of Xinxiang Medical University (ethical batch number: SCXK20190003). All methods were carried out in accordance with relevant guidelines and regulations. All animals were used in strict accordance with the national laboratory animal health regulations. The experimental process strictly followed the animal ethics standards and was approved by the Animal Welfare Ethics Committee (ethical approval number: SCXK (Lu) 20190003).

### *Consent for publication*

All authors final approval of the version to be published.

### *Availability of data and materials*

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### *Statement of conflict of interest*

The authors have declared no conflict of interest.

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