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Electroacupuncture Alleviates Anxiety-like Behaviors and Modulates Gut Microbiota, Neurotransmitters, and Inflammatory Responses in Chronically Stressed Rats

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Abstract

Objective: Based on the gut–brain axis theory, this study aimed to investigate the effects of electroacupuncture (EA) at Baihui (GV20) and Zusanli (ST36) on anxiety-like behaviors, neurotransmitters, inflammatory factors, intestinal neuropeptides, and gut microbiota in rats with generalized anxiety disorder (GAD) induced by chronic restraint stress (CRS). The study sought to evaluate the therapeutic effects of EA on GAD, explore its potential underlying mechanisms, and provide a scientific basis and novel therapeutic insights for the use of EA in GAD treatment.

Methods:

Forty male specific pathogen-free (SPF) Sprague–Dawley (SD) rats were randomly assigned to four groups: a control group, a model group, an EA group, and a drug treatment group. A rat model of GAD was established using CRS. Rats in the EA group received EA at Baihui (GV20) and Zusanli (ST36) (1 mA, 2 Hz, 15 min) 1 h before daily CRS exposure for three consecutive weeks. Rats in the drug treatment group received paroxetine hydrochloride (20 mg/kg) by oral gavage 1 h before daily CRS exposure for three consecutive weeks. Anxiety-like behaviors were assessed using the elevated plus maze (EPM) and Open Field Test (OFT). Enzyme-linked immunosorbent assay (ELISA)

was used to measure the levels of 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), and vasoactive intestinal peptide (VIP) in intestinal tissues, as well as serum levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Western blotting was performed to determine the protein expression levels of γ-aminobutyric acid type A receptor subunit α1 (GABRA1) and brain-derived neurotrophic factor (BDNF) in the hippocampus. Gut microbiota composition was analyzed using 16S rDNA gene sequencing, followed by correlation analyses between microbial taxa and the measured biological parameters.

Results:

Compared with the control group, CRS-exposed rats exhibited pronounced anxiety-like behaviors, accompanied by decreased levels of 5-HT and GABA in intestinal tissues and reduced hippocampal expression of GABRA1 and BDNF, as well as increased serum levels of IL-6 and TNF-α and elevated intestinal VIP levels. Compared with the model group, EA significantly alleviated anxiety-like behaviors, increased 5-HT and GABA levels and GABRA1 and BDNF expression, and decreased IL-6, TNF-α, and VIP levels. 16S rDNA gene sequencing revealed that EA was associated with alterations in gut microbiota composition and richness. Correlation analyses identified associations between *Duncaniella* and members of the family *Erysipelotrichaceae* and selected neurotransmitter and inflammatory markers.

Conclusions:

1. EA increased intestinal 5-HT and GABA levels, upregulated hippocampal GABRA1 and BDNF expression, reduced serum IL-6 and TNF-α levels, and modulated abnormal intestinal VIP levels in GAD model rats. These findings suggest that EA may exert anxiolytic effects by regulating neurotransmitter balance, promoting neuroplasticity, and suppressing inflammatory responses.

2. EA may exert its anxiolytic effects through the gut–brain axis by modulating gut microbial homeostasis and influencing neurotransmitter regulation and inflammatory responses.

Keywords: Generalized Anxiety Disorder; electroacupuncture; Gut–Brain Axis; Gut Microbiota

Introduction

Anxiety disorders (ADs), also known as anxiety neuroses, are a group of psychiatric disorders characterized by excessive fear, anxiety, or avoidance behaviors in response to external or internal stimuli. ADs primarily include GAD, social anxiety disorder, panic

disorder, agorapfhobia, and specific phobias, all of which are characterized by excessive fear or anxiety and related behavioral disturbances(1). GAD is one of the most common subtypes of anxiety disorders, with a 12-month prevalence of 3.1%(2). Patients with GAD commonly experience anxiety, nervousness, irritability, and difficulty concentrating, and these symptoms may lead to significant functional impairment. With increasing social competition and the accelerating pace of modern life, the prevalence of GAD has been reported to be rising, making it an important global public health concern.

Currently, selective serotonin reuptake inhibitors (SSRIs) and serotonin–norepinephrine reuptake inhibitors (SNRIs) are among the most commonly prescribed pharmacological treatments for anxiety disorders(3). However, SSRIs may cause adverse effects such as headache, insomnia, and gastrointestinal discomfort during the initial stages of treatment, whereas SNRIs may lead to mild increases in blood pressure and heart rate in some patients (4). Abrupt discontinuation of either class of medication may result in discontinuation symptoms or symptom rebound(5). Furthermore, treatment responses vary considerably among individuals. Therefore, exploring safer and more effective therapeutic interventions has become an important research priority in clinical medicine and neuroscience.

In recent years, the microbiota–gut–brain axis (MGBA) has attracted considerable attention in research on anxiety and other psychiatric disorders. Bidirectional communication between the gut microbiota and the central nervous system occurs through neural, endocrine, immune, and microbial metabolic pathways (6). Previous studies have demonstrated that chronic stress is associated with alterations in gut microbiota composition, neurotransmitter metabolism, and immune-inflammatory status, which may contribute to the development and persistence of anxiety-related behaviors (7). Among these factors, 5-HT and GABA are important neuroactive substances involved in emotional regulation and gut–brain communication, whereas VIP plays a role in gastrointestinal neural regulation and immune modulation (8). In addition, peripheral inflammatory responses may be associated with anxiety-related neurobiological alterations. IL-6 and TNF-α are important markers for assessing inflammatory status (9, 10). These inflammatory cytokines may influence anxiety-related brain regions, including the amygdala, hippocampus, insula, anterior medial prefrontal cortex, and anterior cingulate cortex, potentially contributing to persistent anxiety symptoms (7, 11). Therefore, the combined analysis of alterations in gut microbiota, neuroactive substances, and inflammatory cytokines may provide further insights into the biological processes

associated with chronic stress.

In addition to alterations in the gut microbiota and peripheral inflammatory responses, changes in inhibitory neurotransmission and neuroplasticity within the central nervous system are closely associated with stress- and anxiety-related behaviors. GABA_A receptors are major inhibitory neurotransmitter receptors in the central nervous system, and their pharmacological properties are closely related to their subunit composition (12-14). Among these subunits, the GABA_A receptor α1 subunit (GABRA1) is an important component involved in GABAergic neurotransmission, and changes in its expression may reflect molecular alterations in the associated receptor system. Furthermore, BDNF plays essential roles in neuronal survival, synaptic plasticity, and adaptation to stress. The regulation of BDNF signaling is closely associated with fear memory, stress sensitivity, and emotion-related behaviors (15, 16). Therefore, examining hippocampal GABRA1 and BDNF protein expression may provide additional evidence for understanding central neurobiological alterations associated with chronic stress and subsequent interventions.

As a form of acupuncture therapy, EA has attracted increasing attention in research on anxiety and other stress-related disorders. Previous systematic reviews have suggested that acupuncture may help alleviate anxiety symptoms in patients with GAD. However, owing to methodological limitations in existing clinical studies, its efficacy and underlying mechanisms require further validation (17). In preclinical research, Bai et al. (18) demonstrated that EA alleviated anxiety-like behaviors in CRS-exposed mice, accompanied by alterations in gut microbiota composition. Furthermore, Duan et al. (19) reported that EA was associated with changes in the gut microbiota and metabolism-related parameters in a rat model of chronic unpredictable mild stress (CUMS). These findings suggest that the gut microbiota may represent an important avenue for investigating the effects of EA on stress-related behaviors. Nevertheless, further studies are needed to integrate evidence from multiple biological levels, including intestinal neurochemical parameters, peripheral inflammatory responses, and hippocampal neurobiological proteins, with particular emphasis on exploring the associations between specific microbial taxa and host biological parameters following EA intervention.

Therefore, this study employed a CRS-induced rat model of GAD to investigate the effects of EA at Baihui (GV20) and Zusanli (ST36) on anxiety-like behaviors, neurotransmitters, inflammatory cytokines, intestinal neuropeptides, and gut microbiota. The study aimed to evaluate the efficacy of EA in alleviating anxiety-like behaviors and

provide experimental evidence for further exploration of its potential anxiolytic mechanisms.

2. Materials and Methods

2.1 Experimental Materials

2.1.1 Experimental Animals

Forty male Sprague–Dawley (SD) rats aged 6–8 weeks and weighing 210–230 g were used in this study. All rats were certified SPF animals (Laboratory Animal Production License No. SCXK [Lu] 2022-0006). The rats were acclimatized for one week under controlled environmental conditions, with a 12-h light/dark cycle and an ambient temperature of 20–22° C, and were provided ad libitum access to food and water. All animals were housed and maintained in accordance with the regulations of the Laboratory Animal Center of Henan University of Chinese Medicine. All experimental procedures complied with animal ethics guidelines and the principles of the 3Rs (Replacement, Reduction, and Refinement), with efforts made to minimize animal stress and suffering. The study was approved by the Institutional Animal Care and Use Committee (IACUC; Approval No. IACUC-202406033).

2.1.2 Animal Grouping and Model Establishment

2.1.2.1 Establishment of the Generalized Anxiety Disorder Model

A CRS protocol was used to induce anxiety-like behaviors in rats over 21 consecutive days. Each morning at 9:00 a.m., the rats were placed in a custom-made restraint device modified from a 500-mL plastic bottle. During the first two days, the rats were restrained for 2 h per day. The restraint duration was subsequently increased by 2 h every two days until it reached 6 h per day. Restraint stress began at 9:00 a.m. each day. Food and water were withheld during restraint, and free access to food and water was restored afterward. The rats were continuously monitored throughout the restraint period to prevent suffocation and minimize unnecessary disturbances. After each restraint session, the devices were thoroughly cleaned to remove excreta and residual odors, dried, and reused.

2.1.2.2 Animal Grouping

Forty SD rats were numbered according to their body weights and randomly assigned to four groups using a randomized block design: the control group (KB), model group (MX), electroacupuncture group (DZ), and drug treatment group (YW), with 10 rats in each group. A blinded assessment procedure was employed in this study. Experimental procedures, outcome measurements, and data analyses were performed by different personnel, and the outcome assessors were blinded to group assignments to ensure the objectivity and reliability of the results.

2.2 Acupoint Selection and Intervention Methods

2.2.1 Acupoint Selection

The acupoints used in this study were located according to Experimental Acupuncture Science (edited by Guo Yi, China Press of Traditional Chinese Medicine, 2021).

Baihui (GV20): Located at the midpoint of the parietal bone.

Zusanli (ST36): Located 5 mm below the head of the fibula, inferolateral to the knee joint of the rat's hindlimb.

2.2.2 Intervention Methods

In this study, interventions in the EA and drug treatment groups were administered 1 h before daily CRS exposure(20-22). The specific interventions were as follows:

1. Control group (KB): Rats were housed under normal conditions without any additional intervention.

2. Model group (MX): Rats were subjected to CRS. Before each daily CRS session, the rats were handled and restrained for 15 min, once daily for three consecutive weeks.

3. EA group (DZ): Rats were subjected to CRS and received daily EA stimulation at Baihui (GV20) and Zusanli (ST36) 1 h before each CRS session. Following needle insertion, the needles were connected to an electroacupuncture device, which delivered electrical stimulation at an intensity of 1 mA and a frequency of 2 Hz for 15 min. The intervention was administered once daily for three consecutive weeks.

4. Drug treatment group (YW): Rats were subjected to CRS and received paroxetine hydrochloride (20 mg/kg) via oral gavage 1 h before each daily CRS session (23). The treatment was administered once daily for three consecutive weeks.

2.3 Biological Sample Collection

Following the final treatment, behavioral assessments were performed, and the rats were weighed. Subsequently, the rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg).

2.3.1 Blood Collection from the Abdominal Aorta

Once fully anesthetized, each rat was placed in the supine position and secured on an operating table. The abdominal cavity was opened layer by layer along the midline using sterile ophthalmic scissors. Under aseptic conditions, the intestines and surrounding tissues were gently displaced to the left to fully expose the abdominal aorta, which appeared relatively pale and was located on the right side of the surgical field. A sterile 5-mL syringe was inserted into the vessel with the needle bevel facing downward at an

angle of 25°–30° and an approximate insertion depth of 5 mm. Blood was slowly withdrawn, with approximately 5 mL collected from each rat. The blood samples were allowed to stand at room temperature for 2 h and then centrifuged at 3,000 rpm for 15 min. The resulting serum was collected and stored at −80°C until analysis.

2.3.2 Fecal Sample Collection

Fecal samples were collected after the final intervention. Each rat was individually placed in a clean, sterilized cage and allowed to defecate naturally. Fresh fecal samples were collected using sterile forceps and transferred into sterile centrifuge tubes. Immediately after collection, the samples were temporarily stored on dry ice or in liquid nitrogen. If urination occurred during collection, the urine was promptly removed using clean paper towels to prevent fecal contamination. Approximately three fecal pellets were collected per sample, with two samples obtained from each rat for subsequent analyses. All samples were clearly labeled and subsequently stored at −80°C until further analysis by high-throughput 16S rDNA gene sequencing.

2.3.3 Colon Tissue Collection

Following blood collection from the abdominal aorta, distal colon tissue samples were excised and longitudinally opened. The intestinal contents were rinsed away with physiological saline. The tissues were then wrapped in aluminum foil and stored at −80°C until further analysis.

2.3.4 Hippocampal Tissue Collection

Whole-brain tissues were collected from a subset of rats, and the hippocampi were rapidly dissected on ice. The isolated hippocampal tissues were transferred into cryogenic storage tubes and stored at −80°C for subsequent Western blot analysis.

2.4 Outcome Measurements

2.4.1 Behavioral Assessments

Following CRS exposure, behavioral tests were conducted in all groups to evaluate changes in anxiety-like behaviors.

1) Open Field Test (OFT)

Before the experiment, a tripod and video recording system were set up and adjusted to ensure clear capture and recording of the rats' movements throughout the test. The open field apparatus (length × width × height: 100 × 100 × 40 cm), with its inner walls painted black, was cleaned with 75% ethanol. At the beginning of the test, each rat was placed in the center of the open field and allowed to explore freely for 5 min for habituation. Subsequently, the number of entries into the central and peripheral zones and the time

spent in each zone were recorded over a 5-min testing period. Fewer entries into the central zone and a shorter duration spent in this zone were considered indicative of greater anxiety-like behavior.

2)Elevated Plus Maze (EPM) Test

The EPM apparatus was elevated 50 cm above the floor and consisted of two open arms (50 cm long × 10 cm wide) and two closed arms (50 cm long × 10 cm wide × 40 cm high). Before the test, each rat was placed in a plastic holding cage for 5 min of habituation. Subsequently, the rat was promptly placed in the center of the EPM, facing one of the open arms, and its movements were recorded using a video camera for 5 min. The behavioral parameters measured included the number of open-arm entries (OE) and the time spent in the open arms. After each test, feces and urine were removed, and the apparatus was cleaned with 75% ethanol to minimize olfactory cues that might interfere with subsequent testing. All rats were transferred to a quiet, dimly lit testing room 1 h before behavioral assessments for acclimatization.

2.4.2 ELISA

ELISA was used to measure the levels of 5-HT, GABA, and VIP in rat colon tissues, as well as serum levels of IL-6 and TNF-α.

Serum samples stored at −80°C were thawed before analysis. Colon tissues were minced and homogenized thoroughly in an appropriate volume of prechilled phosphate-buffered saline (PBS). After centrifugation, the supernatants were collected for analysis. The corresponding ELISA kits were used according to the experimental procedures. Standard, sample, and blank wells were prepared. Following sample addition, incubation, washing, and color development, a stop solution was added to terminate the reaction. Absorbance was measured at 450 nm using a microplate reader, and the concentrations of the analytes were calculated from the corresponding standard curves.

2.4.3 Western Blot Analysis

Western blotting was performed to determine the expression levels of GABRA1 and BDNF proteins in rat hippocampal tissues. Frozen hippocampal tissues were washed with prechilled PBS, minced, and homogenized in RIPA lysis buffer containing protease inhibitors (Servicebio, G2002-100ML). The homogenates were lysed on ice for 30 min and subsequently centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were collected, and protein concentrations were determined using a BCA protein assay kit (Servicebio, G2026-200T). Protein samples were mixed with 5× SDS-PAGE loading buffer at a ratio of 4:1 and denatured in a boiling water bath for 15 min.

The prepared protein samples were separated by 10% SDS-PAGE and subsequently transferred onto 0.45-μm polyvinylidene fluoride (PVDF) membranes (Servicebio, WGPVDF45) at a constant current of 350 mA for 30 min. After transfer, the membranes were blocked with 5% nonfat milk at room temperature for 30 min. The membranes were then incubated overnight at 4°C with primary antibodies against GABRA1 (Servicebio, GB113973; 1:1,000), BDNF (Abcam, ab108319; 1:1,000), and GAPDH (Servicebio, GB15004; 1:10,000), which served as the internal loading control. After three washes with Tris-buffered saline containing Tween 20 (TBST) for 5 min each, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Servicebio, GB23303) at room temperature for 30 min. The membranes were then washed three additional times with TBST for 5 min each.

Protein bands were visualized using enhanced chemiluminescence (ECL) reagents, and images were captured using a chemiluminescence imaging system (Servicebio, SCG-W3000). Band intensities were quantified using AIWBwell™ software. GAPDH was used as the internal loading control, and the relative expression levels of GABRA1 and BDNF were determined by calculating the ratio of the grayscale intensity of each target protein band to that of the corresponding GAPDH band.

2.4.4 16S rDNA gene sequencing Analysis

The sequencing procedures were outsourced to Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.5 Statistical Analysis

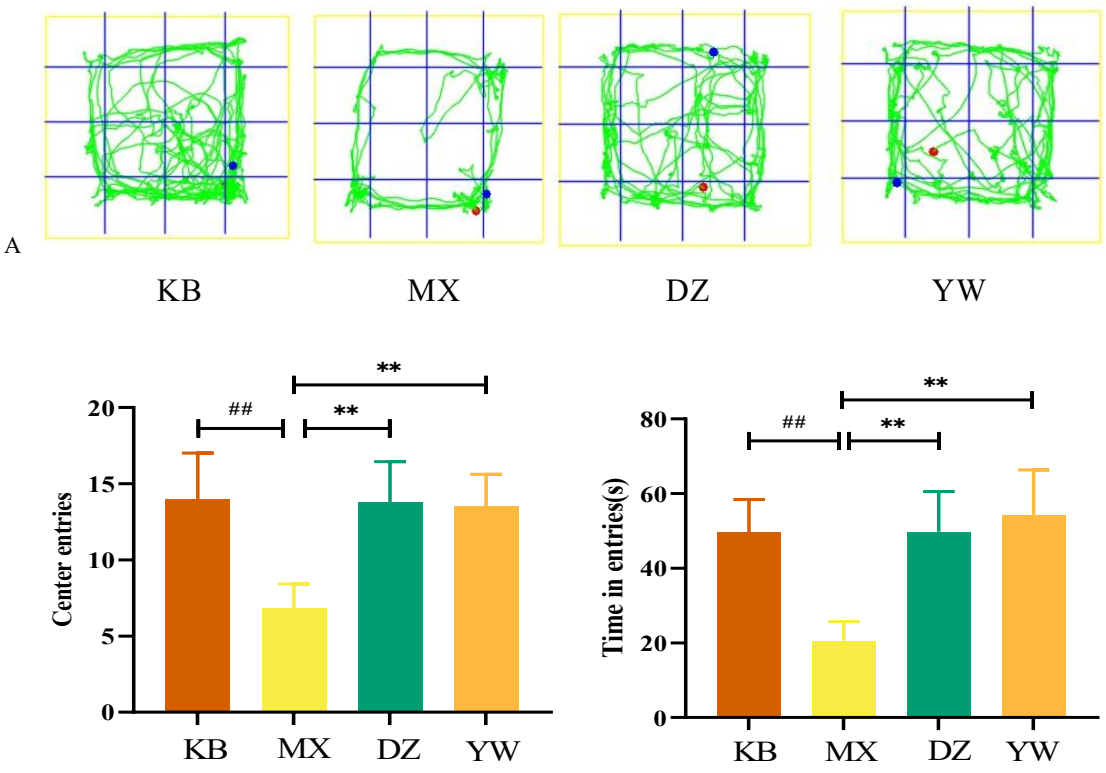
All data were analyzed using SPSS version 21.0, and figures were generated using GraphPad Prism version 9.0. Before performing one-way analysis of variance (ANOVA), data were assessed for normality and homogeneity of variance. For normally distributed data with homogeneous variances, pairwise comparisons among groups were performed using Fisher's least significant difference (LSD) test. For normally distributed data with unequal variances, Tamhane's T2 test was used for pairwise comparisons. These data were expressed as the mean ± standard deviation (SD). For data that did not follow a normal distribution, the Kruskal–Wallis H test was performed, followed by Dunn's post hoc test with Bonferroni correction for multiple comparisons. Non-normally distributed data were presented as the median (P25, P75). A P value < 0.05 was considered statistically significant.

3. Results

3.1 Effects of EA on Anxiety-like Behaviors in GAD Model Rats

3.1.1 OFT

Following the intervention, the OFT results (Figure 1) showed that, compared with the control group, rats in the model group exhibited significantly fewer entries into the central zone and spent significantly less time in this zone ($P < 0.01$). Compared with the model group, both the EA and drug treatment groups showed significant increases in the number of central zone entries and the time spent in the central zone ($P < 0.01$). No significant differences were observed between the EA and drug treatment groups in either parameter ($P > 0.05$).



B. Number of Entries into the Central Zone of the Open Field. C. Time Spent in the Central Zone of the Open Field

Note: $^{\#\#}P<0.01$ vs. the control group;$^{**}P<0.01$vs. the model group.

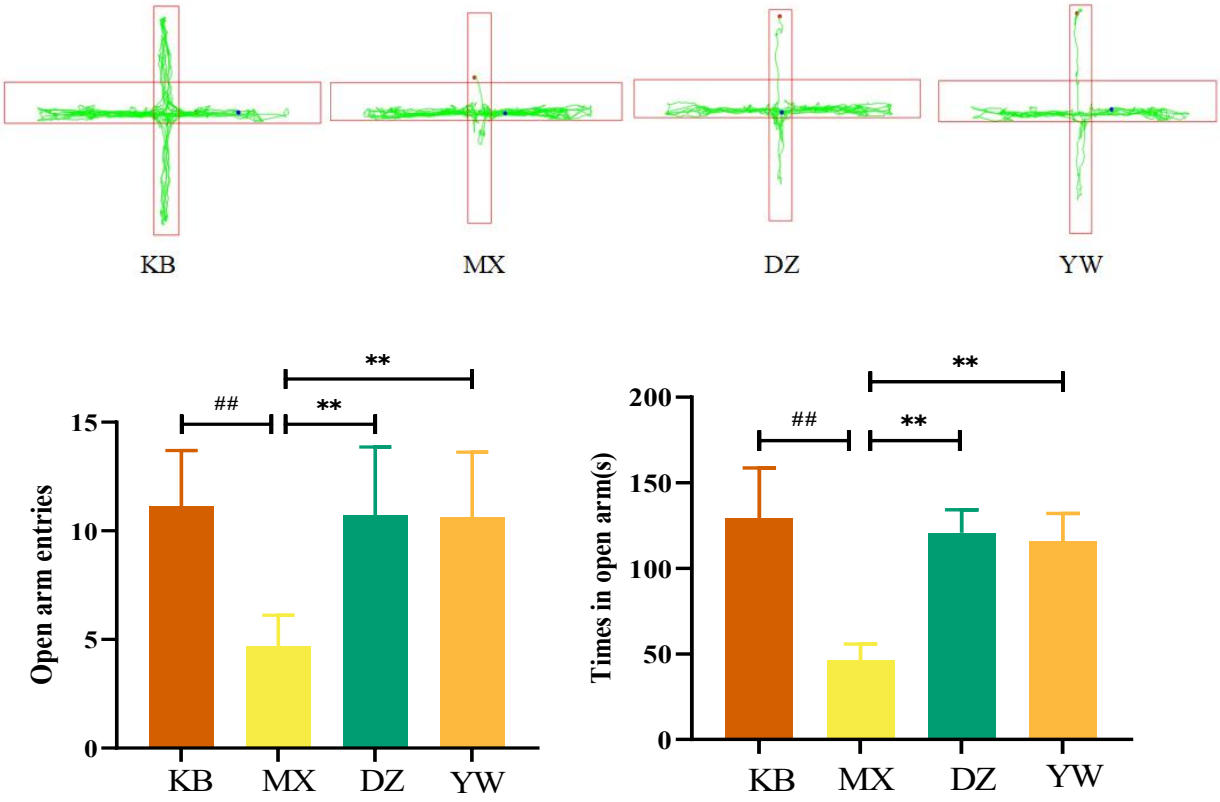
Figure 1. Results of the Open Field Test in Rats

3.1.2 Elevated Plus Maze (EPM) Test

The EPM results (Figure 2) showed that, compared with the control group, rats in the model group exhibited significantly fewer open-arm entries and spent significantly less time in the open arms ($P < 0.01$), indicating that CRS induced pronounced anxiety-like behaviors in rats. Compared with the model group, both the EA and drug treatment groups showed significant increases in the number of open-arm entries and the time spent in the open arms ($P < 0.01$). No significant differences were observed between the EA

and drug treatment groups in either parameter ($P > 0.05$).

A



B. Number of Open-Arm Entries in the EPM C. Time Spent in the Open Arms of the EPM

Note: $^{\#\#}P < 0.01$ vs. the control group; $^{**}P < 0.01$ vs. the model group.

Figure 2. Results of the EPM Test

3.2 Effects of EA on Neurotransmitters, Receptors, and Neurotrophic Factors

3.2.1 Effects of EA on Intestinal 5-HT and GABA Levels in GAD Model Rats

As shown in Table 1 and Figure 3, intestinal 5-HT and GABA levels were significantly decreased in the model group compared with the control group ($P < 0.01$). Compared with the model group, 5-HT levels were significantly increased in the EA group ($P < 0.05$), while GABA levels were significantly increased in both the EA and drug treatment groups ($P < 0.01$). No significant difference in GABA levels was observed between the EA and drug treatment groups ($P > 0.05$).

Table 1. Effects of EA on Intestinal 5-HT and GABA Levels in GAD Model Rats（$\bar{x}\pm s$）

Group	n	5-HT（ng/mg)	GABA(μmol/g)
KB	6	1.65±0.43	0.44±0.02

MX	6	0.69±0.13##	0.17±0.03##
DZ	6	1.14±0.19*	0.25±0.04**
YW	6	0.98±0.20	0.27±0.03**

Note: ##$P < 0.01$ vs. the control group; *$P < 0.05$ and **$P < 0.01$ vs. the model group.

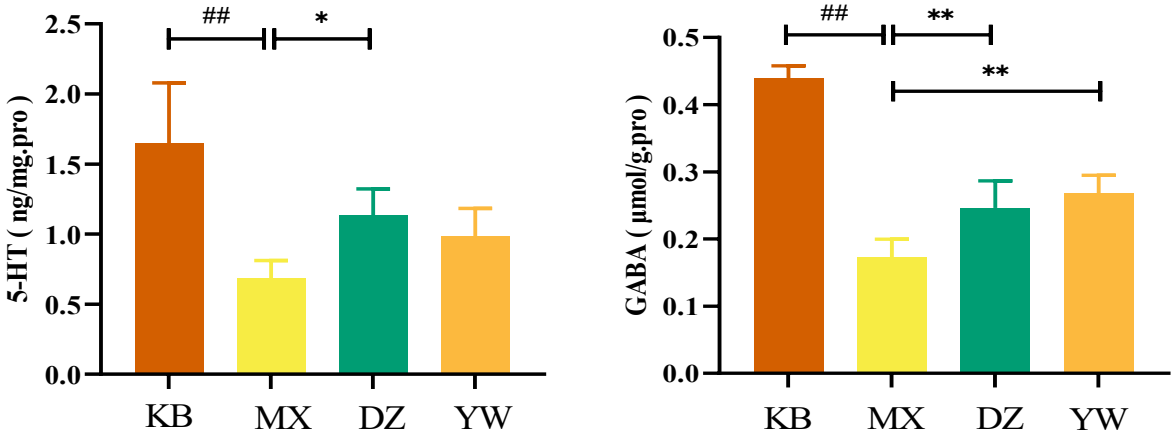


Figure 3. Effects of EA on Intestinal 5-HT and GABA Levels in Rats

3.2.2 Effects of EA on GABRA1 and BDNF Expression in the Hippocampus of GAD Model Rats

Western blot analysis (Table 2 and Figure 4) showed that, compared with the control group, the protein expression levels of GABRA1 and BDNF in the hippocampus were significantly decreased in the model group ($P < 0.01$). Compared with the model group, both the EA and drug treatment groups exhibited significantly increased hippocampal GABRA1 and BDNF protein expression levels ($P < 0.05$, $P < 0.01$). No statistically significant difference was observed between the EA and drug treatment groups in their effects on BDNF expression ($P > 0.05$). However, EA was less effective than drug treatment in upregulating GABRA1 expression ($P < 0.05$).

Table 2. Effects of EA on GABRA1 and BDNF Expression in the Hippocampus of GAD Model Rats

（$\bar{x}\pm s$）

Group	n	GABRA1/GAPDH	BDNF/GAPDH
KB	3	0.53±0.03	0.54±0.05
MX	3	0.29±0.03$^{\#\#}$	0.24±0.047$^{\#\#}$
DZ	3	0.36±0.02*	0.37±0.03*
YW	3	0.44±0.02$^{**\blacktriangle}$	0.45±0.025**

Note: $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$,$^{**}P < 0.01$ vs. the model group; $^{\blacktriangle}P < 0.05$ vs. the EA group.

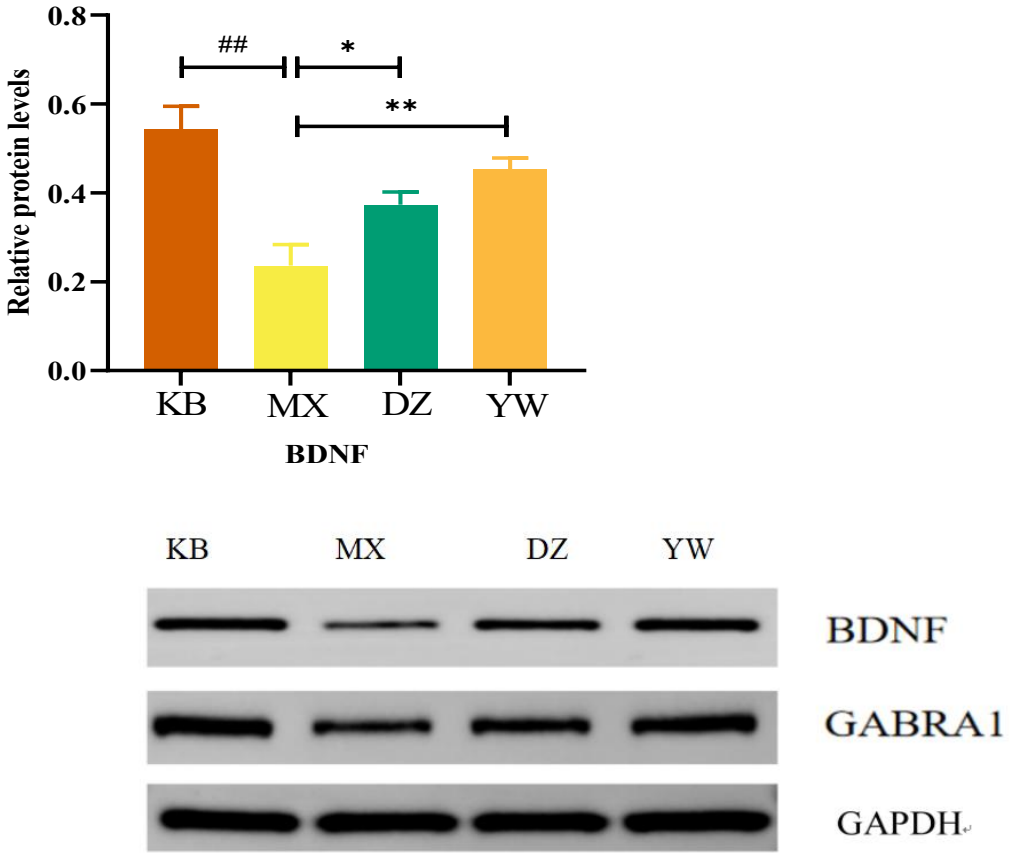


Figure 4. Effects of EA on GABRA1 and BDNF Expression in the Rat Hippocampus

3.3 Effects of EA on Serum IL-6 and TNF-α Levels in GAD Model Rats

ELISA results (Table 3 and Figure 5) showed that, compared with the control group,

the serum levels of the pro-inflammatory cytokines IL-6 and TNF-α were significantly increased in the model group ($P < 0.01$). Compared with the model group, both the EA and drug treatment groups exhibited significantly decreased serum IL-6 and TNF-α levels ($P < 0.05$, $P < 0.01$), indicating that both EA and drug treatment effectively suppressed the abnormal elevation of inflammatory cytokines. Drug treatment was more effective than EA in reducing IL-6 levels ($P < 0.01$), whereas no statistically significant difference was observed between the two groups in their effects on TNF-α levels ($P > 0.05$).

Table 3. Effects of EA on Serum IL-6 and TNF-α Levels in GAD Model Rats（$\bar{x}\pm s$）

Group	n	IL-6	TNF-α
KB	8	27.94±2.21	50.03±4.50
MX	9	38.38±2.70$^{\#\#}$	79.31±4.56$^{\#\#}$
DZ	8	34.58±2.45*	64.63±4.96**
YW	9	29.30±3.09$^{**\blacktriangle\blacktriangle}$	59.75±6.59**

Note: $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$,$^{**}P < 0.01$ vs. the model group; $^{\blacktriangle\blacktriangle}P < 0.05$ vs. the EA group.

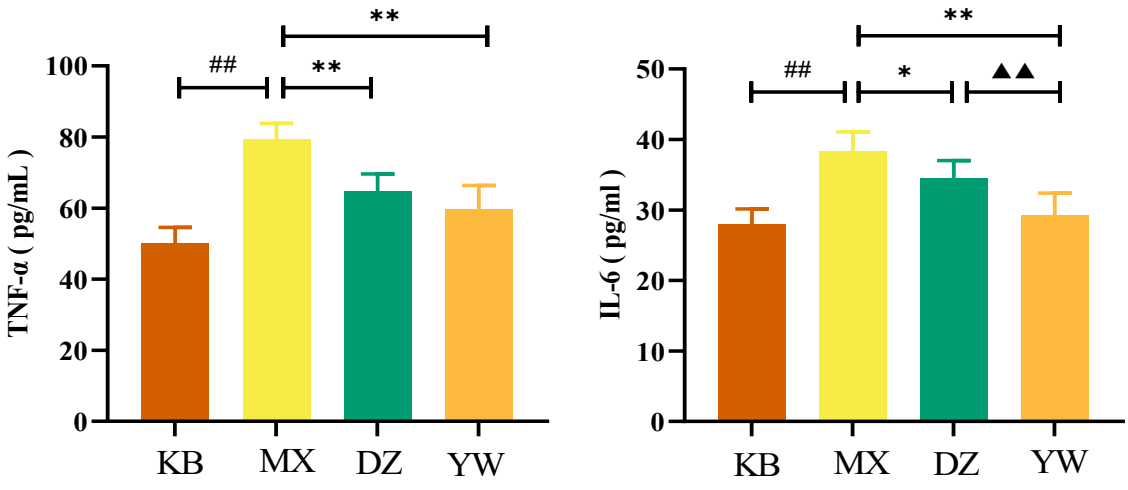


Figure 5. Effects of EA on Inflammatory Factor Levels in GAD Model Rats

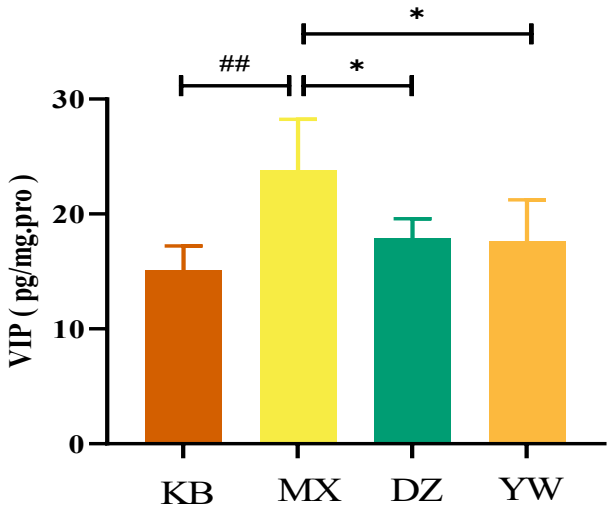
3.4 Effects of EA on VIP Levels

As shown in Table 4 and Figure 6, the VIP level in the intestinal tissue of rats in the model group was significantly increased compared with that in the control group ($P < 0.01$). Compared with the model group, VIP levels were significantly decreased in both the EA and drug treatment groups ($P < 0.05$), suggesting that both EA and drug treatment could alleviate model-induced VIP overexpression and exert regulatory effects on intestinal function. No statistically significant difference in VIP levels was observed between the EA and drug treatment groups ($P > 0.05$).

Table 4. Effects of EA on VIP Levels in Intestinal Tissue

Group	n	VIP（pg/mg）
KB	6	15.12±2.11
MX	6	23.83±4.41$^{\#\#}$
DZ	6	17.88±1.72*
YW	6	17.63±3.61*

Note: $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$ vs. the model group



Note: $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$ vs. the model group

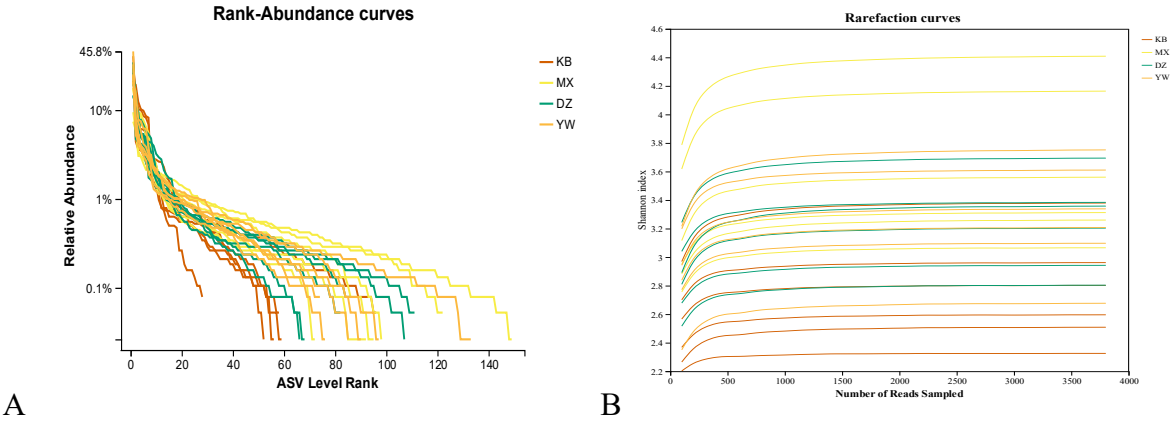
Figure 6. Comparison of VIP Levels in Intestinal Tissue

3.5 Effects of EA on Gut Microbiota Structure (16S rDNA Sequencing)

3.5.1 Sequencing Data Quality, ASV Numbers, Effective Sequence Counts, and R

arefaction Curves

Rank-abundance curves can simultaneously reflect species richness and evenness. Species richness is represented by the length of the curve along the horizontal axis, with a wider horizontal span indicating higher species richness. Species evenness is reflected by the smoothness of the curve, with a flatter curve indicating greater species evenness. As shown in Figure 7A, the curves of samples from each group exhibited a wide horizontal span and eventually tended to stabilize, suggesting that the samples exhibited both high species richness and evenness. Figure 7B shows that as sequencing depth increased, the number of ASVs gradually increased and eventually reached a plateau, indicating that the sequencing data obtained in this experiment were sufficient and could meet the requirements for subsequent analyses.



A B

Figure 7. Analysis of sequencing data quality and ASV counts.

3.5.2 Venn Diagram

To investigate the species composition of each sample, amplicon sequence variant (ASV) clustering and species annotation were performed on all samples, and a Venn diagram was constructed. As shown in Figure 8, the KB, MX, DZ, and YW groups shared 45 ASVs. There were 97 ASVs unique to the KB group, 135 unique to the MX group, 118 unique to the DZ group, and 147 unique to the YW group. These results suggest that GAD leads to changes in the number of ASVs compared with rats in the KB group.

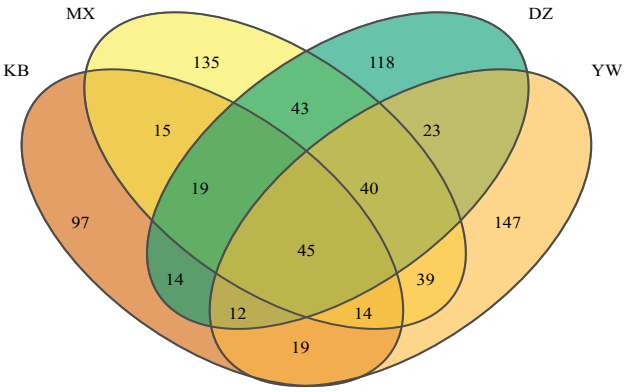
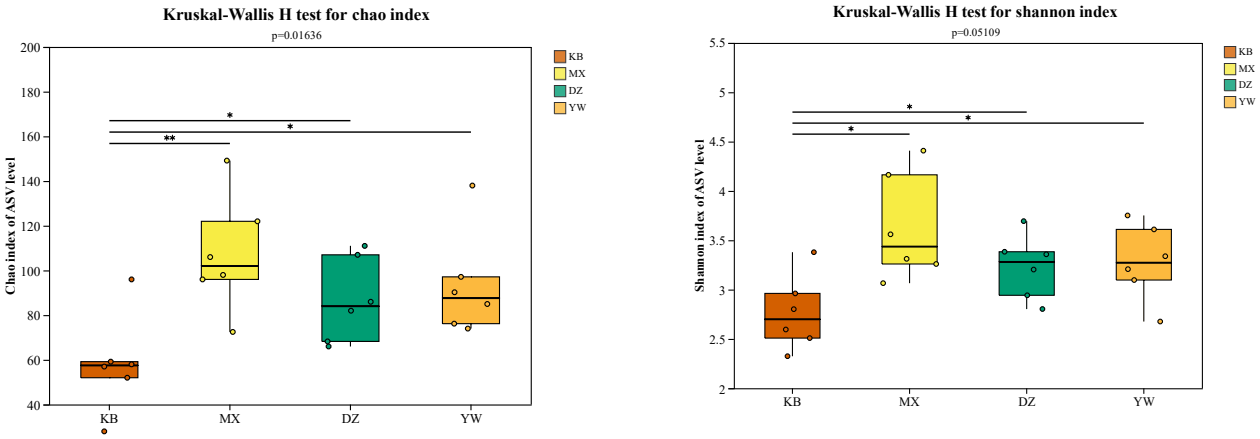


Figure 8. Venn Diagram of ASVs.

3.5.3 Alpha Diversity Analysis

Alpha diversity analysis was performed to evaluate the richness and diversity of microbial communities within individual samples (Figure 9). In this study, the Chao index was used to estimate species richness, while the Shannon index was employed to assess species diversity and evenness. The Chao index analysis (Figure 9A) revealed that species richness was significantly higher in the MX group than in the KB group ($P < 0.01$), suggesting that the anxiety model induced abnormal proliferation and dysbiotic expansion of the gut microbiota. Following EA or pharmacological intervention, the Chao index decreased in both the DZ and YW groups compared with the MX group, exhibiting a trend toward restoration to normal levels. The Shannon index analysis (Figure 9B) showed that microbial diversity was highest in the MX group, whereas both the DZ and YW groups exhibited a decreasing trend in microbial diversity ($P = 0.051$). Taken together, the changes in these two indices suggest that both EA and pharmacological intervention may partially reverse anxiety model-induced gut microbial dysbiosis, promoting the restoration of microbial richness and diversity toward a balanced state.



A. Chao Index B. Shannon Index

Figure 9. Alpha Diversity Analysis.

3.5.4 β-Diversity Analysis

Principal Coordinates Analysis (PCoA) was performed to assess differences in beta diversity among the groups. PCoA is a widely used method for evaluating differences in microbial community structure by comparing microbial community composition across samples. In the PCoA plot (Figure 10), the distances between samples reflect their similarities in microbial community composition, with shorter distances indicating greater similarity in community structure. Specifically, the MX group exhibited clear separation from both the KB and DZ groups, suggesting that the anxiety model induced substantial alterations in gut microbiota composition, whereas EA intervention partially reversed these changes, shifting the microbial community structure toward that of the control group. A certain degree of overlap was observed between the DZ and KB groups, further suggesting that EA exerted a modulatory effect on gut microbial community structure, consistent with the trend toward restoration observed in the YW group.

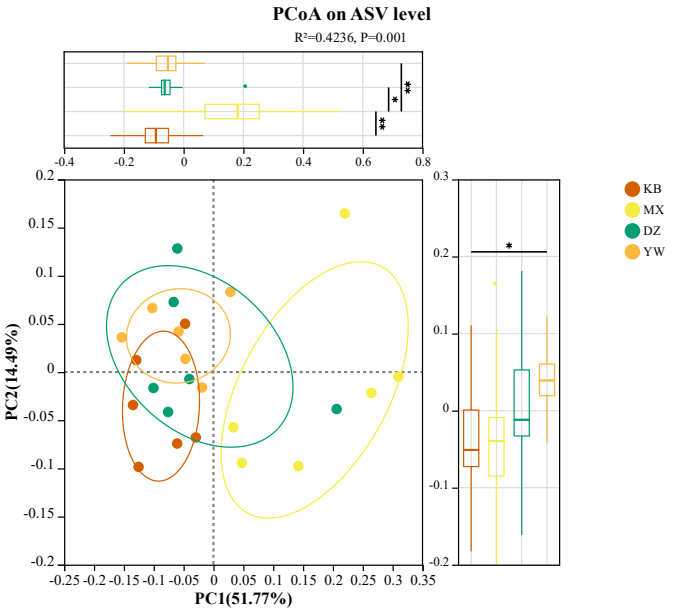


Figure 10.PCoA Plot.

3.5.5 Species Composition Analysis

3.5.5.1 Changes in Gut Microbiota Composition at the Phylum Level in Rats

Based on the taxonomic annotation results, the dominant bacterial phyla in each group were selected to generate a bar plot of relative abundance. As shown in Figure 11, *Bacillota* was the most dominant phylum in the rat gut microbiota, accounting for the largest proportion of the total bacterial community, followed by *Bacteroidota*. In contrast, *Actinomycetota* and *Mycoplasmatota* exhibited extremely low relative abundances. Compared with the KB group, the MX group exhibited marked alterations in gut microbial composition, characterized by a significant decrease in the relative abundance of *Bacillota* and an increase in that of *Bacteroidota*. Furthermore, the relative abundance of *Actinomycetota* increased from nearly undetectable levels to detectable levels. These findings suggest that the anxiety model induced gut microbial dysbiosis, primarily characterized by a reduction in *Bacillota* and an expansion of *Bacteroidota*. Following EA or pharmacological intervention, the gut microbial composition in the DZ and YW groups showed a tendency to return toward that of the control group. Specifically, the relative abundance of *Bacillota* increased, whereas that of *Bacteroidota* decreased, and *Actinomycetota* returned to extremely low levels. These results indicate that both EA and pharmacological treatment may partially reverse the alterations in gut microbial composition at the phylum level induced by the anxiety model.

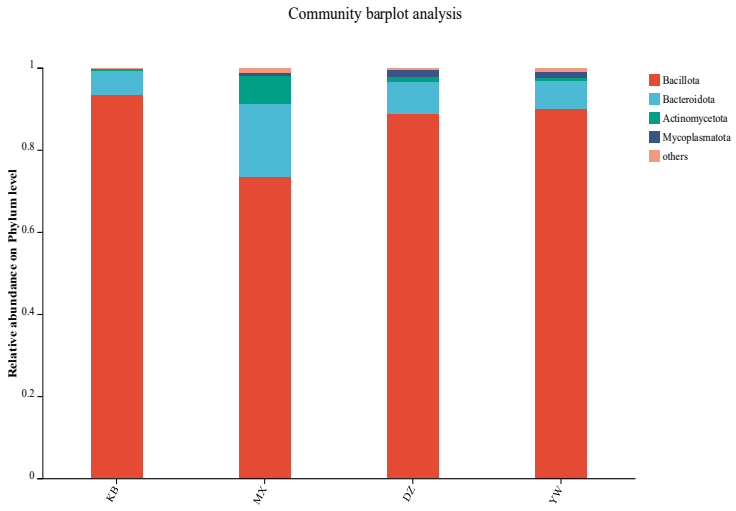


Figure 11. Bar plot of species relative abundance at the phylum level.

3.5.5.2 Changes in Gut Microbiota Composition at the Genus Level in Rats

At the genus level (Figure 12), compared with the KB group, the MX group exhibited significantly reduced relative abundances of several core probiotic-associated genera, including *Ligilactobacillus*, *Lactobacillus*, and *Limosilactobacillus*. The relative abundance of Acetivibrio also decreased, whereas that of potentially pathogenic taxa, such as norank_f_Erysipelotrichaceae, increased, suggesting that the anxiety model was associated with gut microbial dysbiosis. Following EA intervention, the relative abundances of probiotic-associated genera, particularly *Lactobacillus* and *Limosilactobacillus*, increased markedly in the DZ group compared with the MX group. The microbial community shifted from a Ligilactobacillus-dominated profile toward a more balanced composition characterized by the co-dominance of *Lactobacillus* and *Limosilactobacillus*, showing a trend toward the microbial community structure observed in the KB group.

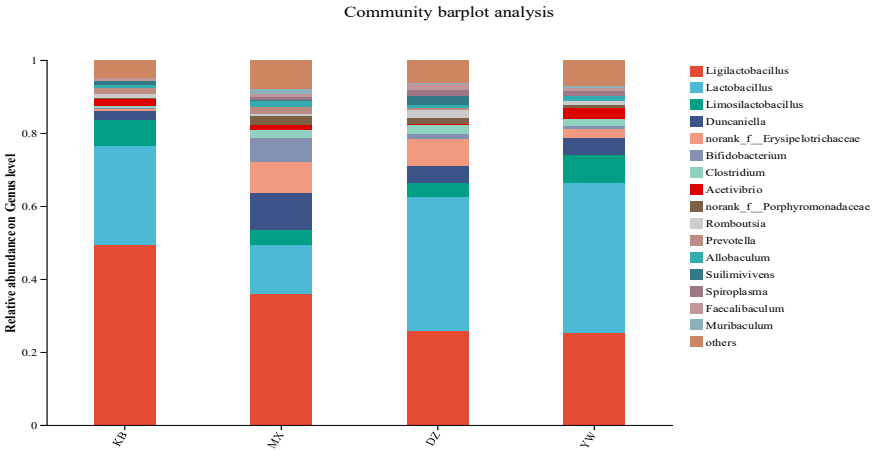


Figure 12. Relative Abundance of Microbial Taxa at the Genus Level.

3.5.6 LEfSe Analysis of Differentially Abundant Microbial Taxa

Linear discriminant analysis effect size (LEfSe) is a method for analyzing differences in microbial community composition based on relative abundance data. By combining nonparametric statistical tests with linear discriminant analysis (LDA), LEfSe considers both statistical significance and biological relevance, enabling the identification of differentially abundant microbial features and their associated taxonomic groups. In the present study, LEfSe analysis was performed to identify differentially abundant microbial taxa among the four groups (KB, MX, DZ, and YW). An LDA score ≥ 3.0 and $P < 0.05$ were used as the screening criteria.

As shown in Figure 13A, the differentially enriched microbial taxa in the KB group included the phylum *Bacillota*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*, norank_p_Bacillota, and Candidatus_Faecenecus. In the MX group, higher relative abundances were observed for the phylum *Bacteroidota*, class *Bacteroidia*, order *Bacteroidales*, class *Erysipelotrichia*, family *Erysipelotrichaceae*, order *Erysipelotrichales*, family *Muribaculaceae*, genus *Duncaniella*, and class *Actinomycetia*. The genus *Clostridium* was identified as a characteristic taxon of the DZ group. In the YW group, the differentially enriched taxa included the genus Akkermansia, phylum *Verrucomicrobiota*, class *Verrucomicrobiae*, order Verrucomicrobiales, and family Akkermansiaceae. As shown in Figure 13B, the concentric circles in the taxonomic cladogram represent taxonomic levels from phylum to genus (or species), progressing from the center outward. The KB and MX groups exhibited distinct distributions across the phylogenetic branches, with characteristic taxa predominantly associated with Bacillota in the KB group and with Bacteroidota and Actinomycetota in the MX group. The YW group was characterized by taxa within the *Verrucomicrobiota* branch. In contrast, relatively few characteristic taxa were identified in the DZ group, with only Clostridium appearing as a characteristic taxon on a terminal branch. Overall, the major differentially abundant taxa were primarily distributed across four phyla: *Bacillota*, *Bacteroidota*, *Actinomycetota*, and *Verrucomicrobiota*.

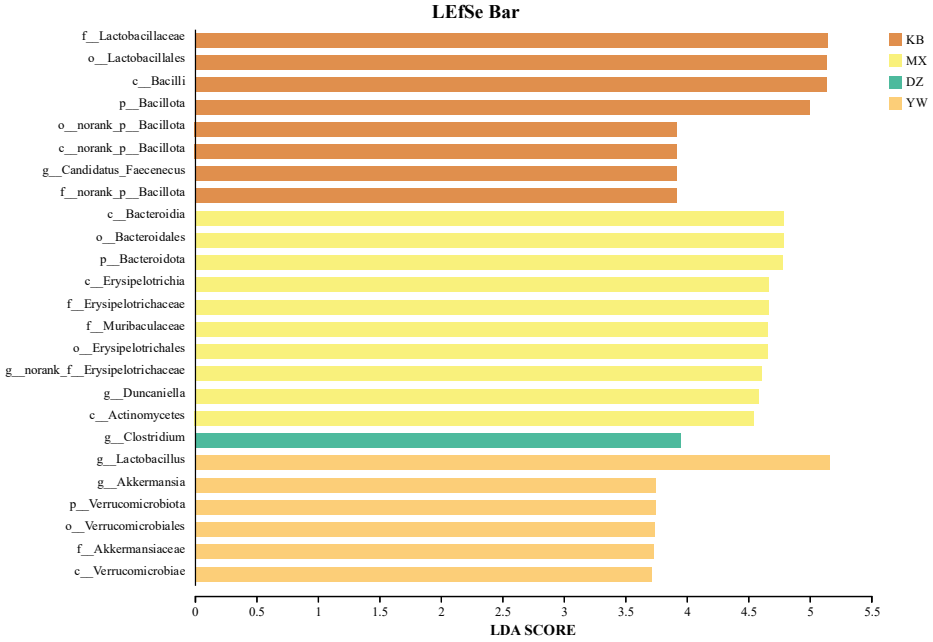


Figure 13A. LDA Bar Plot

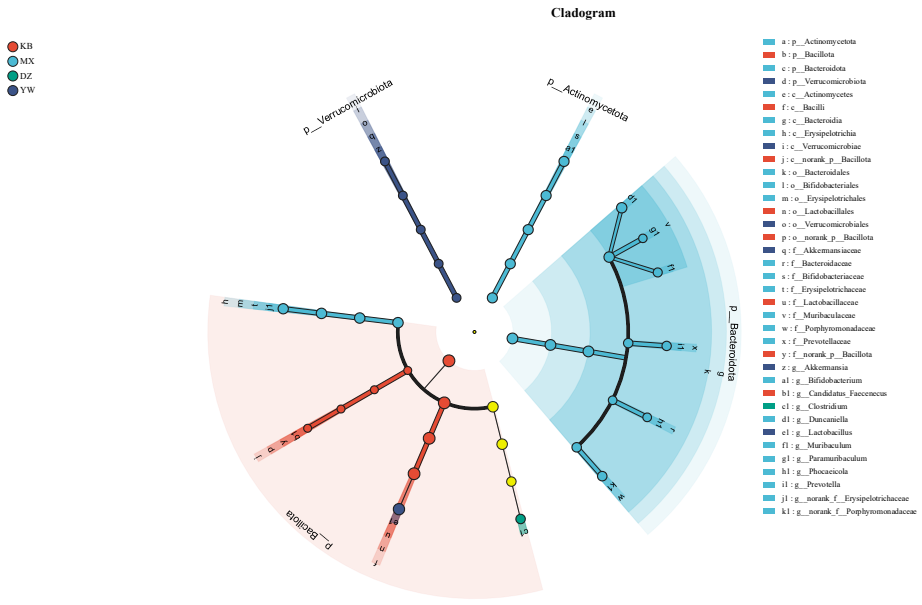


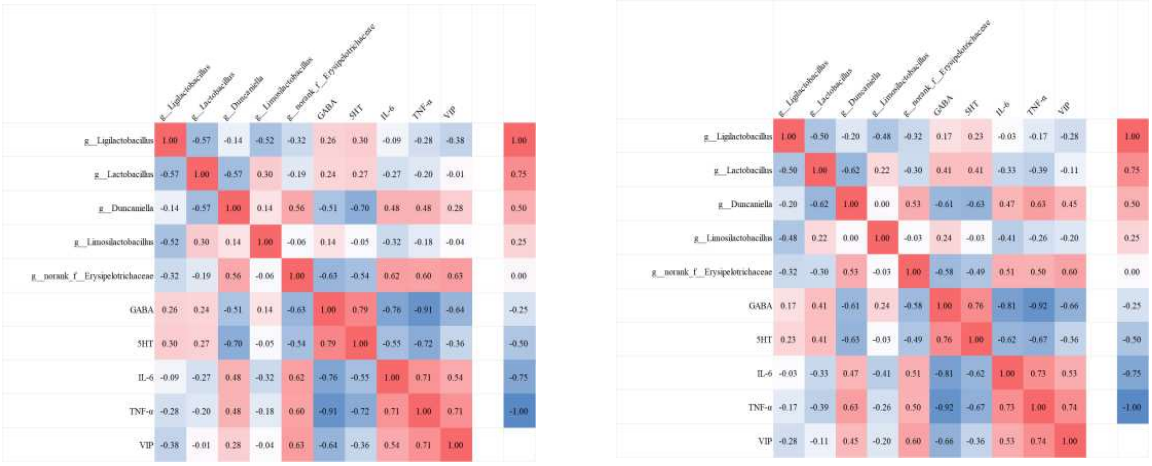
Figure 13B. LEfSe Cladogram

3.5.7 Correlation Analysis Between Gut Microbiota and Inflammatory Factors/Neurotransmitters

Partial correlation analysis was used to control for grouping effects (Figure 14), followed by FDR correction via the Benjamini–Hochberg method ($\alpha = 0.05$). Significant correlations after FDR correction ($q < 0.05$) were as follows: *Erysipelotrichaceae* with GABA ($r = -0.58$, $P = 0.004$, $q = 0.020$) and VIP ($r = 0.60$, $P = 0.003$, $q = 0.015$);

Duncaniella with GABA (r = −0.61, *P* = 0.002, q = 0.012), 5-HT (r = −0.63, *P* = 0.001, q = 0.010), and TNF-α (r = 0.63, *P* = 0.001, q = 0.010).

The above results show that *Erysipelotrichaceae* and *Duncaniella* remained significantly correlated with neurotransmitter levels and inflammatory factors after controlling for grouping factors, suggesting that these two bacterial taxa may be involved in neurotransmitter imbalance and inflammatory responses. Combined with the LEfSe analysis, both taxa were enriched in the model group and markedly decreased after EA intervention. This indicates that EA may exert its anxiolytic effects by regulating gut microbiota dysbiosis, modulating the levels of VIP and inflammatory factors, and restoring neurotransmitter balance.



A Spearman correlation heatmap　　B Partial correlation heatmap

Figure 14 Spearman Correlation Heatmap

4. Discussion

In this study, a CRS-induced rat model of anxiety-like behavior was used to investigate the effects of EA at Baihui (GV20) and Zusanli (ST36) on anxiety-like behaviors and biological parameters associated with the MGBA. The results demonstrated that EA significantly alleviated anxiety-like behaviors in CRS-exposed rats, accompanied by increased intestinal 5-HT and GABA levels, upregulated hippocampal GABRA1 and BDNF protein expression, decreased serum IL-6 and TNF-α levels, and reduced intestinal VIP levels. Furthermore, 16S rDNA gene sequencing revealed that EA was associated with alterations in gut microbiota composition. Subsequent correlation analyses identified significant associations between *Duncaniella* and *Erysipelotrichaceae* and selected neurotransmitter and inflammatory markers. Previous studies have demonstrated that the gut microbiota communicates bidirectionally with the central nervous system

through neural, immune, endocrine, and metabolic pathways, contributing to the regulation of host stress responses and emotional behaviors (24, 25). Taken together, these findings suggest that the improvement in CRS-induced anxiety-like behaviors following EA may be associated with concurrent alterations in gut microbial composition and neuroimmune-related parameters. However, the present findings do not establish a specific causal mediating mechanism.

Chronic stress is an important environmental factor contributing to abnormalities in emotion-related behaviors. Prolonged exposure to stress may disrupt neurotransmitter homeostasis, neuroendocrine function, and signaling pathways associated with neuroplasticity. Liu et al.(26) established a rat model of anxiety-related behaviors using CRS combined with unpredictable empty-bottle stimulation. They found that stressed rats exhibited reduced exploration of the central zone in the OFT and the open arms in the EPM, accompanied by abnormalities in hippocampal neurogenesis-related markers. These findings indicate that sustained stress exposure can induce pronounced anxiety-like behaviors and central neurobiological alterations. Consistent with these findings, the present study demonstrated that rats in the CRS model group exhibited significantly fewer entries into the central zone and spent less time in this zone during the OFT. Similarly, both the number of open-arm entries and the time spent in the open arms were markedly reduced in the EPM, suggesting that CRS induced anxiety-like behaviors. Following EA intervention, these behavioral parameters were significantly improved, indicating that EA may alleviate stress-associated abnormalities in exploratory behavior.

In recent years, increasing attention has been paid to the effects of EA on emotional and behavioral abnormalities associated with chronic stress. Wang et al. (27) demonstrated that EA at Zusanli (ST36) and Tianshu (ST25) alleviated depressive-like behaviors in rats subjected to CUMS, accompanied by alterations in gut microbiota composition. Similarly, Duan et al. (19) reported that EA ameliorated depressive-like behaviors in CUMS-exposed rats and was associated with changes in the gut microbiota and lipid metabolism-related parameters. These findings suggest that EA may exert multifaceted biological effects on chronic stress-related behavioral abnormalities. Notably, previous studies have primarily focused on depressive-like behaviors, whereas the present study investigated CRS-induced anxiety-like behaviors and further assessed intestinal neurotransmitters, peripheral inflammatory cytokines, and hippocampal protein expression. Thus, by examining anxiety-like behaviors alongside multiple gut–brain axis-related parameters, the present study provides additional evidence for understanding the

biological effects of EA intervention under chronic stress conditions.

5-HT and GABA are important neurochemical signals involved in gut–brain axis communication. In addition to its role in emotional regulation, 5-HT contributes to the regulation of gastrointestinal motility, secretion, and local sensory signaling. Yano et al. (28)demonstrated that specific gut microorganisms can regulate 5-HT biosynthesis in colonic enterochromaffin cells, suggesting that the gut microbiota may influence gastrointestinal physiology by modulating host tryptophan metabolism and 5-HT-related metabolic pathways. In the present study, EA reversed the CRS-induced reduction in intestinal 5-HT levels. Given previous evidence that gut microorganisms can regulate 5-HT synthesis in enterochromaffin cells, it is possible that EA modulates intestinal 5-HT synthesis and secretion under chronic stress conditions. However, this study did not assess enzymes involved in tryptophan metabolism, enterochromaffin cell numbers, or 5-HT receptor expression. Therefore, the specific sources and regulatory mechanisms underlying the observed changes in intestinal 5-HT levels remain unclear.

In addition to 5-HT, the present study found that intestinal GABA levels were markedly reduced in the CRS model group but increased following EA intervention. GABA is a major inhibitory neurotransmitter involved in the regulation of neuronal excitability and emotional behaviors. Bravo et al. (29)demonstrated that treatment with a specific strain of *Lactobacillus* rhamnosus (JB-1) influenced emotion-related behaviors and the expression of GABA receptor subunits in different brain regions of mice, whereas vagotomy attenuated some of these effects. These findings provide experimental evidence supporting a potential functional link between the gut microbiota and central GABAergic signaling. Notably, in the present study, the increase in intestinal GABA levels following EA intervention was accompanied by a significant upregulation of hippocampal GABRA1 protein expression. GABRA1 encodes the α1 subunit of the GABA_A receptor, an important component involved in inhibitory neurotransmission. Taken together, these findings suggest that EA intervention is associated with concurrent changes in intestinal GABA levels and hippocampal GABA receptor-related protein expression.

The hippocampus is a key brain region involved in chronic stress responses and emotional regulation, and alterations in its structure and function are closely associated with stress-related behavioral abnormalities. As an important neurotrophic factor, BDNF plays essential roles in neuronal survival, synaptic function, and activity-dependent neuroplasticity. Duman and Monteggia (30)proposed that stress-related mood disorders may be associated with impaired neurotrophic signaling. Reduced BDNF expression

induced by chronic stress may contribute to adaptive changes in the limbic system, while modulation of BDNF signaling by therapeutic interventions may have biological significance. In the present study, hippocampal BDNF protein expression was markedly decreased in the CRS model group but significantly increased following EA intervention, suggesting that EA may modulate hippocampal neurotrophic signaling under chronic stress conditions.

Liu et al. (26)further investigated neurobiological alterations in an animal model of anxiety from the perspective of hippocampal neurogenesis and found that treatment with Danzhi Xiaoyao San was associated with changes in hippocampal neural stem cell differentiation and the Notch signaling pathway. Unlike their study, which employed immunofluorescence to assess neural cell proliferation and differentiation, the present study primarily used Western blotting to examine hippocampal GABRA1 and BDNF protein expression, focusing on molecular changes associated with inhibitory neurotransmission and neurotrophic signaling. Although the two studies differed in their interventions and outcome measures, both suggest that chronic stress-related anxiety-like behaviors may be accompanied by alterations in the molecular and cellular biological status of the hippocampus. Furthermore, the present study found that GABRA1 expression was higher in the paroxetine group than in the EA group, whereas no significant difference in BDNF expression was observed between the two groups. These findings suggest that different interventions may elicit distinct molecular response profiles and that improvements in behavioral outcomes may not necessarily correspond directly to the magnitude of changes in a single molecular marker.

Inflammatory responses represent an important regulatory component of the MGBA. The gut microbiota can interact with neural and endocrine pathways by modulating intestinal mucosal immunity and systemic immune signaling (31). Under chronic stress conditions, alterations in inflammation-related signaling may contribute to host behavioral and physiological responses. Elevated peripheral pro-inflammatory cytokine levels are an important feature of animal models of chronic stress-induced anxiety. In the present study, EA attenuated the CRS-induced increases in serum IL-6 and TNF-α levels, suggesting its potential to modulate peripheral inflammatory responses. However, this study assessed only circulating inflammatory markers and did not evaluate microglial activation or blood–brain barrier integrity. Therefore, the present findings do not establish that EA exerts its anxiolytic effects through central neuroinflammatory pathways.

In addition to inflammatory cytokines, the present study found that intestinal VIP

levels were markedly elevated in the CRS model group but decreased following EA intervention. VIP is a bioactive neuropeptide widely distributed throughout the gastrointestinal tract and nervous system, where it participates in the regulation of gastrointestinal motility, secretion, and neuroimmune responses (32). Iwasaki (33) reported that VIP is involved in gastrointestinal physiological functions and mucosal immune regulation through multiple receptor-mediated signaling pathways, with its biological effects influenced by the tissue microenvironment and pathological conditions. Considering the observed changes in IL-6, TNF-α, and VIP levels, these findings suggest that EA may modulate both peripheral inflammatory status and intestinal neuropeptide signaling under chronic stress conditions. However, whether these two processes are directly linked through specific regulatory mechanisms remains to be determined through further functional studies.

The gut microbiota is an important component of the MGBA, and alterations in the intestinal microbial ecosystem may influence host stress responses through neural, immune, and metabolic pathways (34). In the present study, 16S rDNA gene sequencing was performed to characterize the gut microbiota in CRS-exposed rats. The findings suggest that CRS altered gut microbial richness and that EA partially reversed the stress-induced changes in the Chao richness index. However, no statistically significant improvement was observed in the Shannon diversity index following EA intervention. These findings also highlight that a reduction in microbial richness should not be interpreted as evidence that the gut microbiota has returned to a healthy state.

Beta diversity analysis revealed a clear separation in gut microbial community composition between the model and control groups, whereas the EA and control groups showed partial overlap, suggesting that EA intervention was associated with changes in gut microbiota composition. At the phylum level, the relative abundance of Bacillota decreased and that of Bacteroidota increased in the model group, while EA partially reversed these changes. At the genus level, the relative abundances of *Lactobacillus*, *Limosilactobacillus*, and other bacterial taxa were reduced in the model group but increased following EA intervention. Previous findings by Bravo et al. (29) regarding the associations between specific *Lactobacillus* strains and central GABA receptor expression provide a basis for exploring potential links between Lactobacillus-related taxa and GABA-associated parameters in the present study. However, different strains within the same genus may exhibit distinct metabolic functions, and findings obtained from a specific bacterial strain cannot be directly generalized to all Lactobacillus-related

taxa identified in this study. Furthermore, LEfSe analysis identified Clostridium as one of the differentially abundant microbial taxa characteristic of the EA group.

It should be noted that 16S rDNA gene sequencing data are compositional in nature. Gloor et al. (35) emphasized that an increase in the relative abundance of a particular taxon does not necessarily indicate an increase in its absolute abundance. Therefore, the compositional nature of microbiome data should be considered when interpreting statistical associations among microbial taxa and between microbial taxa and host biological parameters. Overall, the present findings suggest that EA intervention is associated with alterations in gut microbiota composition under CRS conditions.

In addition to analyzing overall gut microbiota composition, the present study further investigated the associations between specific microbial taxa and intestinal neurotransmitters, VIP, and peripheral inflammatory cytokines. After controlling for experimental group effects and applying the Benjamini–Hochberg procedure for false discovery rate (FDR) correction, *Erysipelotrichaceae* was negatively correlated with GABA and positively correlated with VIP. *Duncaniella* was negatively correlated with GABA and 5-HT but positively correlated with TNF-α. Furthermore, both taxa exhibited higher relative abundances in the CRS model group, which decreased following EA intervention. These findings suggest that *Erysipelotrichaceae* and *Duncaniella* may be associated with alterations in intestinal neurochemical parameters and peripheral inflammatory status under chronic stress conditions. Notably, the significant associations of *Duncaniella* with 5-HT, GABA, and TNF-α suggest that this genus may represent a candidate taxon for further investigation of microbiota–host interactions.

Previous studies have demonstrated that alterations in the gut microbiota can influence host emotion-related behaviors under specific experimental conditions. Li et al. (36) reported that transplantation of gut microbiota from chronically stressed mice into recipient mice induced changes in anxiety-like and depressive-like behaviors, accompanied by alterations in inflammatory responses and brain-related biological parameters. Similarly, Kelly et al. (37)demonstrated that transplantation of gut microbiota from patients with depression induced corresponding neurobehavioral alterations in recipient rats. These findings provide functional evidence suggesting that gut microbial communities associated with specific physiological or pathological states may influence host behavioral phenotypes. In the present study, candidate microbial taxa associated with host neurochemical and inflammatory parameters were identified among the EA-associated changes in gut microbiota composition, providing potential targets for future

functional investigations. Further studies employing fecal microbiota transplantation, targeted microbial interventions, and metabolomic analyses are warranted to determine whether these taxa contribute to EA-associated neuroimmune and behavioral changes.

Study Limitations and Future Research Directions

The present study investigated the effects of EA in CRS-exposed rats at multiple levels, including behavioral outcomes, intestinal neurotransmitters, hippocampal protein expression, peripheral inflammation, and gut microbiota. Nevertheless, several limitations should be acknowledged.

First, a sham EA control group was not included in this study. Therefore, the potential contributions of nonspecific effects associated with needle insertion, tactile stimulation at the acupoints, and handling or restraint procedures cannot be completely excluded. Future studies should incorporate a sham EA group to further clarify the specific effects of EA intervention.

Second, only male rats were included in this study, limiting the generalizability of the findings to female animals. In addition, the biological sample sizes for some molecular analyses were relatively small, particularly for the assessment of hippocampal GABRA1 and BDNF protein expression, which may affect the precision of the estimates. Future studies should determine sample sizes based on anticipated effect sizes and comprehensively report randomization, blinding, sample exclusions, and experimental units in accordance with the ARRIVE 2.0 guidelines for reporting animal research(38).

Finally, the present study examined the effects of EA in a rat model of GAD across multiple dimensions, including behavioral outcomes, neurotransmitters, inflammatory cytokines, and gut microbiota. The findings suggest that pathways associated with the gut–brain axis (GBA) may contribute to the anxiolytic effects of EA. However, this study did not employ targeted blockade or manipulation of the vagus nerve, key receptors, or gut microbiota. Therefore, the findings primarily provide correlational evidence supporting the involvement of these mechanisms, and the causal relationships among the different components remain to be fully established. Future studies incorporating pathway-specific blockade or targeted interventions are needed to further elucidate and validate the mechanisms underlying the effects of EA.

Conclusion

1. CRS successfully induced stable anxiety-like behaviors in the model rats. EA stimulation at Baihui (GV20) and Zusanli (ST36) alleviated anxiety-like behaviors in rats with GAD-like symptoms.

2. EA intervention increased intestinal 5-HT and GABA levels, upregulated hippocampal GABRA1 and BDNF protein expression, reduced serum levels of the proinflammatory cytokines IL-6 and TNF-α, and normalized altered intestinal VIP expression in GAD model rats. These findings suggest that the anxiolytic effects of EA may be associated with the restoration of neurotransmitter balance, enhancement of neuroplasticity, and attenuation of peripheral inflammatory responses.

3. The improvement in anxiety-like behaviors following EA intervention was accompanied by changes in gut microbial ecology, neurotransmitter levels, and peripheral inflammatory status. Significant correlations were observed between specific gut microbial taxa and parameters associated with the gut–brain axis, suggesting that the MGBA may contribute to the anxiolytic effects of EA. However, the causal and mediating roles of this pathway require further validation through functional experiments.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics Statement

The animal study was reviewed and approved by the Ethics Committee of Henan University of Chinese Medicine.

Declaration of competing interests

The authors declare no conflicts of interest.

Author Contributions

JX and XW were responsible for the study design. JX and YJ drafted the manuscript. NJ performed data curation and statistical analysis. MY was responsible for data verification. XW provided guidance on animal experiments. JX, YJ, NJ, MY, and YR conducted the experiments. XW supervised the animal experiments and revised the manuscript. All authors have read and approved the final version of the manuscript.

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