# Butyrate Emerges as a Crucial Effector of Zhi-Zi-Chi Decoctions to Ameliorate Depression via Multiple Pathways of Brain-gut Axis

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# Abstract

**Background:** Gut microbiota has emerged as a crucial target of gut-brain axis to influence brain and behavior and also has been closely connected with depression. Zhi-Zi-Chi decoctions (ZZCD), as a classic oral formula in clinic prescribed to clear heat and relieve restlessness traditionally, is widely applied in depression treatment nowadays. However, the underlying mechanism in the antidepressant activity of ZZCD remains largely unknown. Our previous study revealed that isoflavones, the bioactive constituents of *Semen Sojae Praeparatum*, benefited health by regulating the gut microbiota, which introduced the gut microbiota into understanding the mechanism of Traditional Chinese Medicine (TCM). Hence, in the present study, we aimed to investigate the antidepressant mechanism of ZZCD by focusing on the gut microbiota.

**Results:** A classic depression model of chronic mild unpredictable stress (CUMS) was established in rats based on the results of behavioral tests and hippocampal histomorphology. 16S rRNA sequencing analysis indicated that ZZCD could increase short-chain fatty acid-producing and anti-inflammatory bacteria and reduce inflammatory and tryptophan-metabolizing bacteria, which reflected the changes of short-chain fatty acids (SCFAs), inflammation and tryptophan metabolism from the perspective of the gut microbiota. Furthermore, ZZCD reversed the alterations of BDNF, TNF-α, pro-inflammatory cytokines and neurotransmitters in the gut, blood and brain along the brain-gut axis and restored the decrease of butyrate in cecal content caused by CUMS. Then, butyrate was utilized to validate its ameliorative effect on pathological characteristics of depressive rats.

**Conclusions:** Taken together, these results show that ZZCD exhibits antidepressant effect through modulating gut microbiota to facilitate the production of butyrate, which further regulate anti-inflammation, neurotransmitters, endocrine and BDNF along the gut-brain axis. Hence, this study fills the gap of the antidepressive mechanism of ZZCD in the light of the brain-gut axis and established a multi-targets and multi-levels platform eventually for further research into the mechanism of other TCM efficacy.


# 1. Introduction

Depression, characterized by an overwhelming feeling of sadness and hopelessness, has become a leading cause of global disease burden that contributes to suicide and ischemic heart disease. Based on the data recently released by the World Health Organization (WHO), there are more than 300 million people suffering from depression globally[1]. Pharmacological therapy is one of the mainstream treatments for depression, whereas current antidepressants such as selective serotonin reuptake inhibitors (SSRIs) are not universally effective and obvious side effects[2]. Traditional Chinese medicines (TCMs) are regarded as Chinese national treasure and have been widely used for disease management for over 2,500 years in China, which lays a solid foundation for modernization of TCMs. The onset of depression is thought to be associated with liver-qi stagnation resulted from repression of anger and

distress according to TCM theory. Some TCMs are documented to relieve these symptoms and thus are considered to be effective in depression treatment[3, 4].

Gut microbiota, known as an invisible organ, has a significant impact on xenobiotic metabolism[5], immunity[6] and the occurrence and development of diseases. emerging studies have indicated that gut microbiota is a pivotal contributor to the pathophysiology of Parkinson's disease[7], Alzheimer's disease[8], autism[9], depression[10] and other neuropsychiatric disorders with the introduction of the concept of brain-gut axis. Jiang et al.[11] reported that gut microbiota in patients with major depressive disorder exhibited higher levels of *Enterobacteriaceae* and *Alistipes* and a lower level of *Faecalibacterium*, compared with that in healthy controls. Interestingly, transplanting fecal microbiota from depressed patients into a germ-free mouse could trigger depressive-like behaviors and pathological features in the mouse, which further demonstrated the interactive relationship between gut microbiota and depression[11]. However, surprisingly too little is known about the antidepressant mechanism of TCMs based on brain-gut axis theory.

Zhi-Zi-Chi decoction (ZZCD), composed of Gardeniae Fructus (Chinese herbal name is "zhi zi") and *Semen sojae praeparatum* (Chinese herbal name is "dan dou chi"), is a classical prescription that eliminates restlessness and improves insomnia, and is documented in Treatise on Febrile Diseases written by Zhang Zhongjing. The modern clinical application of ZZCD shows that it is effective in regulating and ameliorating depression[12]. Nevertheless, the antidepressant mechanism of ZZCD remains largely unknown thus far. Interested in the antidepressant efficacy of ZZCD, we have been committed to the study of ZZCD and the single herbs in the past years. More specifically, we have conducted qualitative and quantitative studies on the chemical constituents of *Gardeniae Fructus*[13] and *Semen sojae praeparatum*[14] and demonstrated the neuroprotective effect of ZZCD via cell metabolomics[15]. Meanwhile, our previous studies showed that isoflavones in *Semen sojae praeparatum* could significantly influence the composition of gut microbiota in rats, thereby affecting the metabolic characteristics of main iridoids in *Gardeniae Fructus*[16, 17]. Our preliminary work laid the solid foundation for in-depth study on the antidepressant mechanism of ZZCD.

Most TCMs are administered orally. This physiological process inevitably causes TCMs to touch with and further to interact with gut microbiota. The complex chemical composition of TCMs can modulate the structure and composition of gut microbiota and then alter metabolites produced by intestinal bacteria[18], including short-chain fatty acids (SCFAs)[19], long-chain fatty acids[20], amino acids[21], bile acids[22] and indole derivatives[23]. These endogenous metabolites provide an important supplement for the current bioavailability theory of drug and its metabolites due to the properties of mild bioactivity and satisfactory safety. Meanwhile, these medicines, acting by this mechanism, commonly show low content of prototype in plasma to reduce the side effects of organs, such as heart, liver and kidney, and hence may emerge as the development direction of drugs for the treatment of depression in the future. The gut microbiota plays a vital role in the biotransformation of TCMs. The main active components of *Gardeniae Fructus* and *Semen sojae praeparatum* are iridoid glycosides and isoflavones, respectively[24]. They belong to high hydrophilic glycosides and are not readily absorbed by the intestine. Glycosides need

to be metabolized into aglycones with β-glucosidase produced by gut microbiota to enhance bioavailability and exhibit efficacy. Our previous study has shown that the contents of isoflavones in plasma are low after gavage *Semen sojae praeparatum* to rats[25]. Nevertheless, *Semen sojae praeparatum* contains much cellulose and polysaccharide[26, 27], which can be metabolized into SCFAs by gut microbiota.

The pathogenesis of depression is very complex. Nonetheless, previous studies on its mechanism primarily focused on a single factor, such as heredity[28], immunity[29] or endocrine[30], which cannot comprehensively and systematically elucidate the mechanism of this multi-factor disease. Drawing on the close relationship between TCMs and gut microbiota, gut microbiota and depression, the push for exploring the antidepressant mechanism of ZZCD is occurring in the context of brain-gut axis.

Although a wealth of investigations have demonstrated the connections of histomorphology[31], immunity[32], endocrine and gut microbiota with depression so far, this mode is insufficient to fully elucidate the pathogenesis of this multi-targets disease. Instead of focusing on a single factor, a comprehensive exploration may shed light on the antidepressant mechanism of ZZCD by utilizing the material basis of brain-gut axis and combining the perspectives of histomorphology, immunity, endocrine and gut microbiota. On basis of the low bioavailability of isoflavones, our study targeted on the secondary metabolite of gut microbiota, in order to find the antidepressive active substance of ZZCD. The current study therefore aimed to fill the gap of the antidepressant mechanism of ZZCD underlying brain-gut axis and ultimately established a multi-target and multi-level platform for further research into mechanism of other TCM efficacy.

# 2. Materials And Methods

### *2.1. Preparation of ZZCD extract*

The *Gardeniae Fructus* and *Semen sojae praeparatum*, identified by Prof. Lu-Ping Qin (Zhejiang Chinese Medical University), were purchased from Tong Han Chun Tang Chinese Herbal Factory (Shanghai, China) and were prepared as powder. A total of 800 g of powder was weighed according to the ratio of 2:1. The mixture was extracted twice by reflux with 50% ethanol (1:8, w/v) for 4 h and filtered through gauze. The filtrate was concentrated to 0.1 g/mL with rotary evaporator. The sample was loaded in a ratio of 1:1 of medicinal materials (mL)/D101 macroporous adsorption resins (mL), and then the static adsorption was performed for 2 h. The resin was washed with 1BV pure water and ethanol eluent at different concentrations in a range of 10, 20 and 30 (v/v) in turn. 6 BV 40% ethanol eluent was collected and dried by rotary evaporator combined with water bath to obtain the powder of ZZCD extracts.

### *2.2. Animals*

Fifty-six male Sprague-Dawley rats, weighing 200 ± 20 g, were obtained from Experimental Animal Center of Second Military Medical University (Shanghai, China, SCXK2013-0016) and were housed in plastic cages with a 12-h light/dark cycle under controlled temperature (22 ± 2℃) and humidity (55 ± 5%). All

animals were trained to consume 1% sucrose solution for 1 week before the commencement of experiments. After a week for habituation, twenty-eight rats were randomly divided into four groups on average: the control (Ct), CUMS, FLU and ZZCD groups. The rest of rats were also evenly divided into four groups randomly: the control (Ct), CUMS, FLU and the butyrate (BA) groups for following validation experiment. All rats were individually housed except two Ct groups. All experimental procedures in this study were approved by the Ethics Committee of the Second Military Medical University (Shanghai, China).

### *2.3. Chronic unpredictable mild stress (CUMS) and treatment*

The internationally recognized CUMS paradigm was used to establish a chronic depression model[33, 34]. The rats of Ct group acquired water and standard chow diet ad libitum and didn't receive stress stimulations except behavioral tests, while rats of the rest three groups received stress stimulations lasting for 6 weeks. It was not allowed to use a same stressor for 2 consecutive days. The stressors included: wet bedding for 20 h; 45° cage tilting for 24 h; restraint for 2 h; swimming in 4℃ water for 5 min; cage shaking for 15 min; nip tail for 1 min; food or water deprivation for 24 h. Fluoxetine (2.1 mg/kg, Lilly Suzhou Pharmaceutical Co., Ltd.) and ZZCD extract (10 g/kg, equivalent to the weight of raw medicinal materials) were gavaged to the rats of FLU and ZZCD groups from the third week to the end of experiment, respectively. Meanwhile, the equal volume of 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution was gavaged to the rats of Ct and CUMS groups.

### *2.4. Behavioral testing*

#### *2.4.1. Sucrose preference test (SPT)*

The SPT, used to assess anhedonia, was performed at 42th day as described in previous studies with slight modification. All rats were deprived of water and food before SPT. Whereafter, the SPT was conducted for 1 h (19:00-20:00). Each rat had free access to a bottle of tap water and a bottle of 1% sucrose solution to measure the consumed volumes in these two bottles, respectively. The proportion of sucrose preference was calculated as the percentage of the consumed sucrose solution against total amount of liquid intake.

#### *2.4.2. Tail suspension test (TST)*

The test rats were individually suspended by the tail (2 cm from tail tip) on a horizontal bar with sticky tape and the whole test lasted 6 min. The cumulative durations of immobility of rats within the last 4 min was recorded in seconds. Rats were considered to be immobile when they gave up struggle to keep completely motionless.

#### *2.4.3. Forced swim test (FST)*

Rats were individually placed in a cylinder (60 cm × 40 cm diameter) containing 35 cm-depth water at 25℃ and forced to swim for 6 min. The accumulated time of immobility was monitored within the last 4

min. Rats were recognized as immobility when they moved slightly to maintain floating or were stationary in the water without struggling. Water was changed between each rat to remove odors, and the test rats had been wiped dry after the procedure of FST.

### *2.5. Sample harvesting*

After behavioral tests, one rat from each group was anesthetized by intraperitoneal injection with 20% urethane (0.4 mL/100 g). Cardiac perfusion was carried out with phosphate buffer saline (PBS). When lungs, eyeballs and paws displayed white color, 4% paraformaldehyde was perfused to rats until muscles of their upper limbs were stiff. Coronal sections of brain were prepared and fixed in 4% paraformaldehyde for 48 h as soon as possible.

Likewise, the rest rats in each group were anesthetized by intraperitoneal injection with 20% urethane (0.4 mL/100 g) after behavioral tests. The blood was obtained via abdominal aorta and serum was separated by centrifugation at 3500 rpm for 20 min 4℃ after standing for 2 hours at room temperature. Plasma collected by centrifugation at 3500 rpm for 20 min 4℃ immediately. The rats were sacrificed by cervical decapitation, and hippocampus and hypothalamus were carefully stripped from whole brain. The hippocampus, hypothalamus, cecal content and ileum were collected and weighed immediately. All samples were stored at -80℃ for further analysis.

### *2.6. Nissl staining*

Coronal sections of brain, fixed with 4% paraformaldehyde, were embedded in paraffin and sliced into 5-μm-thick sections with a vibratome (Thermo Fisher Scientific, Waltham, MA, USA). Sections were stained in 1% toluidine blue solution for 20 min at 56℃. The morphology of Nissl bodies was observed by microscope (Olympus, Tokyo, Japan) and scanned with the NanoZoomer digital scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan).

### *2.7. DNA Extraction from Cecal Content and 16S rRNA Gene Sequencing*

Total bacterial DNA was extracted from cecal content of all samples using E.Z.N.A® Mag-Bind® Soil DNA Kit (Omega Biotek, Norcross, GA, United States) following the manufacturer's instructions. DNA integrity and concentration were detected by agarose gel electrophoresis. The V3-V4 hypervariable region of bacterial 16S rRNA gene was amplified by PCR using the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) containing specific barcode to tag each PCR product. The procedure of amplification and products' purification had been described previously. Miseq platform (Illumina, San Diego, United States) was used for sequencing by Sangon Biotech Co., Ltd. (Shanghai, China) to generate pair-end reads.

### *2.8. Bioinformatics Analysis*

Usearch (version 10.0.240) was applied to obtain high-quality reads for subsequent bioinformatics analysis through merging pair-end reads, cutting primers, filtering reads and removing redundant. The

acquired high-quality reads were used to generate operational taxonomic units (OTUs) using Unoise3. Whereafter, the taxonomy assignment was performed based on Ribosomal Database Project (RDP) Classifier with a confidence threshold of 80%. OTU abundance information was normalized to the number of sequences in the sample with the least sequences and then the normalized data was used for subsequent analyses of alpha and beta diversity. The index of Shannon e and Simpson was evaluated the species diversity, while the index of Chao1 and Richness was assessed to species richness in alpha diversity. Moreover, Principal Coordinates Analysis (PCoA), based on weighted UniFrac distances, exhibited the differences of samples.

### *2.9. Analysis for SCFAs in cecal content*

The cecal contents were homogenized with methanol and then scrolled 30 min. Thereafter, mixtures were centrifuged at 14000 rpm for 5 min. The supernatant samples were standed for 2 h and then take 800 μL for freeze-drying. The residues were redissolved with 35 μL acetonitrile and vortex 3 min. Mixtures were added 5 μL 1-hydroxybenzotriazole hydrate (HOBT), 5 μL 5-(Diisopropylamino)amylamine (DIAAA) and 5 μL O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) for vortexing 3 min. After 14000 rpm centrifugation for 5 min, the supernatant samples were taken to analyze.

After centrifugation, a Shimadzu LC-40D XS system coupled with a Shimadzu 8045 Triple Quadrupole Mass Spectrometer was used to analyze the supernatants. The chromatographic separation was achieved at 35℃ using a Waters XSelect HSS T3 column (2.1×100 mm, 2.5 μm). The mobile phase, consisting of 0.1% formic acid in water (A) and acetonitrile containing 0.1% formic acid (B), was used with a gradient elution: 0-8 min, 5-95% B; 8-10.9 min, 95% B; 10.9-11 min, 95-5% B, 11-14 min, 5% B. The flow rate was maintained at 0.3 mL/min. The injection volume was 3 μL.

Mass spectrometry analysis was performed by electrospray ionization (ESI) in positive modes. Desolvation line (DL) temperature and heat block temperature were maintained at 250℃ and 400℃, respectively; nebulizer gas, 3 L/min; drying gas, 10 L/min; heating gas, 10 L/min; interface temperature, 300℃; interface voltage, 0 kV. Desolvation temperature was 526℃. Acetic acid: *m/z* 229.23→86.00, collision voltage, 32 kV; propionic acid: *m/z* 243.25→201.00, collision voltage, 8 kV; butyrate: *m/z* 257.26→86.00, collision voltage, 28 kV; valeric acid: *m/z* 271.30→85.90, collision voltage, 25 kV. The dwell time was 100 msec.

### *2.10. Biochemical analysis*

The concentrations of pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and brain-derived neurotrophic factor (BDNF) in the serum, ileum and hypothalamus were evaluated using rat IL-1β, IL-6, TNF-α and BDNF ELISA kits (Mlbio, Shanghai, China) according to their instructions, respectively.

### *2.11. Analysis for neurotransmitters and amino acids levels*

The levels of neurotransmitters and amino acids were examined using 2,6-Dihydroxybenzoic acid (DHBA) as an internal standard. Briefly, the plasma was thawed at room temperature and then was mixed with ice-cold 80% methanol containing 0.1% formic acid and internal standard solution. Meanwhile, the ileum and hippocampus were homogenized with the same solution. Thereafter, mixtures were centrifuged at 14000 rpm for 15 min (4℃). The supernatant samples were centrifuged again for 10 min at the same condition. The collected supernatants were evaporated and the residues were redissolved with 50% methanol. After centrifugation, an Agilent 1290 Infinity LC system coupled with an Agilent 6538 Accurate Mass Quadrupole Time-of-Flight mass spectrometer (UHPLC-Q-TOF/MS) was used to analyze the supernatants.

The chromatographic separation was achieved at 25℃ using a Waters XSelect HSS T3 column (2.1×100 mm, 2.5 μm). The mobile phase, consisting of 0.1% formic acid in water (A) and acetonitrile containing 0.1% formic acid (B), was used with a gradient elution: 0-2 min, 5% B; 2-13 min, 5-95% B; 13-19 min, 95% B. The flow rate was maintained at 0.4 mL/min. The injection volume was 3 μL.

Mass spectrometry analysis was performed by electrospray ionization (ESI) in both positive and negative modes. The parameters were as follows: a capillary voltage of 4.0 kV for positive mode and that of 3.5 kV for negative mode; gas temperature of 350℃; dry gas flow of 11 L/min; nebulizer pressure of 45 psi and fragmentary voltage of 120 V.

After centrifugation, a Shimadzu LC-30AD system coupled with a Shimadzu 9030 Accurate Mass Quadrupole Time-of-Flight mass spectrometer was used to analyze the supernatants. The chromatographic separation was achieved at 40℃ using a Waters XSelect HSS T3 column (2.1×100 mm, 2.5 μm). The mobile phase, consisting of 0.1% formic acid in water (A) and acetonitrile containing 0.1% formic acid (B), was used with a gradient elution: 0-2 min, 3% B; 2-10 min, 5-95% B; 10-11 min, 95% B, 11-15 min, 3% B. The flow rate was maintained at 0.4 mL/min. The injection volume was 3 μL.

Mass spectrometry analysis was performed by electrospray ionization (ESI) in both positive and negative modes. The interface voltage was 4.5 kV for positive mode and −3.5 kV for negative mode; nebulizer gas, 3 L/min; heating gas, 10 L/min; interface temperature, 300℃; drying gas, 10 L/min; desolvation line (DL) temperature and heat block temperature were maintained at 250℃ and 400℃, respectively.

### *2.12 Animal experiment design of the validation study*

After adaption for a week, twenty-eight rats for validation were evenly divided into four randomly: the control (Ct), CUMS, FLU and BA groups. The chronic depression model was established according to the procedure described above. Fluoxetine (2.1 mg/kg) and butyrate (30 mg/kg, Sigma-Aldrich) were gavaged to the rats of FLU and BA groups from the fourth week to the end of experiment, respectively. Meanwhile, the equal volume of 0.5% CMC-Na aqueous solution was gavaged to the rats of Ct and CUMS groups. After behavioral testing, samples were collected for further study. Nissl staining, biochemical analysis and detection of neurotransmitters and amino acids were conducted according to the procedure described above.

### *2.13. Statistical Analysis*

With regard to data obtained from the experiments of behavioral testing, biochemical analysis, analysis for neurotransmitters levels and alpha diversity, one-way ANOVA followed by Tukey's post hoc was used to assess these data using GraphPad Prism software (version 5.02). Data were presented as mean ± SEM. As for the gut microbiota data, Welch's t-test was used to identify taxonomic changes with significant difference between two different groups in STAMP software. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### *3.1. Behavioral effects and Nissl staining of ZZCD extract following CUMS*

The depression-like behaviors induced by CUMS were evaluated by SPT, TST and FST. The former used to assess anhedonia is characterized by depression of the consumption of sweet solution, while the latter two are represented by immobility time. As shown in Figure 1A, 6-week CUMS procedure caused a reduction of proportion of sucrose preference ($P<0.001$) and significantly extended the immobility times in TST and FST ($P<0.001$, $P<0.001$) compared with Ct rats, which indicated that the CUMS-induced depressive model had been successfully established. After 4-week treatment, the administration of ZZCD extract or Fluoxetine both ameliorated the above three indicators compared with CUMS group. However, there was no significant effect of Fluoxetine administration on TST.

The histopathological changes of tissue can be observed directly with the Nissl staining. We examined Nissl bodies in hippocampal neurons in the CA1 subfield of the hippocampus by Nissl staining. The arrangement of hippocampal neurons was disordered and some neurons exhibited atrophy in CUMS group (Figure 1B). Meanwhile, their Nissl bodies were reduced or absent. On the contrary, there were more hippocampal neurons which had normal morphology and arranged in an orderly fashion, and their Nissl bodies were clearly visible in the Ct, FLU and ZZCD groups. These findings suggest that ZZCD extract has the anti-depression activity to the CUMS-induced rats.

### *3.2. ZZCD extract reverses CUMS-induced gut dysbiosis*

To analyze the effect of ZZCD extract on the gut microbiota, the V3-V4 hypervariable region of 16S rRNA of bacteria in cecal content was sequenced. A total of 1,000,550 raw reads were generated from 24 samples and 915,745 high quality sequences were obtained after quality filtering. There were 267 unique OTUs acquired with the use of Unoise3.

The alpha diversity was evaluated by the indexes of Shannon_e, Simpson, Chao1 and Richness. It illustrated that there was no significant differences among Ct, CUMS, FLU and ZZCD groups (Table S1). The unconstrained principal coordinate analysis (PCoA) based on weighted UniFrac distance was then used to discover the overall structural changes of gut microbiota among four groups. The result exhibited an apparent clustering in microbial composition for each group (Figure 1C). In particular, the CUMS group

could be distinguished with the other three groups along the negative direction of the first axis. The difference between treatments and Ct group is smaller than that between CUMS-induced rats and normal ones, which indicated that ZZCD extract and Fluoxetine ameliorated microbial composition of CUMS-induced rats. The first two principal component scores accounted for 49.16% and 12.74% of total variations, respectively.

The microbial composition of different cecal contents was exhibited in significant differences at multiple taxonomical levels. The most primary phyla were Firmicutes, Bacteroidetes and Proteobacteria, accounting for > 95% of the microbial compositions in all samples (Figure 1D). As shown in Figure 1F, the relative abundance of two phyla was significantly altered between the CUMS group and the Ct group. Compared with normal rats, the procedure of CUMS resulted in a decrease in the relative abundance of Firmicutes and an increase in that of Proteobacteria. Meanwhile, compared with CUMS group, Fluoxetine obviously ameliorated the dysbiosis of Proteobacteria induced by CUMS and increased the relative abundance of Unassigned, whereas ZZCD extract increased the relative abundances of Firmicutes and Candidatus_Saccharibacteria significantly. The microbial composition of cecal content was then analyzed at the genus level. There were 5 genera exhibiting remarkably difference between the groups of Ct and CUMS (Figure 1E, G). Among these data, the relative abundances of *Lactobacillus* and *Romboutsia* were lower in the CUMS group than in the Ct group, while the relative abundances of 3 genera (*Oligella*, *Alistipes* and *Desulfovibrio*) showed enriching effects in the CUMS group. The relative abundance of *Lactobacillus* was increased whereas the relative abundances of *Oligella*, *Bacteroides* and *Corynebacterium* were decreased in the FLU group compared with the CUMS group significantly. Moreover, ZZCD extract remarkably increased the relative abundances of *Saccharibacteria_genera_incertae_sedis*, *Barnesiella* and *Lachnospiracea_incertae_sedis* and decreased the relative abundance of *Streptococcus* compared with the CUMS group. Although without significant differences, the most interesting result was that ZZCD extract ameliorated the dysbiosis of 5 genera induced by CUMS (Table S2). Collectively, these results demonstrate that ZZCD extract is able to modulate the dysbiosis of bacterial ecosystem in cecal content induced by CUMS.

### *3.3. ZZCD extract restored butyrate production reduced by CUMS*

We used cecal contents to measure SCFAs, including acetic acid, propionic acid, butyric acid and valeric acid and data was described as peak area. As shown in Figure 1H, butyric acid was the only one significantly reduced by CUMS among these SCFAs ($P$<0.001). Interestingly, ZZCD extract and Fluoxetine inproved this change induced by CUMS.

### *3.4. ZZCD extract and butyrate normalizes altered concentrations of cytokines and BDNF in brain-gut axis caused by CUMS*

To investigate ZZCD extract regarding its effects against cytokines and BDNF, IL-1β, IL-6, TNF-α, and BDNF levels were measured in the serum, ileum and hippocampus using ELISA (Figure 2A, B).

In the serum, the concentrations of TNF-α ($P$<0.001), IL-1β ($P$<0.001) and IL-6 ($P$<0.001) were significantly higher; while the level of BDNF was significantly lower in the CUMS group than in the Ct group ($P$<0.001). Fluoxetine treatment significantly reduced the concentrations of TNF-α ($P$<0.001) and IL-1β ($P$<0.001) and increased the level of BDNF ($P$<0.01). Meanwhile, ZZCD extract remarkably modulated the abnormities of all four indexes.

In the ileum, similar to what was observed in the serum, the procedure of CUMS caused the abnormities of the above all indexes and ZZCD extract was able to normalize altered all indexes. Nevertheless, Fluoxetine increased the concentration of BDNF with no statistically significant.

In the hypothalamus, compared with the Ct group, the levels of TNF-α ($P$<0.01) and IL-1β ($P$<0.001) were significantly increased in the CUMS group, which was obviously reversed by the treatment of ZZCD extract ($P$<0.01, $P$<0.001), while the level of BDNF was significantly decreased in the CUMS group ($P$<0.05). However, the abnormities of the four indexes were slightly modulated by Fluoxetine without significant differences. Taken together, these findings indicate that both ZZCD extract and Fluoxetine ameliorated the changes of cytokines and BDNF induced by CUMS, yet the former displayed a stronger effect than the latter.

To examine the ameliorate effect of butyrate for depression, it was administered to rats and some indexes were analyzed. Butyrate restored hippocampal neurons and the proportion of sucrose preference and remarkably increased the immobility times in TST and FST ($P$<0.05, $P$<0.001) compared with CUMS rats (Figure 3A). Biochemical indexes in serum, ileum, and hypothalamus, including TNF-α, IL-1β, IL-6 and BDNF, exhibited a significantly better trend in BA group compared with the CUMS group (Figure 3F, G).

### *3.5. ZZCD extract and butyrate regulates altered levels of some neurotransmitters and amino acids induced by CUMS*

Given the potential consequence of serotonin (5-HT) and dopamine (DA) in depression, we detected the effect of ZZCD extract on the levels of tryptophan (Trp), kynurenine (Kyn), 5-HT, 5-hydroxyindole acetic acid (5-HIAA), phenylalanine (Phe) and DA in plasma, ileum and hippocampus. The data were represented by the relative peak area.

In the hippocampus, compared with the Ct group, the levels of 5-HIAA ($P$<0.05), Kyn ($P$<0.01), 5-HIAA/5-HT ($P$<0.01) and Kyn/Trp ($P$<0.01) were significantly increased, while the level of 5-HT was significantly decreased in the CUMS group ($P$<0.05), which was obviously reversed by the treatment of ZZCD extract. In addition, our results indicated that ZZCD extract increased the level of DA (Figure 3C).

In the ileum, the rats in the CUMS group displayed lower levels of 5-HT and higher levels of 5-HIAA, Kyn, 5-HIAA/5-HT, Kyn/Trp and Phe, which were reversed in the ZZCD group. (Figure 3D).

In the plasma, the levels of 5-HIAA ($P$<0.01), Kyn ($P$<0.05) and 5-HIAA/5-HT ($P$<0.001) were significantly higher, while the relative peak areas of 5-HT ($P$<0.001) and 5-HT/Trp ($P$<0.0001) were significantly lower in the CUMS group than in the Ct group. Fluoxetine treatment significantly reduced the levels of 5-HIAA

($P$<0.05) and 5-HIAA/5-HT ($P$<0.01). Meanwhile, ZZCD extract remarkably modulated the abnormities of 5-HIAA ($P$<0.01), Trp ($P$<0.05), Kyn ($P$<0.01), 5-HIAA/5-HT ($P$<0.01), Kyn/Trp ($P$<0.05) (Figure 3E).

We simultaneously examined the levels of neurotransmitters such as Trp in the hippocampus, ileum, and plasma. These results showed that the levels of Trp, spaglumic acid, GABA, epinephrine, norepinephrine, proline, and phenylalanine were significantly decreased in the CUMS group, whereas the levels of Kyn and Kyn/Trp were elevated compared with the Ct group. Interestingly, butyrate was able to reverse the levels of these neurotransmitters.

# 4. Discussion

Although clinical studies have shown that ZZCD plays a positive role in the treatment of depression[12], the mechanism of this TCM focused on gut microbiota had not been investigated. In this study, ZZCD extract and Fluoxetine were able to markedly modify behavior relevant to depression in rats. Additionally, ZZCD extract had capability to alleviate the disorder of intestinal microecology triggered by CUMS. Importantly, changes of ZZCD extract on gut microbiota related to inflammation and tryptophan were consistent with the results of biochemical indexes on the brain-gut axis. Together, these data support the hypothesis that ZZCD extract targets gut microbiota to make contributions to depressive behavior.

Our previous studies have illustrated that isoflavones, the main active components of *Semen sojae praeparatum*, were able to alter the composition of gut microbiota in rats, which induced changes in pharmacokinetic profiles of geniposide and genipin in rats administrated with isoflavones and thereby exerting the effect of detoxification[35]. However, the bioavailability of isoflavones is low. Meanwhile, *Semen sojae praeparatum* contains large quantities of cellulose and polysaccharide, which can be metabolized into SCFAs by gut microbiota. Hence, we take gut microbiota as the target of our study to further explore the antidepressive effect of ZZCD based on the brain-gut axis and SCFAs.

Concomitant with the improved behavioral indexes of depression, we discovered ZZCD extract could reverse CUMS-induced gut dysbiosis. After analyzing the microbial composition in cecal content of rats, we discovered that the ZZCD extract could improve the imbalance of gut microbiota of depressed rats caused by CUMS at the phyla level and the genus level. At the phyla level, Firmicutes, Bacteroidetes and Proteobacteria are the most important components of rat and human gut microbiota[11]. Zhang et al. found that mice showed depressive symptoms after social defeat stress and the relative abundance of Firmicutes in their feces decreased[36]. In the clinical study, the fecal microbiota of patients with depression also showed an abnormality. The relative abundance of Bacteroidetes and Proteobacteria increased remarkably, while the relative abundance of Firmicutes markedly decreased[37]. In this study, we also observed that the relative abundance of Firmicutes in the cecal contents of rats in the model group markedly decreased, yet the relative abundance of pathogenic Proteobacteria increased obviously. The improvement of depression by ZZCD extract is mainly achieved by restoring the level of Firmicutes, but the effect of ZZCD extract on depression by increasing the level of Candidatus_Saccharibacteria remains to be further studied.

In this study, the relative abundance of *Lactobacillus* and *Romboutsia* in the model group reduced significantly, while the relative abundance of *Oligella*, *Alistipes* and *Desulfovibrio* increased observably. More recent studies have shown that depression can reduce the level of intestinal *Lactobacillus*[38, 39] and increase the level of pathogenic bacteria, while taking probiotics can improve depressive behavior[40, 41]. *Romboutsia* is closely related to the health status of patients and decreases sharply in carcinogenic mucosa and adenomatous polyps. It can be used as a new biomarker of early tumor formation[42], but the relationship between *Romboutsia* and depression has not been reported. Bacteria such as *Oligella* and *Streptococcus* can be isolated in the urine of female patients with urgent urinary incontinence, but not in the urine of female volunteers without the disease[43]. Meanwhile, *Oligella ureolytica* is one of *Oligella*, which can cause septicemia[44]. The relative abundance of *Alistipes* which belongs to Bacteroidetes significantly increased in gut microbiota of patients with chronic fatigue syndrome[45] and irritable bowel syndrome[46], which is suggesting that *Alistipes* may be related to inflammation. Besides, *Alistipes* contain tryptophan enzyme which can metabolize tryptophan to indole. This process affect the metabolism of tryptophan, which further influences the level of 5-HT. Jiang et al found that the relative abundance of *Alistipes* in stool of patients with depression also raised significantly[11]. *Desulfovibrio* is the dominant group of sulfate reducing bacteria, which can reduce sulfate to hydrogen sulfide. The increased of *Desulfovibrio* will lead to the endogenous hydrogen sulfide rise, thus affecting cell respiration, inducing apoptosis and chronic inflammation[47]. In the ZZCD group, ZZCD extract can trigger the disorder of cecal microbiota in depressed rats induced by CUMS and elevate the level of beneficial bacteria. Among them, *Barnesiella* can produce butyric acid, isobutyric acid and other short-chain fatty acids[48]. Its abundance is related to the number of a variety of immune cells, thus playing an anti-inflammatory effect[49]. In addition, as far as we know, we clarified the relationship between Candidatus_Saccharibacteria phylum, *Romboutsia* and *Oligella* and CUMS-induced depression in rats for the first time. Taken together, this study can provide a reference for the treatment of depression targeting gut microbiota in the future.

On the basis of elevating the level of gut bacteria producing short-chain fatty acids by ZZCD extract, we further measured SCFAs in cecal content and found that ZZCD extract restored the disorder of butyrate induced by CUMS. Butyrate, as a star molecule of SCFAs, is a key product of gut microbiota through fermentation of dietary fiber and shows interesting implications for a variety of diseases, including diabetes[50], obesity[51], cancer[52] and neurological diseases[53]. It regulates host immune functions and energy metabolism to play an important role in host-microbe crosstalk. Previous studies have shown that butyrate ameliorate depressive-like behaviour through some pathways, such as inhibiting histone deacetylase and exerting anti-inflammatory effects[54]. Next, we conducted experiments on the antidepressant effects of ZZCD and butyrate based on brain-gut axis, hoping to observe the impact of the release of butyrate induced by ZZCD on depression.

Maintaining the normal life activities of the human body is the result of the coordination and unification of various parts of the nervous system, immune system and endocrine system. The occurrence of disease cannot be caused by a single factor. With the introduction of the concept of "brain-gut axis", we found that the process of bidirectional regulation between the brain and the intestinal tract involves many

single mechanisms previously proposed[55, 56], which can provide a more comprehensive understanding of the antidepressive mechanism. Based on the study that ZZCD extract can reverse the changes of gut microbiota in depressed rats by reducing the relative abundances of inflammatory bacteria, affecting the level of tryptophan metabolic bacteria and increasing the level of anti-inflammatory bacteria, it is suggested that gut microbiota affect the process of inflammation and 5-HT metabolism. Moreover, in the process of studying the pathogenesis of depression, although the exact pathophysiological mechanism of depression is not clear, more and more evidence shows that the inflammatory process in vivo and the imbalance of brain-derived neurotrophic factor and 5-HT are closely related to the pathophysiology of depression[57, 58]. Some animal studies have shown that the mice showed depressive symptoms after injection of IL-1β or IL-6 into the hippocampus, while the depressive symptoms were improved after injection of corresponding antibodies[59, 60]. For Nrf2 knockout mice with depressive symptoms, the level of pro-inflammatory cytokines in serum was higher than that in wild-type mice, while the expression of BDNF in hippocampal CA3 and DG region was lower than t

s is one of the most common and dangerous factors leading to depression. It is well known that in wild-type mice[61].

Psychological streshat stress can activate inflammatory response, so inflammation plays an important role in stress-induced depression and other diseases[62]. Animal experiments have shown the role of inflammatory response in depression[63]. In addition, a large number of literatures have confirmed the increase of pro-inflammatory cytokines such as TNF-α and IL-6 in peripheral blood of patients with depression[64, 65]What's more, many studies have found that there is a significant correlation between the concentration of inflammatory factors in peripheral blood and the severity of depressive symptoms[66, 67]. Besides, body steady state is guaranteed by the interaction, coordination and unity of various tissues, organs and systems through neuro-regulation and humoral regulation. Zhang et al found that intravenous injection of anti-IL-6 receptor antibody could induce rapid and lasting antidepressant effect in susceptible mice after social defeat stress, normalize gut microbiota and significantly increase the ratio of Firmicutes to Bacteroidetes as well as the level of *Oscillospira*[36]. Other studies have shown that the expression of *Clostridia* associated with inflammatory phenotype increases in the gut microbiota of susceptible rats with depression-like behavior[68]. Our results indicated that the rise of inflammatory bacteria in the intestinal tract of rats in the CUMS group resulted in increased levels of TNF- α, IL-1β and IL-6 in serum, ileum and hypothalamus. Interestingly, ZZCD extract reversed abnormal changes of inflammatory factors in different parts of the brain-gut axis by reducing the relative abundances of inflammatory bacteria and increasing levels of anti-inflammatory bacteria, which was even better than that of positive drugs. Similarly, butyrate has shown significant anti-inflammatory effects.

Clinical and animal studies have shown that depression is associated with the loss of neurons in specific brain regions such as the hippocampus and cerebral cortex, which is related to changes in the level of neurotrophic factors in specific brain regions[69]. Among different types of neurotrophic factors, BDNF is one of the most important neuronal growth and differentiation factors. In animal experiments, various stresses such as CUMS can reduce the level of BDNF in the hippocampus of rats, which can be reversed

by antidepressant treatment[70, 71]. Similarly, in most clinical studies, the level of BDNF in peripheral blood of patients with depression is lower than that of healthy people[72]. The content of BDNF in hippocampus of germ-free rats is lower than that of SPF rats[56]. In this study, CUMS-induced depression rats showed low levels of BDNF in serum, ileum and hypothalamus, while ZZCD extract and butyrate could reverse the decreases of BDNF caused by depression.

The monoamine neurotransmitter hypothesis is another classical hypothesis to study the pathogenesis of depression[73]. This hypothesis holds that the monoamine neurotransmitters play a key role in antidepression and are related to the pathogenesis of depression when they decrease in synaptic gap. At present, the commonly used selective serotonin reuptake inhibitors belong to the second generation of antidepressants. They maintain the activity of the central nervous system by inhibiting the reuptake of serotonin in the presynaptic membrane and increasing the concentration of serotonin in the synaptic gap. However, the pathogenesis of depression cannot be explained by a single theory. There is also a certain relationship between neurotransmitters and gut microbiota. Knockout of rat serotonin transporter gene can cause more obvious changes in gut microbiota of maternal isolated rats[74]. The disorder of gut microbiota affects the metabolism of phenylalanine and tryptophan[75], thus affecting the levels of dopamine and 5-HT. Lower dopamine levels can lead to irritability or depression[76, 77]. The mouse model of chronic restraint stress showed that the production of 5-HT was inhibited and the metabolic pathway of Kyn was activated. Tryptophan reacted under the catalysis of IDO to produce Kyn, which was then metabolized into neurotoxic 3-HK and QA[78]. Clinical studies have shown that the ratio of serum Kyn/Trp in patients with depression is increased[79] In this study, the results showed that the content of phenylalanine in the ileum and hippocampus increased and the level of DA decreased in CUMS group, suggesting that phenylalanine degradation was inhibited and DA synthesis decreased, while ZZCD extract could improve it. The increase of relative abundance of *Alistipes* in intestinal tract of CUMS-induced depressive rats promoted the indole metabolic pathway of tryptophan and the transition from tryptophan to kynurenine. As a result, the levels of 5-HIAA and kynurenine increased, while the level of 5-HT significantly decreased. The ZZCD extract could not only improve the gut microbiota, but also reverse the increase of 5-HIAA and kynurenine in plasma, ileum and hippocampus of CUMS rats and decrease the levels of 5-HIAA/5-HT and Kyn/Trp. In addition, the level of 5-HT in ileum and hippocampus of CUMS rats significantly increased, which caused the increase of 5-HT/Trp in ileum. In addition, butyrate could also decrease the level of Kyn/Trp. The above results suggested that proinflammatory cytokines, BDNF and neurotransmitters play important roles in the pathophysiology of depression. Balancing the release of these factors can improve the symptoms of depression. In the meantime, it clarifies the protective effect of butyrate on the nervous system. Therefore, butyrate may be a potential neuroprotective compound used to explore anti-depression mechanism of ZZCD

# 5. Conclusion

In summary, this study provided the first evidence that the antidepressive regulatory effect of ZZCD extract is mediated by gut microbiota to upregulate the level of butyrate, which further regulate anti-inflammation, neurotransmitters, endocrine and BDNF along the gut-brain axis (Figure 4). We discovered

that ZZCD extract could not only regulate the disturbance of microbiota related to inflammation and 5-HT metabolism, but also increase the level of other beneficial bacteria to play a better antidepressant effect. Moreover, further results indicated that ZZCD extract ameliorated the disorder of pro-inflammatory cytokines, BDNF, some neurotransmitters and amino acids induced by CUMS perhaps through regulating the production of butyrate. Given the brain-gut axis, the convergence of gut microbiota, histomorphology, immunity and endocrine has comprehensively investigated the antidepressive mechanism of ZZCD to provide the theoretical basis for its clinical research and has established a multi-targets and multi-levels platform to lay the foundation for further research into the mechanism of other TCM efficacy as well.

# Declarations

### Ethical Approval and Consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The data that support the findings of this study are openly available in NCBI SRA at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA787405. The accession number for the data is PRJNA787405.

### Competing interests

The authors declare that there is no conflict of interest.


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### Authors' contributions

Jialin Liu and Tingting Zhou conceived the project. Jialin Liu, Yichao Fang and Lixun Cui conducted the major part of the experiments. Lixun Cui, Congcong Gao and Wen Ge assisted in animal experiments. Zhongzhao Wang and Yusha Luo assisted in the extraction of herbs. Taohong Huang provided technical support. Jialin Liu and Yichao Fang wrote the manuscript. Jun Wen and Tingting Zhou reviewed the manuscript.


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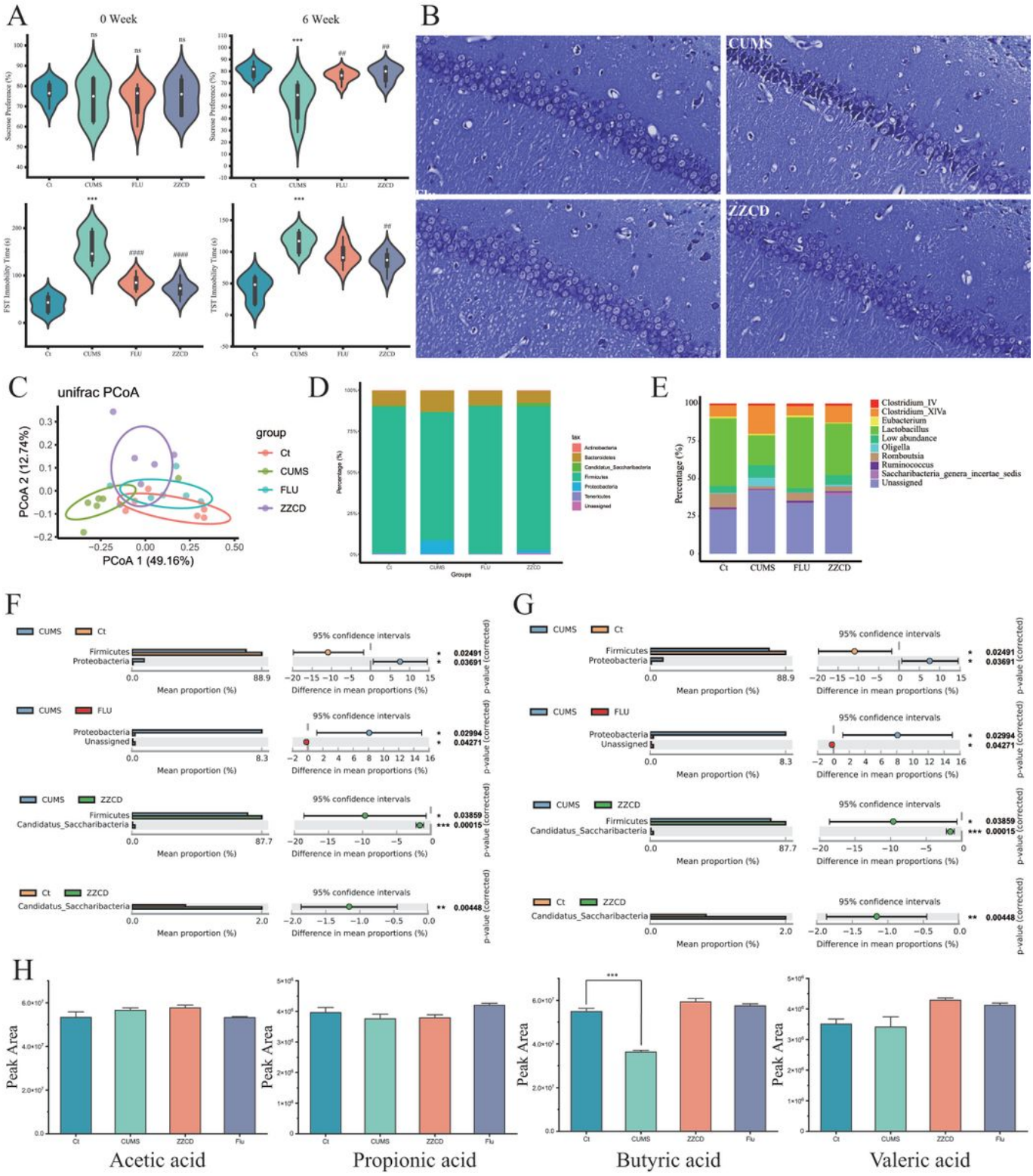
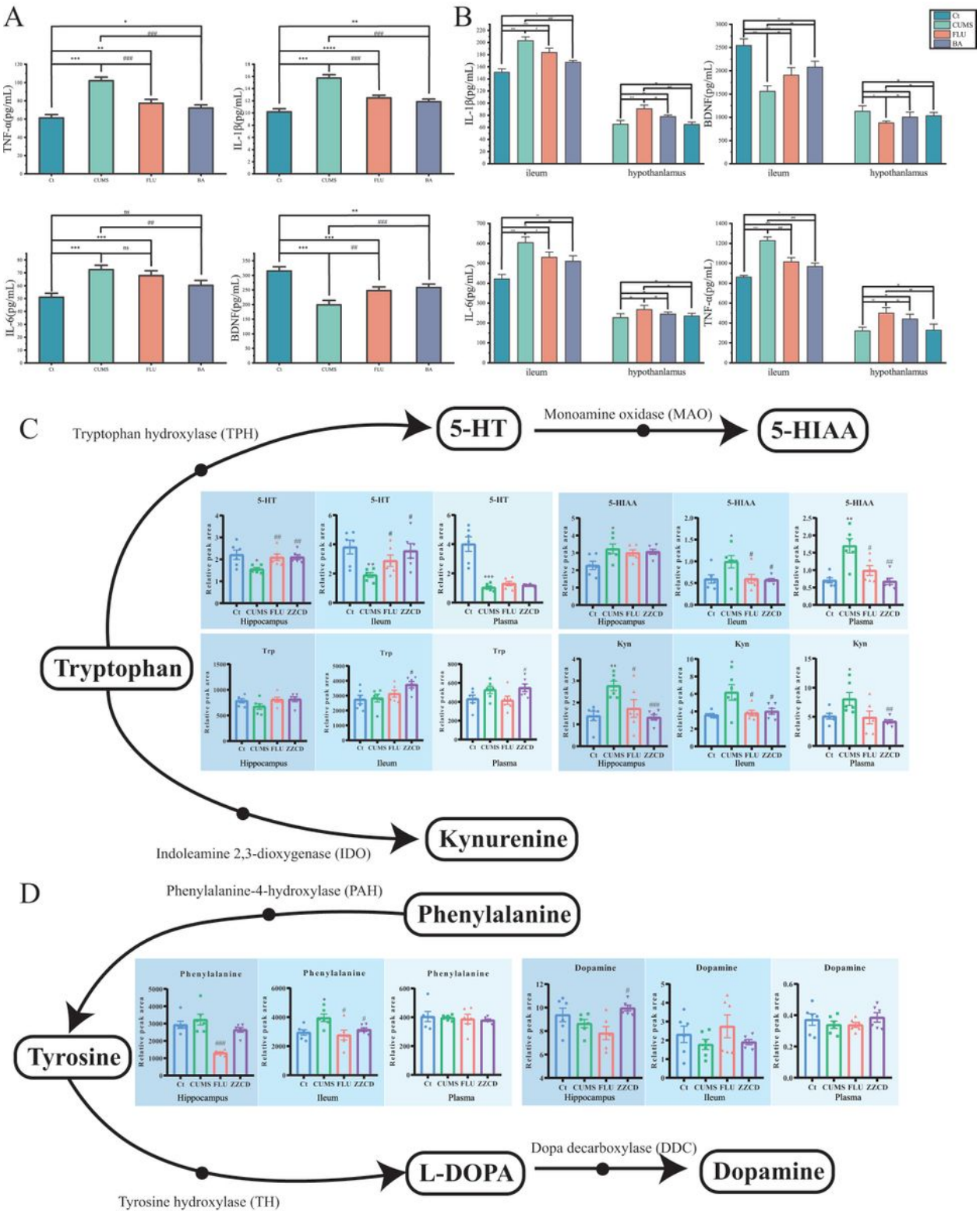
# Figures




## Figure 1

ZZCD extract alleviated CUMS-induced depression. (A) Effect of ZZCD extract on the behavioral tests of CUMS rats (n=7) and (B) results of Nissl staining in the CA1 region of rats' hippocampus (×20). (C) Plot of unconstrained principle coordinate analyses based on weighted UniFrac distances. (D) The composition of microbiota in cecal contents between four groups at phylum level. (E) The composition of microbiota in cecal contents between four groups at genus level. (F) The relative abundance of

microbiota in the cecal contents between the four groups compared in pairs at the phylum level. (G) The relative abundance of microbiota in the cecal contents between the four groups compared in pairs at the genus level. (H) CUMS altered the levels of SCFAs, including acetic acid, propionic acid, butyric acid and valeric acid in cecal contents and were restored with ZZCD. Data are expressed as Mean±SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the Ct group; $^{\#\#}P < 0.01$ and $^{\#\#\#\#}P < 0.0001$, compared with the CUMS group.



## Figure 2

Effect of the ZZCD extract on inflammatory factors and brain-derived neurotrophic factor in serum (A), ileum and hypothalamus (B) of depressed rats (n=6). (C) ZZCD extract modulate tryptophan metabolism. (D) ZZCD extract modulate tyrosine metabolism. Data are expressed as Mean±SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$, compared with the Ct group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with the CUMS group.

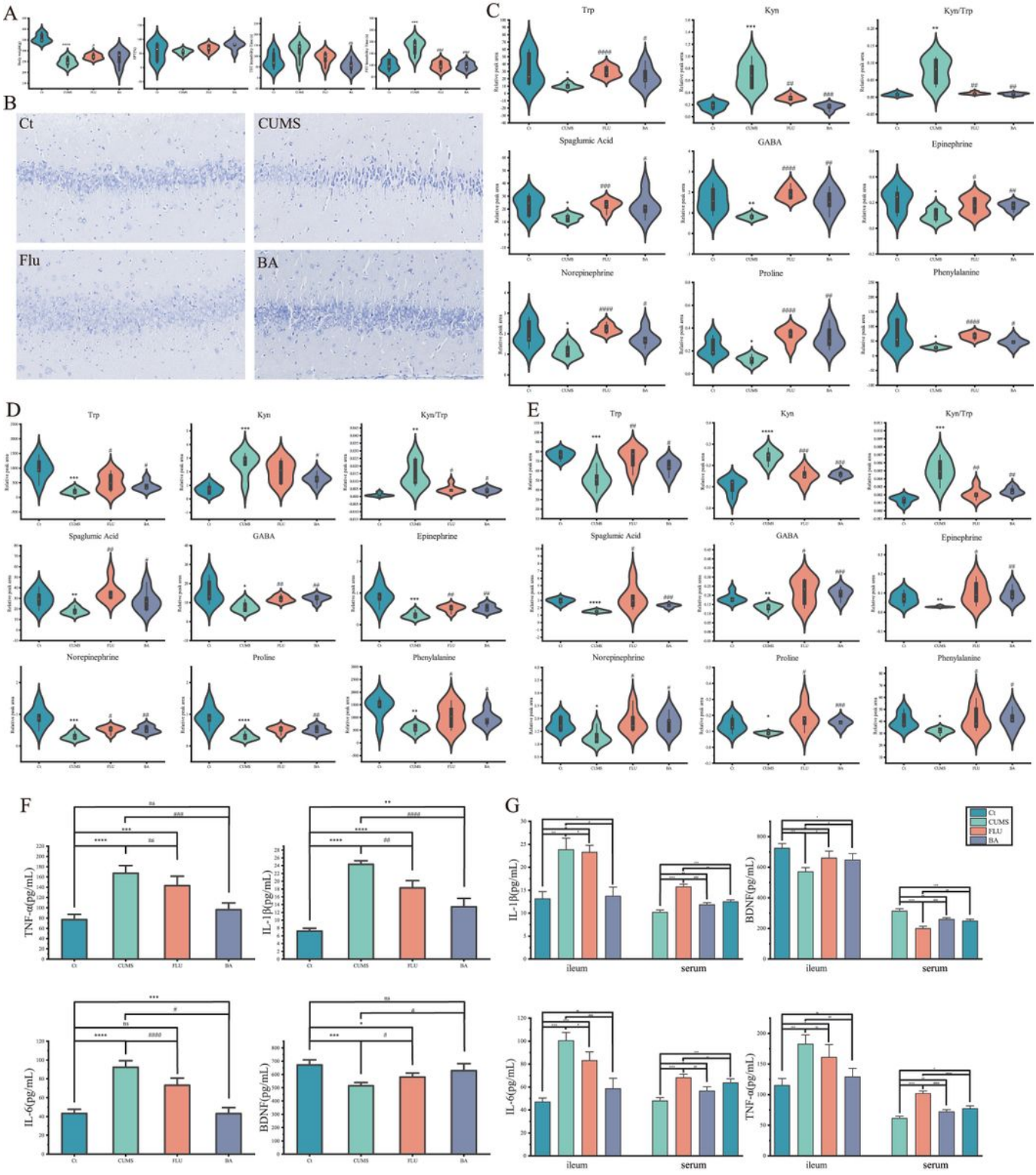


**Figure 3**

Butyrate alleviated CUMS-induced depression. (A) Effect of butyrate on the behavioral tests of CUMS rats and (B) results of Nissl staining in the CA1 region of rats' hippocampus (×20). Butyrate modulated the levels of neurotransmitters and amino acids in hippocampus (C), ileum (D) and plasma (E). Effect of the butyrate on inflammatory factors and brain-derived neurotrophic factor in hypothalamus (F), ileum and serum(G) of depressed rats. Data are expressed as Mean±SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$, compared with the Ct group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$, compared with the CUMS group.

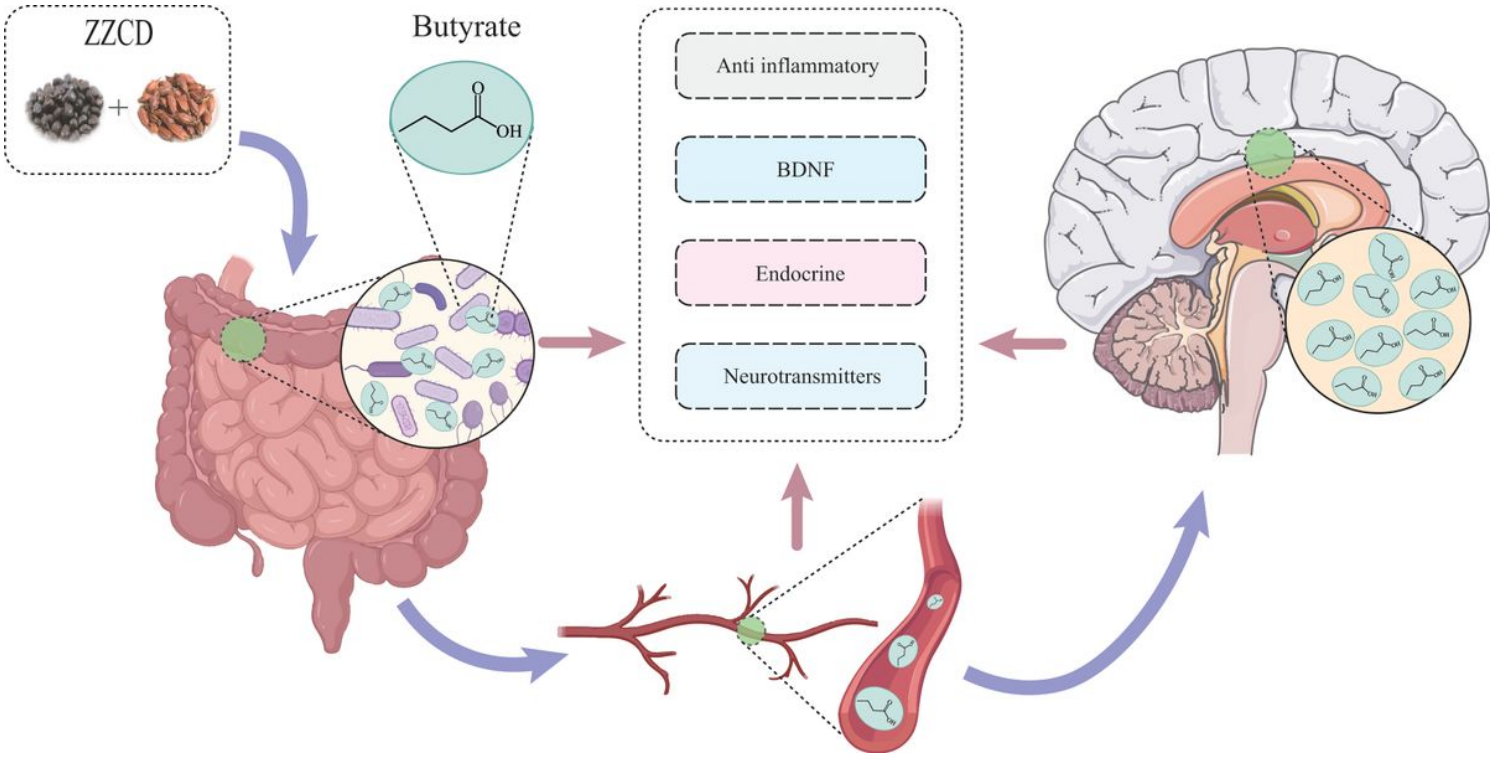



**Figure 4**

The underlying mechanisms of ZZCD to alleviate depression through regulation gut microbiota.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- TableS2.docx