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# Genistein improves depression-like behavior in rats by regulating intestinal flora and altering glutamate gene expression

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ABSTRACT

Depression is a mental disorder, and genistein is known to have antidepressant effects, but its mechanism of action is still unclear. Here, the mechanism of genistein improving depression based on gut microbiota was explored using classic behavioral indicators of depression combined with genomic technology. The behavioral evaluation showed that rats gavaged with 20–40 mg/kg genistein showed an increase in body weight, glucose preference, absenteeism score, body temperature, and 5-hydroxytryptamine (5-HT) content, while a decrease in adrenocorticotropic hormone (ACTH) and corticosterone (CORT) content compared to the depression rat model group, but there was no significant difference compared to the positive control (fluoxetine). The results of high-throughput sequencing showed that genistein increased the relative abundance of Firmicutes and Actinobacteriota and decreased the relative abundance of Bacteroidota at the phylum level. At the genus level, the abundance of *Bifidobacterium*, a short-chain fatty acid producing bacterium, was increased. Furthermore, metagenome results revealed that the antidepressant effect of genistein can be achieved by promoting glutamate metabolism, increasing glutamic acid decarboxylase (GAD) expression levels, promoting γ-aminobutyric acid (GABA) synthesis, and indirectly increasing 5-HT levels.

## 1. Introduction

Depression is a common psychological disorder characterized by significant and persistent depression (Qiao et al., 2014). Its symptoms include negative thinking, lack of pleasure, anxiety and irritability, inattention, abnormal appetite, and self-destructive tendency (Sim et al., 2015). Fierce social competition is the leading cause of depression. According to the World Health Organization statistics (2023), more than 300 million people are suffering from depression, which is highly correlated with suicide mortality and is accompanied by other chronic diseases (Krueger et al., 2013; Nicolau et al., 2020). Therefore, it has become a significant factor in the global disease burden (Gerhard et al., 2016). Currently, the treatment of depression mainly includes drug therapy, physiotherapy and psychotherapy. Antidepressants are the most used clinical treatment, but this method will lead to drug dependence, high side effects and low remission rate (Kappelmann et al., 2020). Compared with drug therapy, physiotherapy has the advantages of mild side effects and high tolerance, but it may have the risk of high recurrence rate and acute cognitive impairment (Rossini et al., 2010). The curative effect of psychotherapy is slow, and human factors often limit the therapeutic effect, so this treatment method is often used as adjuvant therapy to drug therapy and physiotherapy (Cohen and Sclar, 2013; Swartz et al., 2018).

The pathogenesis of depression is complex, and the classic pathogenesis mainly includes the hypothesis of monoamine neurotransmitters and the hypothesis of the hypothalamic-pituitary-adrenal axis (HPA). The monoamine neurotransmitter hypothesis suggests that depression is caused by a decrease or deficiency of monoamine neurotransmitters, such as serotonin, norepinephrine, and dopamine in the central nervous system of patients (Huang et al., 2019). These neurotransmitters mainly participate in various neural activities, such as emotions, appetite and emotions. The central neurotransmitters of the body will change under stress, leading to mental illnesses (such as depression). The HPA hypothesis suggests that the activity of the HPA axis is mainly controlled by

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the secretion of corticotropin releasing factor and vasopressin from the hypothalamus. When pressure is perceived by the cerebral cortex and transmitted to the hypothalamus, the corticotropin releasing factor (CRF) is released, thereby activating the secretion of ACTH by the pituitary gland. The final stimulation of the adrenal cortex to secrete glucocorticoids activates the HPA axis, which affects brain function (Nikkheslat et al., 2020). That is, stress is a state in which the sympathetic nervous system and HPA axis are jointly activated (Chrousos, 1995). Psychological factors generated by stress can interfere with the activity of the HPA axis, affecting host's disease progression. In addition, the bidirectional signal pathway between the brain and intestinal microflora has recently attracted widespread attention (Tian et al., 2020). This interaction may lead to changes in human physiological and pathological behaviour. Intestinal microflora can regulate host health in various ways, and the increasing evidence suggests that intestinal microorganisms can communicate with the central nervous system through the interaction of neural, endocrine, and immune signal mechanisms. Some studies have shown that chronic stress can lead to disruption of the gut microbiota in adolescents, which in turn leads to long-term HPA axis dysregulation (Freimer et al., 2022). Notably, the gut microbiota influences the HPA axis, produces neuroactive substances, such as gamma-aminobutyric acid (GABA) and SCFAs, and affects the immune system and gut barrier (Ait-Belgnaoui et al., 2012). Alterations in the composition of the gut microbiota may help to promote the release of cytokines and the synthesis of bioactive small molecules, certain of which are potent activators of the HPAZ axis (Misiak et al., 2020). Thus, the concept of the microbiota-intestinal-brain axis was introduced, and its dysfunction had been shown to be the major cause of depression (Hu et al., 2019).

Intestinal microflora constitutes the genome of many human intestinal cells. These symbiotic microorganisms communicate with each other and their hosts and play an essential role in human health. It has been shown that changes in the composition of intestinal microorganisms increased the permeability of the intestinal barrier, activated systemic inflammation and immune response (Fung et al., 2017), regulated the release of monoamine neurotransmitters (Mittal et al., 2017), changed the activity and function of the HPA and the abundance of brain-derived neurotrophic factor. And these reactions are closely related to depression (Teitelbaum et al., 2008). Moreover, many factors affect the composition of intestinal microorganisms, from the mode of delivery (Chong et al., 2018) and the way of feeding (Cong et al., 2016) to the diet and drug use of different age groups. Therefore, it is necessary to search for materials with the same origin of medicine and food to alleviate symptoms of depression (Ni et al., 2014).

*Genistein* is an isoflavone compound derived from soybean. Genistein has been found that have many physiological and pharmacological properties, such as regulating insulin, anti-tumour, bacteriostasis, and reducing blood lipids, which make genistein a potential drug for the prevention and treatment of a variety of chronic diseases. Atteritano et al. found that taking genistein in postmenopausal women with osteopenia can effectively improve the quality of life and depression (Atteritano et al., 2014). With the deepening of the study, the antidepressant activity of genistein extended to non-ovariectomized animals (Kageyama et al., 2010). Daily intragastric administration of genistein and antidepressant amitriptyline had significant antidepressant effects. These studies confirmed the antidepressant effect of genistein; however, the association between intestinal flora and depression, and as well as how genistein affects glutamate gene expression have not been reported yet.

In this study, a depression rat model was established, and three doses of genistein were administered orally to rats. Behavioral and serum indicators were measured at different stages, and colon HE staining and infrared imaging were observed. The antidepressant effect of genistein was explored based on 5-HT content, HPA axis hyperactivity, colon function, and body temperature. Moreover, the effects of different doses of genistein on the gut microbiota of model rats and the differential metabolic pathways between groups were studied. The effect of genistein on the gut microbiota structure of the chronic unpredictable mild stress (CUMS) model rats was explored, and the correlations between microbiota and behavioral, biochemical indicators were established. Finally, the antidepressant effects of genistein were explored using metagenomic technology. The research objective is to provide theoretical support for the possible mechanism of genistein in alleviating depression and to provide new targets for the treatment of depression.

## 2. Materials and methods

### 2.1. Materials

Genistein (the batch numbers is 190822R) (purity ≥98%) was purchased from Nanjing Dausf Biotechnology Co., Ltd. (Nanjing, China). Rat serotonin (5-HT, the batch numbers is MM-0981R2), rat corticotropin (ACTH, the batch numbers is MM-0565R2) and rat corticosterone (CORT, the batch numbers is MM-0559R2) kit were all purchased from Jiangsu Enzyme Immunity Industry Co., Ltd. (Jiangsu, China). Saline (0.9%) was provided by Shandong Kelun Pharmaceutical Co., Ltd. (Shandong, China), and fluoxetine hydrochloride was provided by Lilai Suzhou Pharmaceutical Co., Ltd. (Suzhou, China). Paraformaldehyde tissue fixing solution was provided by Shanghai Yuanye Biotechnology Co., Ltd. (China, Shanghai). Dimethyl sulfoxide (DMSO) was provided by Tianli Chemical Reagent Co., Ltd. (Tianjin, China). The hematoxylin-eosin staining reagent and neutral gum were obtained from Tianjin BASF Chemical Co., Ltd. (Tianjin, China). The Fast DNA SPIN extraction kits were obtained from Shanghai Abe Medical Equipment Co., Ltd. (Shanghai, China). The PicoGreen dsDNA detection kits were obtained from Tianjin Pinsinuo Biotechnology Co., Ltd. (Tianjin, China).

### 2.2. Animal trials and treatments

The experiment was conducted on healthy male sprague-dawley (SD) rats (180–200 g). Rats, rat foods, and corncob cushions were purchased from Changchun Yisi Experimental Animal Technology Co., Ltd. (Changchun, China). The water for feeding was ordinary domestic water. The study was performed under experimental protocols approved by the Harbin University of Commerce Ethical Committee. The housing and experimental procedures were in accordance with the European Union Directive of September 22, 2010 (2010/63/EU).

Briefly, 72 male SD rats fed adaptively for 7 days, and then were divided into six experimental groups (12 rats in each group) receiving: blank group, CUMS model group, fluoxetine group, genistein group with high, medium and low dose. Except for the blank group, the other 5 groups of rats were fed in a single cage. Each group of rat was treated as described below.

(1) Blank group: rats did not receive any stimuli, and they were fed and watered normally at the end of adaptive feeding.
(2) CUMS model group: CUMS model group was established according to the method of Ma et al. with slightly modifications (Ma et al., 2011). At the end of fed adaptively for 7 days, rats received chronic, unpredictable and mild stimuli to simulate the chronic low-intensity stress received by humans in daily life. One stimulus was randomly selected to treat the rats every day, consecutively without repetition (Table 1), with the aim of making the rats unable to anticipate the stimulus, thus establishing a rat CUMS model. After modeling, 1 mL of physiological saline was administered by gavage for 21 days.
(3) Fluoxetine group (positive control): fluoxetine hydrochloride capsules was fixed by distilled water after removing the outer capsule coat, and the bottle was subjected to ultrasonic dissolution to make an instillation solution with a drug concentration of 0.5 mg/mL. The CUMS rats were gavaged with 1 mL of fluoxetine gavage solution (the dose of fluoxetine hydrochloride used in

**Table 1**
Stimulation methods for modeling CUMS.

| Day | Stimulation methods | Day | Stimulation methods |
|---|---|---|---|
| 1 | Fasting | 2 | Empty bottles |
| 3 | Pintail | 4 | Vibrating rat cage |
| 5 | Moist bedding material | 6 | Tilting mouse cage |
| 7 | No water | 8 | Empty bottles |
| 9 | Pintail | 10 | Tilting mouse cage |
| 11 | No water | 12 | Vibrating rat cage |
| 13 | Moist bedding material | 14 | Fasting |
| 15 | Pintail | 16 | No water |
| 17 | Vibrating rat cage | 18 | Moist bedding material |
| 19 | Fasting | 20 | Empty bottles |
| 21 | Tilting mouse cage | 22 | No water |
| 23 | Vibrating rat cage | 24 | Pintail |
| 25 | Empty bottles | 26 | Moist bedding materia |
| 27 | Tilting mouse cage | 28 | Fasting |

humans was 20 mg/kg d, which was converted to 1.8 mg/kg d administered to rats) for 21 days.

(4) Genistein group: genistein were dissolved in 1 mL of DMSO, and then normalized with saline to prepared gavage solutions of 10 mg/kg (low dose), 20 mg/kg (medium dose), and 40 mg/kg (high dose). The CUMS rats were gavaged with 1 mL of genistein gavage solution for 21 days to obtained low-dose group (10 mg/kg d), medium-dose group (20 mg/kg d) and high-dose group (40 mg/kg d) respectively.

Subsequent experiments were conducted on day 0, day 7, and day 21 of gastric lavage in rats. The animal experiment flowchart is shown in Supplementary Fig. S1. All rats were euthanized by cervical dislocation.

### 2.3. Behavioral evaluation of rats

#### 2.3.1. Weight changes and sugar water preference test (SPT) of rats

Weight changes and sugar water preference test were carried according to the method of Liu et al. with slightly modifications (Liu et al., 2023). The body weight of the rats was measured with analytical balance at 0 d, 7 d and 21 d in the process of gavage. Rats were trained for 1 day with two bottles, the one with 1% (w/v) sucrose water, and the others with distilled water. The position of the two bottles was changed every 4 h. After 1 day of fasting and water fasting, 200 mL each of 1% (w/v) sucrose water and distilled water were prepared and placed in the rat cages, and the consumption of distilled water and sucrose water was recorded after 24 h. The sugar-water preference was calculated as the following equation:

$$\text{Sugar water preference}\,(\%) = \frac{\text{Cane sugar water consumption}}{\text{Cane sugar and distilled water consumption}} \times 100$$

#### 2.3.2. Open field test (OFT) of rats

The open field test was conducted according to the method of Liu et al. (2016a). A self-made open box (110 cm × 110 cm × 40 cm and divided into 25 cm × 25 cm compartments) was used in the experiment, and each rat was placed in the center for 2 min to adapt. And then, the number of autonomous activities was recorded and scored within 5 min. The rat entering a square with three feet at the same time was scored 1 point, while two forefeet were lifted or climbing the wall of the box was marker as 1 point. At the end of the test, 75% alcohol was used to remove the smell.

### 2.4. Determination of 5-HT, ACTH and CORT in rats

The contents of 5-HT, ACTH, and CORT in rat serum were determined according the ELISA kit according the reference of Fan et al. (2022). All rats were euthanized by cervical dislocation. Blood of 5 mL was taken from the abdominal aorta with a negative pressure vacuum glass tube, and the blood was centrifuged at 3818×*g* for 20 min at 4 °C using a high-speed freezing centrifuge (Symefeishier Technology Company). The supernatant was taken and stored in a refrigerator at −20 °C. The content of 5-HT, ACTH, and CORT in rat serum were measured according to the instructions of the ELISA kit.

### 2.5. Hematoxylin-eosin (HE) staining of rats colon

HE staining of rat colon was done according the method of Wang et al. as preciously discribed (Wang et al., 2020). The rats were euthanized by cervical dislocation at the 0, 7th and 21st day and their colons were taken. Blood was collected from the abdominal aorta, and the stripped colon tissue (about 2 cm of colon tissue was cut) was fixed in 4% paraformaldehyde for 24 h. Then the colon tissue was trimmed, dehydrated and embedded in paraffin, and cut into 5 μm slices. Slices were soaked in xylene I, xylene II and ethanol for 2 min, and finally washed with tap water for 2 min to achieve dewaxing. And then, the slices were stained with hematoxylin for 2 min, washed with running water, and stained with ammonia return blue and eosin stain for 5 min. The transparent tissue sections were dripped with neutral resin, sealed with coverslips, examined microscopically with a light microscope. Image J software (National Institutes of Health, USA) with 100× magnification was used for image acquisition and analysis.

### 2.6. Determination of rats temperature

The rats in each group were photographed by a thermal infrared imager (FORTIC 320pro, USA) at the 0 day, 7 th day and 21 st day after modelling. The tail of rats was fixed, and the brain and body were photographed. The brain and body temperature of the rats were analyzed and recorded by AnalyzIP software (JL Technology, Shanghai). The final result was taken as the average of the body temperatures of the four rats.

### 2.7. Determination of the mechanism of genistein in improving depression

#### 2.7.1. 16S rRNA amplicon pyrosequencing

The 16S rRNA amplicon pyrosequencing was carried out using the method of Zhang et al. with slightly modifications (Zhang et al., 2023). The DNA SPIN Extraction Kit was used to extract and isolate the DNA of rat intestinal microorganisms. The V3-V4 region of the 16S rRNA gene was amplified using the forward primer 338F (5′-ACTCCTACGGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGTWTCTAAT-3′). The sample-specific 7-bp barcode was added to the primers for multiplex sequencing. Sample-specific 7-bp barcodes were added to the primers to complete the amplification. PCR components included 5 μL of Q5 reaction buffer (5 × ), 5 μL of Q5 high-fidelity GC buffer (5 × ), 0.25 μL of Q5 high-fidelity DNA polymerase (5 U/mL), 2 μL of dNTPs (2.5 mmol/L), 1 μL each of the forward and reverse primers (10 μmol/L), 2 μL DNA template and 8.75 μL dd$H_2O$. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified with PicoGreen dsDNA Analysis Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification, equal amounts of amplicons were pooled together and then subjected to paired-end 2 × 300 bp sequencing using the Illumina MiSeq platform and MiSeq Reagent Kit v3 at Shanghai Personal Biotech. (Shanghai, China).

#### 2.7.2. Bioinformatics analysis

Bioinformatics analysis refers to the method of Wang et al. (2019). Sequence data were analyzed primarily using the QIIME and R software packages (v3.2.0). The OTU table in QIIME was used to calculate OTU-level α-diversity indices. The α-diversity can be used to characterize species diversity; for example, Chao1 can respond to microbial abundance, and Shannon and Simpson indices can characterize the

diversity of microbial communities. OTU-level abundance ranking curves were generated to compare the richness and evenness of OTUs in different samples.

*2.7.3. Metagenomic analysis*

Metagenomic analysis was carried out reference the method of Yao et al. (2023). The standard Illumina TruSeq DNA library preparation experiment (Illumina TruSeq DNA Sample Preparation Guide) was used

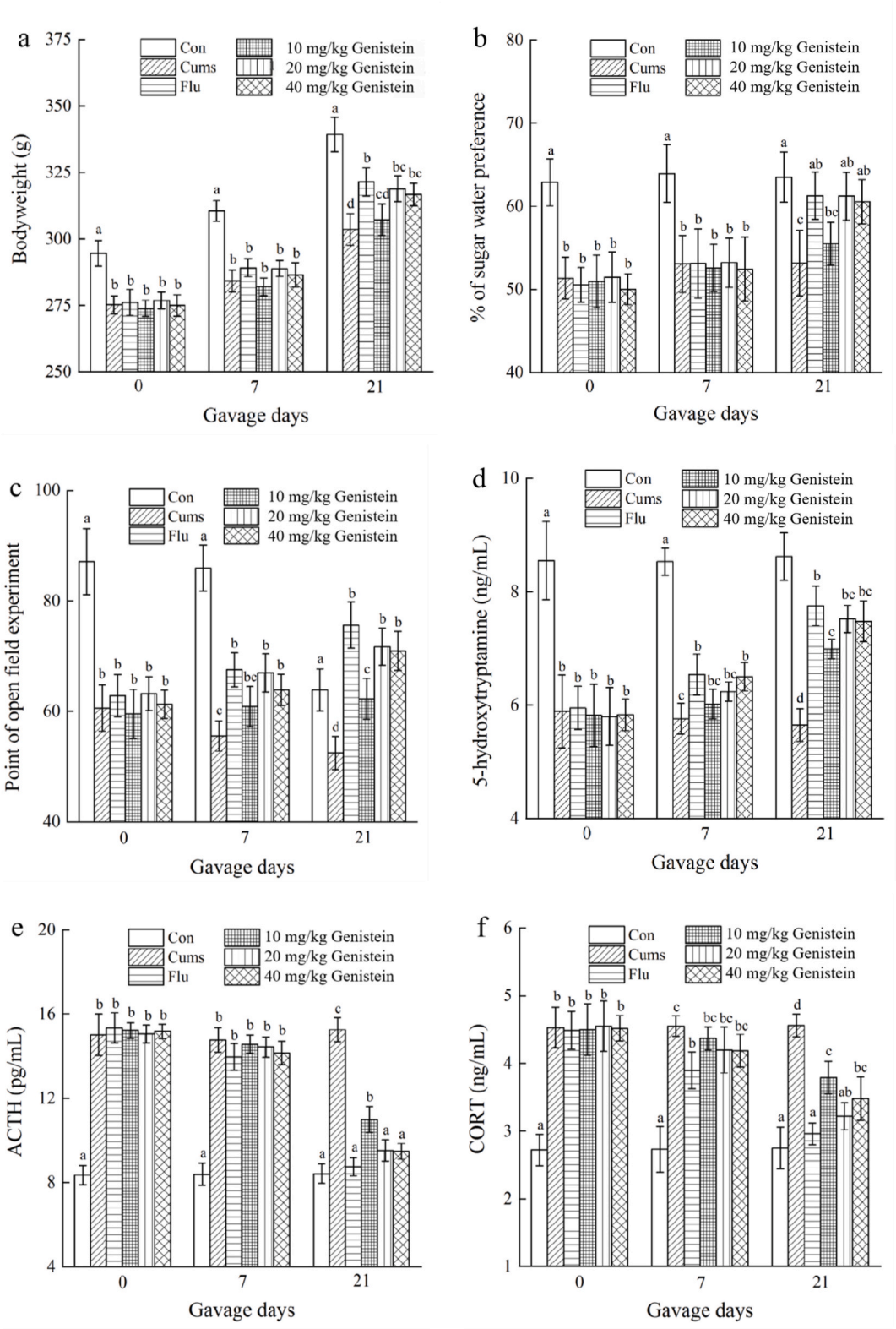


**Fig. 1.** Antidepressant effect of genistein on depressed rats after 0 d, 7 d and 21 d of instillation. (a) Changes in body weight of rats. (b) Sugar-water preference test of rats (c) Mineral field score of rats (d) Serum 5-HT content of rats (e) Serum ACTH content of rats (f) Serum CORH content of rats. Different lowercase letters above the same set of bar graphs indicate means One-way ANOVA differences are statistically significant ($p < 0.05$).

to construct the genomic library. The mixed library (10 nmol/L) was diluted to 4–5 pmol/L and sequenced. Filter the original data to get the effective data (Clean Data), and then assemble the metagenome; the scaftigs assembled from a single sample were predicted by Meta Gene Mark, and the predicted genes of each sample were put together to remove redundancy and construct unigenes. Based on unigenes, the clean data of each sample was synthesized and obtain the abundance information of unigenes in each sample.

### 2.8. Statistical analysis

The results are based on three independent experiments or assays, and are presented as means ± standard deviations. With the statistical program for social sciences 16.0 software package (SPSS Inc., Chicago, IL, USA) and duncan multiple comparisons, the one-way analysis of variance (ANOVA) was used to determine the significance between the groups ($P < 0.05$).

## 3. Results

### 3.1. The antidepressant effect of genistein on CUMS depression rats

#### 3.1.1. The behavioral evaluation of depressed rats

The body weight of the rats after gavage for 0 d, 7 d, 21 d is shown in Fig. 1a. The average body weight of the control was 294.64 ± 4.81 g before gavage, while that of the model group was only 275.31 ± 3.31 g. There were significant differences between them, indicating that the modelling was successful (Ge et al., 2024). After intragastric administration of different doses of genistein for 7 d, there was no significant difference ($P>0.05$) in the other groups that had slow growth of body weight as compared to the blank group, which probably because the time was too short for the action of the drug to effective. After intragastric administration of different doses of genistein for 21 d, the weight gain of the positive druggroup, middle-dose group, and high-dose group increased significantly ($P<0.05$) compared with the model group, while that of the low-dose group had no significant difference ($P>0.05$). Notably, there was no significant difference in antidepressant effect between the middle/high-dose groups and the positive drug (fluoxetine group) ($P>0.05$), but they were significantly higher than the model group, and the difference between the two was significant compared with the control group ($P<0.05$). The above results indicated that the medium and high doses of genistein had good antidepressant effects on rats.

The results of the sugar water preference experiment showed (Fig. 1b) that the sugar water intake of the model rats (51.37 ± 2.51% vs) was significantly lower than that of the control group (62.87 ± 2.91%) before gavage. Therefore, the model rats were suffer from depression. The use of fluoxetine hydrochloride and different doses of genistein on rats by gavage for 7–21 d increased the sugar and water intake, and it is worth mentioning that the medium dose of genistein (20 mg/kg) had the best effect (51.48–61.22%), which was comparable to that of the positive group (50.56–61.28%). These results suggested that genistein can be applicated as a food source component in antidepressants.

Open field test is another effective method for evaluating animal behavior. From the results of the open field test (Fig. 1c), the model rats showed the typical symptoms of decreased autonomic activity and exploratory behaviors, and there was a significant decrease compared to the control group (60.58 ± 4.21 vs 87.12 ± 5.98 before gavage). The scores of rats treated with 10–40 mg/kg genistein were 59.52–63.19, 60.88–66.95, 62.29–71.69 at gavage for 0, 7 and 21 d, respectively, which were not significantly different from those of rats treated with fluoxetine hydrochloride (62.85–75.62). However, it was significantly higher than that of the model with the scores of 52.45–60.58 after continuous intragastric administration for 0–21 days. The results showed that genistein could significantly improve the depressive state of model rats with decreased autonomic activity and exploratory behaviors, and its effect was not significantly different from that of fluoxetine hydrochloride drug treatment. However, the dosage of genistein significantly affected the improvement of the depressive state of rats.

### 3.2. Effect of genistein on the contents of 5-HT, ACTH and CORT in depressed rats

The 5-HT content of rats after gavage for 7–21 d is shown in Fig. 1d. The 5-HT content in the model group (5.65–5.89 ng/mL) was significantly lower than that of the control group (8.55–8.62 ng/mL), suggesting that prolonged stimulation affects the serum 5-HT content in rats. After 7–21 d of continuous intragastric injection of genistein, the 5-HT content of rats in the fluoxetine hydrochloride and genistein treatment group was significantly higher than that of the model group (Fig. 1d). This study confirmed that both fluoxetine hydrochloride and genistein administered continuously intragastrically for more than 7 d could positively affect the depressive state of rats. The effects of medium and high doses of genistein have not significantly different (5.80–7.52 ng/mL and 5.83–7.48 ng/mL), when it were compared to those of positive group (5.95–7.75 ng/mL).

Significantly higher levels of ACTH (Fig. 1e) and CORT (Fig. 1f) were found in the model compared to the control group. These results are consistent with previous findings that when ACTH and CORT levels are increased, it causes a stress response that increases stress and leads to depression in rats (Chen et al., 2021; Bai et al., 2022a). Administration of fluoxetine hydrochloride or genistein to rats for 7–21 d resulted in a significant decrease in ACTH and CORT levels compared to the model group, suggesting that these drugs can alleviate depressive symptoms. After 21 d of intragastric administration, ACTH and CORT content decreased significantly, and there were differences in the effects of different doses of genistein. It was also shown that 20 mg/kg of genistein (9.52 ± 0.51 pg/mL and 3.22 ± 0.20 ng/mL for ACTH and CORT contents) was the most similar to fluoxetine hydrochloride (8.75 ± 0.44 pg/mL and 2.96 ± 0.16 ng/mL for ACTH and CORT contents) and had the best effect.

### 3.3. Results of HE staining of the colon

The colonic HE staining in rats is depicted in Fig. 2. In the control group, the overall structure of the colon tissue was complete, the intestinal glands were arranged well, the mucosal cells were arranged regularly, the columnar epithelium was arranged into a tube, and many goblet cells were sandwiched between the epithelial cells. In the model group, the intestinal glands decreased, the arrangement of mucosal epithelial cells was disordered, noticeable exfoliation and necrosis could be seen, prominent edema could be seen in the submucosa, and inflammatory cells infiltrated the mucous membrane.

Compared with the control group, the rats treated with intragastric administration (the model, fluoxetine hydrochloride, and genistein group) for 7 d showed that the intestinal glands were irregular and tight, and there was still inflammatory cell infiltration in the mucous tissue membrane. However, after continuous intragastric administration of fluoxetine hydrochloride or 20–40 mg/kg genistein for 21 d, HE staining showed that the infiltration of inflammatory cells in rats decreased significantly, indicating glandular injury was decreased and medium and high doses of genistein could improve the histological changes of the colon in rats with depression induced by chronic stress.

### 3.4. Results of brain temperature and body temperature

The brain temperature and body temperature of the model rats were lower than that of the control group, as shown in Fig. 3. Brain temperature might be related to the disturbance of hypothalamus function caused by chronic stress and the disorder of body temperature (Nakamura et al., 2022). Compared with the model group (18.8 °C), the

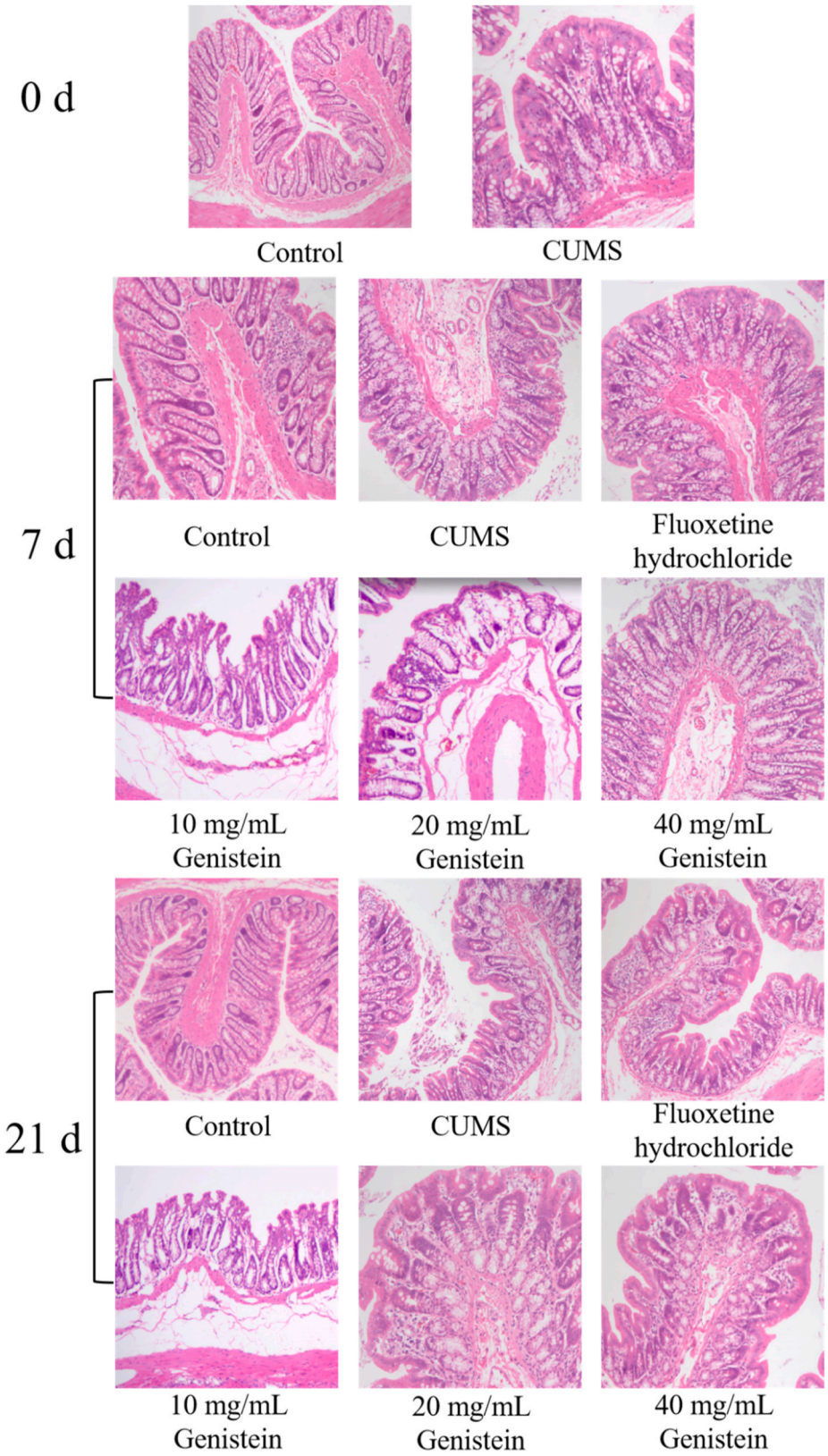


**Fig. 2.** HE staining of rat colon tissue ( × 100).

body temperature of rats treated with fluoxetine hydrochloride (21.3 °C) and genistein (21.9 °C, 16.6 °C, 19.5 °C) did not increase significantly after intragastric administration for 7 d. However, after 21 d of intragastric administration, the body temperature of rats treated with 20–40 mg/kg genistein increased significantly (24.4 °C and 24.8 °C), indicating that medium and high doses genistein could increase the body temperature of depressed rats, and close to the body temperature of normal rats.

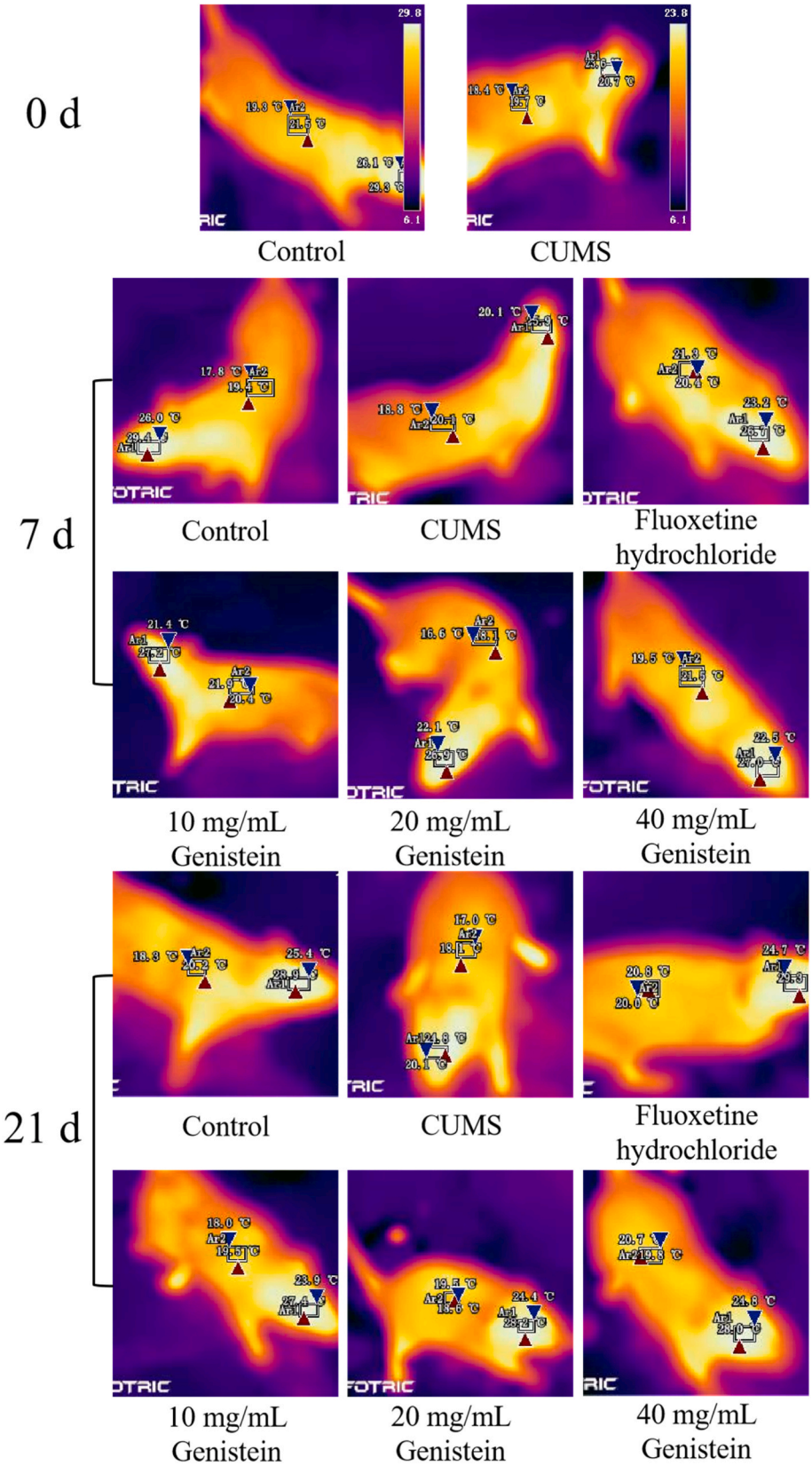


**Fig. 3.** Brain temperature and body temperature of rats under thermal infrared imaging.

### 3.5. High-throughput sequencing results of genistein on intestinal flora in rats

The basic information of sample sequencing and alpha diversity indices of samples shows in Table 2. The average sample length was 87361bp. The effective fragments of sample 16S rRNA between 80058 and 93087, which was greater than 50000. Therefore, this batch of samples met the sequencing quality requirements (GC% > 50%, Q30 > 85%). The abundance of OTU can show the richness of the sample species. Fig. 4a showed the operating taxon results of the sequence at 99% similarity, with 348 species coexisting. The number of OTU in the CUMS group was higher than that in control (972 vs 749), but the number of OTU decreased after intragastric administration of fluoxetine hydrochloride and genistein. The Chao1, observed species Index, Shannon, and Simpson indices in model group were 886.953, 766.6, 6.552, and 0.969, which were higher than those of the control group (687.331, 566.2, 4.915 and 0.911) (Table 2). After intragastric administration of fluoxetine hydrochloride or genistein, the Alpha diversity index of rat intestinal flora decreased significantly.

The relative abundance of gut microbiota at the portal level in each group of rats is shown in Fig. 4b. Studies have shown that the ratio of Firmicutes to Bacteroidetes can reflect the degree of depression (Gao et al., 2023). Compared with the control, continuous stress significantly decreased the abundance of Firmicutes and Actinobacteria dominant bacteria in CUMS, while significantly increased the abundance of Bacteroidota. In genistein-treated rats, the abundance of Actinobacteria and Firmicutes increased, and the abundance of Bacteroidota decreased.

Relative abundances of intestinal microbiota at the genus level for each group of rats (top 50 heat map and top 20 histogram) is shows in Fig. 4c and d. The genus level is mainly concentrated in the genera Lactococcus spp., Lachnospiraceae spp., Prevotella spp., Bifidobacterium spp., etc. Compared with the control group, the levels of genera of Trichoderma spp., *Przewalski* spp, *Ricinella* spp, *Muribaculaceae* spp, *Ruminalococcus* spp., and *Clostridium* spp. were elevated in the CUMS group, whereas the levels of *Rönbutzia spp.*, *Streptococcus* spp, and *Faecalococcus* spp. were reduced. After gavage of fluoxetine, the levels of *Prevotella* spp. and *Ruminalococcus* spp. *UCG-005* were significantly increased and the levels of *Trichoderma* spp. and *Lactobacillus* spp. were significantly decreased. It was also found that the levels of *Lactococcus* spp., *Bifidobacterium* spp., and *Streptococcus* spp. were increased and the levels of T*richoderma* spp. and *Prevotella* spp. were decreased after gavage of low and medium doses of genistein. In contrast, high doses of genistein did not increase the levels of *Bifidobacterium* spp. but elevated the levels of *Lactobacillus* spp. and *Streptococcus* spp. while decreasing the levels of *Spiroplasma* spp. and *Przewalski* spp. In addition, the abundance of *Lachnospiraceae NK4A136* was significantly increased in the genus level of the CUMS group and decreased after gavage of genistein with fluoxetine.

The experimental results showed that *Bacteroides* was positively correlated with body weight, sugar preference, open test and 5-HT level, and negatively correlated with serum ACTH and CORT levels ($P<0.05$) (Fig. 5). The abundance of *Bacteroides* in the rats increased, and biochemical and behavioral indicators in the rats changed, leading to depressive symptoms. The abundance of *bifidobacterium* and *actinomyces* decreased in the CUMS group compared with the control group. Gastric administration of genistein upregulated the abundance of both bacteria.

#### 3.5.1. Results of amino acid metabolism of genistein to improve depression

Glutamate is a substrate for GAD. After decarboxylation, GABA can be formed (Lie et al., 2018). The level of genes encoding GAD, a key enzyme in glutamate metabolism, in each group of rats is shown in Fig. 6. The number of genes encoding for GAD enzyme was significantly lower in the model group (508) than in the blank group (627) ($P<0.05$), which may be due to the decrease in the expression of GAD proteins in the hippocampus of the rats under chronic stress, which increases the content of Glu and decreases the synaptic release of GABA. Whereas after the gavage of genistein, the number of genes encoding for GAD enzyme (998) was significantly increased compared with the model group and the blank group ($P<0.05$). Therefore, genistein can exert its antidepressant effect by regulating glutamate metabolism levels.

## 4. Discussion

### 4.1. Effect of genistein on behavior in depressed rats

Behavioral experiments can reflect the degree of depression (Guo et al., 2020), therefore, body weight changes, preference for sugar water, and mineral field scores were tested to reflect behavioral assessments of depressed rats in the CUMS depression model. The results of the study showed that the CUMS model could lead to a decrease in body weight, sugar water preference, and mine field score in rats. These metrics can reflect anxiety and depression in rats (Xu et al., 2022), and rats with these characteristics can be considered to have depression successfully modeled (Gu et al., 2020). Based on the results of behavioral tests in this experiment, genistein promotes increased body weight gain in depressed rats, which may be due to the fact that genistein flavonoids regulate the expression of hypothalamic orexin, a neuropeptide substance involved in the regulation of the physiological process of food intake in organisms. In addition, orexin promotes brain information feedback to increase appetite, which in turn increases food intake in rats (Lu et al., 2023). Sucrose preference is considered to be an indicator of pleasure-deficit-like behaviors such as pleasure deficit and loss of responsiveness to reward (Dhingra and Bansal, 2014). After genistein treatment, the sugar water preference and mine field scores of rats improved to some extent and approached those of normal rats. This suggested that genistein treatment alleviated the symptoms of depression in rats, and furthermore, medium to high doses of genistein resulted in better improvement. One study confirmed that the use of Lactobacillus plantarum to treat depressed rats was effective in improving their depressive behavior (Zhao et al., 2023). This phenomenon suggested that genistein is beneficial exogenous factors for treating depression.

### 4.2. Effect of genistein on the content of 5-HT, ACTH and CORT in depressed rats

The onset of depression is intricately linked to a deficiency in 5-HT, a crucial monoamine neurotransmitter in the brain, and alterations in its functioning have the potential to precipitate symptoms of depression, anxiety, or mania (Wang et al., 2023). In this experiment, the rats model of depression established by CUMS resulted in reduced levels of 5-HT, a

**Table 2**
Basic information on sequenced sequences and alpha diversity indices of samples.

| Sample | Clean_paired_reads | GC (%) | Q30 (%) | Chao1 Index | Observed species Index | Shannon Index | Simpson Index |
|---|---|---|---|---|---|---|---|
| Control | 81093 | 52 | 97.09 | 687.331 | 566.2 | 4.915 | 0.911 |
| CUMS | 93087 | 53 | 96.62 | 886.953 | 766.6 | 6.552 | 0.969 |
| Fluoxetine hydrochloride | 93090 | 52 | 96.87 | 782.815 | 637.8 | 5.746 | 0.924 |
| 10 mg/kg Genistein | 89058 | 53 | 96.50 | 815.577 | 732.9 | 6.451 | 0.961 |
| 20 mg/kg Genistein | 80058 | 52 | 96.74 | 759.315 | 650.5 | 5.771 | 0.934 |
| 40 mg/kg Genistein | 87780 | 53 | 96.65 | 760.356 | 630.7 | 5.479 | 0.931 |

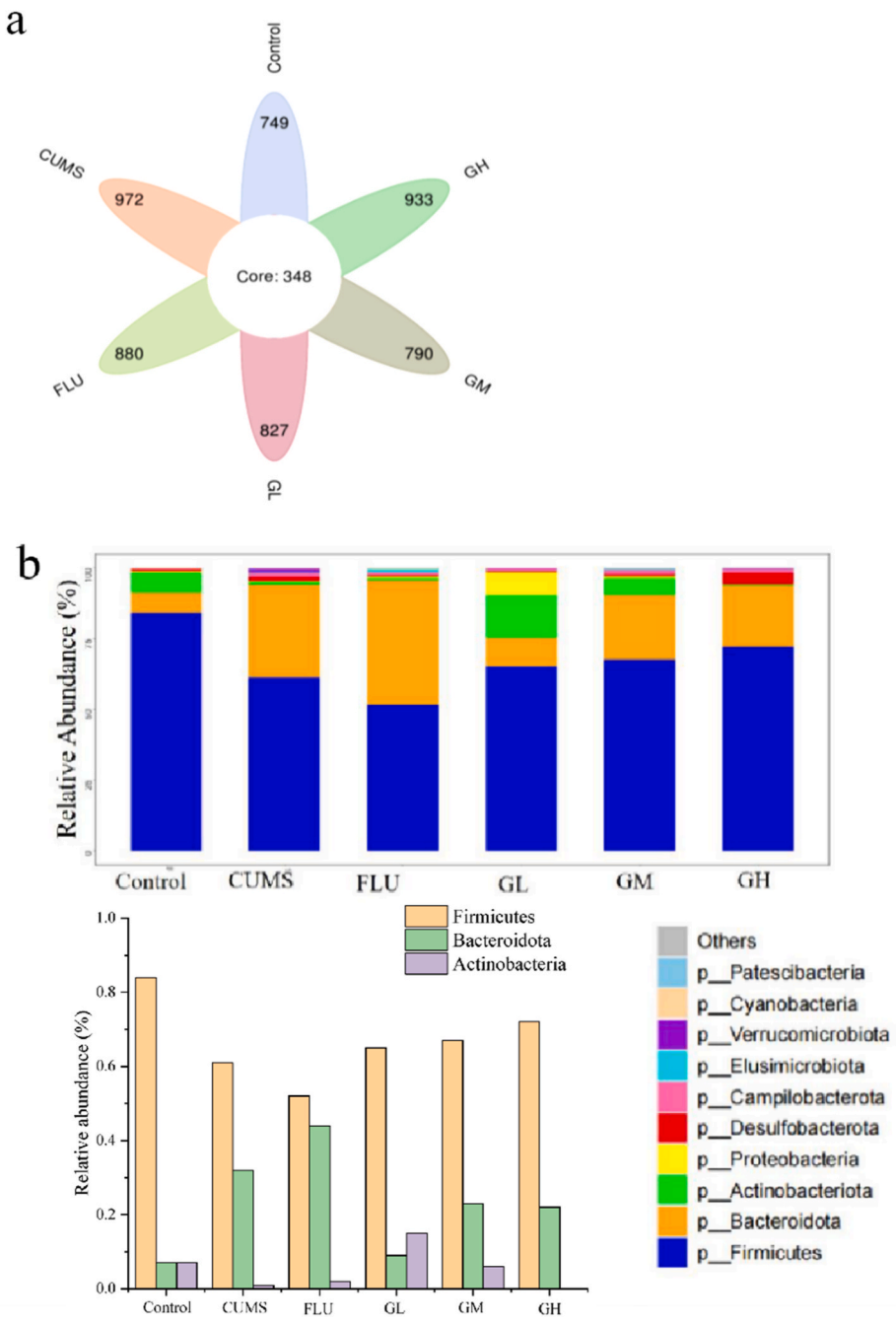


**Fig. 4.** Effects of genistein on intestinal flora in rats (a. OTU Venn diagram for each group of samples; b. Relative abundance of gut microbiota at the gateway level in various groups of rats; c&d. Relative abundance of intestinal microbiota at the genus level for each group of rats - top 50 heat map and top 20 histogram).

group of mood-related symptoms in which the monoamine 5-HT in the neural center is thought to be strongly associated with depression (Lima-Ojeda et al., 2018). The findings of our experiment indicated that genistein may have the potential to elevate and regulate 5-HT levels, which is consistent with previous studies (Liu et al., 2016b). The majority of existing antidepressant medications function by enhancing monoamine neurotransmitter levels within the synaptic cleft, including Selective serotonin reuptake inhibitors (SSRIs) and Serotonin-norepinephrine reuptake inhibitors (SNRIs), with fluoxetine falling into the latter category (Cryan et al., 2002). According to the monoamine hypothesis of depression, genistein was believed to regulate the levels of monoamine neurotransmitters, specifically 5-HT, in the body. This modulation of neurotransmitter levels may contribute to the improvement of depressive symptoms and the production of antidepressant effects. Studies on neurotransmitter levels have indicated a negative correlation between neurotransmitter content and the severity of depression, suggesting that genistein may increase neurotransmitter expression as a potential mechanism for treating depression.

The HPA axis is an important component of the neuroendocrine system, and ACTH and CORT are negatively correlated with the regulation of the HPA axis (García-Cáceres et al., 2010). Hyperactivity of the HPA axis may damage hippocampal monoamine neurons, which may lead to decreased levels of monoamine neurotransmitters (Chevalier et al., 2020). Hyperactivity of the HPA axis is the most common neurobiological change in depressed patients, and excessive CORT indicates reduced resistance to negative motivation (Chi et al., 2020).

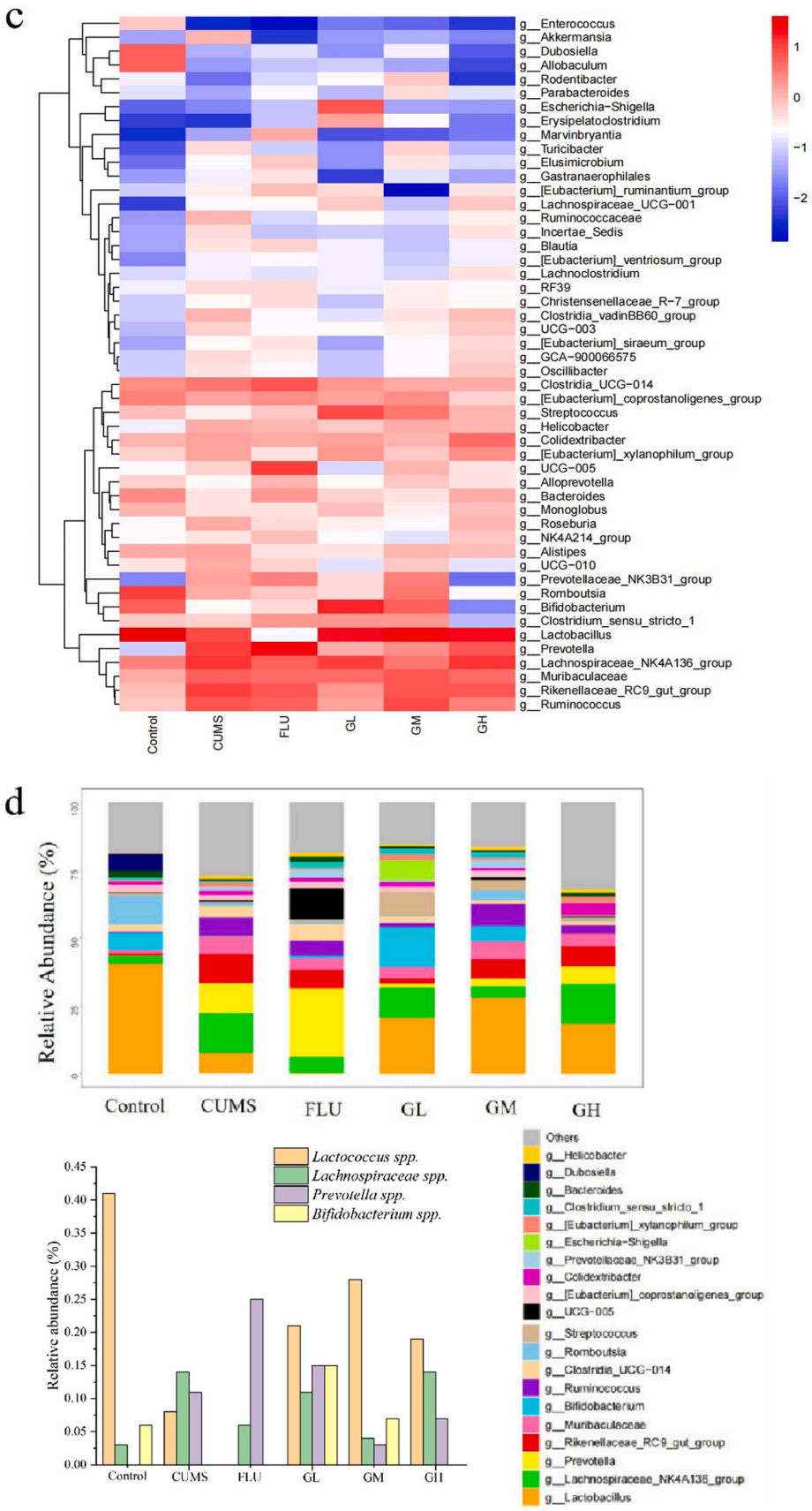


**Fig. 4.** (*continued*).

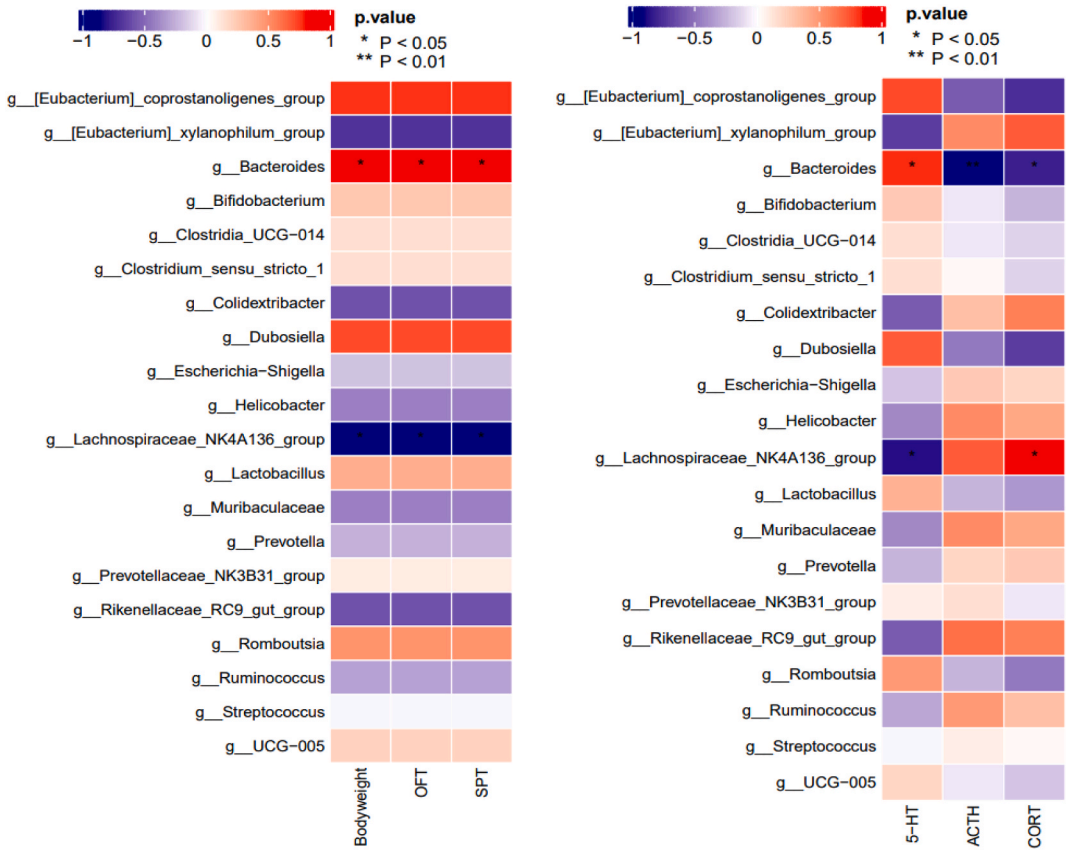


**Fig. 5.** Correlation between intestinal flora, biochemical and behavioural indicators.

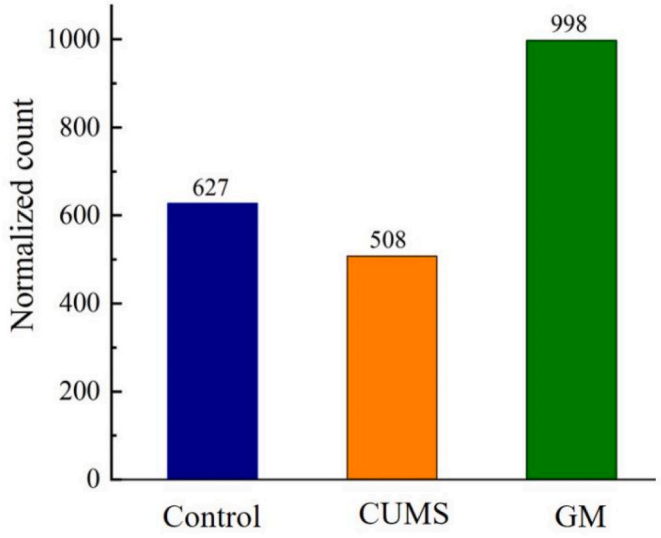


**Fig. 6.** The number of glutamate decarboxylase GAD genes in each group of rats.

Following the administration of genistein via gavage, levels of ACTH and CORT in rats were observed to increase as a result of the release of CRF into the pituitary portal system, thereby stimulating ACTH secretion in response to stress (Mikołajczyk and Złotkowska, 2019). Genistein attenuates HPA axis hyperactivity by reducing ACTH and CORT levels. Combined with the previous behavioral experiments, the levels of ACTH and CORT were positively correlated with the degree of depression, which is consistent with previous studies (Chen et al., 2021; Bai et al., 2022b). This indicated that the antidepressant properties of genistein are related to its inhibition to the HPA axis.

### 4.3. *Effect of genistein on colon HE staining in depressed rats*

Colonic dysfunction is recognized as a common symptom associated with depression (Ding et al., 2020). Furthermore, research has demonstrated that prolonged stress can result in compromised gut barrier integrity in the individual, ultimately triggering significant inflammation within the intestines via neural communication or alterations in microbiota composition (Jiang et al., 2020; Wei et al., 2020). Therefore, colon HE staining experiments can observe the therapeutic effect of genistein on colon. In the current investigation, genistein administration was found to mitigate intestinal barrier impairment in rats subjected to CUMS. This finding is significant as compromised intestinal barrier function can facilitate the infiltration of pathogenic microorganisms and toxic substances into the systemic circulation (Stevens et al., 2018). Invasion of pathogens into the internal environment triggers a cascade of inflammatory responses that propagate from the gastrointestinal tract to the central nervous system, ultimately leading to activation of the HPA axis (Schachter et al., 2018), thereby affecting mood and behavior. Based on HE staining experiments, it was determined that rats exhibited a notable decrease in inflammatory cell infiltration following genistein administration via gavage, indicating a mitigation of glandular injury. Additionally, the administration of medium and high doses of genistein was found to ameliorate histological alterations in the colons of rats experiencing depression induced by chronic stress. The mechanism may be that genistein flavonoids inhibit macrophage infiltration into the colonic mucosa, produce anti-inflammatory activity in the intestine, and increase the disease activity index (Castanho et al., 2014). Studies have shown that treatment of depressed rats with rhamnosus is also effective in treating the colon of rats, which is consistent with this study (Xu et al.,

2022). It is postulated that genistein flavonoids in the experimental group may exhibit antidepressant properties through the regulation of intestinal flora, modulation of intestinal dysfunction, and subsequent enhancement of colonic morphology.

### 4.4. Effect of genistein on body temperature in depressed rats

Research has demonstrated that numerous symptoms of depression are marked by disturbances in circadian rhythms, including alterations in sleep patterns, body temperature, and levels of circulating hormones and neurotransmitters (Edgar and McClung, 2013). Depressed patients are associated with severe circadian rhythm disturbances, which correlate with the severity of depression and increase the range of body temperature fluctuations (Castanho et al., 2014). CUMS significantly reduced circadian range of activity and body temperature in rats, and these changes are directly correlated with depressive behaviors. In our experiments, genistein increased body temperature of depressed rats and approached that of normal rats. A study has shown that 5-HT receptor antagonists and melatonin receptor agonists are involved in regulating body temperature (Klerman et al., 2002). Thus, genistein may regulate body temperature in CUMS rats by modulating 5-HT receptor expression.

### 4.5. Effect of genistein on intestinal flora in depressed rats

As the basis of the microbe-gut-brain axis, the intestinal flora provides the environment for genistein to participate in gut-brain axis communication. The dynamic homeostasis of the intestinal environment is maintained through the interrelationships and interactions between beneficial and pathogenic microorganisms in the gut. This disease can cause long-term health hazards (Ma et al., 2023). In recent years, several studies have also confirmed that intestinal flora was strongly associated with depression (Fukuda et al., 2012; O'Callaghan and van Sinderen, 2016). In the current investigation, the combined effects of chronic stress and genistein administration via gavage were found to induce notable alterations in the gut microbiota composition of rats. However, after gavage of genistein, indices relating to alpha diversity in stressed rats were reduced but did not return to the level of rats in the blank group, demonstrating the severe damage caused by stress and the limited effect of genistein.

In order to delve deeper into the impact of genistein on the intestinal microbiota of rats subjected to CUMS, an examination was conducted to analyze variations in the relative abundance of the intestinal microbiota at both the phylum and genus levels. At the level of phylum, significant changes in the levels of the phylum Firmicutes, Actinobacteriota and Bacteroidota were found in depressed rats. The use of genistein normalized the levels of the Firmicutes and Actinobacteriota phylum in depressed rats. 90% of intestinal flora belong to Firmicutes and Bacteroidetes. Firmicutes can convert carbohydrates into short-chain fatty acids, which induce T cell differentiation and promote inflammation, leading to central neuronal inflammation and depression (Cao et al., 2020). Actinobacteriota can modulate depression by affecting the body's neurotransmitter levels through the production of short-chain fatty acids that stimulate the central nervous system and intestinal tract (Evrensel and Ceylan, 2015; Galland, 2014). It has been shown that the severity of depressive symptoms is directly proportional to the abundance of Bacteroidota, which explains the improvement in depression-like behavior observed in this study (Yang et al., 2020). In this study, it was found that the abundance of Bacteroidota increased in rats under chronic stress, which made their body disordered, the biochemical and behavioral indexes of rats changed, and the rats showed depressive symptoms. This is because Bacteroidota play an important role in the interaction between intestinal flora and host. Studies have shown that Bacteroidota affect the host metabolic pathway and immune system (Maier et al., 2014), and induce the host to produce cytokines. Schiepers et al. (2005) found that increased Bacteroidota abundance could lead to increased levels of peripheral cytokines and hormones and increased inflammation in patients with depression. These results indicate that genistein can change the abundance of specific intestinal microbiota in depressed rats, thereby maintaining intestinal microbiota homeostasis and achieving antidepressant effects.

At the genus level, gavage of genistein elevated the genus levels of *Prevotella* and *Ruminococcus*, while decreasing the genus levels of *Lachnospiraceae* and *Lactococcus*. In addition, a significant increase in the abundance of *LachnospiraceaeNK4A136* at the genus level in the CUMS group and a decrease in the abundance of *LachnospiraceaeNK4A136* after gavage of genistein were observed. As a potential butyrate-producing bacterium, the LachnospiraceaeNK4A136 group has been shown to have protective and anti-inflammatory effects (Ma et al., 2020). In addition, the abundance of *Bifidobacterium* and *Actinobacteria* phylum in the CUMS group in this study was reduced compared to the control group. *Eubacterium coprostanoligenes* and *Bifidobacterium* can be involved in the synthesis of intestinal short chain fatty acids (SCFAs), which are the end products of gut flora metabolism, and many studies have demonstrated that SCFAs can regulate the structure and function of the brain, of which acetic acid can cross the blood-brain barrier and directly participate in cellular metabolism in the central nervous system (Yunes et al., 2016). SCFAs can also regulate the integrity of the blood-brain barrier (Burokas et al., 2017a). *Bifidobacterium* and its symbiotic bacteria are able to promote the production of acetic acid, which in turn synthesizes SCFAs (Fukuda et al., 2012; Jiang et al., 2015). Lactobacillus and Bifidobacterium have been shown to produce GABA, and it has been observed that the abundance of bifidobacterium (a GABA-producing bacterium) is inversely associated with depression (Yunes et al., 2016). Supplementation of prebiotics to chronic stress mice effectively prevented the decrease of bifidobacteria and lactobacillus abundance in depressed mice (Burokas et al., 2017b). In summary, it can be inferred that genistein regulates the level of SCFAs through the blood-brain barrier, thus maintaining the internal homeostasis of the intestinal flora.

### 4.6. Effect of genistein on amino acid metabolism in depressed rats

The pathogenesis of depression is usually associated with abnormal expression of GABA and its receptors in the central nervous system, and drug studies targeting glutamate have become one of the main strategies for the treatment of depression (Yao et al., 2020). To further investigate the *in vivo* metabolic impacts of genistein on depressed rats, its effects on glutamate metabolism were analyzed. Glutamate is an inhibitory neurotransmitter that is abundant and widely distributed in the central nervous system (Hashimoto, 2009). The CUMS model resulted in a reduction in the expression of GAD-encoding genes in rats, while administration of genistein via gavage lead to an elevation in GAD expression levels towards baseline levels. This phenomenon could be attributed to the down regulation of GAD protein expression and elevation of Glu levels in the hippocampus of rats subjected to prolonged stress, resulting in diminished GABA ergic neurotransmission. The research revealed that elevated expression of the GAD gene was associated with a higher likelihood of Glu conversion inactivation, leading to decreased Glu levels and elevated GABA levels in the hippocampus (Li et al., 2018). Previous studies have shown that antidepressant SSRIs can modulate GABA by affecting 5-HT receptors to upregulate 5-HT levels (El Mansari et al., 2005). This indicates that the regulation of glutamate metabolism levels plays a role in exerting antidepressant effects (Battaglioli et al., 2003). The findings from previous studies indicating that genistein elevated blood levels of 5-HT, suggesting a potential mechanism by which genistein alleviate symptoms of depression was 5-HT-mediated alterations in GABA.

## 5. Conclusion

In conclusion, genistein can effectively improve depression and anxious behaviors in chronic stress rats, increase the level of

neurotransmitter 5-HT, alleviate HPA axis hyperfunction, and improve colon histology and body temperature of depressed rats in chronic stress rats. Studies on intestinal flora showed that genistein can reduce the number of species in the intestinal flora of CUMS rats, promote the proliferation of *Bifidobacterium* spp. in the phylum Firmicutes, restore the balance of intestinal flora in CUMS rats. In addition, metagenomic results showed that genistein significantly increased the gene level encoding the key enzyme GAD in glutamate metabolism. The antidepressant mechanism is related to its promotion of glutamate metabolism, increasing of GAD expression level, promoting synthesis of GABA, and indirect increasing of 5-HT levels. This study provides theoretical support for the anti-depression mechanism of genistein based on gut microbiota and metagenomics.

## CRediT authorship contribution statement

**Chun-min Ma:** Conceptualization, Writing – review & editing, Supervision, Writing – review & editing. **Fu-shun Zhang:** Data curation, Writing – original draft. **Xin-huai Zhao:** Conceptualization, Investigation, Writing – review & editing. **Yang Yang:** Methodology, Supervision. **Bing Wang:** Methodology, Supervision. **Yan Wang:** Conceptualization. **Xiao-fei Liu:** Conceptualization. **Xin Bian:** Conceptualization. **Zi-Xuan Xu:** Formal analysis. **Guang Zhang:** Formal analysis. **Li-zhe Qu:** Formal analysis. **Na Zhang:** Funding acquisition, Project administration.

## Ethic approval

The study was performed under experimental protocols approved by the Harbin University of Commerce Ethical Committee. The housing and experimental procedures were in accordance with the European Union Directive of September 22, 2010 (2010/63/EU). The study was carried in accordance with the “Principles of Labora-tory Animal Care”.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2025.101020.

## Data availability

The authors do not have permission to share data.

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