Lactobacillus yogurts display antidepressant-like effects in CUMS mice via inhibition of NF-κB pathway, activating CREB-BDNF pathway and regulating gut-brain axis

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Abstract

In this work, we compared the effects of probiotics (Lactobacillus reuteri and Lactobacillus bulgaricus) fermented yogurt on chronic unpredictable mild stress (CUMS)-induced anxious and depressive-like symptoms in mice as well as discussed potential mechanism. The behavioral test results showed that L. reuteri and L. bulgaricus fermented yogurt significantly improved anxious- and depressive-like symptoms. We also found that Lactobacillus fermented yogurt presented an overall neuroprotective effect on hippocampus as well as maintained the cerebral activity. L. reuteri and L. bulgaricus fermented yogurts also were observed to ameliorate the levels of monoamine neurotransmitters and inflammatory cytokines via inhibiting the activation of NF-κB pathway accompanying with elevating the CREB-BDNF pathway. The Our findings suggested that L. reuteri and L. bulgaricus fermented yogurts may be involved in modulating gut microbiota and metabolite composition, and thereby performing neuroprotective effects and ameliorating depressive behaviors in mice by at least partially microbiota-gut-brain axis.

1. Introduction

Anxiety and depression are widespread mental disorders characterized by low mood, loss of interest, and decreased energy [2]. In 2021, approximately 359.2 million people have anxiety, and 332.4 million people suffer from depressive disorders worldwide [35]. Recently, notably during the COVID-19 pandemic, the incidence and mortality rates of depression have continuously increased, becoming an increasingly heavy burden on individuals and society [54]. The prevalence of depression and/or anxiety was higher than any physical comorbidity in 2020 (60%) and in 2030 (64%) [3]. By 2030, depression is expected to be the largest contributor to disease burden, according to the prediction of The World Health Organization [40]. Currently, antidepressants are not universally effective and frequently result in a range of undesirable side effects [96]. Researchers are actively seeking affordable and holistic treatments for depression and anxiety that minimize side effects.

The gut-brain axis serves as a vital communication system that connects the functions of the gut and the brain [22]. It has sparked widespread interest due to its potential impact on overall well-being [61]. Research suggests that microorganisms, particularly the gut microbiota, play a crucial role in shaping social behaviors and neurodevelopment across various animal species [90]. Furthermore, nutritional interventions, including probiotics, have been proven to positively influence the gut-brain axis and provide notable health benefits. Consequently, this axis stands as a compelling connection between diet quality and the prevalence of depression.

Fermented foods are typically produced through the controlled growth of microbes and enzymatic conversions of various food components. Fermented foods are rich in probiotic bacteria and enzymes to the gut and intestinal health microbiota, thereby maintaining digestive and immune system health [66]. Yogurt is the main product resulting from the fermentation of milk from different sources by specific microorganisms or complex microbial communities [64]. It is estimated that yogurt contains more than 109 viable bacteria per milliliter of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [112]. Previous studies have assessed yogurt, the main sources of probiotics in human diets, and have reported associations of yogurt with improved immune health [39] and reduced risks of various diseases, including metabolic disorders [94], type 2 diabetes [8], cardiovascular diseases [21], cancers [108], and mental diseases [62]. Despite the extensive studies have been conducted on the link between consuming yogurt and the risk of depression and anxiety [77; 81; 87; 88; 89], the underlying mechanism remains unclear.

Herein, we investigated the effects of probiotics (*Lactobacillus reuteri and Lactobacillus bulgaricus*) fermented yogurts on alleviating the anxiety- and depression-like behaviors in chronic unpredictable mild stress (CUMS) mice via a series of behavioral tests. Furthermore, the modulation of yogurts on monoamine neurotransmitters and inflammatory cytokines in the serum, and the signaling pathways in the hippocampus of CUMS mice was evaluated. We also assessed the maintaining cerebral activity and protection on the neuronal survival in hippocampus of yogurt with a combination of [18F]-FDG PET/CT imaging *in vivo* and Nissl staining. To gain insights into the potential mechanism of yogurt impacting the microbiota-gut-brain axis and alleviating depression and anxiety, we utilized full-length 16S rRNA sequencing and untargeted metabolomic analysis

to conduct gut microbiota composition and fecal metabolites in CUMS mice. The results presented in this work may provide valuable insights into the mechanism study of probiotics fermented yogurt for the treatment of depression and anxiety.

2. Materials and Methods

2.1 Experimental animals and chemical reagents

Thirty-six male ICR mice (28–34 days old, 20–25 g) were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. with production license number SCXK (Shandong) 2022 0006. The animals were adapted to the laboratory conditions for 1 week before the experiment and comply with the guidelines for the care and use of laboratory animals as described by the U.S. National Institutes of Health. The animal study was reviewed and approved by the Medical Ethics Committee of Taian Central Hospital (Approval number: No. 2024-05-70).

Mice food was purchased from Keao Xieli Feed (Beijing, China) with the food guarantee analysis of 10.0% humidity, 4.0% fat, 8.0% ash, 1.5% calcium, 20.0% protein, 5.0% fiber, and 0.6% phosphorus. The content is as follows: corn, fish meal, yeast powder, vegetable oil, salt, vitamins, and minerals. Fluoxetine hydrochloride capsules were purchased from Patheon (20 mg/tablet, France). Carprofen and RIPA buffer containing a protease and phosphatase inhibitor cocktail were purchased from Merck Science & Technology (Shanghai, China); IP cell lysis buffer, BCA assay and toluidine blue were purchased from Servicebio Biotechnology (Wuhan, China); ELISA kits for tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), lipopolysaccharides (LPS), 5-hydroxytryptamine (5-HT), dopamine (DA), primary antibodies anti-brain derived neurotrophic factor (BDNF), anti-recombinant signal transducer and phosphorylated activator of transcription 3 (p-STAT3), anti-cAMP-response element binding protein (CREB), anti-neuron-specific enolase (NSE), anti-nuclear factor kappa-B (NF-κB), anti-β-actin and the horseradish peroxidase–linked secondary antibody were purchased from Abways Technology (Shanghai, China).

Fresh cow milk was purchased from Taian Dairy (Taian, China). The fresh milk was fermented in warm water at 40 ℃ for 6h, and then was put into the refrigerator at 4 ℃ for 12 h after reaching the solidified state. The determination of the end point of yoghurt fermentation was the is solidified state and there was no excessive whey separation. *L. reuteri* and *L. bulgaricus* were purchased from Zhenjiang Tianyi Biotechnology (Jiangsu, China). The 1.2 g colonies of *L. reuteri* and *L. bulgaricus thermophilus* was added to 110 mL of fresh cow milk. The fluoxetine solution was prepared by 20 mg of fluoxetine dissolving in 20 mL of Milli-Q water to a final concentration of 1 mg/mL. Mice in the fluoxetine-treated group were gavage with 0.1 mL of fluoxetine solution per day (5 mg/kg/day).

2.2 Chronic unpredictable mild stress (CUMS)

The CUMS experiment was conducted as reported by Katz et al [48]. Thirty-six mice were randomly and averagely divided into six groups. They were non-stressed on normal diet group (control group, [Con]), stress group ([CUMS]), stress fed fluoxetine group (5 mg/kg/day, [CUMS-F]), stress fed milk group (nightly free feeding, [CUMS-M]), stress fed *L. reuteri* yogurt group (nightly free feeding, [CUMS-R]) and stress fed *L. bulgaricus* yogurt group (nightly free feeding, [CUMS-B]). For [Con] group, six mice were housed in cage. For [CUMS], [CUMS-F], [CUMS-M], [CUMS-R] and [CUMS-B] group, one mouse was initially housed in a cage (320 mm × 210 mm × 160 mm) in a room under normal conditions (25 ± 1°C, 50 ± 2% humidity, and a 12 h light/12 h dark cycle) with free access to food and water. Single-housed stress elicited a range of social isolation-related behavioral and neurobiological abnormalities [58; 70].

The CUMS group was housed separately, and the following stressors were applied for 7 weeks: food deprivation (24 hours), water deprivation (24 hours), tail pinch (1 cm from the tail end, 1 min), forced swimming (4°C ± 2°C, 5 min), inversion of day/night light cycle, cage tilt (45°C, 8 hours), cage shaking (180 rpm, 10 min), moist bedding (200 mL, 8 hours), and captivity (placing the mouse in a 50 mL centrifuge tube for 30 min. These nine stressors were arranged in a sequenced order every day to produce an unexpected mild stress effect and repeated throughout the 7-week experiment [17; 33]. A variety of stresses were randomly arranged one or two kinds every day for 7 weeks. From the fifth week, the mice in the fresh cow milk and

yogurt groups were given the prepared cow milk and yogurt from 8 p.m. to 8 a.m. the next day for 3 weeks. At the same time, the mice in the drug group were given fluoxetine hydrochloride by gavage at a dose of 5 mg/kg/day for 3 weeks. From the seventh week, the behavioral tests were carried out between 8:30 am and 11:30 am for 1 week. The behavioral tests timeline for CUMS-induced mice in this study was shown in Scheme 1.

2.2.1. Sucrose preference test (SPT)

The sucrose preference test (SPT) was conducted as previously described [107]. Briefly, the mice were cut off food and water 24 h before the experiment. Then, mice were given a bottle of 1.0% (wt/vol) sucrose water and a bottle of tap water for 24 h. The weight of each bottle was recorded before and after the SPT. Sucrose preference was calculated as follows: consumption (%) = [sucrose consumption / (sucrose consumption + water consumption)] × 100%.

2.2.2. Tail suspension test (TST)

The mice were elevated by their tails at a height of 40 cm above the floor for 6 min, and an adhesive tape was placed 1 cm from the tail tip. The time of immobility was recorded during the final 4 min of the test [45]. Stillness refers to the absence of any movement of the body during suspension.

2.2.3. Forced swimming test (FST)

Each mouse was individually forced into an open vertical cylindrical container (35 cm in height and 12 cm in diameter) containing tap water at 25 ± 1°C and 20 cm in depth. The experiment lasted 6 min and the immobility time of mice, referring to mice that floated in the water and only kept their nose above the water, was scored over the last 4 min [106].

2.2.4. Elevated plus-maze test (EPM)

The EPM apparatus consisted of two open arms (50 × 10 cm), two enclosed arms (50 × 10 cm), connected by a central area (10 × 10 cm), and raised to a height of 50 cm above the floor. On the day of the EPM test, mice were placed on the center platform of an instrument facing an open arm and allowed to explore the maze freely for 5 min [95]. The percentage of time spent in open-arm were calculated.

2.2.5. Hole-board test (HBT)

The testing apparatus was consisted of a nontransparent white plastic box with a raised floor containing the arena (100 × 100 cm, opaque black) with 16 holes (4 cm in diameter) located at equal distances from one another, arranged by four in four rows. The holes were restricted to a central zone of the arena (75 × 75 cm) whereas the outer zone (12.5 cm in width) was situated along all the walls (40 cm of height). Each single mouse was put into the middle point of the floor and enabled to move freely for 10 min. The number of head-dipping (both eyes hidden in the hole) were calculated.

2.2.6. Light-dark transition test (LDTT)

The testing apparatus was composed of an open chamber with white floor (30 cm × 60 cm) equipped with infrared light sensors and one equivalent dark chamber with black walls and lid, which is connected by an opening (13 cm × 5 cm) for mice to shuttle freely. The apparatus was placed directly below the camera and the light chamber was illuminated by two 40-watt light bulbs. Mice were released in the center of the light chamber and allowed to explore freely for 5 min. Time spent in the light chamber was recorded to measure the anxiety-like behavior of mice.

2.2.7. Open field test (OFT)

The open field apparatus was a box (50 × 50 × 28 cm) with a floor divided into 25 squares. The nine central squares were defined as the center. Each mouse was placed in the center area of the box for 10 min, and its movements were digitally recorded using a camera fixed above the floor and analyzed with a video-tracking system. The total number of the entries into the central area, activity trajectory, traveled distance as well as time spent in the central area were evaluated.

2.3. Nissl staining

Nissl staining was conducted as previously described [23]. Tissue sections were prepared 5 μm in thickness. Paraffin the sections were dewaxed with xylene (3 times, 10 min each time), graded alcohol solutions (100%, 90%, 70%, once for 5, 2, and 2 min, respectively), and distilled water (5 min). Then, the tissue sections were stained with 0.5% toluidine blue at room temperature for 1 h, dehydrated with 95% ethanol, baked at 65°C for 4 h, cleared with xylene for 10 min, and sealed with neutral gum. Subsequently, the slides were finally mounted and covered with coverslips. The total number of cell hippocampus was observed and counted using an optical microscope (×400, Nikon E100, Japan), and the number of surviving cells was counted using ImageJ. The numbers of surviving Nissl cells were counted served as neuronal density.

2.4. PET/CT scanning

Prior to the PET scans, the mice were fasted for 12 h. Each mouse was injected approximately 0.5 mCi (18.5 MBq) [18F]-FDG in less than 0.5 ml via tail vein. The injection was completed in less than 1 min and the injection site was pressed for 30 s to prevent leakage. The exact injection time and the radio activity of the syringe both before and after the injection, together with their measure time, were recorded for dosage calibration. After an uptake period of 55 min, the mouse was anaesthetized with a gas mixture of 1% isoflurane and oxygen (1 L/min) and then prone positioned in the gantry with a mask covering its mouth to ensure continuous gas inhalation throughout the scan. The PET signals were acquired from approximately 60 min post-injection for 10 min 67000 IRIS PET/CT scanner (Inviscan, France) designed for high resolution imaging of small laboratory animals. The following CT scan was performed for localization and attenuation correction. For data analysis, the region of interest (ROI) was manually drawn on the CT images, and the ROI was copied to the corresponding PET images. The mean standard uptake value (SUV mean) of the ROIs were recorded.

2.5. ELISA

Before euthanasia, mice were weighed and injected with 5 mg/kg carprofen as an analgesic. Then, mice were anesthetized (isoflurane 2–3% mixed with 30% O_2 and 70% N_2O) and the blood samples were collected from the inferior vena cava. The whole blood was incubated at 37°C for 20 min, kept at 4°C for 2 h, and then centrifuged (3500rpm, 4°C, 15 min). The serum was collected and stored at -80°C until use. Subsequently, the mice were immediately killed by decapitation, and the brain tissues were removed and stored at -80°C until use. The hippocampus was recovered and homogenized with ice cold np40 cell lysis buffer. The hippocampus levels of the 5-HT, DA, TNF-α, IL-6, IL-10, LPS were determined using appropriate ELISA kits according to the manufacturer's instructions.

2.6. Western Blot

Hippocampal brain tissues were homogenized in RIPA buffer containing a protease and phosphatase inhibitor cocktail and then quantified by BCA assay according to the manufacturer’s protocol. The quantified proteins (2 μg/lane) were separated on 10% SDS-PAGE and then blotted onto polyvinylidene fluoride membranes. After blocked with 5% skim milk for 1 h at room temperature, the membranes were then incubated with a primary antibody, BDNF, CREB, p-STAT3, NSE, NF-κB and anti-β-actin overnight at 4°C in 5% skim milk in TBST. After incubation with the horseradish peroxidase-linked secondary antibody for 2 h, immunoreactive proteins were detected using a chemiluminescence detection system (LI-COR Biosciences, Lincoln, NE, USA) and then analyzed using ImageJ [56].

2.7. 16S rRNA analysis of fecal microbiota

Fecal samples of mice for DNA sequencing were collected after 7 weeks, with a uniform sampling time between 10:00 and 11:00 am to avoid heterogeneity. 2 mL Safe-lock tubes (Eppendorf, Netherlands) were used to collect fecal samples for storage at -80 ℃ after being snap frozen in liquid nitrogen.

About 100–200 mg of fecal matter per sample was used for genomic DNA isolation with the PSP® Spin Stool DNA Kit (STRATEC Molecular GmbH, Berlin, Germany) as per the manufacturer’s instructions for difficult to lyse bacteria. Genomic DNA integrity and purity were verified by 1% agarose gel electrophoresis, and the DNA concentration and purity were measured using a Nano Drop One instrument. Genomic DNA was used as a template for PCR amplification and electrophoretic detection of the product, with V3V4 selected as the sequencing region. Gene Tool analysis software (version

4.03.05.0, Syn Gene) was used to compare the concentrations of PCR products, calculate the volume required for each sample, and mix the PCR products for each group. The E.Z.N.A. gel extraction kit was used to recover PCR products and TE buffers were used for elution and recovery of DNA fragments. The sequencing library was constructed according to the standard NEBNexti lula library preparation kit and a high-throughput sequencing platform (Illumina Hiseq) was used for sequencing.

For 16S rDNA gene sequencing analysis, one-way ANOVA was used to determine the differential alpha diversity (Chao1 index and ACE index) of different groups. β diversity was calculated based on the Bray Curtis algorithm and visualized using principal coordinate analysis (PCoA) and distance. Significant differences between sample groups were determined using the ANOSIM test. Taxonomic changes at the phylum and genus levels between different groups are presented as histograms using R language. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify differential microbiota between groups.

2.8. Untargeted metabolomics analysis

For metabolomic analysis, 120 μL of methanol was added to 20 mg of fecal sample, then homogenized for 1 min and centrifuged at 14000 r/min for 10 min at 4 ℃. The supernatant (100 μL) was collected and centrifuged at 14000 r/min for 10 min at 4 ℃. The supernatant (50 μL) was then collected for analysis by UPLC-Q-TOF-MS. In addition, 10 mg of each fecal sample was treated as a quality control sample according to the above method. Prior to analysis, the sample was injected continuously three times to ensure good stability of the instrument. During analysis, the injection of 6 test samples was always followed by the injection of one control sample.

The chromatographic separation parameters were as follows: American Waters corporation BEH C8 chromatographic column (1.7 μm, 2.1 × 100 mm), mobile phase is formic acid aqueous solution (A) - acetonitrile (B), gradient elution: 0–1 min, 5% B; 1.1–11 min, 5%-100% B; 11.1–13 min, 100% B; 13.1–15 min, 5% B. The volume flow rate is 0.35 ml/min and the injection volume is 5 μL. Mass spectrometry parameters: ionization modes are electrospray positive ion mode and negative ion mode; Spray Voltage is 3.8 kV; Sheath gas flow rate is 35 Arb; Aux gas flow rate is 8 Arb; Mass range (m/z) is 70-1050; TopN is 5; NCE/stepped NCE are 20 and 40; Capillary temperature is 320 ℃; Aux gas heater temperature is 350 ℃; S-lens RF level is 50; Full ms resolution is 70,000; MS/MS resolution is 17,500. All other parameters were left at the default setting.

2.9. Statistical analysis

Statistical analysis was performed using ANOVA and Student-Newman-Keuls *post hoc* test for one-way analysis of variance and two-sample comparisons using Graphpad prism 6.0 statistical software. Results are presented as mean ± standard error SEM. Differences were considered statistically as ****$p < 0.0001$, ***$p < 0.0005$, **$p < 0.01$, *$p < 0.05$ and $^{\#}p > 0.05$. Black sign (*) is the difference of [CUMS] compared with [Con], [CUMS-F], [CUMS-M], [CUMS-R] and [CUMS-B]. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

3. Results

3.1. Lactobacillus fermented yogurts alleviate depressive behavior in mice

The body weight and sucrose consumption were monitored during for CUMS treatment, and the forced swimming test (FST) as well as tail suspension test (TST) were performed to evaluate depressive behavior. As shown in Fig. 1A, the change of body weight in mice between groups showed significant differences after 7 weeks [$F(5, 30) = 139.1$; $p < 0.0001$], and the critical weight loss of mice was observed in the CUMS ([CUMS]) group as compared to that of the control ([Con]) group ($p < 0.0001$), which suggested that depression was induced with CUMS in mice. The body weight gain significantly increased in the milk ([CUMS-M], $p = 0.0005$), *L. reuteri* ([CUMS-R], $p < 0.0001$) and *L. bulgaricus* yogurt group ([CUMS-B], $p = 0.0026$) compared with the fluoxetine ([CUMS-F]) group (Fig. 1A insert). On the other hand, the [CUMS-R] group mice gained a

significant body weight ($p < 0.0001$) compared to [CUMS-M] and [CUMS-B] groups. No significant difference between the [CUMS-M] and [CUMS-B] groups ($p = 0.1365$) was observed.

The sucrose consumption reduced significantly in [CUMS] mice, and [CUMS-R] and [CUMS-B] treatments could alleviate this reduction as the antidepressant fluoxetine did ([$F(5, 30) = 55.91$; $p < 0.0001$], Fig. 1B). However, no difference in the sucrose intake preference through the experiment between the groups was observed.

After 6 weeks of CUMS, the behavior tests including the FST and TST were used to evaluate the influence of the CUMS interventions. As seen from Figs. 1C and 1D, [CUMS-M], [CUMS-R] and [CUMS-B] groups had significantly reduced immobility time when compared with mice in the [CUMS] group in the FST ([$F(5, 30) = 129.0$; $p < 0.0001$]) and TST ([$F(5, 30) = 169.3.0$; $p < 0.0001$]). There was no significant difference between *Lactobacillus* yogurt groups and [CUMS-F] treatment in the FST ($p = 0.0568$ for [CUMS-R] and $p = 0.0053$ for [CUMS-B]) and TST ($p = 0.0084$ for [CUMS-R] and $p = 0.0621$ for [CUMS-B]) tests. The results indicated that the *Lactobacillus* fermented yogurt treatment might alleviate the depressive behaviors in mice like fluoxetine did.

3.2. Lactobacillus fermented yogurts alleviate anxiety behavior in mice

The Elevated plus-maze test (EPM), hole-board text (HBT), light-dark transition test (LDTT) and open field test (OFT) were used to evaluate anxiety behavior and the results were shown in Fig. 2. Noteworthy, the locomotor activities for the mice treated with *Lactobacillus* yogurt enhanced, such as the time spent in the open arms ([$F(5, 30) = 14.7$; $p < 0.0001$]), the frequency of exploring in the hole board ([$F(5, 30) = 232.1$; $p < 0.0001$]), the time spent in the light box ([$F(5, 30) = 57.29$; $p < 0.0001$]), and the frequency of light/dark transition ([$F(5, 30) = 17.03$; $p < 0.0001$]) (Figs. 2A-D). While, there was no significant difference for the time spent in the open arms, the frequency of exploring in the hole board, time spent in the light box and the frequency of light/dark transition in the [CUMS-R] and [CUMS-B] group was not significantly different compared to the [CUMS-F] group in the EPM, HBT and LDTT.

Similarly, as per OFT results (Figs. 2E-H), the number ([$F(5, 30) = 15.16$; $p < 0.0001$]) and time ([$F(5, 30) = 34.69$; $p < 0.0001$]) of entering into the central area as well as the total distance ([$F(5, 30) = 9.211$; $p < 0.0001$]) were significantly increased in the milk and *Lactobacillus* yogurt groups when compared to the [CUMS] group. Notably, the mice from the [CUMS-R] and [CUMS-B] group had a higher frequency and longer distance entering the central area in OFT that those from the [CUMS-F] group. No significant difference for the time ($p = 0.0316$), number ($p = 0.3991$) and distance ($p = 0.4903$) in the OFT between [CUMS-R] and [CUMS-B] groups was observed.

The above results showed that *Lactobacillus* fermented yogurt and fluoxetine treatment might play a similar role in alleviating the anxiety-like behavior in CUMS mice even better in the OFT.

3.3. Lactobacillus fermented yogurts protect neuronal survival in hippocampus and maintain cerebral activity

To further elucidate the effect of probiotics fermented yogurt on neuronal damage in CUMS mice, the Nissl staining was utilized to investigate changes in the morphology of pyramidal neurons in the hippocampus. Normal neurons presented with round and pale stained nuclei, with this morphology disappearing in the dead cells. As shown in Fig. 3A and Fig. S1 (Supporting Information), the Nissl blue-stained neurons in the [CON] group showed circle and intensity cells with clear cell membranes, nuclei, and discrete nucleoli when compared with the [CUMS] group that presented a typical damage feature in the hippocampus. The milk and *Lactobacillus* yogurt groups displayed a significant reduction in neuronal deterioration compared with the [CUMS] group.

The [18F]-FDG PET scanning method was used to gain a comparison of whole-brain glucose uptake was performed to detect neuronal activity at the regional level. The color represents the amount of uptake, and the areas of interest included the hippocampus and prefrontal cortex. The standardized uptake value (SUV, g/mL) was used to evaluate glucose metabolism in the hippocampus and prefrontal cortex regions.

As shown in Fig. 3B, compared with the [CON] group, [CUMS] group had a significant decreased glucose uptake on brain [18F]-FDG signal in the both hippocampus ([F(5, 30) = 1079; p < 0.0001]) and prefrontal cortex ([F(5, 30) = 1260; p < 0.0001]). Treatment with *Lactobacillus* yogurt completely reversed the decreases in [18F]-FDG signal. Especially, it was found that the SUVs level of the prefrontal cortex in the *Lactobacillus* yogurt group were significantly higher than those in the [CUMS-F] group (p = 0.002 for [CUMS-B] and p < 0.0001 for [CUMS-B]) and the SUVs level in the hippocampus was no significant difference from those of the [CUMS-F] group (p > 0.05). No difference was seen in the SUVs level of the hippocampus and prefrontal cortex between [CUMS-R] and [CUMS-B] groups (p > 0.05). The results suggested that the *Lactobacillus* yogurt treatment might have an overall protective effect on neuronal survival hippocampus and reverse the reduction of the cerebral activity in CUMS mice by increasing FDG uptake in the brain.

3.4. Lactobacillus fermented yogurts ameliorate levels of monoamine neurotransmitters and inflammatory cytokines

The effects of *Lactobacillus* yogurt on the levels of the monoamine neurotransmitters 5-HT and dopamine in the hippocampus of CUMS mice were measured by ELISA. As seen from Figs. 4A-B, the ELISA analysis revealed significant effects of treatments in the content of 5-HT [F(5, 30) = 53.12; p < 0.001] and DA [F(4, 35) = 12.34; p < 0.001] among groups. The concentration of 5-HT and dopamine was notably higher in the hippocampus of CUMS mice, whereas the [CUMS-R] (p < 0.0001 for both 5-HT and DA) and [CUMS-B] (5-HT, p < 0.0001; DA, p = 0.0002) treatment prevented the decrease of 5-HT and dopamine. Even, the mice in the [CUMS-R] and [CUMS-B] group showed a noteworthily higher levels of 5-HT (p = 0.0006 and p = 0.0043) than those in the [CUMS-F] group. Interestingly, the DA level of [CUMS-B] treatment was slightly higher than that of [CON] group (p = 0.1969). No significant difference was found between [CUMS-R] and [CUMS-B] groups (5-HT, p = 0.6287; DA, p = 0.0064). The results demonstrated that *Lactobacillus* yogurt might reverse the reduction of monoamine neurotransmitters levels in the hippocampus of CUMS mice.

We further measured the levels of and proinflammatory cytokines (IL-6, TNF-α and LPS) and anti-inflammatory cytokine (IL-10) in the blood serum and results shown in Figs. 4C-4F. It was suggested notable effects of treatments in the levels of IL-6 [F(5, 30) = 23.52; p < 0.0001], TNF-α [F(5, 30) = 41.59; p < 0.001], LPS [F(5, 30) = 14.92; p < 0.001], and IL-10 [F(5, 30) = 37.26; p < 0.0001] among groups. The IL-6, TNF-α and LPS levels of the [CUMS-R] and [CUMS-B] groups were significantly lower than that of the [CUMS] group (p < 0.0001, each), while no significant difference of these proinflammatory cytokines levels in [CUMS-R] (IL-6, p = 0.2272; TNF-α, p = 0.2737 and LPS, p = 0.0355) and [CUMS-B] (IL-6, p = 0.2751; TNF-α, p = 0.0062 and LPS, p = 0.0084) treatment was observed when compared with [CUMS-F] group. It is worth noting that we observed a slight increase in the IL-6 and LPS levels in the [CUMS-R] (p = 0.6625 and p = 0.0189) and [CUMS-B] (p = 0.002 and p = 0.0007) groups compared with [CON] group. As compared with the [CUMS] group, a remarkable increase in the anti-inflammatory cytokine IL-10 level of the [CUMS-R] (p < 0.0001) and [CUMS-B] (p = 0.0002) groups was observed. Moreover, there was no significant difference IL-10 level in between the *Lactobacillus* yogurt treatment ([CUMS-R], p = 0.0473 and [CUMS-B], p = 0.0071) and [CUMS-F] group. The above results indicated that *Lactobacillus* yogurt treatment might ameliorate the inflammatory cytokines levels changes in the blood serum of CUMS mice.

3.5. Lactobacillus fermented yogurts modified pathway expression in CUMS mice

To estimate possible mechanism of *Lactobacillus* fermented yogurts alleviating the anxiety- and depressive-like behavior in CUMS mice, the CREB, BNDF, NSE, p-STAT3 and NF-κB p65 expression were detected. As seen from Fig. 5, the treatments had significant effects on the expression of CREB (F(5, 30) = 94.44; p < 0.0001), BNDF (F(5, 30) = 167.3; p < 0.0001), NSE (F(5, 30) = 90.39; p < 0.0001), p-STAT3 (F(5, 30) = 456.3; p < 0.0001) and NF-κB p65 (F(5, 30) = 439.0; p < 0.0001) among groups. Moreover, the expression of CREB, BNDF, NSE and p-STAT3 in the *Lactobacillus* fermented yogurts groups (p < 0.0001, each) were significantly higher than those in [CUMS] group. Interestingly, the CREB, BNDF, NSE and p-STAT3 levels showed a significant enhance in the [CUMS-B] group (p < 0.0001, each) as compared with [CUMS-F] group. Moreover, the [CUMS-B] treatment upgraded the downregulation of CREB, BNDF, NSE and p-STAT3 expression more effective than that of [CUMS-R] treatment (p < 0.0001, each). The *Lactobacillus* fermented yogurts downregulated remarkably the NF-κB p65 expression that

was upregulated in the [CUMS] group ($p < 0.0001$, each). Even, the NF-κB expression in the [CUMS-R] and [CUMS-B] treatments was lower notably than that of [CON] ($p = 0.0017$ and $p = 0.0002$) and [CUMS-F] group ($p < 0.0001$, each). No significant difference was observed in NF-κB p65 expression between *Lactobacillus* fermented yogurts groups ($p = 0.0409$). The results indicated that *Lactobacillus* fermented yogurts might reverse the reduction of CREB, BNDF, NSE and p-STAT3 expression in the hippocampus and the enhancement of NF-κB p65 expression in the blood serum of CUMS mice. And *L. bulgaricus* fermented yogurt might have a better performance in reversing these changes that fluoxetine.

3.6. Fermented yogurt modulated the gut microbiota composition in CUMS mice

To evaluate the effect of *Lactobacillus* fermented yogurt, which can alleviate the depression and anxiety behaviors, on the modulation of gut microbiota, 16S rRNA sequencing analysis was performed on the fecal samples in all the above groups.

The sequence counts in the [CON], [CUMS], [CUMS-F], [CUMS-M], [CUMS-R], [CUMS-B] groups were 31,754, 30,551, 32,700, 30,281, 33,466 and 30,305, respectively (Fig. S2), while the observed species (OTU counts) were 5,630, 3,106, 3,543, 3,625, 4,698 and 5,023, respectively (Fig. 6A). While alpha diversity analysis, as measured by richness (Chao1 index and ACE index) in the gut microbiota of the [CUMS] treatment mice were significantly lower than those in the [CON] group ($p < 0.0001$, Fig. 6A), suggesting that CUMS intervention significantly reduced microbial community richness. Interestingly, the [CUMS-R] and [CUMS-B] groups notably ($p < 0.0001$, each) reversed the decreased trend of ACE and Chao1 index found in [CUMS] group. Compared with [CUMS-F] group, the Chao1 and ACE index also significantly increased in the *Lactobacillus* yogurt groups ($p < 0.001$, each). The beta diversity of the microbial community using Principal Coordinate Analysis (PCoA) and distance based on Bray-Curtis dissimilarity clustering of the intergroup differences was further assessed (Fig. 6B). The conclusion validated by ANOSIM testing result ($R = 0.498$, $p < 0.001$) demonstrated that the gut microbiota of each group clustered significantly. The assessment of microbial community structural differences distance heatmap shown in Fig. 6C suggested the significant differences in the microbial composition and structure among groups ($p < 0.001$). The results suggested that the *Lactobacillus* yogurt might modulate notably the gut microbiota composition of CUMS mice. As modulations in the dominant gut microbiota after probiotic intervention may be responsible, further analyses are required to find out whether the composition and abundance changes of gut microbial taxa may be related to anxiety and depression-like behaviors.

As seen from the species abundance cluster heatmap (Fig. 6D), at the phylum level, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria* and *Deferribacteres* predominated the [CON] group. The [CUMS] treatment increased the relative abundance of *Bacteroidetes*, but down-regulated the relative abundance of the other bacterial phyla mentioned above. Compared with [CUMS] group, the gut of mice in the [CUMS-R] and [CUMS-B] groups presented more *Firmicutes* but less *Bacteroidetes*, *Proteobacteria* and *Deferribacteres* with and a higher *Firmicutes/Bacteroidetes* (F/B) ratio.

At the genus level (Fig. 6E), *Kineothrix, Duncaniella* and *Dysosmobacter* were dominated in the [CON] group. And the [CUMS] group upregulated the abundance of *Ligilactobacillus*, *Eisenbergiella*, *Odoribacter*, but down-regulated the abundance of *Akkermansia*, *Herbinix* and *Muricomes*. Moreover, *Schaedlerella*, *Odoribacter* and *Kineothrix* were most abundant in the [CUMS-R] group, while *Kineothrix*, *Blautia* and *Acetatifactor* dominated in the [CUMS-B] group. Compared with [CUMS] group, the abundance of *Schaedlerella Lacrimispora* and *Streptococcus* in the [CUMS-R] group and the abundance of *Blautia*, *Faecalimonas* and *Streptococcus* were upregulated in the [CUMS-B] group. Additionally, that of *Ligilactobacillus* and *Eisenbergiella* was decreased in both yogurt groups.

As seen from Fig. 6H, at the family level, the relative abundance of and *Lactobacillaceae* and *Odoribacteraceae* were elevated, while the *Lachnospiraceae* and *Oscillospiraceae* declined in the [CUMS] group as compared with [CON] group. The fermented yogurt treatment groups reversed these [CUMS]-related changes. The genera *Lactobacillus*, *Lachnospiraceae*, and *Oscillospiraceae*, belong to the Firmicutes phylum, and the genera *Odoribacteraceae* belong to the *Bacteroidetes* phylum, were altered by CUMS, and these changes were found to be improved by fermented yogurt treatment. Although microbiota composition was changed after CUMS treatment and by fermented yogurt supplementation, the difference between the [CUMS-R] and [CUMS-B] groups were not significant. The relative abundance heatmap shown in Fig. 6F also indicated the

abundance similarity clustering of each species among groups at the phylum level, suggesting that the microbial composition disparities might be responsible for the *Lactobacillus* fermented yogurt induced ecological shifts.

Functional prediction using PICRUSt2 at level 2 revealed significant differences in the relative abundance of KEGG functional pathways among groups. As shown in Fig. 6G, enrichment in pathways related to human disease (such as neurodegenerative disease, cancer and infectious disease) and cellular activity (amino acid metabolism, membrane transport, signal transduction and cell motility) in *Lactobacillus* fermented yogurt groups suggested potential mechanistic associations with treatment effects. These results indicated distinct metabolic capacities and ecological functions in the two *Lactobacillus* fermented yogurts.

3.7. Fermented yogurt modulated gut metabolites and metabolic pathway in CUMS mice

To investigate the modulation of *Lactobacillus* fermented yogurt on the metabolomic profiling of CUMS mice. chronic stress upon the metabolomic profiling, untargeted metabolomic analysis was performed to analyze the metabolite composition in the fecal samples from all groups of mice using LC-MS/MS. There was a total of 228 metabolites were identified under positive and negative ion detection modes in TIC, in which 74 compounds were annotated to the Kyoto Encyclopedia of Genes and Genomes (KEGG), 120 compounds were annotated to Human Metabolome Database (HMDB) and 32 compounds were annotated to Lipid maps, respectively. As shown in Fig. 7A, the organic oxygen compounds, organic acids and derivatives, lipids and lipid-like molecules predominated in the metabolites, accounting for 31.98%, 25.51%, 19.84% and 7.69%, respectively. To evaluate differences and clustering patterns among groups, Principal Component Analysis (PCA) was performed (Fig. 7B). The result showed a clear separation between the [CUMS] group and [CUMS-R]/[CUMS-B] groups, indicating significant alterations of fermented yogurt treatment on the metabolites of CUMS mice. And, there were three distinct clusters among the milk and fermented yogurt groups, suggesting samples from different treatments were clearly distinguished.

The metabolic differences of between the [CON], [CUMS-F], [CUMS-R], [CUMS-B] group and the [CUMS] group were further identified and shown in Fig. 7C. The differential metabolites between [CON] group and [CUMS] group were detected as 86, in which 48 metabolites were upregulated and 38 metabolites were downregulated. A total 85 differential metabolites with were determined between [CUMS-R] group and the [CUMS] group, in which 47 metabolites were upregulated and 38 metabolites were downregulated. For the [CUMS-B] group, the differential metabolites were identified as 93 as compared with [CUMS] group, in which 67 compounds were upregulated and 26 compounds were downregulated. Notably, the heatmap of top 20 differential metabolites [CON], [CUMS-F], [CUMS-R], [CUMS-B] group and the [CUMS] group were presented in Fig. 7D. Among them, 9 compounds belonged to the organic oxygen compounds, 8 belonged to the organic acids compound. We found that the levels of fatty acids (oleic acid, linoleic acid, myristic acid, gentisic acid) in the feces of the CUMS mice were all increased by *Lactobacillus* fermented yogurt. After the classification of the matched metabolites to their relevant pathways annotated by KEGG (summarized in Table 1), it has been determined that the upregulated and downregulated metabolites were associated with energy, lipid, carbohydrate, nucleotide, amino acid metabolism. And the metabolites were also responsible for nervous, immune, endocrine, digestive and circulatory systems (Fig. 7E). Especially significant was the involvement of long-term depression, inflammatory mediator regulation of tryptophan channels, microbial metabolism in diverse environments, longevity regulating pathway, cell growth and death pathway. Moreover, the upregulated metabolites associated pathway, such as the GnRH and Fc epsilon RI signaling pathway and Fc gamma R-mediated phagocytosis, involving immune system; the biosynthesis of phenylpropanoids, alkaloids, terpenoids and steroids and polyketides, involving anti-inflammation, antioxidant, and anti-Alzheimer's disease; the GnRH and Oxytocin signaling pathway, relating with the modulation of the autonomic nervous system via the vagal pathway, serotonergic and dopaminergic synapse as well as retrograde endocannabinoid signaling. These results might suggest the potential mechanism of the *Lactobacillus* fermented yogurt alleviated the anxiety- and depression-like behaviors via changing the metabolites in CUMS mice.

Table 1

Summary of differential metabolites of [CUMS] group compared with [CON], [CUMS-F], [CUMS-M], [CUMS-R] and [CUMS-B] groups. Differential metabolites screening threshold, *p*-value ≤ 0.05.

Sample	Up-regulated compounds	Pathway	Down-regulated compounds	Pathway
[CUMS] *vs* [CON]	Mucic acid, maltose, maleic acid	Carbohydrate metabolism	Salicin, L-malic acid, succinic acid, xylitol, sorbitol	Carbohydrate metabolism
	creatine acid, Sulfuric acid, maleic acid, indole-3-acetic acid	Amino acid metabolism	Glutaric acid, succinic acid, sarcosine, 3,4-dihydroxyphenylacetic acid, succinic acid	Amino acid metabolism
	Dihydroxyacetone	Lipid metabolism, Energy metabolism, Carbon metabolism	Glutaric acid, behenic acid, lignoceric acid	Lipid metabolism
	Sulfuric acid, maleic acid, dihydroxyacetone	Microbial metabolism in diverse environments	Glutaric acid, pyridoxine, L-malic acid, 3,4-dihydroxyphenylacetic acid, succinic acid	Microbial metabolism in diverse environments
	Sulfuric acid, inosine	Nucleotide metabolism	Adenine	Nucleotide metabolism
	maleic acid	Nicotinate and nicotinamide metabolism	Succinic acid	Nicotinate and nicotinamide metabolism
	sulfuric acid, mannitol, inosine, maltose	ABC transporters	Sorbitol, biotin, xylitol	ABC transporters
	maltose	Carbohydrate digestion and absorption	3,4-Dihydroxyphenylacetic acid, succinic acid	Nervous system, GABAergic synapse, Dopaminergic synapse, Degradation of aromatic compounds
	Sulfuric acid, maltose	Biosynthesis of secondary metabolites	Pyridoxine, succinic acid	Metabolism of cofactors and vitamins
	Sulfuric acid, maltose	Biosynthesis of secondary metabolites	L-Malic acid, succinic acid	Energy metabolism, lipid metabolism, glucagon signaling pathway, proximal tubule bicarbonate reclamation
			Pyridoxine, biotin	Biosynthesis of cofactors
			Ferulic acid, salicin, L-malic acid, succinic acid, succinic acid, 4-vinylphenol, adenine	Biosynthesis of unsaturated fatty acids, phenylpropanoids, alkaloids
			succinic acid	cAMP signaling pathway
[CUMS] *vs* [CUMS-F]	Pyruvic acid, indole-3-acetic acid, L-malic acid, mucic acid	Amino acid metabolism, Carbohydrate metabolism	Succinic acid, glutaric acid, 3,4-dihydroxyphenylacetic acid	Amino acid metabolism
	Pyruvic acid, L-malic acid	Biosynthesis of phenylpropanoids, terpenoids and steroids, alkaloids, signal transduction	Salicin, succinic acid, xylitol, sucrose, maltose	Carbohydrate metabolism

Sample	Up-regulated compounds	Pathway	Down-regulated compounds	Pathway
	Cholesterol	Digestive system, lipid metabolism	Salicin, succinic acid	Biosynthesis of phenylpropanoids, terpenoids, steroids and alkaloids
	Pyruvic acid	HIF-1 signaling pathway	Sucrose, maltose, biotin, phosphate	Digestive system
	Pyruvic acid, cholesterol	Metabolism of cofactors, vitamins, terpenoids and polyketides	Succinic acid, phosphate	Endocrine system, Energy metabolism
	Pyruvic acid, L-malic acid cholesterol	Endocrine system, energy metabolism, excretory system, microbial metabolism in diverse environments	Glutaric Acid, pyridoxine, 3,4-dihydroxyphenylacetic acid, succinic acid	Microbial metabolism in diverse environments
			Glutaric acid, behenic acid, ergosterol, lignoceric acid	Lipid metabolism
			Phosphate, biotin, xylitol, sucrose, maltose	ABC transporters
			Pyridoxine, succinic acid	Metabolism of cofactors and vitamins
			3,4-Dihydroxyphenylacetic acid, succinic acid	Nervous system, GABAergic synapse, Dopaminergic synapse
			succinic acid	cAMP signaling pathway
[CUMS] *vs* [CUMS-M]	Maleic acid	Amino acid metabolism, Metabolism of cofactors and vitamins	succinic acid, sarcosine, glutaric Acid, 3,4-dihydroxyphenylacetic acid	Amino acid metabolism
	Mucic acid, maleic acid	Carbohydrate metabolism	L-malic acid, succinic acid, xylitol, sorbitol, sucrose	Carbohydrate metabolism
	Mannitol	ABC transporters	L-Malic acid, succinic acid, 4-hydroxybenzoic acid	Biosynthesis of phenylpropanoids, terpenoids, steroids and alkaloids
	Cholesterol	Digestive system	Sucrose, biotin	Digestive system
	Cholesterol, maleic acid, 4-hydroxy-3-methoxybenzyl alcohol	Microbial metabolism in diverse environments	L-malic acid, succinic acid	Endocrine system, energy metabolism
	Stearic acid, myristic acid, cholesterol, arachidonic acid	Lipid metabolism	Adenine	Nucleotide metabolism

Sample	Up-regulated compounds	Pathway	Down-regulated compounds	Pathway
	Arachidonic acid	Cell growth and death, circulatory system, endocrine system, immune system, retrograde endocannabinoid signaling serotonergic synapse	4-Hydroxybenzoic acid, pyridoxine, biotin	Biosynthesis of cofactors
			Glutaric acid, ergosterol, behenic acid, lignoceric acid	Lipid metabolism
			Sorbitol, biotin, xylitol, sucrose	ABC transporters
			4-Hydroxybenzoic acid, pyridoxine, succinic acid	Metabolism of cofactors and vitamins
			Succinic acid, 3,4-dihydroxyphenylacetic acid	Nervous system, GABAergic synapse, dopaminergic synapse
			Succinic acid	cAMP signaling pathway
[CUMS] *vs* [CUMS-R]	Creatine, maleic acid, gentisic acid, indole-3-acetic acid	Amino acid metabolism	Succinic acid, sarcosine, methylmalonic acid, glutaric Acid	Amino acid metabolism
	Salicin, L-malic acid, mucic acid, maleic acid	Carbohydrate metabolism	Methylmalonic acid, xylitol, succinic acid	Carbohydrate metabolism
	Arachidonic acid	Cell growth and death, circulatory system, GnRH and Fc epsilon RI signaling pathway, Fc gamma R-mediated phagocytosis (Immune system), retrograde endocannabinoid signaling serotonergic synapse, long-term depression, inflammatory mediator regulation of TRP channels	Succinic acid	Biosynthesis of phenylpropanoids, terpenoids, steroids and alkaloids, Glucagon signaling pathway, Energy metabolism, Carbon metabolism, Degradation of aromatic compounds, GABAergic synapse, cAMP signaling pathway
	Salicin, L-malic acid	Biosynthesis of phenylpropanoids, alkaloids, terpenoids and steroids	Pyridoxine	Biosynthesis of cofactors
	Cholesterol, biotin	Digestive system	Xylitol	ABC transporters
	Cholesterol, L-malic acid, arachidonic acid	Endocrine system	Pyridoxine, succinic acid	Metabolism of cofactors and vitamins
	L-Malic acid	Energy metabolism, excretory system, carbon metabolism	Glutaric Acid, ergosterol, lignoceric acid	Lipid metabolism
	Cholesterol, maleic acid, 4-hydroxy-3-methoxybenzyl alcohol, L-malic acid, gentisic acid	Microbial metabolism in diverse environments	Methylmalonic acid	Nucleotide metabolism
	Uracil	Nucleotide metabolism	Glutaric Acid, pyridoxine, succinic acid	Microbial metabolism in diverse environments
	Biotin	Biosynthesis of		

Sample	Up-regulated compounds	Pathway	Down-regulated compounds	Pathway
		cofactors		
	Myristic acid, cholesterol, arachidonic acid	Lipid metabolism		
	Biotin, mannitol	ABC transporters		
	Maleic acid, uracil, cholesterol	Metabolism of cofactors, vitamins, terpenoids and polyketides		
[CUMS] *vs* [CUMS-B]	Oleic acid	Longevity regulating pathway	sulfuric acid, inosine, maltose	ABC transporters
	3,4-Dihydroxyphenylacetic acid	Tyrosine metabolism, degradation of aromatic compounds	Dihydroxyacetone, Pyruvic acid	Energy metabolism, carbon metabolism
	Salicin	Carbohydrate metabolism	Maltose	Digestive system
	Arachidonic acid	Cell growth and death, circulatory system, GnRH and xxytocin signaling pathway, Ovarian steroidogenesis, Endocrine system, Platelet activation, Fc epsilon RI signaling pathway, Fc gamma R-mediated phagocytosis, Inflammatory mediator regulation of TRP channels	Pyruvic acid	Gluconeogenesis, TCA cycle, pentose phosphate pathway, pentose and glucuronate interconversions ascorbate, aldarate, starch, sucrose, pyruvate, butanoate, glyoxylate and dicarboxylate metabolism, biosynthesis of phenylpropanoids, terpenoids, steroids and alkaloids, insulin secretion, metabolism of cofactors, vitamins, terpenoids and polyketides glucagon, HIF-1 and AMPK signaling pathways
	Oleic acid, linoleic acid, salicin	Biosynthesis of plant secondary and phenylpropanoids	Dihydroxyacetone, sulfuric acid, pyruvic acid	Microbial metabolism in diverse environments
	4-Hydroxy-3-methoxybenzyl alcohol, 3,4-dihydroxyphenylacetic acid	Microbial metabolism in diverse environments	Inosine, sulfuric acid	Nucleotide metabolism
	Oleic acid, linoleic acid, arachidonic acid	Lipid metabolism	Ergosterol, dihydroxyacetone	Steroid biosynthesis, glycerolipid metabolism
	Arachidonic acid, 3,4-dihydroxyphenylacetic acid	Retrograde endocannabinoid signaling, serotonergic synapse, dopaminergic synapse, long-term depression	Sulfuric acid, sarcosine, pyruvic acid	Amino acid metabolism

4. Discussion

In the present study, we investigated and compared the potential antidepressant and antianxiety effects of probiotics fermented yogurt on the chronic unpredictable mild stress (CUMS) mice. We found that the *L. reuteri* and *L. bulgaricus* fermented yogurts alleviated the depressive and anxious behaviors in CUMS-induced mice, as assessed by behavioral tests, and exhibited an overall protective effect on neuronal survival hippocampus as well as maintained the cerebral activity. Moreover, *L. reuteri* and *L. bulgaricus* fermented yogurts ameliorated the levels of monoamine neurotransmitters and inflammatory cytokines. Interestingly, the effect of yogurts is comparable to or even better than that of fluoxetine. Additionally, the gut microbiota composition and gut metabolic pathways associated with depression that could be modulated by *L. reuteri* and *L. bulgaricus* fermented yogurts. Our findings suggested that *L. reuteri* and *L. bulgaricus* fermented yogurts may be involved in modulating gut microbiota and metabolite composition, and thereby performing neuroprotective effects and ameliorating depressive behaviors in mice by microbiota-gut-brain axis. In the following sections, the effect of *Lactobacillus* fermented yogurt on depression at four levels.

Studies have shown that anxiety and depression are strongly associated with changes in body weight, and anxiety and depression can lead to weight loss by increasing energy expenditure through activating adaptive thermogenesis and basal metabolism via sympathetic nervous system activation [78]. *Lactobacillus* has been extensively utilized in clinical research and dairy products to prevent and treat a range of gastrointestinal infections and inflammatory conditions. Its protective effects help maintain the integrity of the intestinal mucosa [41]. Multiple research findings highlighted the significant impact of *Lactobacillus* spp. on microbiota composition and metabolism of gastrointestinal cells, thereby exerting an indirect impact on energy expenditure, storage, and weight change [25]. Considerable evidence suggested that *Lactobacillus* species improve the absorption of nutrients and process in the gut, particularly in relation to carbohydrate metabolism and its potential impact on weight change [7]. And the carbohydrate metabolism of *Lactobacilli* associated with multiple proteins involved in fructose, mannose, starch, and sucrose metabolism were not encoded by *Lactobacillus genomes* resulting in the weight gain [24]. In this work, the body weight gain significantly increased in the [CUMS-M], [CUMS-R] and [CUMS-B] groups compared with the [CUMS] group, and mice in the [CUMS-R] group gained more body weight than those in the [CUMS-M] and [CUMS-B] group. However, interestingly, the sucrose consumption showed he sucrose intake preference through the experiment between the *Lactobacillus* fermented yogurt and [CUMS-M] groups. On the one hand, this was lined with our previous results that cow milk and goat milk treatment reversed the reduction of body weight in CUMS mice without significant difference between two groups [96]. Moreover, it was indicated that *Lactobacillus* spp. had a significant effect on microbiota and host digestion, and consequently, on weight change as compared to milk treatment. It is generally accepted that proteins in yogurt are more easily digested than those in milk due to the limited pre-digestion that may result from the proteolytic activity of lactic acid bacteria during fermentation [71]. On the other hand, studies showed that some specific strains of *Lactobacillus* supplements had an anti-obesity weight-loss effect through several mechanisms including decreasing insulin resistance, and increasing satiety [34; 79]. This might explain the results in this work that the *L. reuteri* fermented yogurt treatment increased significantly the body weight of CUMS mice but no notable enhancement in sucrose consumption. The different performance within *L. reuteri* and *L. bulgaricus* demonstrate the fact that *Lactobacillus* genus is extremely diverse and may therefore explain the different effects on weight and sucrose consumption induced by the different strains [25].

The most noteworthy finding of this study is that *Lactobacillus* fermented yogurt treatment remarkably reversed loss of neuronal survival in hippocampus and maintain cerebral glucose metabolism of [18F]-FDG PET/CT. Many brain regions, especially the widespread and functionally interactive limbic-cortical network, are involved in the pathogenesis of depression and anxiety, where the prefrontal cortex is related to emotional experience, initiating and modifying emotion, as well as controlling emotional expression [104]. The hippocampus plays a crucial role in regulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the secretion of adrenocorticotropic hormone as well as the spatial learning and memory [65]. In major depressive disorder, decreased blood flow and metabolism have been regularly described in multiple areas of the prefrontal cortex with occasional changes in the hippocampal region [84]. Reduced volume and decreased neuronal number in the hippocampus have been found in depressed patients as well as depression model animals, and effective antidepressants block or reverse the neuronal deficits [27; 49]. The imaging studies provide indirect evidence for alterations in cell number or morphology in the hippocampus in anxiety and depression. The induction of neurons by *Lactobacillus*

fermented yogurt in CUMS mice showed no difference when compared with fluoxetine treatment, consistent with the effect for the therapeutic action of this medication.

PET has been used extensively to measure cerebral glucose metabolism and to evaluate the activity of discrete brain regions in depressed patients and model animals [11; 15; 22; 82; 100]. The [18F]-FDG signal (regional quantification using standardised uptake value (SUV)) increases with heightened synaptic activity, and decreases with reduced brain activity, such as in cases of synaptic dysfunction, neural degeneration, or decreased physical activity [100]. Accumulating evidence indicates that bipolar patients in a depressive episode or patients with major depressive disorder have globally lowered brain metabolism or decreased glucose metabolism in the prefrontal cortex (PFC), dorsal anterior cingulated cortex (dACC) [32; 91].

In the [18F]-FDG-PET study, we found that the *Lactobacillus* fermented yogurt treatment completely reversed the decreased glucose metabolism characterizing in the hippocampus and prefrontal cortex of CUMS mice even closed to the levels of control and fluoxetine groups. These findings partly agree with the results of Hu et al, who also reported a reduction in glucose metabolism in the piriform cortex, septal nuclei, left colliculus and periaqueductal grey matter; and an increase in [18F]-FDG uptake in the left auditory cortex in CUMS mice [42]. While, Zhao et al. found that a decrease in [18F]-FDG uptake in the nucleus of the inferior colliculus, the retro-splenial agranular area, secondary sensory and primary auditory cortices, thalamic postero-medial nucleus and globus pallidus in CUMS mice [117]. The impact of CUMS induced depression and anxiety on [18F]-FDG uptake varies among studies due to differences in stress protocols, such as timing and sequence of stressors, leading to an unpredictable model and contributing to variability in results[100]. And different stressors require responses from different brain areas and thus might contribute to the variability found in these studies. Taken together, the *Lactobacillus* fermented yogurt presented neuroprotective effects of increasing the number of neurons and maintaining the brain activity.

Neurotransmitters have predominated in the study of the pathogenesis of depression. Depletion of monoamine neurotransmitters leads to depression [9]. At present, the primary focus of pharmacological treatments for depression is addressing neurotransmitter deficiency in monoaminergic synapses [83]. The monoamine hypothesis has suggested that serotonin (5-HT) and dopamine (DA) are the significant neuro transmitters involved in the depressive disorder [74]. As a critical monoamine neurotransmitter in the brain, 5-HT plays a regulatory role in neurobehavior and emotional regulation, and that the reduction in 5-HT level or alteration of 5-HT activity in the brain might lead to depression, anxiety, or mania [19]. DA plays a key role in the perception of pleasure and reward, as well as motivated behavior. Many studies have shown a closed correlation between DA receptor polymorphisms and the absence of pleasure in individuals suffering from depression [115]. One of preclinical investigation suggest a role for dopaminergic alterations in depression that the chronic mild stress animal model of depression has shown reduced DA neuron activity [67]. And increase in the DA level might partially alleviate chronic social frustration and depression in mice [29]. According to the meta-analyses of clinical literature, treatment with dopamine receptor partial agonists ameliorated depressive symptoms, such as aripiprazole [93] and cariprazine [85], as well as the dopamine agonist pramipexole [99]. In the present study, the monoamine neurotransmitters such as 5-HT and DA were downregulated in CUMS mice, and *Lactobacillus* fermented yogurt treatment elevated these neurotransmitters. This study is in line with the previous studies, where the monoamine neurotransmitter levels were up-regulated by the supplement of antidepressant drugs [18].

Both preclinical and clinical evidence show that neuroinflammation is closely implicated in the anxiety and depression [98]. The levels of Pro-inflammatory cytokines (IL-6 and TNF-α), and anti-inflammatory cytokines (IL-8 and IL-10) are well-characterized indications of neuroimmune inflammation [96]. Lipopolysaccharide (LPS) has been considered a key mediator of a low-grade inflammatory state [38]. Patients with major depressive disorder may exhibit high levels of LPS in their bloodstream (endotoxemia), associated with systemic inflammation and neuroinflammation [75]. Psychosocial stressors cause elevated levels of pro-inflammatory cytokines, like IL-6 and TNF-α in brain and cause a loss of hippocampal neurogenesis, leading to sickness behavior syndrome. And the antidepressants treatment might reverted the elevated pro-inflammatory cytokines in depressive symptom patients back to the normal level [13]. Growing studies have shown that

Lactobacillus strains can affect colonic motility, immunity maintenance, and anti-inflammatory properties [1; 103; 111; 112]. Metabolites secreted by *Lactobacillus casei* were able to block NF-κB activation and IL-6 production [63], which presented an anti-inflammatory effect on the host, and then alleviate stress-induced depression and anxiety. Particularly, the levels of IL-6 and TNF-α of suicide depressed victims are markedly increased than normal subjects, suggesting a vital role of IL-6 and TNF-α in the pathophysiology of suicidal behavior [95]. Thereby, the inflammation may contribute to the progression of anxiety and depression in multiple ways. Consistent with previous studies [1], we found that *Lactobacillus* fermented yogurt treatment decreased the levels of IL-6, TNF-α and LPS in CUMS mice accompanying with an elevated IL-10 level, indicating that the antidepressant-like effect of *Lactobacillus* fermented yogurt may be related to inhibiting neuroinflammation. Noteworthy, *Lactobacillus* fermented yogurt treatment decreased the levels of IL-6, TNF-α and LPS in CUMS mice, indicating that *Lactobacillus* fermented yogurt showed an antidepressant-like effect may be related to inhibiting neuroinflammation. This overall increase in inflammation contributes to depression by activating the HPA axis, as well as reducing the availability of neurotransmitter precursors and altering neurotransmitter metabolism. The alteration of inflammation cytokines may lead to improved modulation of the HPA axis and neurotransmitter activity [101]. Mohammadi et al [68]. reported that whereas *Lactobacillus acidophilus* ad *Bifidobacterium lactis* fermented yogurt displayed significant benefits on mental health by positively modulating the HPA axis, while conventional *Streptococcus thermophilus* and *Lactobacillus bulgaricus* fermented yogurt did not have any benefit, which may be correlated with the differences of methodology, diagnostic criteria and grouping criteria, etc [75].

Another widely accepted hypothesis of depression claims that the damage of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)-brain derived neurotrophic factor (BDNF) signaling pathway in the brain plays a critical role in the development of depression [10; 16]. The BDNF gene contains a CRE, which binds phosphorylated CREB thereby enhancing transcription [18]. Changes in the expression levels of BDNF, a molecule downstream of CREB and functioning as a stimulator for growth and survival of neurons, are suggested in the serum samples and the brain tissues of both experimental animals and patients with depression [10; 37; 43]. The mechanisms of antidepressants modulating the CREB-BDNF signaling pathway under stressed treatment have been reported in several studies, in which ketamine [109], coadministration of rolipram with imipramine [28], and fluoxetine [72], have been reported to bind and stabilize tyrosine kinase receptor (TrkB) in synaptic membranes [97]. Moreover, nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, which regulates a large array of genes involved in different processes of the neuroprotective effects [63], immune and inflammatory responses [60]. NF-κB nuclear translocation, production of TNF-α and IL-6 were associated with decreased the expression of neuroprotective BDNF in hippocampus [76]. Activation of NF-κB reduced expression of CREB signaling involved in regulation of spatial memory formation, synaptic transmission, and plasticity [47]. The activated NF-κB signaling has been considered as a critical mediator of neuroinflammation in CUMS animals [92]. Thereby, the interaction between NF-κB and the CREB-BDNF signaling pathway implies that NF-κB could serve as an indicator of the antidepressant effects. Notably, western blot analysis in this work has confirmed the upregulation of BDNF and CREB levels and downregulation of NF-κB expression by treatment of *Lactobacillus* fermented yogurts. And *L. bulgaricus* fermented yogurt might have a better performance in reversing these changes that fluoxetine. Therefore, *Lactobacillus* fermented yogurts may alleviate the anxiety- and depression-like behavior in CUMS mice via activating the CREB-BDNF signaling pathway along with inhibition of NF-κB to reduce inflammation as similar to that of classic antidepressants, ketamine [92] and fluoxetine [14] treatment, inhibiting the activation of NF-κB accompanying with elevating the CREB-BDNF pathway.

NSE, a putative marker of neuronal damage, first described in 1965 [69], is a soluble protein present mainly in the cytoplasm of neurons, both in the cortex and in subcortical regions, and constitutes a significant fraction of the total soluble protein of the brain [5]. Clinical neuropathological screening results revealed that serum levels of NSE significantly was decreased in major depression disorder penitents, suggesting that peripheral neuronal specific enolase may be a useful marker drug-naive major depression disorder [105]. Furthermore, signal transducer and activator of transcription 3 (STAT3) is one of the transcription factors for cytokines expression [30; 52], such as IL-6 [50], IL-10 [114] and TNF-α [51]. As mentioned above, these cytokines have been identified as mediators for the neuroimmune system of depression. We found the levels of NSE and p-STAT3 in the fermented yogurt groups was reversed significantly the reduction of that in [CUMS] group, indicating neuroprotective effects and enhanced immunity of *Lactobacillus* fermented yogurt for depression mice induced by CUMS.

It has been accepted generally that the pathophysiology of depression involves four dimensions, the brain dysfunction, the hypothalamus-pituitary-adrenal (HPA) axis, the immune system, and the gut-brain axis [55]. Evidence obtained in the last decades reveals a strong and bidirectional relationship between the gut and the brain, and the gut microbiota has an essential role in the anxiety and depression disorders [31]. Antidepressive mechanisms of probiotics and their therapeutic potential via modifications to the gut microbiota has been studied and reviewed widely [6; 46; 73; 110]. The impact of probiotics on gut microbiota and the subsequent relationship with brain function has been a subject of investigations both in healthy volunteers and in patients with depression, bipolar disorder, or anxiety [113]. Mounting evidence suggests that the gut microbiota composition of patients with depression is different from that of healthy individuals namely decrease in microbiota richness and diversity [57]. It has been reviewed that the levels of *Lactobacillus*, *Bifidobacterium*, *Firmicutes*, *Faecalibacterium* and *Ruminococcus* decrease, while the levels of *Provotella*, *Bacteroides* and *Proteobacteria* increase [55]. Moreover, the microbiota of animals in a variety depression model have similarities with those of depressive patients; such as, the richness of *Bacteroidetes* increases while the richness of *Firmicutes* decreases and the abundance of *Lactobacillus* declines [55; 59; 102]. These results are highly in coincidence with our work that the gut of mice in the [CUMS-R] and [CUMS-B] groups presented more *Firmicutes* but less *Bacteroidetes*, *Proteobacteria* and *Deferribacteres* with and a higher *Firmicutes/Bacteroidetes* (F/B) ratio as compared with [CUMS] group. It has been reported that *Proteobacteria* contain several genes for the biosynthesis of LPS, which can activate macrophages and trigger an immune response [12]. These results suggest that gut microbiota disturbance, particularly increased *Proteobacteria* population, and stresses may increase the occurrence of anxiety and depression, which can change the secretion of neuroactive and immunological molecules in brains by unrelating NF-κB activation and prohibiting BDNF expression; these effects might be reversed by correcting gut microbiota composition and structure [44].

Although all these studies have shown the gut microbiota abnormalities of depressed animal and human, it is almost impossible to obtain the consistent results of microbiota composition as compared with reported literature, the definite distinctions between that of patients and controls are still in debate, which may be correlated with the methodology, differences of diagnostic criteria, grouping criteria, etc [31; 55].

Metabolism is a significant pathway through which gut microbiota affects depression through the brain gut axis. Both direct changes in key metabolites and indirect changes in circulating serum metabolites can modulate anxiety and depression symptoms in the central nervous system. Untargeted metabolomic analysis studies confirmed that *L. reuteri* and *L. bulgaricus* fermented yogurts alleviated CUMS-induced metabolic dysfunction and significantly regulate 85 and 93 metabolites, respectively. The results of metabolic pathway analysis were remarkably in line with microbiome function predictions. Subsequently, KEGG functional enrichment analysis suggested that the differentially abundant metabolites back-regulated by *Lactobacillus* fermented yogurt were mainly enriched in several metabolic pathways that are closely related to neuroprotective effects. Especially crucial is being involved long-term depression, inflammatory mediator regulation of tryptophan channels, microbial metabolism in diverse environments, longevity regulating pathway, cell growth and death pathway. Moreover, the upregulated metabolites associated pathway, such as the GnRH and Fc epsilon RI signaling pathway and Fc gamma R-mediated phagocytosis, involving immune system; the biosynthesis of phenylpropanoids, alkaloids, terpenoids and steroids and polyketides, involving anti-inflammation, antioxidant, and anti-Alzheimer's disease; the GnRH and Oxytocin signaling pathway, relating with the modulation of the autonomic nervous system via the vagal pathway, serotonergic and dopaminergic synapse as well as retrograde endocannabinoid signaling. The retrograde endocannabinoids are key modulators of synaptic function, and converging preclinical and clinical data revealed a key role for endogenous cannabinoid signaling in the modulation of anxiety and depression [80]. *Lactobacillus* fermented yogurt also upregulated significantly levels of fatty acids as compared with CUMS treatment. Numerous studies revealed that fatty acids metabolism disorder may be partially responsible for the development of depression. Studies have suggested that fatty acids are able to cross the blood-brain barrier (BBB) and enter the brain to be taken up by endothelial cells, glial cells and neurons [86]. Oleic acid, a monounsaturated omega-9 fatty acid, was previously reported to be elevated in the hippocampus with imipramine and fluoxetine treatment and was associated with a reduced risk of severe depressed patients and improved depression-like behavior in CUMS model animal [116]. It has been reported that gut microbiota-triggered inflammation and its implications in shifting the tryptophan metabolism towards kynurenine biosynthesis will disrupt the serotonergic signaling, resulting in

mental health problems including depressive symptoms [26]. Luisa et al reported that dietary supplementation with linoleic acid relieves depressive-like symptoms in mice by modulation of the Nrf2 pathway signaling [20]. It has been suggested that medium and long chain fatty acids (C12:0-C18:0) contributed to regulate the GABAergic system by action on the $(GABA)_A$ receptor and probably by modulating the membrane fluidity by the incorporation of fatty acid in the lipid bilayer. Long-chain unsaturated fatty acids has also been reported to activate G protein-coupled receptor 40 in the brain, ultimately altering emotional behavior and modulating the immune-inflammatory process [53]. Study has shown that redox active quinonoid acetylsalicylic acid (ASA) and its metabolite gentisic acid, an endogenously produced siderophore with much more potent antioxidant effects, may act as adjunctive agents in the treatment of psychiatric disorders [4]. These results indicated that fatty acids may be a candidate to help determine the interaction between the gut microbiota and depression [36]. All these studies could provide more insight into clarifying the mechanisms of *Lactobacillus* fermented yogurt alleviating the CUMS-induced anxiety and depression disorders and the gut-brain axis may propose as a potential link between fermented dairy foods and depression [62].

5. Conclusion

In conclusion, this study provides evidence for the antidepressant and antianxiety effects of *Lactobacillus* fermented yogurt alleviating the depressive and anxious behaviors in CUMS-induced mice, as assessed by behavioral tests, and presenting an overall protective effect on neuronal survival in hippocampus as well as maintained the cerebral activity. *L. reuteri* and *L. bulgaricus* fermented yogurts also were found to ameliorate the levels of monoamine neurotransmitters and inflammatory cytokines via intervening on brain dysfunction and improving immunity. Our findings suggests that *L. reuteri* and *L. bulgaricus* fermented yogurts may be involved in modulating gut microbiota and metabolite composition, and thereby performing neuroprotective effects and ameliorating depressive behaviors in mice by at least partially microbiota-gut-brain axis. We speculate that *Lactobacillus* yogurts display antidepressant-like effects in CUMS mice via inhibition of NF-κB pathway, activating CREB-BDNF pathway and regulating gut-brain axis (Scheme 2). These findings have important implications for understanding the pharmacodynamic mechanisms of poorly absorbable antidepressants that target the intestinal microbiota and provide a promising new avenue for the development of novel treatments for depression.

Declarations

Conflict of interest statement

The authors declare no conflict of interest.

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Data curation, Conceptualization, Methodology, Software, Writing, review, and editing, supervision.

Xiulian Li

Sample preparation and experiment operation.

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Data curation, Conceptualization, Methodology, Software, data interpretation and manuscript editing, supervision.

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Author Contribution

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Schemes

Schemes 1 and 2 are available in the Supplementary Files section.

Figures

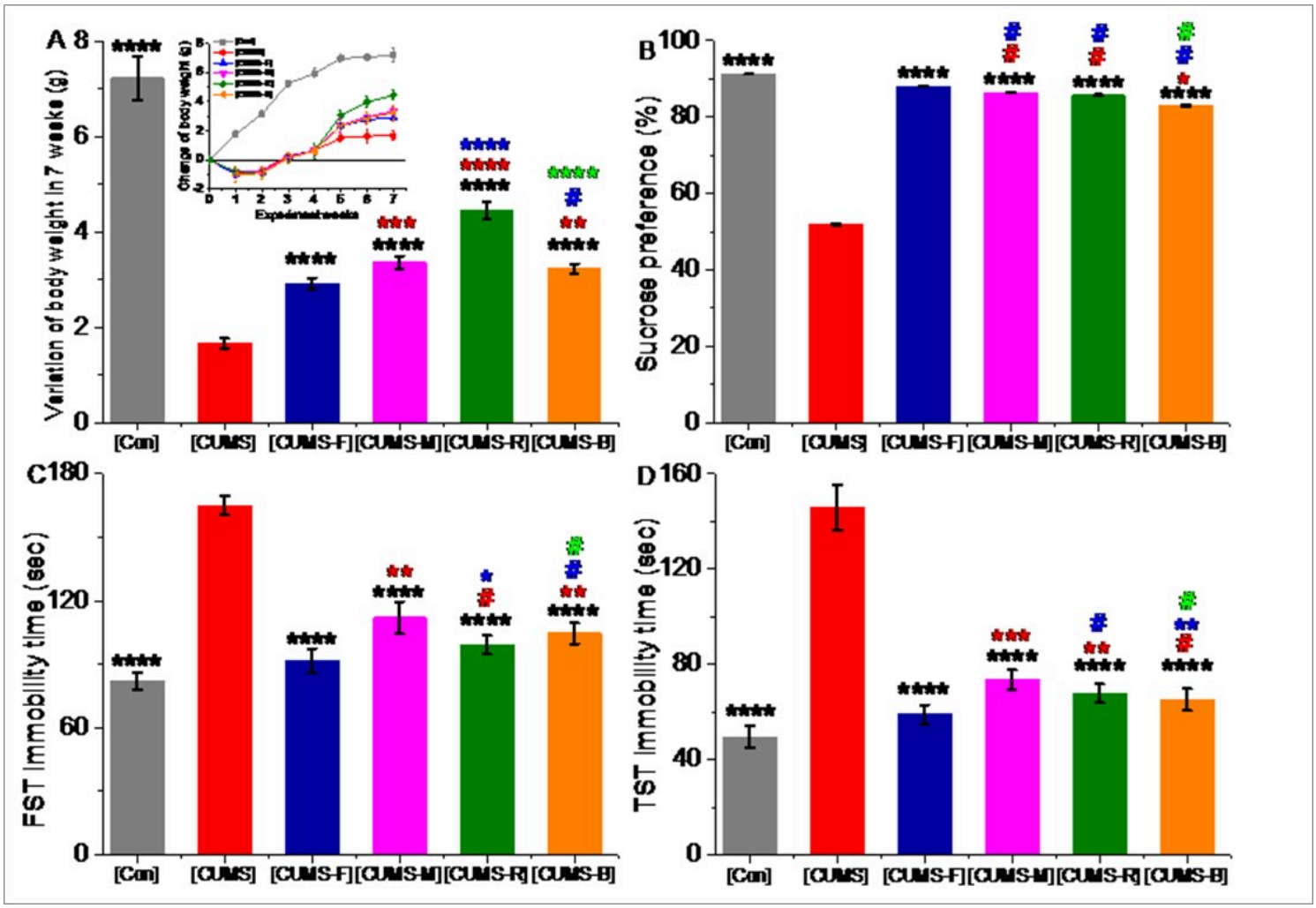

Figure 1

Treatment with probiotics (*L. reuteri* and *L. bulgaricus*) fermented yogurts alleviate CUMS-induced depression behavior in mice. (A): body weight gain and change during 7 weeks. (B): Sucrose preference in sucrose consumption test. (C): Time of immobility in FST. (D): Time of immobility in TST. Results are presented as mean ± SEM (n = 6, per group). ****p<0.0001, **p < 0.01, *p < 0.05 and $^{\#}p$ > 0.05. Black sign (*) is the difference of [CUMS] compared with [Con], [CUMS-F], [CUMS-M], [CUMS-R] and [CUMS-B]. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

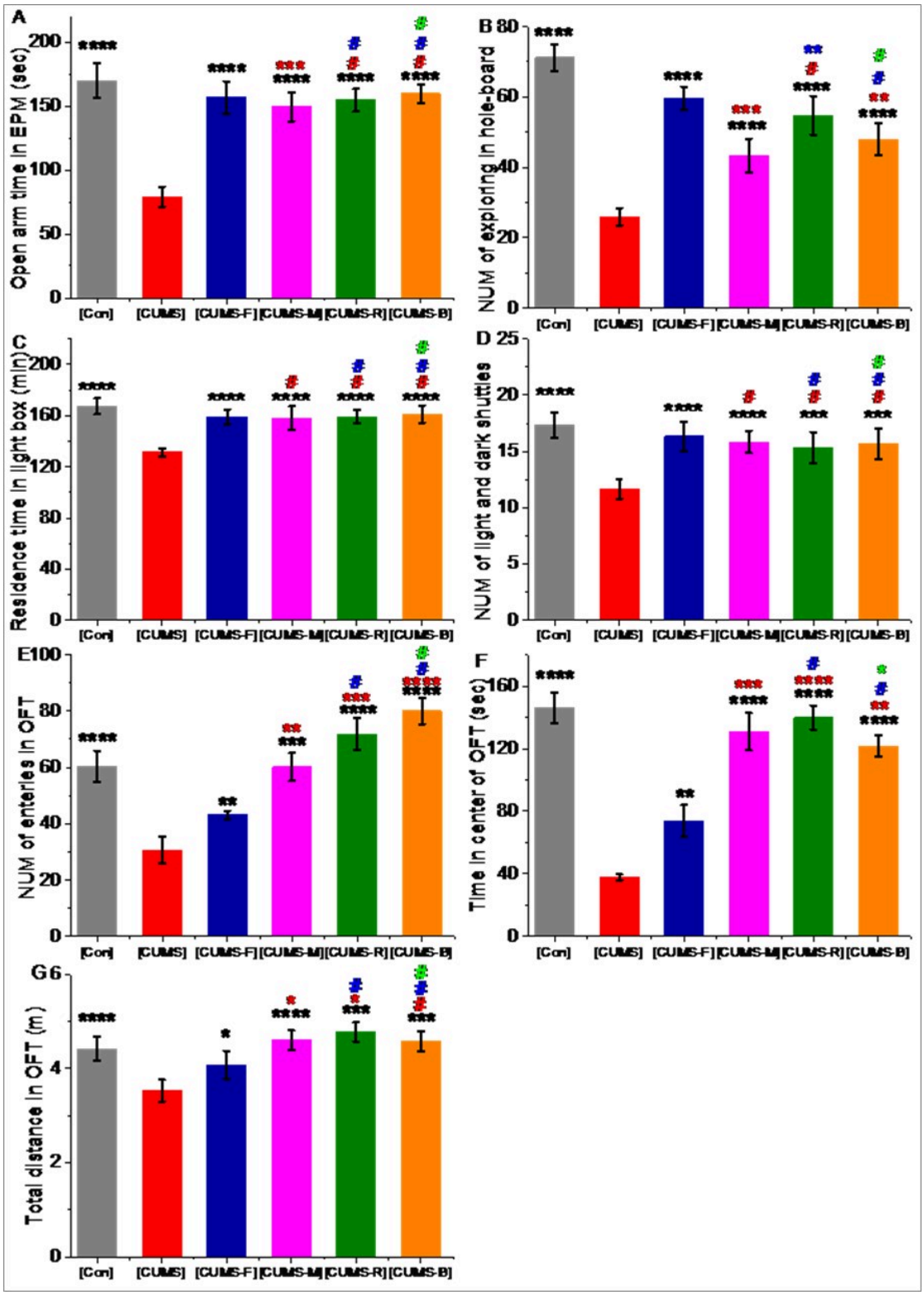

Figure 2

Treatment with probiotics (*L. reuteri* and *L. bulgaricus*) fermented yogurts alleviate CUMS-induced anxiety behavior in mice. (A): elevated plus-maze test (EPM); (B): hole-board test, (C and D): light-dark transition test, (E): open field test (OFT). (H): Activity tracks in the OFT, (a-f): [Con], [CUMS], [CUMS-F], [CUMS-M], [CUMS-R] and [CUMS-B] group, respectively. Results are presented as mean ± SEM (n = 6, per group). ****p<0.0001, ***p<0.001, **p < 0.01 and #p > 0.05. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

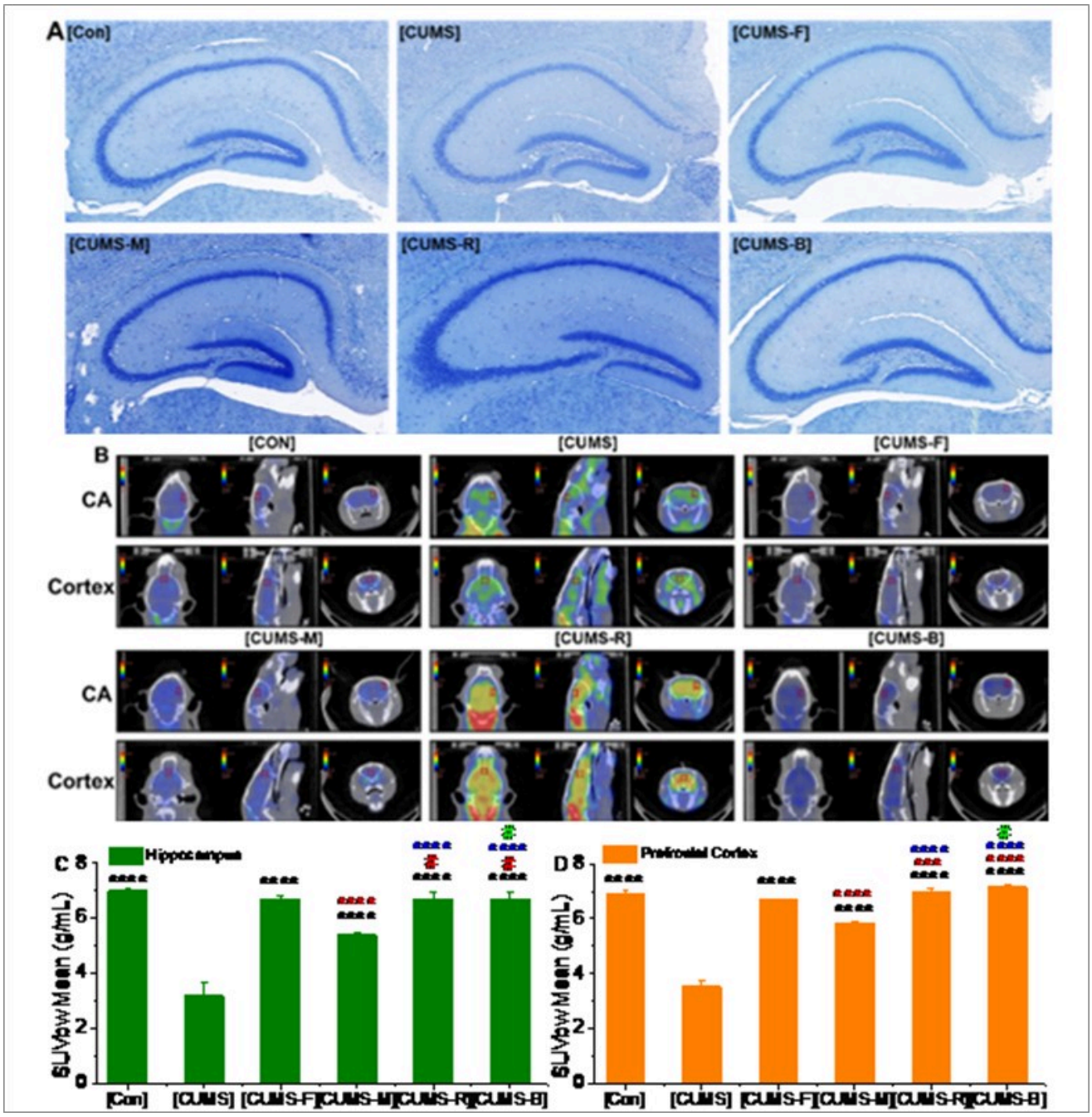

Figure 3

A: Effects of probiotics (*L. reuteri* and *L. bulgaricus*) yogurt on the hippocampus region in CUMS mice were measured by Nissl staining with 40 magnifications. B: The reconstruction of PET-CT images demonstrated a remarkable [18F] FDG uptake in the prefrontal cortex and temporal cortex in CUMS mice. C and D: Standard intake value (SUV) chart of the prefrontal cortex, temporal cortex and hippocampus, respectively. Results are presented as mean ± SEM (n = 6, per group). ****p<0.0001, ***p<0.001, **p < 0.01 and #p > 0.05. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

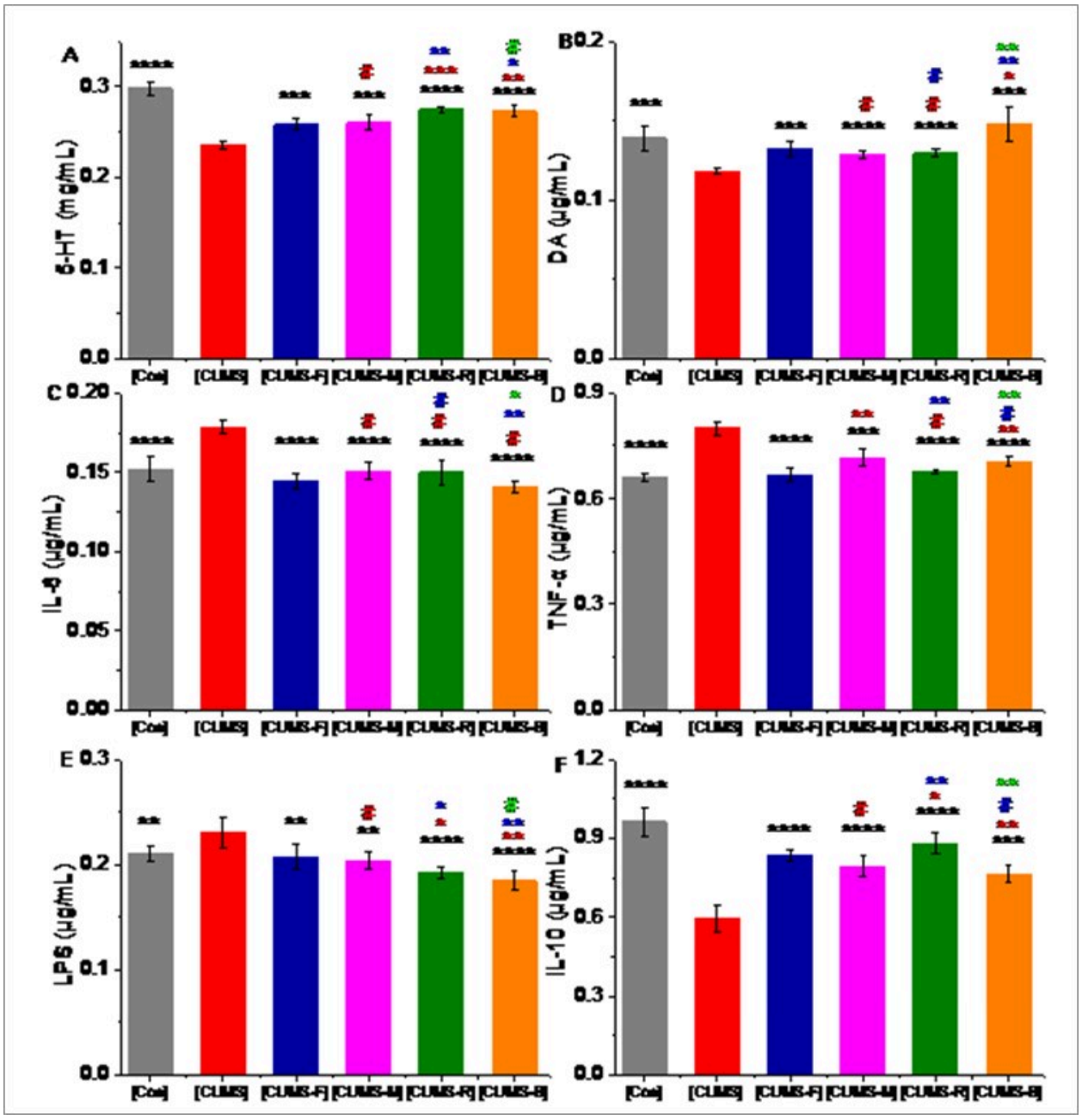

Figure 4

Effects of probiotics (*L. reuteri* and *L. bulgaricus*) yogurt on the levels of monoamines neurotransmitters in hippocampus and inflammatory cytokines in blood serum of CUMS mice measured by ELISA. (A): 5-hydroxytryptamine (5-HT); (B): dopamine (DA); (C): interleukin-6 (IL-6); (D): tumor necrosis factor (TNF-α); (E): lipopolysaccharide (LPS); (F): interleukin-10 (IL-10). Results are presented as mean ± SEM (n =6, per group). ****p<0.0001, ***p<0.001, *p<0.05 and $^{\#}p > 0.05$. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

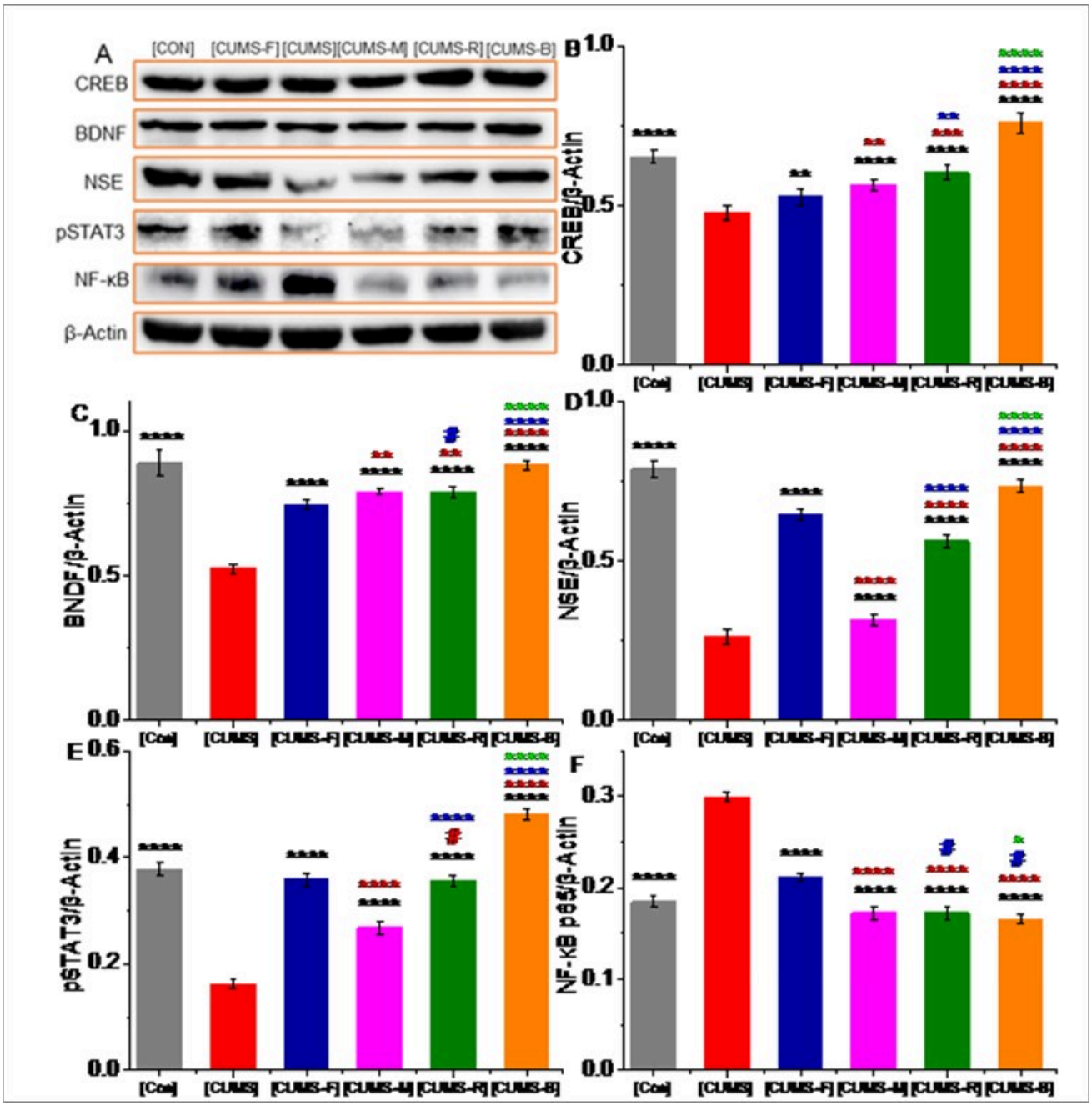

Figure 5

A-E: Effects of probiotics (*L. reuteri* and *L. bulgaricus*) yogurt on the expressions of cAMP-response element binding protein (CREB), brain derived neurotrophic factor (BDNF), nuclear factor kappa-B (NF-κB), neuron-specific enolase (NSE) **and** phosphorylated activator of transcription 3 (p-STAT3) in the hippocampus of CUMS mice was analyzed by Western Blotting**, respectively.** F: The corresponding protein levels of in hippocampus were measured by Western Blotting. Results are presented as mean ± SEM (n = 6, per group). ****p<0.0001, ***p<0.001, *p<0.05 and $^{\#}p$ > 0.05. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

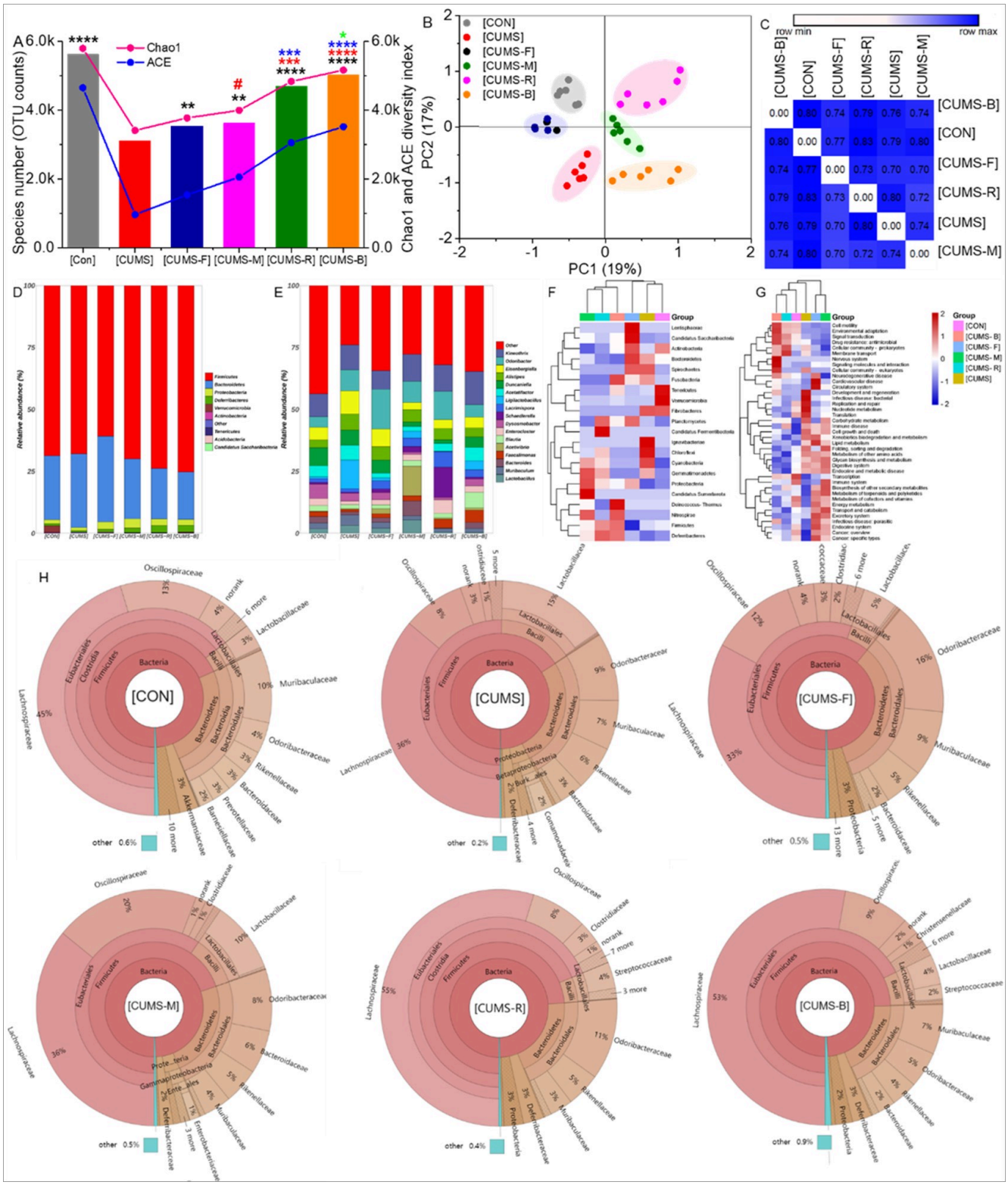

Figure 6

Effects of *Lactobacillus* fermented yogurt on the gut microbiota composition of CUMS mice. (A) Species number (OTU counts) and species richness estimates, Chao1 and ACE diversity index. (B) Unweighted PCoA analysis. (C) Heatmap of beta diversity distance. (D) Relative abundance of gut microbiota species at the phylum level. (E) Relative abundance of gut microbiota species at the genus level. (F) Heatmap of relative abundance of species at the genus level. (G) Heatmap of PICRUSt2 functional annotation clustering across groups based on the relative abundance of KEGG pathways in each group at level 3. (H) Dominant bacteria taxa in different group determined by KRONA. The discriminative bacteria species were

identified based on LDA score >2.5. Results are presented as mean ± SEM (n = 6, per group). ****p<0.0001, ***p<0.001, *p<0.05 and $^{\#}p$ > 0.05. Red sign (*) is the difference of [CUMS-F] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Blue sign (*) is the difference of [CUMS-M] compared with [CUMS-M], [CUMS-R] and [CUMS-B]. Green sign (*) is the difference of [CUMS-R] compared with [CUMS-B].

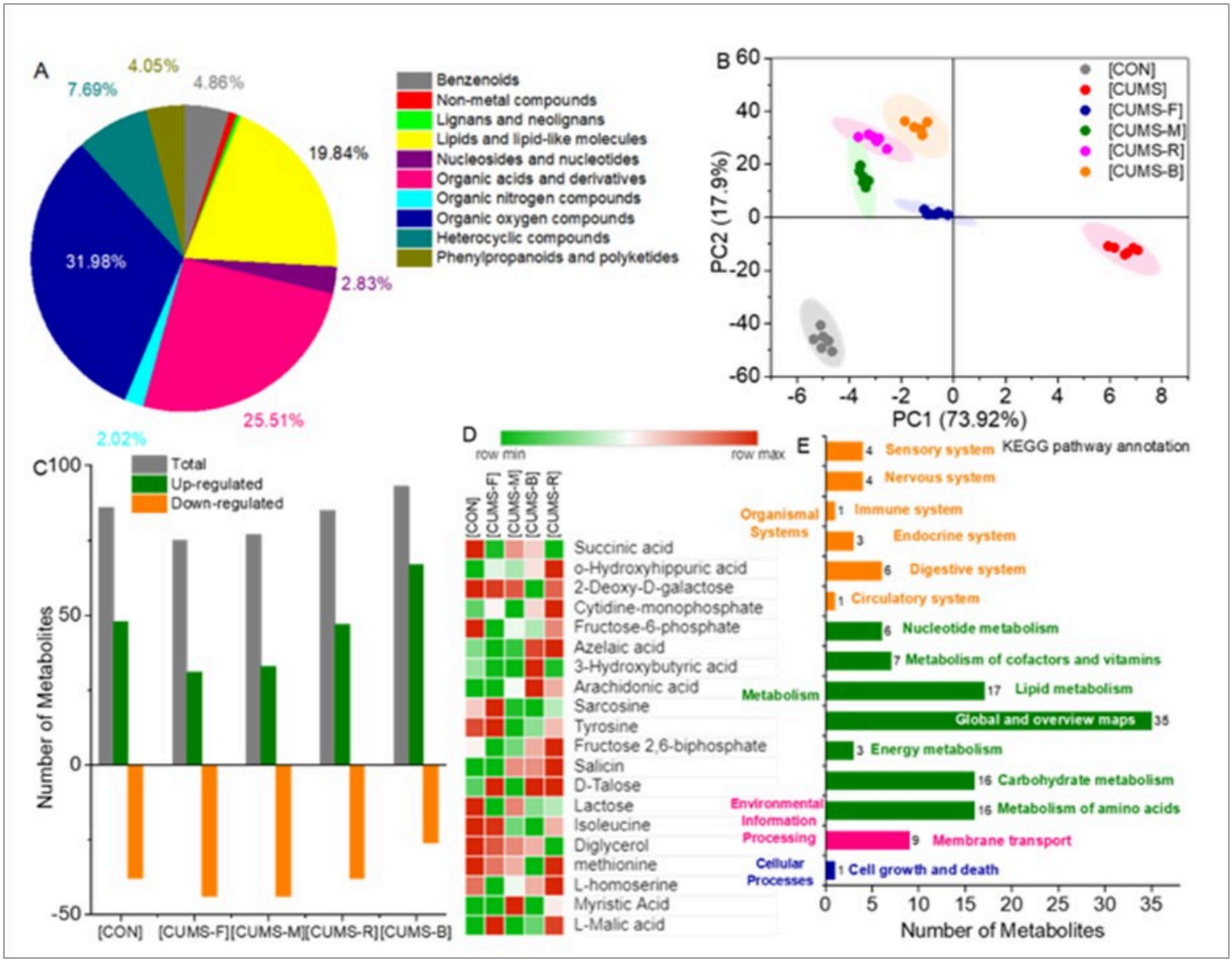

Figure 7

Effects of probiotics (*L. reuteri* and *L. bulgaricus*) yogurt onthe untargeted metabolic analysis. (A) pie-chart of metabolite composition compound class. (B) PCA analysis of positive ion model data. (C-D) Number of total differential, up-regulated and down-regulated metabolites, as well as the top twenty differential metabolites of [CUMS] group compared with [CON], [CUMS-M], [CUMS-F], [CUMS-R] and [CUMS-B] group ($p \leq 0.05$). (E) Biological signaling pathways were identified by using KEGG pathway analysis.

Supplementary Files

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

- Scheme1.png
- Scheme2.png