

# Effects of regulating gut microbiota on the serotonin metabolism in the chronic unpredictable mild stress rat model

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## Abstract

**Objective:** This study was to inspect the antidepressant-like effect of prebiotics and probiotics, and to explore the effect of modulating gut microbiota on the serotonin (5-HT) metabolism.

**Methods:** Fifty rats were separated into control and other four groups randomly. The four groups underwent the chronic unpredictable mild stress (CUMS) intervention with or without prebiotics and probiotics (*Bifidobacterium longum*, *L. rhamnosus*) treatment. After weighted, the animals underwent a series of behavioral tests comprising the sucrose preference test (SPT) and the forced swimming test (FST). Central and colonic serotonin levels and relative metabolism factors were measured and analyzed. Microbiota was examined by 16S rRNA gene pyrosequencing.

**Results:** CUMS intervention caused a decrease in body weight, an increase in FST, and a decrease in SPT. Prebiotics and probiotics all ameliorated the CUMS-induced loss of weight and depressive-like behaviors to a certain extent, especially *L. rhamnosus*. Compared with the group of CUMS intervention, the rats of probiotics and prebiotics treatment had a tendency to reduce colonic 5-HT and increase 5-HT in frontal cortex and hippocampus. However, there was no significant difference in peripheral blood 5-HT among these groups. Furthermore, CUMS caused noteworthy gut microbiota variations at the phylum and other levels in rats. Remarkably, there were considerable relations of perturbed gut microbiota with the changed metabolism of 5-HT.

**Conclusion:** In conclusion, these findings implied that prebiotics and probiotics have antidepressive effects, and a considerable effect on the regulation of 5-HT metabolism, especially *L. rhamnosus*.

## KEYWORDS

depression, gut microbiota, gut-brain axis, prebiotics, probiotics, serotonin

## 1 | INTRODUCTION

Depression is a predominant, fetal and extremely recurring mental illness described by anhedonia, depressed mood, plus great suicide

rates.<sup>1</sup> As per the World Health Organization (WHO), depressive illnesses are the utmost heavy sicknesses in the general public, besides they may perhaps become the principal foundation of disability globally.<sup>2</sup> In latest periods, numerous concepts have made an effort

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to clarify the pathogenesis of major depressive disorder (MDD), containing neurotransmission insufficiency,<sup>3</sup> neurotrophic changes,<sup>4</sup> endocrine-immune system dysfunction,<sup>5</sup> and neuroanatomical irregularities.<sup>6</sup> However, there have not been any globally approved theories. Therefore, there is a need to determine the etiology of depression and neurobiological mechanisms for the avoidance and treatment of this illness.

Multiple data support that gut microbiota has an effect on gastrointestinal physiology and central nervous system (CNS) function plus behavior via the microbiota-gut-brain axis.<sup>7-9</sup> Serotonin (5-hydroxytryptamine, 5-HT) is a kind of neurotransmitter both in the central nervous system and in the peripheral nervous system (CNS/PNS), which has been implicated in the etiology of numerous disease states, including depression, anxiety, social phobia, schizophrenia, obsessive-compulsive, and panic disorders. Latest research recommended that some prebiotic and probiotic treatment could result in reversal of behavioral deficits, adjust the composition of gut microbiota, rise in peripheral levels of the serotonin precursor tryptophan, and alter 5-hydroxyindoleacetic acid (5-HIAA) and dihydroxyphenylacetic acid (DOPAC) levels in the brain of animal models of depression and chronic stress.<sup>10</sup> Precisely, the lack of GI microbes in rats leads to decreased expression of brain-derived neurotrophic factor in the cortex and hippocampus, in addition to an inflated hypothalamic-pituitary-adrenal (HPA) axis reaction to stress.<sup>11</sup> Given the capability of the gut microbiota to influence serotonin and its precursor, tryptophan,<sup>12</sup> control the stress response<sup>13</sup> plus control cognition<sup>14</sup> in addition to behavior,<sup>15</sup> the potential prominence of the gut microbiota to psychiatry in general and to depression definitely is obvious. In-depth research is desired to cross-examine this fascinating proposal. Numerous lines of proof, comprising the current reports from Hsiao and colleagues,<sup>16</sup> prove that, in the gut, microbial-derived metabolites have the impact on the creation of serotonin which in turn influences host physiological functions.<sup>17</sup>

As not all probiotics are beneficial in depression, we selected *L. rhamnosus* and *Bifidobacterium longum*. It has been reported that they can advance expressive behavior in animals.<sup>18,19</sup> In this study, we selected prebiotics (fructo-oligosaccharide and galactooligosaccharide, FOS/GOS) and probiotics (*Bifidobacterium longum* and *L. rhamnosus*) and intended to identify the difference in probable antidepressant properties of them in the chronic unpredictable mild stress (CUMS) rat model of depression on the adult behavioral phenotype. Especially, the serotonin and crucial systems participating in depression along with brain-gut communication were the focus of this study.

## 2 | MATERIALS AND METHODS

### 2.1 | Animal handling

Fifty male Wistar rats (The Animal Center of Qingdao Medicine University, China) weighing 200 ~ 220 g were kept ten per cage in the polycarbonate cages, in a regulated environment (temperature

### Key Points

- The aims of this study were to explore the effects of intestinal flora on host behavior and serotonin metabolism.
- The peripheral blood, brain tissue, intestinal tissue and contents of 50 rats were detected and analyzed by microflora analysis and statistical software.
- The results showed that prebiotics and probiotics did have effects on the metabolism of serotonin in intestinal and brain tissues.
- It is useful to understand the mechanism of the 'microbiota-gut-brain axis' and the potential value of prebiotics and probiotics.

22 ± 2°C; humidity, 55 ± 5%; 12-hour light/dark cycle) with availability of regular chow and water. The animals were given 7 days for proper adaptation, and their tails were marked. Next, they were weighed and randomly allocated into five groups (10 rats per group). According to whether CUMS intervention was implemented and whether probiotics and prebiotics were added, the five groups were named as follows: control group (non-CUMS and non-pre/probiotics), CUMS group (CUMS and perfusion saline without pre/probiotics), FOS/GOS group (CUMS + perfusion fructo-oligosaccharide and galactooligosaccharide, FOS & GOS), BL group (CUMS + perfusion *Bifidobacterium longum*), and Lr group (CUMS + perfusion *L. rhamnosus*). All animal experimentations were done as per the Guidelines for the Care and Use of Laboratory Animals by the National Institute of Health. Approvals for the study were acquired from the ethics committee of Qingdao Medicine University (Qingdao, China).

### 2.2 | Rat Model of CUMS

CUMS is one of the behavioral models leading to depression-related behaviors.<sup>20-22</sup> Except the control group, the CUMS process was done among other groups for four weeks. In brief, the stimuli included 45° cage tilt for 12 hours (6:30 AM to 6:30 PM, hard to get food and water), tail pinching for 3 minutes with a clip (8 AM to 10 AM, just whine without skin damage), cage shaking for 5 minutes (8 AM to 10 AM), swimming in 4°C cold water or 45°C hot water for 5 minutes (8 AM to 11 AM, using 50-cm high plastic drum and 20 cm in diameter, the water depth was determined by the rats' toes reaching the bottom of the container), reversed light/dark cycle for 24h (dark for 8 AM to 8 PM, light for 8 PM to 8 AM), housed in an empty squirrel cage with no padding for 15 hours (5 PM to 8 AM of next day), damp bedding for 15 hours (5 PM to 8 AM of next day), and lack of food or water for 24 hours (8 AM to 8 AM of next day). These stressors were applied in random order without repetition in one week. Each stressor was repeated two or three times during the four weeks of stress period.

### 2.3 | Treatments and behavioral test

Except the control group, rats in the other groups were orally gavaged with normal saline (CUMS group) or with fructo-oligosaccharide (FOS) and galactooligosaccharide (GOS) [8%, 1 mL per 100g weight; FOS/GOS] or with *Bifidobacterium longum* [ $1 \times 10^9$  cfu per 100 g weight; BL] or with *L. rhamnosus* [ $1 \times 10^9$  cfu per 100 g weight; Lr] during the CUMS molding. The FOS and GOS were provided by Quantum Hi-Tech Biological Co., Ltd. *Bifidobacterium longum* and *L. rhamnosus* were provided by SHAANXI SCIPHAR NATURAL PRODUCTS CO., LTD. This process was done every day between 8:00 and 9:00 for 28 consecutive days. All animals of each group were weighed weekly before and after the process of CUMS. After the CUMS intervention and prebiotics and probiotics treatment, various behavioral test such as the sucrose preference test (SPT) and the forced swimming test (FST) was done on all animals. These results were used to investigate the degree of anhedonia and behavioral despair. All behavioral tests were done by the investigator who was not aware of the treatment of each animal.

### 2.4 | Forced swim test (FST)

An altered type of the FST defined earlier by Cryan et al<sup>23</sup> was implemented here. Concisely, rats were kept into a Perspex cylinder comprising 30 cm of water heated at 25°C for 15 minutes before the test on day one. The next day, test periods lasting 5 minutes were noted. The immobility time was recorded when the animals were floating in the water with no struggle at all and they only moved to maintain their heads above the water level.

### 2.5 | Sucrose preference test (SPT)

SPT was done as explained with slight alterations after the CUMS.<sup>24</sup> All rats were taught to acclimate to the 1% (w/v) sucrose solution: First they were exposed for 24 hours to two bottles of sucrose solution. Next, they were exposed to one bottle of sucrose solution plus one bottle of water for another 24 hours; then, the rats were not given food and water for 12 hours. SPT was done for 12 hours. During this course, the rats were kept in separate cages with easy access to two bottles (1% sucrose solution and water bottle). The locations of the bottles in the cage were interchanged after 6 hours to evade probable side-preference impacts. The intakes of the sucrose solution, water, and total consumption of liquids were assessed by weighing the bottles. The inclination for sucrose was noted as a fraction of the ingested sucrose liquid comparative to the entire volume of liquid consumption. Following equation was used to calculate the sucrose preference value: Preference value (%) = sucrose intake / (sucrose intake + water intake) × 100%.

### 2.6 | Animal sacrifice and tissue dissection

All of the animals were weighed and knocked out by injecting 3% sodium pentobarbital (20 mg/kg body weight) intraperitoneally. Blood samples were taken from the aorta ventralis puncture and split into microtubes. The blood samples were centrifuged, and the

supernatants were put in storage at -20°C for consequent serum serotonin (5-HT) by enzyme-linked immunosorbent assay (ELISA) kit (MultiSciences Biotech, PRC). The head was rapidly removed, and the brain areas were dissected to acquire the frontal cortex and hippocampus, which were preserved at -80°C for consequent examination. The part of colon was collected and separated into two sections; one segment was made from tissue homogenates; then, serotonin levels were identified in supernatants by ELISA as per the supplier's protocol (Cloud Clone Corp.). The other section was stored at -80°C for immunofluorescent detection. The contents of the cecum were taken out for 16S rRNA Gene Sequencing and Analysis.

### 2.7 | Immunofluorescent detection of the distribution of colonic enterochromaffin cells (ECs) and 5-HT

Tissue was implanted in paraffin and cut into 5-mm sections. After deparaffinization and a categorized ethanol series, heat-induced antigen recovery on slides was done in pH 6.0 or pH 9.0 unmasking liquid (Vector Laboratories). They were blocked by 5% normal goat serum and then incubated overnight at 4°C with primary antibodies: chromogranin A (rabbit anti-CgA; Abcam) and 5-HT (goat polyclonal antiserotonin; Abcam). Secondary antibodies were smeared for 1 hour at room temperature (RT), followed by 4',6 diamidino-2-phenylindole dihydrochloride (Life Technologies) to stain cell nuclei. CgA + ECs and 5-HT + cells were calculated by the Countess II Automated Cell Counter (Thermo Fisher Scientific).

### 2.8 | Fluorogenic quantitative PCR detection

The expression of tryptophan hydroxylase 2 (TPH2) and indoleamine 2,3-dioxygenase (IDO) mRNA in hippocampus and frontal lobe and the expression of colonic tryptophan hydroxylase 1/glyceraldehyde-3-phosphate dehydrogenase (TPH1/GAPDH) mRNA and serotonin transporter gene SCL6A4/GAPDH mRNA were determined by SYBR GreenI Real-Time PCR. Total RNA was extracted from brain tissues by

**TABLE 1** Primers used for RT-PCR

Gene	Sequence (sense, antisense: 5'-3')	Size (bp)
TPH	CTTGGGGTGTGTGTTTCG TACTTGGTCAGCAGGGGGA	91
IDO	GACACCTTTTCCACGTTCTTC TCACCAACGCATGCTTTATTC	568
β-actin	CTTGCATCCCTCAGCACCTT TCCTGTGGACAATGGATGGA	140
TPH1	GGCGCGATCAGGATCACTG ACTTTTTTCAAACATACGT	263
SLC6A4	GGGTACAGGAGAGAGGATTG GTGCAATTTAAACCTTATAC	108
GAPDH	GGGGCTGGGAAGGAACCACG CGGTAAGGACTATATAATGT	72

TRIzol (Invitrogen, Cat# 15596-026). Total RNA (1  $\mu$ g) was reverse transcribed into cDNA by TIANScript RT KIT (TIANGEN, Cat# KR104-02) as per the supplier's protocol. List of primers is mentioned in Table 1 (TaKaRa Biotechnology Co., Ltd).  $\beta$ -Actin was used for normalization. Real-time PCR was done as published earlier.<sup>22</sup>  $2^{-\Delta\Delta Ct}$  method was preferred for the comparison of the relative expression levels.

## 2.9 | Western blot examination

Total proteins were extracted from rat brain, and protein concentrations were identified by a BCA Protein Assay Kit. 10 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run to separate the proteins. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 5% skim milk for 2 hours at RT. The membranes were treated overnight at 4°C with the following primary antibodies: rabbit anti-TPH (Tryptophan Hydroxylase, Beijing Biosynthesis Biotechnology CO., LTD), rabbit anti-IDO (Zymed Laboratories), and rabbit anti-TH (Serotonin, Abcam).  $\beta$ -Actin (Sigma) was considered as a loading control. Next, the membranes were incubated with respective secondary antibodies. Image-Pro Plus 6.0 software (Media Cybernetics) was used for densitometry analysis of the obtained bands.

## 2.10 | Bacterial diversity analysis

The contents of the colon were taken and stored at  $-80^{\circ}\text{C}$ . Total genome DNA was extracted by CTAB/SDS method. The V4 regions of the 16S rRNA gene were PCR amplified; then, PCR products were purified by means of Qiagen Gel Extraction Kit. The 16S ribosomal RNA (rRNA) gene was evaluated to assess the bacterial assortment by the Illumina HiSeq2500 platform (Novogene Bioinformatics Technology Co., Ltd.). Operational taxonomic units (OTUs) were picked by pick\_de\_novo\_otus.py. Sequence analyses were performed using the UPARSE software (Uparse v7.0.1001). Sequences with  $\geq 97\%$  similarity were assigned to the same operational taxonomic units (OTUs).

Additionally, based on the normalized OTUs, alpha diversity using Shannon index was applied to analyze the complexity of species diversity for the samples.<sup>25</sup> A high  $\alpha$  diversity indicates a high richness of genera within the sample. MRPP analysis was used to evaluate differences among the samples in species complexity. Metastats analysis was carried out under various classification levels (Phylum, Class, Order, Family, Genus and Species) by R software (Version 2.15.3) to obtain  $P$  value, which was then modified by Benjamini and Hochberg false discovery rate method to obtain  $q$  value.

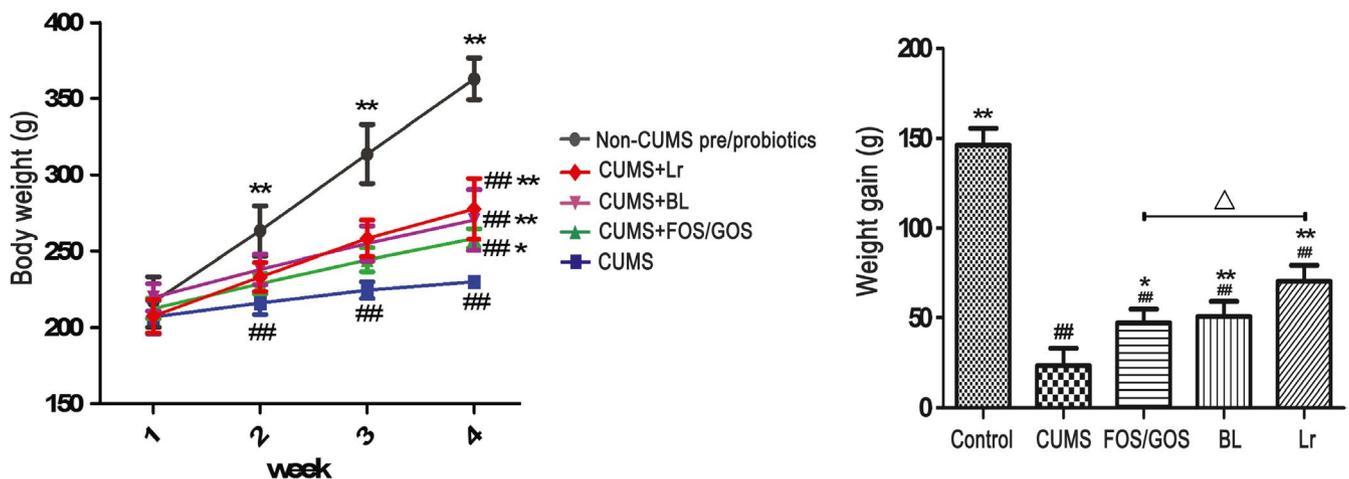
## 2.11 | Statistical analysis

Quantitative data with normal distribution were expressed as means  $\pm$  standard deviation (SD). Quantitative data were analyzed by the one-way ANOVA for comparison between multiple groups after the normality test, and the LSD  $t$  test was used for comparison between every two groups; the Pearson correlation analysis was used for correlation analysis of measurement data after the normality test. All statistical tests were 2-sided, and  $P < .05$  was regarded as significant. Statistical analyses of data were performed using SPSS 20.0 software.

## 3 | RESULTS

### 3.1 | Prebiotics (FOS/GOS) and probiotics (Bifidobacterium longum and L rhamnosus) restore the decreased body weight in rats subjected to CUMS

There was no significant difference in body weight among each group before the experiment ( $F = 0.822$ ,  $P > .05$ ), but body weights were significant differently after CUMS intervention ( $F = 46.675$ ,  $P < .01$ ). As presented in Figure 1, probiotics (BL and Lr) and prebiotics (FOS/GOS) indeed improved the decreased body weight in rats subjected to CUMS, but still could not restore the body weight compared with control group. In terms of body weight gain comparisons, the weight



**FIGURE 1** Effect of probiotics and prebiotics on body weight of rat and weight gain of each group. Values are stated as the mean  $\pm$  standard deviation.  $^{\#}P < .05$ ;  $^{\#\#}P < .01$  compared with the control group;  $^*P < .05$ ;  $^{**}P < .01$  compared with the CUMS group;  $\Delta P < .05$  compared with the FOS/GOS group

gain of each group after CUMS intervention was less than that of the control group. However, CUMS intervention combined with probiotics or prebiotics resulted in more weight gain of the pre/probiotic groups than in the CUMS group. Pairwise comparison by LSD t test showed that the weight gain of the Lr group was more obvious than that of the FOS/GOS group ( $P = .016$ ), but there was no significant difference between the BL group and the FOS/GOS group ( $P = .590$ ).

### 3.2 | Prebiotics (FOS/GOS) and probiotics (*Bifidobacterium longum* and *L. rhamnosus*) alleviate CUMS-induced depressive-like behavior

There were significant differences among the groups in FST ( $F = 19.824$ ,  $P < .01$ ) and SPT ( $F = 21.431$ ,  $P < .01$ ) after CUMS intervention and pre/probiotic treatment. As displayed in Figure 2, the immobility time in the FST was significantly increased in the CUMS group compared with the control group ( $P < .01$ ), which was greatly reduced by treatment with FOS & GOS, *Bifidobacterium longum*, and *L. rhamnosus* compared with the CUMS group. Pairwise comparison by LSD t test showed that the immobility time of FOS/GOS group was still longer than that of the control group ( $P = .012$ ), but there was no significant difference in the BL group and Lr group compared with the control group ( $P = .154$ ;  $P = .535$ ). Compared with the FOS/GOS group, the effect of the Lr group was more obvious ( $P = .035$ ). As demonstrated in Figure 2, the CUMS group displayed a significant reduction in sucrose ingestion compared with the control group, while treatment with FOS & GOS, *Bifidobacterium longum*, and *L. rhamnosus* obviously augmented sucrose ingestion in rats exposed to CUMS. Pairwise comparison by LSD t test showed that there was significant difference between the control group and FOS/GOS group ( $P = .012$ ), FOS/GOS group and Lr group ( $P = .035$ ). All of these observations suggested that CUMS-treated rats have increased immobility and decreased sucrose preference, and that these effects are mitigated with probiotic and prebiotic treatment. Furthermore, the effect of treatment with *L. rhamnosus* is obviously better than FOS/GOS.

### 3.3 | Effects of prebiotics (FOS/GOS) and probiotics (*Bifidobacterium longum* and *L. rhamnosus*) on serotonin levels and relative factors

As demonstrated in Figure 3A, B, compared with the control group, there were not noteworthy alterations in serum 5-HT levels of each

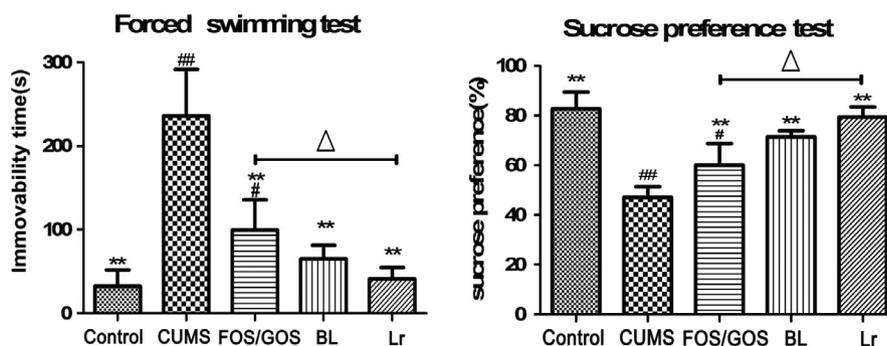
group ( $F = 1.518$ ,  $P > .05$ ), but there were significant differences in colonic 5-HT levels of each group ( $F = 32.026$ ,  $P < .01$ ). Colonic 5-HT in the CUMS group were increased after CUMS compared with the control group ( $P = .014$ ). However, colonic 5-HT levels were decreased after treatment with prebiotics (GOS/FOS) and probiotics (*Bifidobacterium longum* and *L. rhamnosus*) compared with the CUMS group; colonic 5-HT levels of probiotics groups were even lower than the control group. Additionally, the decline of colonic 5-HT of these groups treated with *Bifidobacterium longum* and *L. rhamnosus* was more pronounced than the FOS/GOS group.

Furthermore, we found the expression of TPH1/GAPDH mRNA was increased in the CUMS group; inversely, the expression of SCL6A4/GAPDH mRNA was decreased in the CUMS group compared with the control group, but the trend was reversed in the groups of prebiotics (GOS/FOS) and probiotics (*Bifidobacterium longum* and *L. rhamnosus*) compared with the CUMS group. Similarly, the variation tendency of the group provided with *L. rhamnosus* was more obvious than the FOS/GOS group ( $P < .05$ ).

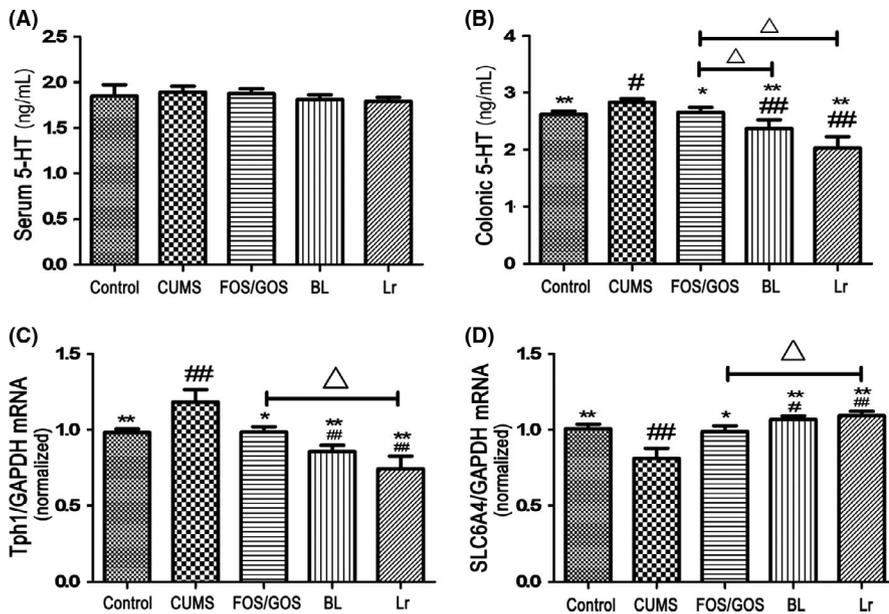
Most of serotonin (5-HT) is well known to derive from the GI tract, and recent report suggests that microbiota mainly regulate colonic 5-HT and the level of colonic 5-HT is confined to colonic chromogranin A-positive (CgA+) enterochromaffin cells (ECs). We found the levels of colonic 5-HT were consistent with the abundance of 5-HT + cell (Pearson  $r = 0.866$ ,  $P < .01$ ; Figure 4C). The abundance of CgA + cell was no different after the CUMS intervention and the treatment of probiotics and prebiotics ( $F = 2.133$ ,  $P > .05$ ; Figure 4D). The 5-HT + cell of the CUMS group evaluated, but the counts of other group decreased after treatment with prebiotics and probiotics. The ratio of 5-HT + cell/CgA + cell showed the similar changes. Furthermore, the ratio of the Lr group was lower than the FOS/GOS group (Figure 4E).

### 3.4 | Effects of prebiotics and probiotics on serotonin levels and metabolites in frontal cortex and hippocampus

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the biosynthesis of the biogenic monoamine serotonin. TPH2 is accountable for the production of 5-HT in the brain. Neuronal 5-HT is a key regulator of mood and behavior, and its deficiency has been implicated in a variety of neuropsychiatric disorders, for example, depression and anxiety. 99% of brain tryptophan



**FIGURE 2** Effect of probiotics and prebiotics on FST and SPT. Values are stated as the mean  $\pm$  standard deviation. # $P < .05$ ; ## $P < .01$  compared with the control group; \* $P < .05$ ; \*\* $P < .01$  compared with the CUMS group;  $\Delta P < .05$  compared with the FOS/GOS group



**FIGURE 3** Comparison of serum 5-HT and colonic 5-HT, colonic expression of TPH1/GAPDH mRNA and SCL6A4/GAPDH mRNA of each group. A, The serum 5-HT of each group. B, The colonic 5-HT of each group. C, Colonic expression of TPH1/GAPDH mRNA. D, Colonic expression of SCL6A4/GAPDH mRNA. Data are standardized to expression levels in the control group. Values are stated as the mean  $\pm$  standard deviation. # $P < .05$ ; ## $P < .01$  compared with the control group; \* $P < .05$ ; \*\* $P < .01$  compared with the CUMS group.  $\Delta P < .05$  compared with the FOS/GOS group

metabolism via its degradation to kynurenine (KYN) catalyzed by indoleamine 2,3-dioxygenase (IDO). Examination of frontal cortex and hippocampus tissues displayed significant differences in the contents of 5-HT, TPH2, and IDO (Table 2). Compared with the control group, the intervention of CUMS prompted a reduction in 5-HT ( $P < .05$ ) and TPH2 ( $P < .05$ ), an increase in IDO ( $P < .01$ ) in frontal cortex. Treatment with probiotics and prebiotics significantly augmented the levels of 5-HT and TPH2 and diminished the levels of IDO in frontal cortex relative to the control and CUMS groups. Furthermore, the effect of the group treatment with *L. rhamnosus* was much better than the prebiotic group (FOS/GOS). No differences in IDO content of the hippocampus were found between the CUMS group vs control group and FOS/GOS group vs CUMS group. Explicitly, concentrations in the *L. rhamnosus* group were significantly different compared with the FOS/GOS group both in frontal cortex and in hippocampus.

### 3.5 | Gut microbiota changes in CUMS intervention and treatment of probiotics and prebiotics

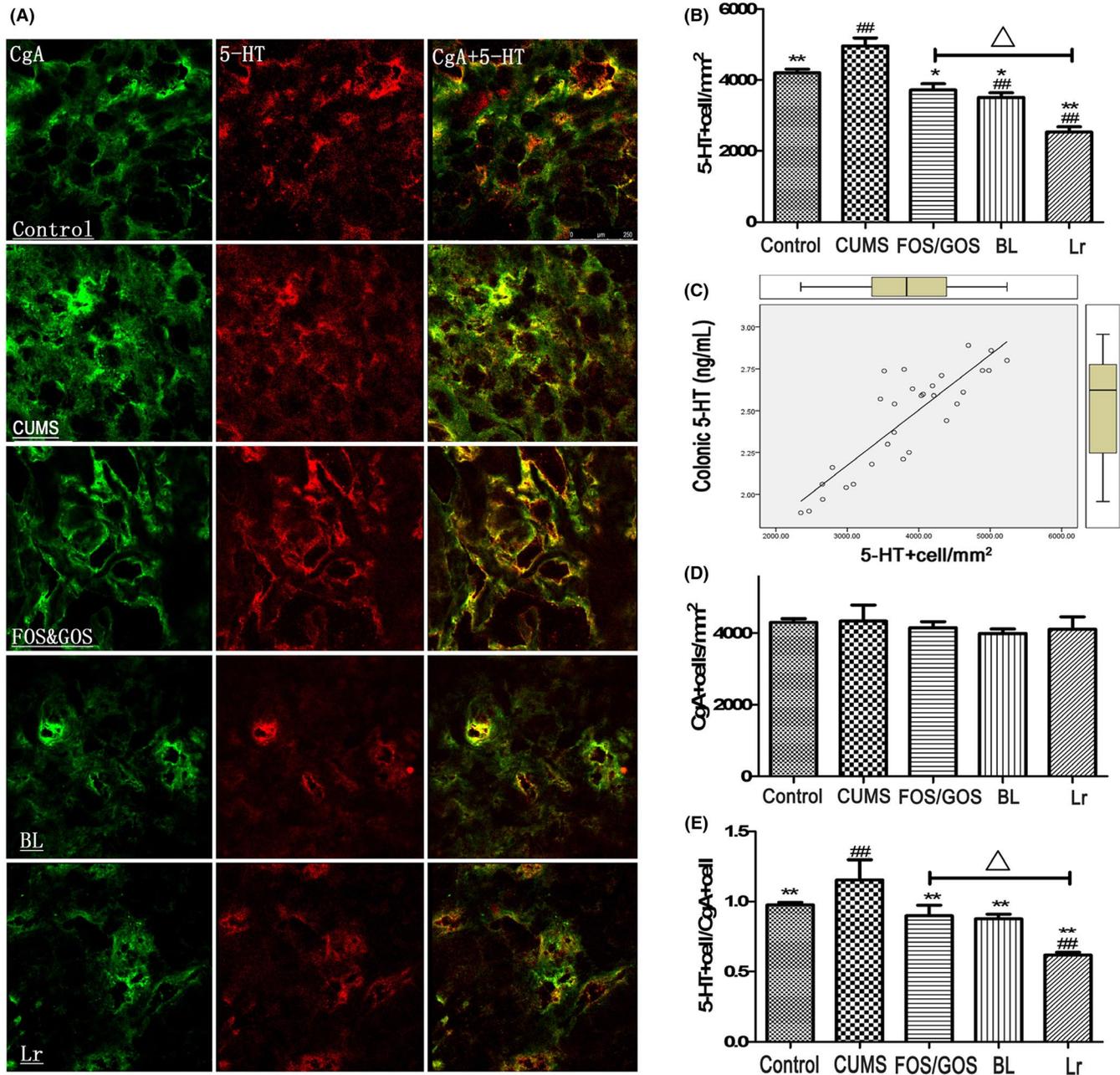
Colonic microbiota composition profiles were examined by the 16S high-throughput gene sequencing-based method. Compositional examination of gut microbiota structure by pyrosequencing revealed that CUMS intervention and pre/probiotic treatment can influence gut microbiota structure. According to the species abundance cluster heat map at phylum level, CUMS intervention caused higher relative abundance of Clostridia, bacilli, and mollicutes. The colonic microbiota structure changed greatly after the treatment with prebiotics and probiotics, especially the *L. rhamnosus* group (Figure 5A). Alterations were detected on the phylum level and on the levels of order, class, family, and genus (Figure 5B; Appendix S1). A noteworthy diminution in the large quantity of Bacteroidetes and an increase in Firmicutes (phylum) were detected in the CUMS group after the intervention

with the CUMS procedure. Shannon index based on the genera profile was calculated to estimate the within-sample ( $\alpha$ ) diversity. The  $\alpha$  diversity at the genus level was much higher in CUMS group ( $P = .047$ , control vs CUMS;  $P = .042$ , CUMS vs FOS/GOS; Tukey Kruskal-Wallis test; Figure 5C). The increased richness of genera in the CUMS group suggested possible increased pathogenic microflora.

Figure 5 Comparative gut microbiota abundance at the phylum and genus level and species abundance clustering map at the phylum level. Data are stated as mean percentage values from each group ( $n = 8$  per group). (a) The difference in cecal bacterial structure at the Phylum level among different groups. The color bar denotes the z-scores. (b, c) The top 10/30 species of each group in terms of maximum abundance on the Phylum/Genus level. (d)  $\alpha$  diversity (as accessed by Shannon index) of each group.

The result of MRPP analysis showed that the differences in microbial community structure between groups was significant ( $P < .05$ ), except control-FOS/GOS and BL-Lr ( $P = .36$ ,  $P = .182$ ; Table 3). Furthermore, a value of all groups greater than 0 indicated greater difference between groups than within groups. The values of Observed-delta and Expected-delta of control-FOS/GOS were both smaller than other groups, which indicated smaller differences within and between the groups. The values of Observed-delta and Expected-delta of Model-Lr were both larger than other groups, which indicated larger differences within and between the groups.

On phylum level, Firmicutes conquered the microbiota of the control group and the CUMS group demonstrating 56% and 66% of all read out, correspondingly, followed by Bacteroidetes, which denoted 36% and 25%. In the microbiota of both prebiotic- and probiotic-fed groups, Firmicutes were suggestively decreased ( $P < .05$  for the CUMS group comparisons, FOS/GOS vs Lr group and control vs BL/Lr group), while Bacteroidetes were augmented ( $P < .05$  for both comparisons, control vs BL/Lr, CUMS vs BL/Lr, FOS/GOS vs BL/Lr). Moreover, feeding *Bifidobacterium longum* and *L. rhamnosus* decreased the proportion of Tenericutes (phylum) compared with the CUMS group.



**FIGURE 4** A, Typical descriptions of colons stained for chromogranin A (CgA) (left), 5-HT (center), and merged (right). B, Quantification of 5-HT + cell. C, Quantification of CgA + cell. D, Ratio of 5-HT + cells/CgA + cells. All cell number are per area of colonic epithelial tissue. Values are stated as the mean  $\pm$  standard deviation.  $^{\#}P < .05$ ;  $^{\#\#}P < .01$  compared with the control group;  $^*P < .05$ ;  $^{**}P < .01$  compared with the CUMS group.  $\Delta P < .05$  compared with the FOS/GOS group

Similarly, a decreased abundance of Clostridia (class), Clostridiales (order), Lactobacillales (order), Lachnospiraceae (family), Ruminococcaceae (family), Coriobacteriales (order) and a raised profusion of Prevotellaceae (family), Prevotellaceae-NK3B31-group (genus), etc, were identified in the *L. rhamnosus*-treated group compared with the CUMS group (Figure 6; Appendix S1).

Figure 7. Correlation between the rate of Firmicutes in total gut microbiota and colonic 5-HT (a); correlation between the rate of Firmicutes in total gut microbiota and 5-HT in frontal cortex (b).

Pearson correlation analysis displays a noteworthy positive association of the ratio of Firmicutes in total gut microbiota versus

colonic 5-HT (Pearson  $r = 0.923$ ;  $P = .000$ ,  $n = 25$ ); Pearson correlation analysis displays a noteworthy negative correlation of the ratio of Firmicutes in total gut microbiota versus frontal CX 5-HT (Pearson  $r = -0.879$ ;  $P = .000$ ,  $n = 25$ ).

## 4 | DISCUSSION

The pathophysiology of depression is complex and involves several different signaling pathways. 5-HT, as a neurotransmitter, is confirmed to be involved in depression.<sup>26</sup> Several studies have established that

**TABLE 2** Concentrations of serotonin (5-HT) and their metabolites in frontal cortex and hippocampus (ng/mg)

Brain area	5-HT		TPH2		IDO	
	Frontal Cx	Hippocampus	Frontal Cx	Hippocampus	Frontal Cx	Hippocampus
Control	409.7 ± 49.7	495.3 ± 38.1	561.3 ± 60.7	495.3 ± 74.3	831.3 ± 24.0	783.7 ± 70.7
CUMS	231.67 ± 66.7 <sup>#</sup>	317.3 ± 68.1 <sup>#</sup>	178.3 ± 28.7 <sup>#</sup>	308.7 ± 91.2 <sup>#</sup>	941.7 ± 58.6 <sup>##</sup>	893.3 ± 72.3
FOS/GOS	629.7 ± 65.2 <sup>##,***</sup>	695.7 ± 111.5 <sup>##,***</sup>	636.7 ± 104.6 <sup>##,***</sup>	685.3 ± 100.6 <sup>##,***</sup>	740.7 ± 42.8 <sup>##,***</sup>	716.7 ± 97.7
BL	690.7 ± 92.2 <sup>##,***</sup>	770.3 ± 70.2 <sup>##,***</sup>	690.7 ± 115.9 <sup>##,***</sup>	761.7 ± 90.2 <sup>##,***</sup>	617.7 ± 32.1 <sup>##,***,ΔΔ</sup>	639.0 ± 54.8 <sup>*</sup>
Lr	815.3 ± 72.9 <sup>##,***,ΔΔ</sup>	890.3 ± 40.5 <sup>##,***,ΔΔ</sup>	827.7 ± 108.2 <sup>##,***,Δ</sup>	890.7 ± 40.7 <sup>##,***,Δ</sup>	261.0 ± 14.5 <sup>##,***,ΔΔ</sup>	261.7 ± 41.6 <sup>##,***,ΔΔ</sup>
F(4,39), P	32.65, .000	31.03, .000	27.11, .000	23.31, .000	145.39, .000	17.90, .000

Note: Data are stated as the mean ± standard deviation (SD). Statistically noteworthy values are emphasized in grey.

<sup>#</sup>P < .05;

<sup>##</sup>P < .01 compared with the control group.

<sup>\*</sup>P < .05;

<sup>\*\*</sup>P < .01 compared with the CUMS group.

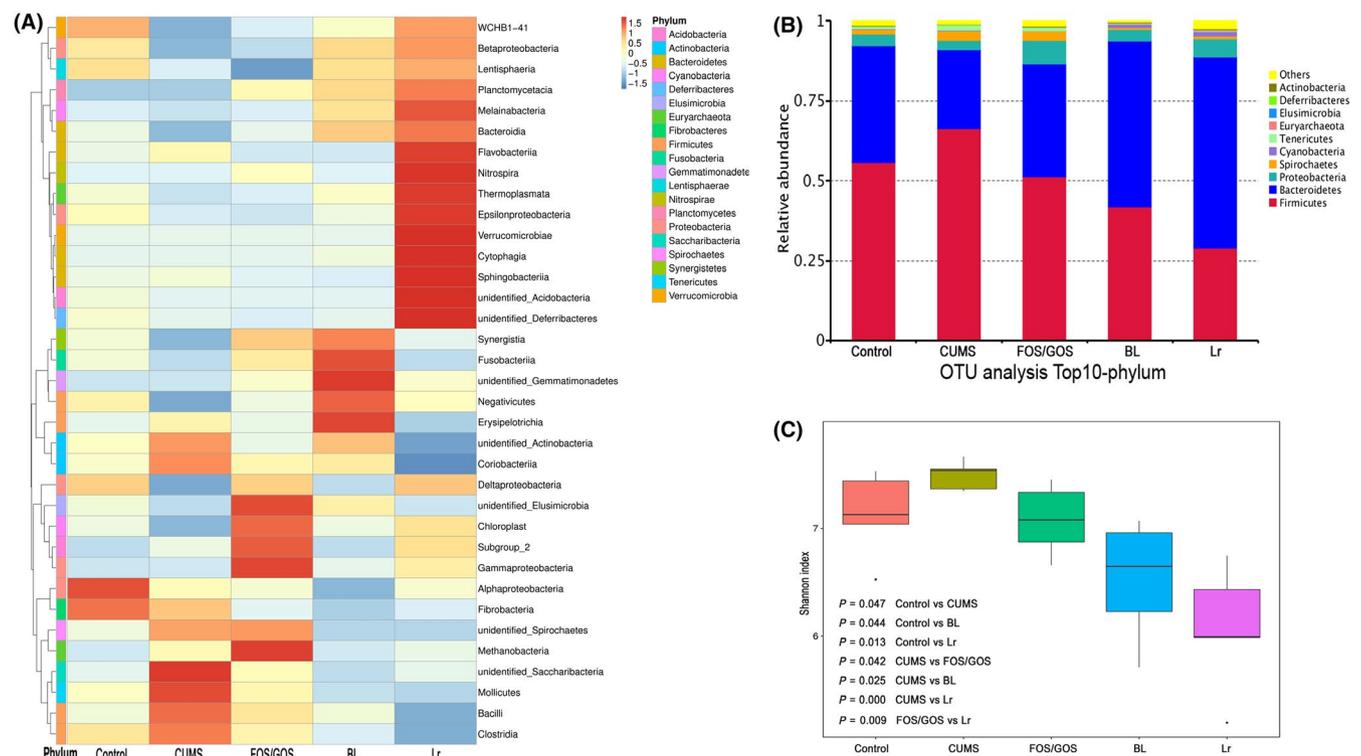
<sup>ΔΔ</sup>P < .01;

<sup>Δ</sup>P < .05 compared with the FOS/GOS group.

the lack of gut microbiota encourages depression-like behavior in rats,<sup>27,28</sup> adjusting the composition of gut microbiota could effectively improve the behavior of CUMS rats through providing probiotics and prebiotics.<sup>29</sup> Jessica M. Yano has previously reported that Indigenous bacteria from the gut microbiota modulate metabolites which stimulate colon 5-HT biosynthesis.<sup>16</sup> In the present study, we selected the common prebiotics (GOS/FOS) and probiotics (*Bifidobacterium longum*, *L. rhamnosus*) compared with the CUMS and control groups, and detected a series of behavioral tests and the monoamine level in

peripheral blood and brain of CUMS rats following 4-week treatment. Furthermore, the colon content of rats was isolated and performed sequencing and data analysis was performed.

The outcomes of this study are constant with previous trials examining that rats subjected to CUMS have a depressive-like behavior, including decreased body weight, sucrose preference percentages, and longer immobility time in FST and SPT. Prebiotic (FOS/GOS) and probiotic (*Bifidobacterium longum* and *L. rhamnosus*) supplements alleviated these changes. However, we found that the



**FIGURE 5** Comparative gut microbiota abundance at the phylum and genus level and species abundance clustering map at the phylum level. Data are stated as mean percentage values from each group (n = 8 per group). A, The difference in cecal bacterial structure at the Phylum level among different groups. The color bar denotes the z-scores. B, The top 10 species of each group in terms of maximum abundance on the Phylum level. C, The  $\alpha$  diversity (as assessed by Shannon index) of each group

**TABLE 3** MRPP analysis of differences between groups

Groups	A	Observed-delta	Expected-delta	Significance
Control-Model	0.04248	0.4112	0.4111	0.04
Control-BL	0.1162	0.4215	0.4769	0.021
Control-Lr	0.08496	0.4293	0.4691	0.022
Control-FOS/GOS	0.001911	0.3937	0.412	0.36
Model-FOS/GOS	0.03615	0.4424	0.4589	0.028
Model-BL	0.1666	0.4527	0.5431	0.011
Model-Lr	0.1756	0.4883	0.5585	0.009
BL-FG	0.09702	0.4702	0.5207	0.02
FG-Lr	0.09394	0.478	0.5275	0.01
BL-Lr	0.0241	0.4604	0.5003	0.182

A smaller value of Observed-delta indicates a small difference within the group, and a larger value of Expected-delta indicates a large difference between the groups. A value greater than 0 indicates greater difference between groups than within groups, while A value less than 0 indicates greater difference within groups than between groups. *P* value of <.05 indicates a significant difference.

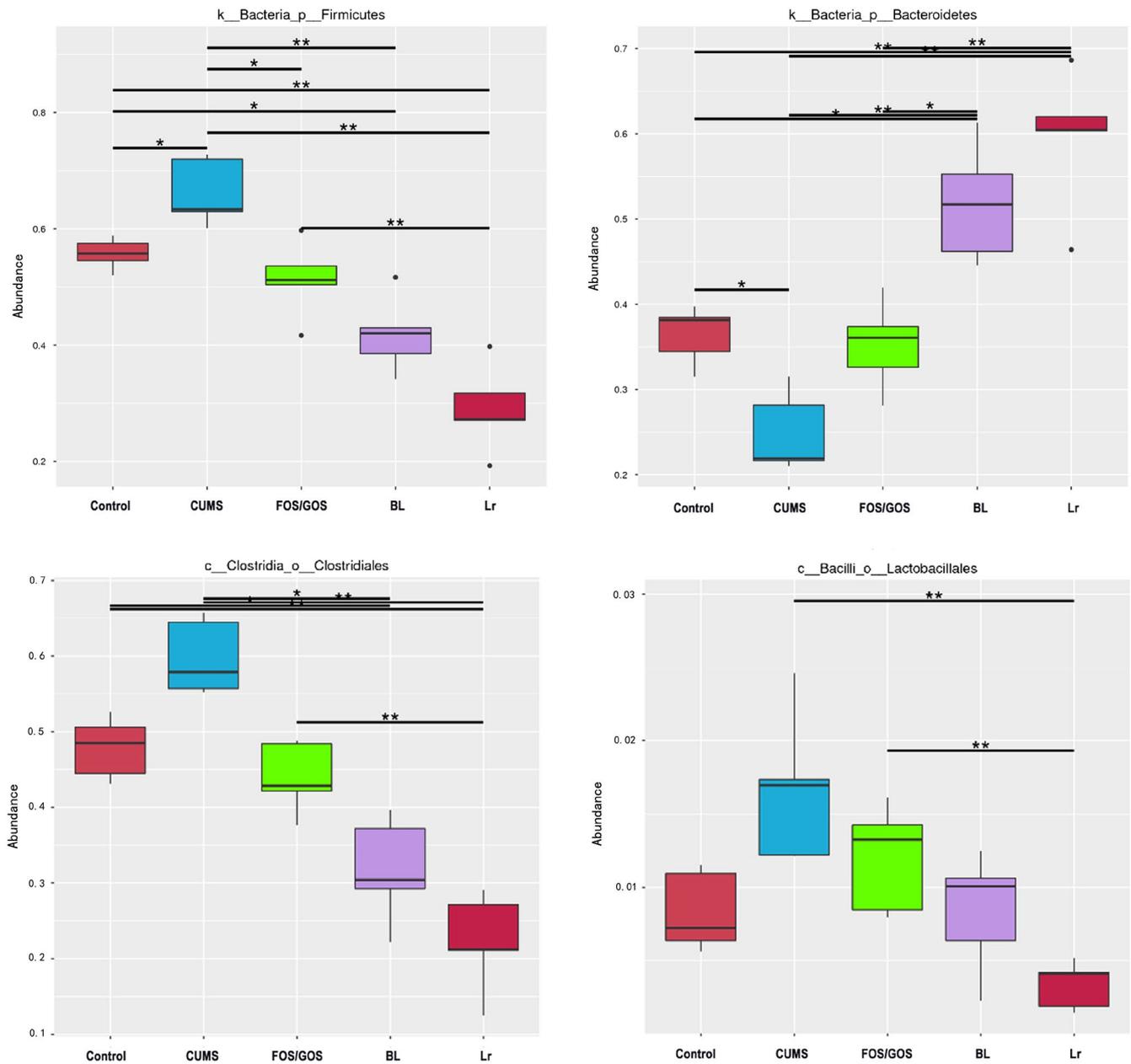
effect of *L. rhamnosus* was significantly better than others through the comprehensive comparison.

There are controversial results of blood 5-HT. Some studies reported that platelet 5-HT levels did not change in CUMS rats,<sup>30-32</sup> whereas some studies reported reduced blood 5-HT levels could be a marker for depression.<sup>34-36</sup> The present study found there were no noteworthy changes in serum 5-HT levels of each group. The reason might be related to blood-brain barriers (BBB). The tight junctions' transmembrane proteins claudins, tricellulin, and occluding restrict paracellular diffusion of water-soluble substances from blood to the brain.<sup>37</sup> Consistent with previous studies, we deduced that peripheral blood 5-HT did not correlate with CUMS because of the complex influential factors.

Previous studies have suggested that gut microbiota played a special role in regulating colonic 5-HT.<sup>16</sup> In our study, we removed a piece of colon tissue and detected the serotonin levels by ELISA. We found that CUMS group exhibited increased levels of colonic 5-HT compared with the control group. To identify stages of 5-HT metabolism which might be affected by the microbiota, important intermediates of the 5-HT pathway were evaluated in colon from control vs other groups. The results suggested that CUMS colons exhibit increased expression of TPH1 (Figure 3C), which was the rate-limiting enzyme for 5-HT biosynthesis in enterochromaffin cells (ECs).<sup>38</sup> CUMS rats also showed decreased colonic expression of the 5-HT transporter SLC6A4 (Figure 3D), manufactured largely by enterocytes to allow 5-HT uptake.<sup>39</sup> The same change trend was found between colon 5-HT and the abundance of 5-HT + cell, while the abundance of CgA + cell showed no difference among (Figure 4). When probiotics and prebiotics were given to in CUMS rats for 4 weeks, both colonic 5-HT and TPH1 all went down correspondingly, whereas SLC6A4 depressed the opposite situation. Maximum of the body's serotonin (~95%) exist in the gut. Inside the bowel, serotonin is produced by the enterochromaffin (EC) subtype of enteroendocrine cell and by serotonergic neurons of the myenteric plexus.<sup>40-43</sup> Studies have shown that tryptophan in the gut can cross

the blood-brain barrier to participate in serotonin synthesis.<sup>44</sup> From these data, we deduced CUMS intervention and pre/probiotic treatment affected the level of colonic 5-HT mainly through serotonin metabolic and transport-related factors were not involved in the changed abundance of EC cells. Furthermore, the decrease in intestinal 5-HT synthesis under the intervention of probiotics might make more tryptophan participate in the synthesis of 5-HT through the blood-brain barrier.

Using 16S rRNA gene sequencing, we found that the phyla Bacteroidetes were significantly decreased in profusion, while Firmicutes were evidently increased in the CUMS group compared with the control group. Compared with the CUMS group, we found that both the BL and Lr groups showed a decreased abundance of Firmicutes (phylum), Clostridia (class), Clostridiales (order), etc. Furthermore, these changes in the Lactobacillus group were significantly different from those in the prebiotic group. Several researches have stated that depression influences the structure of the gut microbiota. Recent study has established noteworthy changes in profusion levels among phyla and genera in the gut microbial community; in the meantime, the metabolism of tryptophan and bile acids was also disturbed after chronic variable stress intervention.<sup>45</sup> Recently, Stephanie Cheung et al reported that the largest number of differentiating taxa was within phylum Firmicutes; nine genera were higher in major depressive disorder.<sup>46</sup> Jessica M et al have reported that indigenous spore-forming bacteria control metabolites that stimulate colon 5-HT biosynthesis from colonic enterochromaffin cells.<sup>16</sup> Clostridium is categorized as a genus under the phylum Firmicutes and class Clostridia, plus includes 221 types till date (September 2017).<sup>47</sup> Clostridium spp. are Gram-positive spore-forming anaerobes, which are found all over the place in the atmosphere and the abdominal tract of humans plus animals.<sup>48</sup> Furthermore, we found there was a noteworthy positive association of the ratio of Firmicutes in total gut microbiota versus colonic 5-HT through Pearson correlation analysis (Pearson *r* = 0.923; Figure 7). All above, we deduced that CUMS could cause gut microbiota perturbations



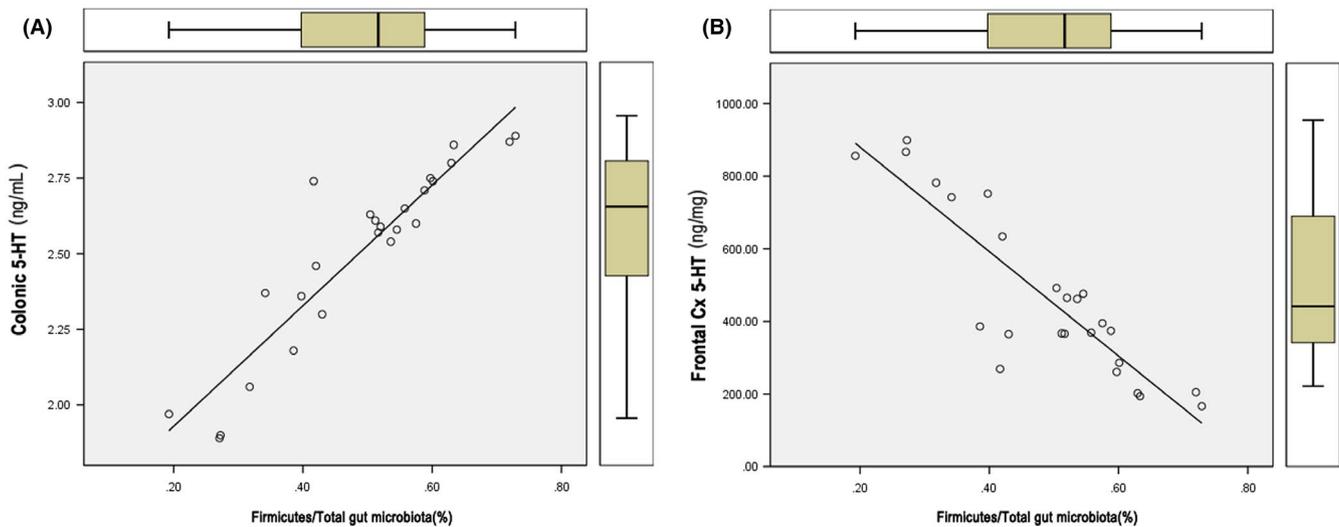
**FIGURE 6** The abundance distribution boxplots of different species between groups. The MetaStat method was used to recognize the suggestively decreased or raised of each group. k, kingdom; p, phylum; c, class; o, order; f, family; g, genus; All values are mean  $\pm$  SEM (n = 5). \*adjusted P value <.05; \*\*adjusted P value <.01

and the composition of gut microbiota is significantly altered, especially Firmicutes significantly evaluated in abundance which promote colonic 5-HT biosynthesis. Probiotics and prebiotics could modulate gut microbiota disturbed by CUMS. The phylum Firmicutes and class Clostridia were markedly decreased after probiotics treatment, corresponding colonic 5-HT decreased.

Central serotonergic neurons are detached from peripheral serotonergic neurons, platelets, and EC cells by the blood-brain barrier, which is resistant to serotonin.<sup>39</sup> Two brain regions including frontal cortex and hippocampus involved in emotional, motivational, and mnemonic processes associated with depression were explored.<sup>29,49</sup> Our results showed that 5-HT levels in frontal cortex

and hippocampus were decreased after the 4 weeks of CUMS procedure. However, the level of 5-HT in frontal cortical and hippocampal significantly increased compared with the CUMS and control groups after treatment with probiotics and prebiotics for 4 weeks. Furthermore, the metabolic enzymes associated with 5-HT showed an increase in TPH2 and a decrease in IDO. The serotonergic hypothesis of depression and several studies suggested that depressive symptoms were related to a reduced 5-HT concentration in the brain synapse and an enhancement in the concentration of this neurotransmitter might be able to induce antidepressive action.<sup>50-53</sup>

There have been various preceding studies with focus on the effects of the "microbiota-gut-brain axis".<sup>54,55</sup> To search the role of



**FIGURE 7** Correlation between the rate of Firmicutes in total gut microbiota and colonic 5-HT (A); correlation between the rate of Firmicutes in total gut microbiota and 5-HT in frontal cortex (B)

“microbiota-gut-brain” axis in CVS-induced depression, we recognized the association of frontal Cortex 5-HT levels and gut microbiota phylum Firmicutes by Pearson's correlation analysis (Pearson  $r = -.879$ ; Figure 7). The result suggested that frontal Cortex 5-HT exhibited negative associations with the ratio of Firmicutes in total gut microbiota, which suggested there might be a negative feedback mechanism between gut microbiota and neurotransmitter. Although the mechanism of the observed antidepressant effect of probiotics is still unclear whether it is due to reestablishment of tryptophan metabolism, or lessening in serotonin turnover. The current data point to a strong link between intestinal inflammation, disruption of serotonin signaling and the consequent alteration in gut motility, and development of depression. Presently, there were some studies, which implied another mechanism by the creation of the neuro-suppressive indole-derivatives<sup>56</sup>; alternative probable mechanism might be through stimulation of the vagal afferent fibers.<sup>57</sup> Overall, additional studies are necessary for a complete understanding of the interaction of this bacterial organism and depression.

This study has various limitations. Firstly, even though we were capable to recognize the concerned gut microbial communities of CUMS rats, we did not inspect extra depression model intervention. Secondly, we only selected the common probiotics (*L. rhamnosus* and *Bifidobacterium longum*) and common prebiotics (FOS/GOS), without other probiotics and prebiotics. Third, only central metabolite signatures of prefrontal cortex and hypothalamus were recognized in our studies. Hence, additional studies involving other brain parts are desired to further examine the central nervous system-based metabolite variations related to modifications in gut microbiota.

## 5 | CONCLUSIONS

The results implied that probiotics and prebiotics exert antidepressive effects in mouse model of CUMS-induced depression. We

established in our experiment that regulating gut microbiota through probiotics and prebiotics has a considerable impact on the modulation of tryptophan metabolism, especially *L. rhamnosus*. 16S rRNA gene sequencing showed that CUMS-induced depression suggestively changed not only the composition of gut microbiota but also the abundance of phylum Firmicutes and other levels. Furthermore, correlation analysis exposed that phylum Firmicutes were strongly correlation with changed colonic and Frontal CX 5-HT metabolites. Overall, these outcomes specify that CUMS-induced depression disturbs the gut microbiota at the profusion level and modifies the host 5-HT metabolism. Probiotics and prebiotics have an effect of regulating the intestinal flora composition and 5-HT metabolism, especially *L. rhamnosus*. Generally, the controlled gut microbiota-associated 5-HT metabolites might be possible biomarkers to review the functional impacts of depression. Regulating the gut microbiota configuration by adding *L. rhamnosus* might be a treatment for depression. However, further studies are required to substantiate the clinical use of probiotics.

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## CONFLICT OF INTERESTS

The authors have no conflict of interests.

## AUTHOR CONTRIBUTIONS

Dianliang Zhang and Huawei Li designed the research study and analyzed the data; Huawei Li, Luqiao Huang and Ping Li performed the research; Huawei Li and Peng Wang analyzed the data and wrote the paper.

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