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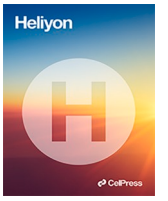

Research article

Electroacupuncture intervention alleviates depressive-like behaviors and regulates gut microbiome in a mouse model of depression

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ABSTRACT

Electroacupuncture (EA) is a neuroregulatory therapy for depression. Nonetheless, the effects of EA on the gut microbiome in mice models of depression are not well established. Here, using a chronic unpredictable mild stress (CUMS) model in mice, we evaluated the antidepressant effects of EA and changes in gut microbiota with behavioral tests and 16S rRNA gene sequencing. The results found that EA increased the time spent in the center area of the open-field test and the percentage of sucrose preference and reduced the immobility time in the tail suspension test in CUMS-treated mice. Furthermore, the genus *Lachnoclostridium, Ruminococcaceae_UCG-002* and *Rikenellaceae_RC9_gut_group* were enriched in the CUMS group, which was positively correlated with depressive-like behaviors. Whereas phylum *Actinobacteria* and genus *Allobaculum*, *Bifidobacterium*, *Dubosiella*, *Rikenella* and *Ileibacterium* were enriched in the EA and CUMS + EA groups, all of which were negatively correlated with depressive-like behaviors. This study characterizes gut microbiota under EA treatment and provides new insights into the association of antidepressive-like effects of EA and gut microbiota.

1. Introduction

Depression is a widespread mental disorder and a leading cause of disease disability and burden [1,2]. The lifetime risk of depression is 15–18 % [3], and it is a well-established risk factor for suicide [4]. Previous studies elucidate that about 56–88 % of patients with depression have suicidal ideation [5], among which 15 % have recurrently attempted suicide [6]. However, despite a

Abbreviations: EA, Electroacupuncture; CUMS, chronic unpredictable mild stress; FMT, fecal transplantation; OFT, Open-field test; TST, Tail suspension test; SSRIs, selective serotonin reuptake inhibitors; MAC, minimum alveolar concentration; SPT, sucrose preference test; PERMANOVA, permutational multivariate analysis of variance; OTUs, operational taxonomic units; PCOA, principal co-ordinate analysis; LEfSe, linear discriminant analysis (LDA) effect size; MDD, major depressive disorder; GV20, governing vessel 20; TSC, time spent in the central area; SP, sucrose preferences; IT, immobility time; GV29, governing vessel 29; lncRNAs, long noncoding RNAs; miRNAs, exosomal microRNAs.

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battery of standardized psychological or pharmacological treatments, such as antidepressant medications or cognitive behavioral therapy, only about 74 % of patients show improvement in symptoms [7] and around 20 % do not respond to any intervention [8]. Thus, the identification of the additional pathogenesis and the development of novel treatment strategies for depression are urgently needed.

Electroacupuncture (EA) comes together with the advantages of electrophysiological stimulation and acupuncture. The antidepressant effect of EA has attracted wide attention. Previous clinical work found that the antidepressant effects of EA were the same as that of selective serotonin reuptake inhibitors (SSRIs) [9], and better anti-depressive efficacy was presented when the two were combined [10,11]. Furthermore, EA also improved the depressive symptoms in methamphetamine addicts during abstinence and

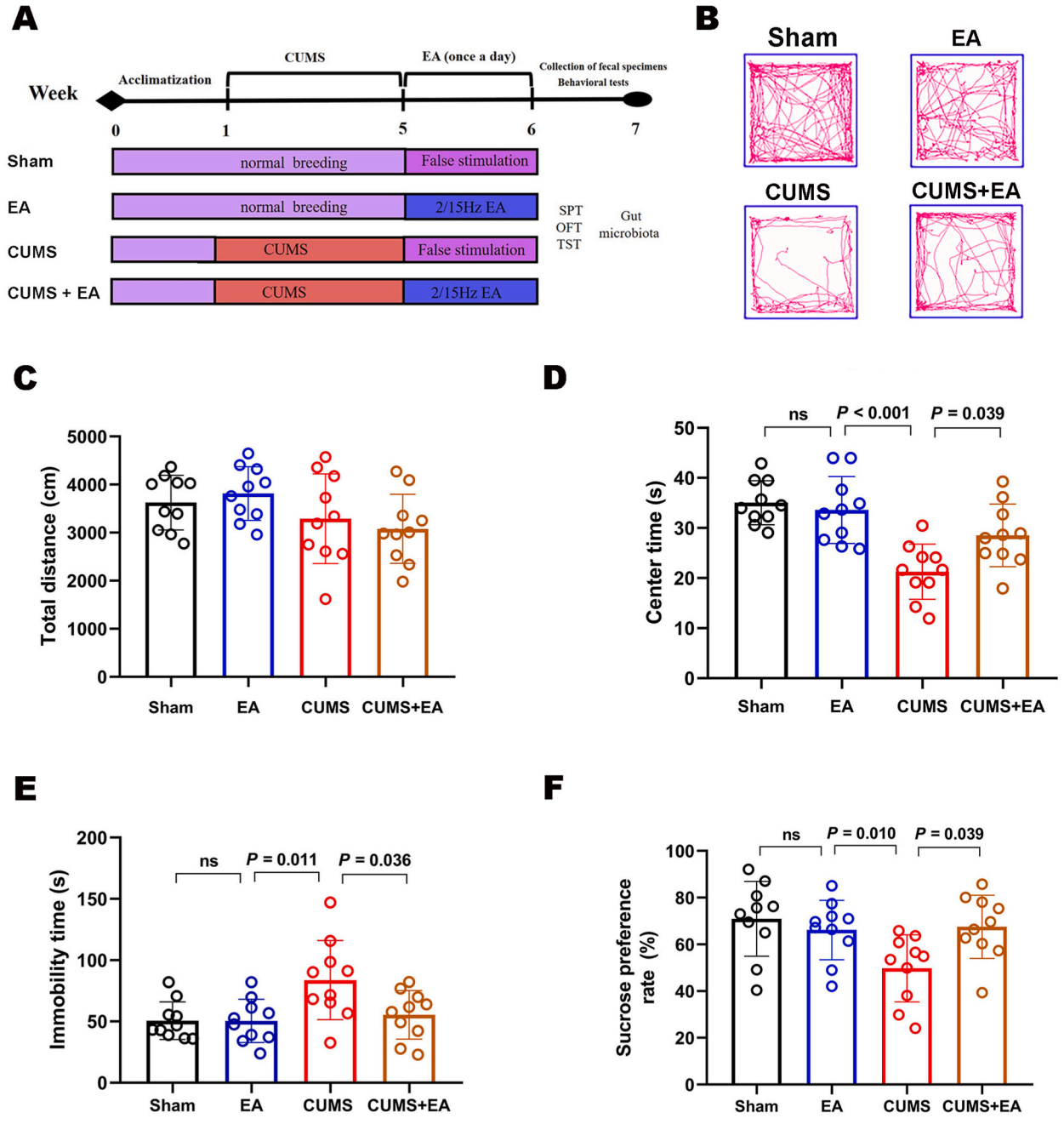

Fig. 1. EA ameliorates depressive-like behaviours in CUMS-treated mice. (A) Schematic diagram of CUMS exposure. (B) Representative open field movement traces. (C) The total distance traveled in the OFT. (D) Time spent in the center of the OFT. (E) Percentages of sucrose consumption in the SPT. (F) Immobility in the TST. The data are presented as means ± SEM (n = 10 mice/group). The circle represents one value from individual mice (C–F). *P*-values were determined by one-way analysis of variance with the Bonferroni post-hoc test to compare the means of different groups.

patients with polycystic ovarian syndrome [12,13]. However, the selection of electroacupuncture stimulation sites and stimulation parameters still lacks a biological basis, and the potential biological mechanisms underlying the effects of specific acupoints are still unclear.

Recently, accumulated evidence indicates that intestinal dysbacteriosis is involved in the development of depression. For instance, compositional differences between healthy controls and patients with depression in gut microbiota were observed [14–16]. Meanwhile, preclinical research also found that gut microecology was imbalanced, which was related to depressive-like behavior [17]. Moreover, several antidepressants have antimicrobial effects [18]. On the other hand, gut microbiota dysfunction leads to low resilience to depression [19,20] and the changes in microbiota in depression are related to quality-of-life indicators [21]. Correspondingly, probiotics and prebiotics have anti-depressive effects [22] and fecal transplantation (FMT) induces depressive-like behavior in recipient germ-free mice [23,24]. These results suggest that gut microbiota dysfunction has contributed to the pathogenesis of depression, and the improvement of the function of the gut-brain axis might have some potential therapeutic effects for depression [25,26]. Importantly, a previous study found that EA decreased plasma adrenocorticotropic hormone and cortisol levels, and mRNA of hypothalamic corticotropin-releasing hormone in chronic unpredictable mild stress (CUMS)-treated rats [27], which are involved in the regulation of gut function and microecology. Furthermore, recent studies elucidated that EA regulated the expression of brain-gut peptides in patients with Parkinson's disease [28] and relieved experimental colitis by modulating the gut microbiota [29,30]. Together, current evidence strongly indicates that the regulation of the gut microbiome was involved in the neuroregulation effects of EA. Nevertheless, whether EA could regulate the gut microbiome in animal models of depression remains to be elucidated.

CUMS is one of the most valid rodent models for mimicking human depression, and CUMS-treated male C57BL/6 mice have been widely used for the pathogenesis of depression in the preclinical testing, such as the neural circuit, neuroimmune regulatory, and gut microbiota dysfunction of depression [31,32]. In this context, the present study aims to investigate the influence of EA on the composition of gut microbiota in CUMS-treated mice identify the microbiota signatures specific for EA treatment and observe the correlation between the gut microbiota and depressive-like behavior as well.

2. Materials and methods

2.1. Animals

The experimental procedures were under the guidelines and approved by the Animal Use and Protection Committee of the Xi'an Gaoxin Hospital (No. 2023KY011). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines (National Research Council 2003, "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research"). Adult male C57BL/6 mice, weighing 18–22 g and aged 8 weeks, were group-housed in cages with five mice per cage. The mice followed a 12-h light/dark cycle, with lights on from 8:00 a.m. to 8:00 p.m. They had unrestricted access to water and food and were kept in an environment with temperatures ranging from 20 to 25 °C. All experiments were conducted during the light phase excluding for CUMS experiment.

2.2. Experimental design

After acclimatization for 7 days, 40 mice were randomly assigned to Sham, EA, CUMS, and CUMS + EA groups (n = 10 per group). Mice in the Sham group were maintained in their home cages while mice in the CUMS and CUMS + EA groups were subjected to CUMS for 4 weeks. Subsequently, mice in the EA and CUMS + EA groups were treated with EA stimulation and mice in the Sham and CUMS groups were subjected to false stimulation (received anesthesia and acupuncture) for 7 days and the behavioral tests were conducted 24 h later (Fig. 1A).

2.3. CUMS

The CUMS experiment was performed as per a previous study [33]. Briefly, mice were subjected to two randomly selected stressors daily and each of the stressors was equally administered 2 or 3 times during the treatment for 4 weeks. The stressors were as follows: (a) continuous lighting (12 h); (b) physical restraint (1.5 h); (c) paired housing (24 h); (d) 24 h cage tilt (30°); (e) forced swim (8 °C for 5 min); (f) food or water deprivation (24 h); (g) wet bedding for 24 h; and (h) cold stress at 4 °C for 10 min.

2.4. EA treatment

Mice received daily session EA application (dilatational waveform, 2/15 Hz, 1 mA) for 30 min for 7 days under anesthesia (isoflurane, 1.5 minimum alveolar concentration (MAC)) [34], by using an instrument (Qingdao Xinsheng Ltd., Serial Number: 227033). The acupuncture needle was inserted into the acupoint Governing Vessel 20 (GV20) and another end was connected to an electrode clamped onto the tail to complete the electrical circuit. The same acupoint was used but without the application of electricity for the false stimulation.

2.5. Behavioral tests

Mice were acclimatized to the separate room prior to each behavioral test for at least 30 min. The area was cleaned with 75 %

ethanol between tests. The sucrose preference test was conducted first. Then mice were exposed to an open-field test and 2 h later, the tail suspension test was performed.

2.5.1. Sucrose preference test (SPT)

SPT is a reward-based test used to reflect anhedonia [35]. Mice were single-housed and habituated with two bottles of water for 24 h, and then the water was replaced with 1 % sucrose for another 24 h. Next, they were deprived of water and food for 24 h. After that, water and 1 % sucrose were placed in pre-weighed bottles, and the mice could consume the fluid freely for 2 h. According to a previous study, the consumption of each fluid was measured and the sucrose preference was calculated [36].

2.5.2. Open-field test (OFT)

The locomotor activity and exploratory and anxiety-like behavior were detected by OFT [37]. Mice were placed in the center and their activity was recorded for 5 min in an open-field box (40 cm × 40 cm × 40 cm). The time and distance spent in arena were analyzed by the Top Scan software (Clever Sys Inc., USA) [38].

2.5.3. Tail suspension test (TST)

TST is a paradigm that evaluates learned helplessness [39]. TST was performed according to a previous study [40]. Mice were hung with standard laboratory tape and allowed to hang for 6 min. The immobility was analyzed via Freeze Scan (Clever Sys, Inc., USA).

2.6. 16S rRNA microbiome sequencing

Fecal samples were collected before the behavioral tests [41]. E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, USA) was used to perform DNA extraction and the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the 16S rRNA gene based on previous studies by polymerase chain reaction [42,43]. Amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and extracted from 2 % agarose gels. Amplicons were quantified by Quantus™ Fluorometer (Promega, USA), and then were pooled in equimolar and paired-end sequenced (2 × 250 bp) on an Illumina MiSeq PE300 platform. The ribosomal database project classifier algorithm (http://rdp.cme.msu.edu/) was used to analyze the taxonomy of each 16S rRNA gene sequence and the associations between behavioural parameters and microbial composition and the comprehensive microbial phenotypes were evaluated by permutational multivariate analysis of variance (PERMANOVA).

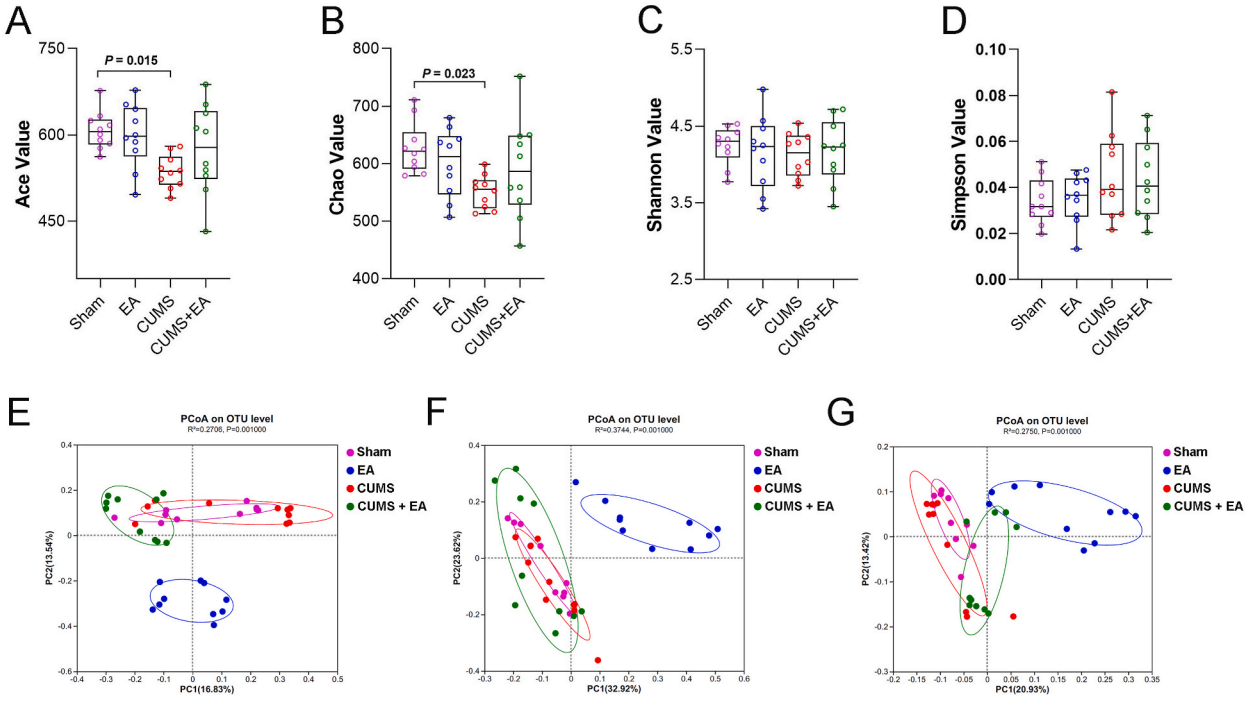

Fig. 2. Comparison of α and β diversity analysis between each group. (A–D) α diversity including the (A) Ace, (B) Chao, (C) Shannon and (D) Simpson index. (E–G) A clear separation among each group by principal co-ordinate analysis (PCoA) based on bray-curtis (E), weighted UniFrac (F) and unweighted UniFrac (G) distance index on OTU level. The circle represents one value from individual mice.

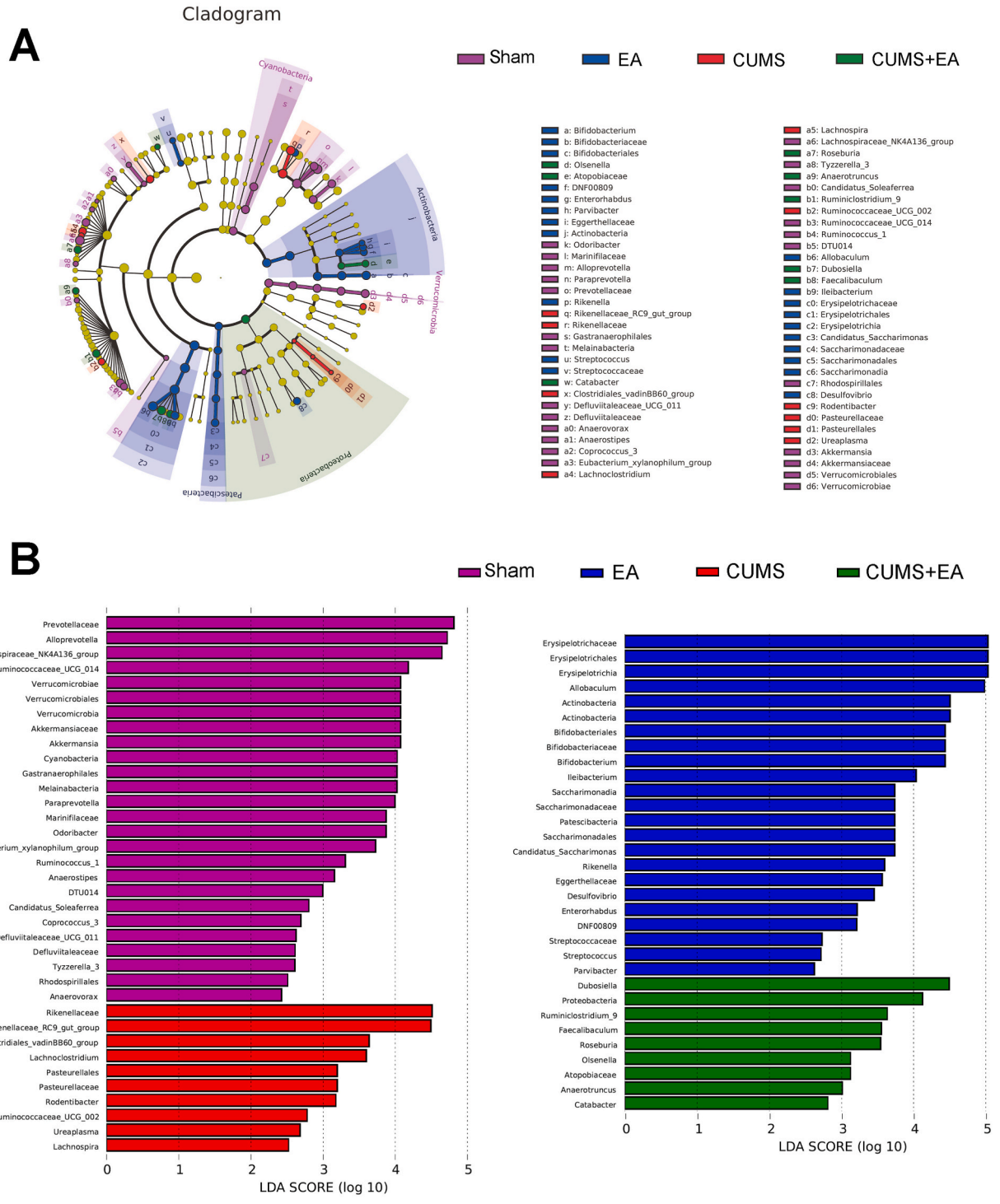

Fig. 3. Taxonomic cladogram generated based on LEfSe and LDA scores among Sham, EA, CUMS, and CUMS + EA groups. (A) Bacterial taxa enriched in the Sham (purple dots), EA (blue dots), CUMS (red dots), and CUMS + EA group (green dots). (B) LDA scores indicate taxa enriched in the Sham (purple), EA (blue), CUMS (red), and CUMS + EA group (green), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.7. Statistical analyses

Statistical analyses were performed using SPSS v.19.0 software (SPSS Inc., Chicago, IL, USA) and R software package (http://www.R-project.org/). Data were analyzed by nonparametric test (Kruskal-Wallis, did not satisfy normal distribution) or one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test to compare means of different groups. Spearman's rank correlation coefficient was used to calculate the associations of behavioural tests with the differential flora at the genus level as well as group-enriched operational taxonomic units (OTUs). The microbial community was shown by the heat map and the correlation network diagram was drawn using Cyberscape software.

3. Results

3.1. The influence of EA on depressive-like behavior

Depressive-like behavior was obtained 24 h after the final EA simulation. There was no significant difference in the total distance traveled in OFT ($F_{3,\ 36} = 2.161$, $P = 0.110$; Fig. 1B and C). Meanwhile, significant differences were observed in the time spent in the central area (TSC, $F_{3,\ 36} = 11.56$, $P < 0.001$; Fig. 1D) of OFT, the sucrose preferences (SP) in the SPT ($F_{3,\ 36} = 4.433$, $P = 0.009$; Fig. 1E), as well as immobility time (IT) displayed in the TST ($F_{3,\ 36} = 5.113$, $P = 0.005$; Fig. 1F). CUMS markedly reduced the time spent in the center of the OFT and sucrose preferences rate but increased the immobility time significantly (CUMS *vs.* Sham, $P < 0.01$).

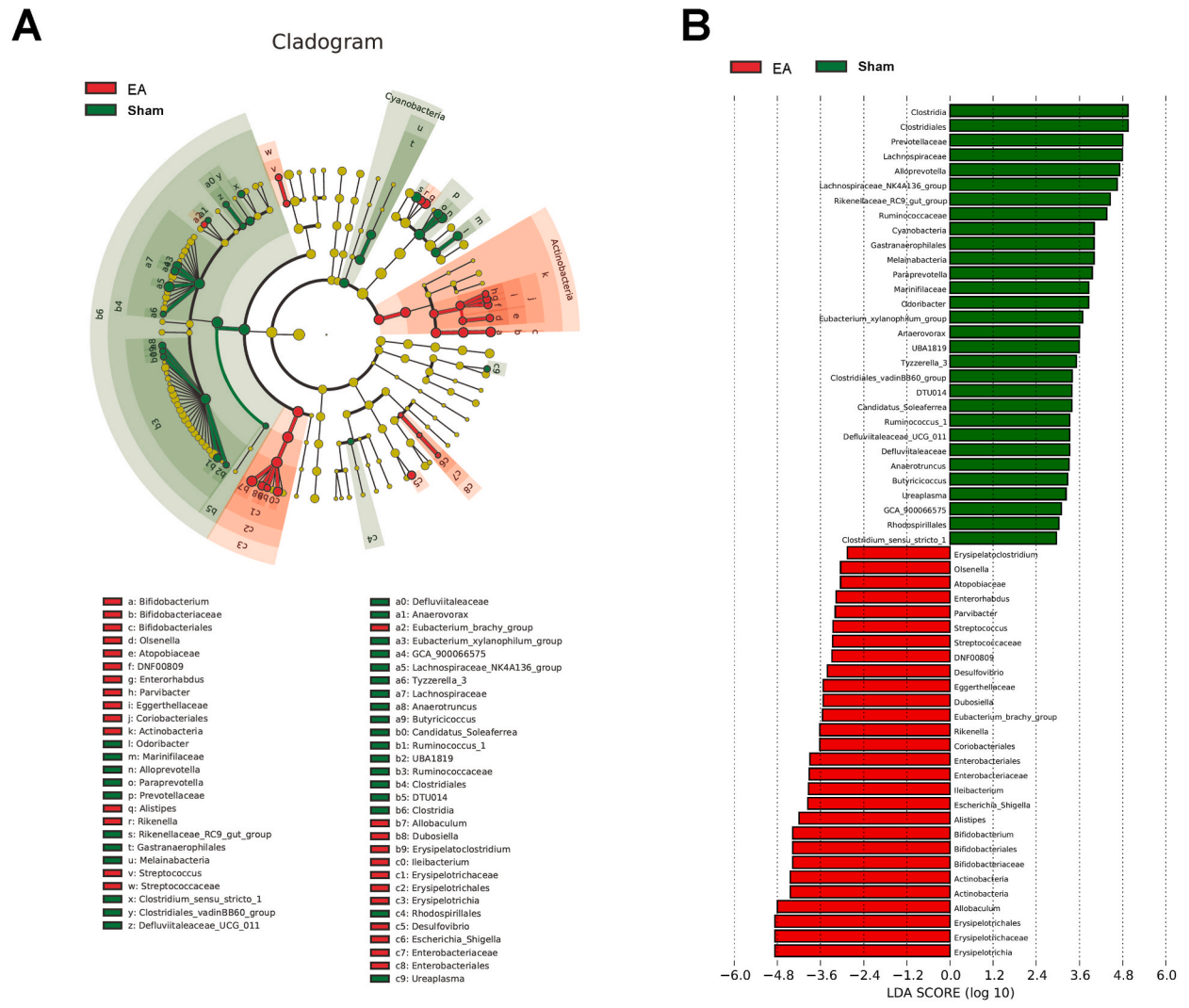

Fig. 4. Taxonomic cladogram and LDA scores between the Sham and EA group. (A) Bacterial taxa enriched in the EA group (red dots) and Sham (green dots). (B) LDA scores indicated taxa enriched in the Sham or EA group, with a positive LDA score (green) and negative LDA score (red), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

EA treatment increased TSC, SP rate and decreased IT (CUMS *vs.* CUMS + EA, $P < 0.05$). These results suggest that EA could ameliorate the depressive-like behavior of CUMS-treated mice.

3.2. Microbial diversity and distinct microbiome signatures

We obtained a total of 926,967,577 bases and 2,210,842 high-quality 16S rRNA gene sequences, which were subsequently clustered into 1068 OTUs after downstream analysis. The α-diversity values, including community richness indices (Ace and Chao) and community diversity indices (Shannon and Simpson) were observed (Fig. 2A–D). There were significant differences in the indices of Ace ($H = 10.912$, $P = 0.014$) and Chao ($H = 8.715$, $P = 0.031$) but not Shannon ($F_{3,\ 36} = 0.194$, $P = 0.890$) and Simpson ($F_{3,\ 36} = 1.457$, $P = 0.265$) among the four groups. Meanwhile, Ace and Chao were decreased in the CUMS group when compared with the sham group ($P < 0.05$). β-diversity analysis showed that the microbiomes were divided into clusters by principal co-ordinate analysis (PCoA) based on bray-curtis ($R^2 = 0.270$, $P < 0.01$), weighted UniFrac ($R^2 = 0.374$, $P < 0.01$) and unweighted UniFrac ($R^2 = 0.275$, $P < 0.01$) on OTU level (Fig. 2E–G). Together these findings suggest that there were differences in bacterial diversity among the four groups.

The relative abundance of microbial compositions was compared at the phylum, order, class, and family levels (Fig. 3A and B). The abundance of phylum *Cyanobacteria* and *Verrucomicrobia*; class *Melainabacteria* and *Verrucomicrobiae*; order *Rhodospirillales, DTU014, Gastranaerophilales* and *Verrucomicrobiales*; and family *Defluviitaleaceae*, *Marinifilaceae*, *Akkermansiaceae* and *Prevotellaceae* were enriched in the Sham group. Meanwhile, the abundance of phylum *Patescibacteria* and *Actinobacteria*; class *Saccharimonadia*, *Actinobacteria* and *Erysipelotrichia*; order *Saccharimonadales*, *Bifidobacteriales* and *Erysipelotrichales*; and family *Streptococcaceae*, *Eggerthellaceae*, *Saccharimonadaceae*, *Bifidobacteriaceae* and *Erysipelotrichaceae* were enriched in the EA group. Although the abundance of order *Pasteurellales* and family *Pasteurellaceae*, *Clostridiales_vadinBB60_group* and *Rikenellaceae* were enriched in the CUMS group, there was no enriched member of phylum and class observed. Moreover, the abundance of phylum *Proteobacteria* and family *Atopobiaceae* were enriched in the CUMS + EA group. To characterize the shared and distinct microbial compositions at the genus level, we further found that 14 genera were enriched in the Sham group, 10 genera were enriched in the EA group, 6 genera were enriched in the CUMS group, and 7 genera were enriched in the CUMS + EA group. The detailed information is depicted in Supplementary Table 1.

3.3. Microbiota signatures specific for EA treatment

To investigate microbial signatures able to discriminate EA treatment, the microbial compositions between the sham and EA groups were compared (Fig. 4A and B). At the phylum level, the relative abundance of *Cyanobacteria* was enriched in the sham group, and *Actinobacteria* was enriched in the EA group. Meanwhile, the abundance of *Defluviitaleaceae*, *Clostridiales_vadinBB60_group*, *Marinifilaceae*, *Ruminococcaceae*, *Lachnospiraceae,* and *Prevotellaceae* was enriched in the sham group; whereas *Topobiaceae*, *Streptococcaceae*, *Eggerthellaceae*, *Enterobacteriaceae*, *Bifidobacteriaceae,* and *Erysipelotrichaceae* were enriched in the EA group at the family level. Moreover, 17 genera were enriched in the sham group, and 15 genera were enriched in the EA group (Supplementary Table 2).

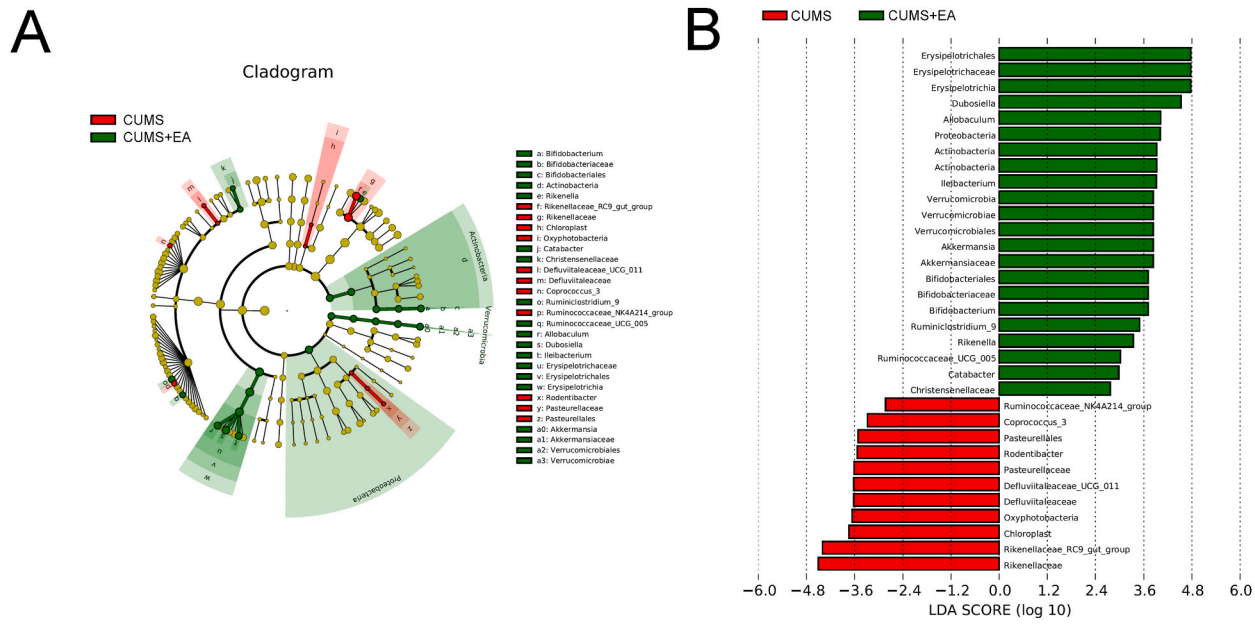

Fig. 5. Taxonomic cladogram and LDA scores between the CUMS and CUMS + EA groups. (A) Bacterial taxa enriched in the CUMS + EA (green dots) and CUMS group (red dots). (B) LDA scores indicated taxa enriched in the CUMS + EA or CUMS group, with a positive LDA score (green) and negative LDA score (red), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

As shown in Fig. 5A and B, at the phylum level, *Verrucomicrobia*, *Actinobacteria,* and *Proteobacteria* were enriched in the CUMS + EA group. *Pasteurellaceae*, *Defluviitaleaceae,* and *Rikenellaceae* were enriched in the CUMS group, while *Christensenellaceae*, *Bifidobacteriaceae*, *Akkermansiaceae,* and *Erysipelotrichaceae* were consistently higher in the CUMS + EA group at the family level. Furthermore, *Ruminococcaceae_NK4A214_group*, *Coprococcus_3*, *Rodentibacter*, *Defluviitaleaceae_UCG_011* and *Rikenellaceae_RC9_gut_group* were enriched in the CUMS group, while *Catabacter*, *Ruminococcaceae_UCG_005*, *Rikenella*, *Ruminiclostridium_9*, *Bifidobacterium*, *Akkermansia*, *Ileibacterium*, *Allobaculum* and *Dubosiella* were enriched in the CUMS + EA group at the genus level (Supplementary Table 3). Together, these findings indicate that EA intervention can regulate gut microbiota composition. Importantly, phylum *Actinobacteria;* family *Bifidobacteriaceae* and *Erysipelotrichaceae;* and genera *Rikenella*, *Dubosiella*, *Bifidobacterium*, *Ileibacterium* and *Allobaculum* were enriched in both the EA and CUMS + EA groups, suggesting that these bacteria may reflect the biological regulation of EA.

3.4. Altered OTUs and the association of microbiota and depressive-like behavior

Co-occurrence network analysis illustrated the statistical covariation among altered OTUs (Fig. 6). CUMS-enriched and CUMS + EA-enriched OTUs were less interconnected than EA-enriched and sham-enriched OTUs. Meanwhile, the correlation between CUMS-enriched and CUMS + EA-enriched OTUs, EA-enriched and sham-enriched OTUs, as well as CUMS + EA-enriched and sham-enriched OTUs were mainly negative, while the correlation between EA-enriched and CUMS + EA-enriched, and sham-enriched and CUMS-enriched, OTUs were mainly positive.

We assessed the association of gut microbiota at the genus level and depressive-like behaviors (Fig. 7). The abundance of *Allobaculum*, *Ileibacterium*, *Ruminococcaceae_UCG−002*, *Alloprevotella*, *Gastranaerophilales_norank*, *Lachnoclostridium*, *Bifidobacterium*, *Dubosiella*, *Ruminiclostridium*, *Rikenella*, *GCA−900066575*, *Clostridiales_vadinBB60_group_norank*, *Lachnospiraceae_unclassified* and *Rikenellaceae_RC9_gut_group* was negatively correlated; while the abundance of *Ruminiclostridium_9*, *Enterorhabdus*, *Olsenella*,

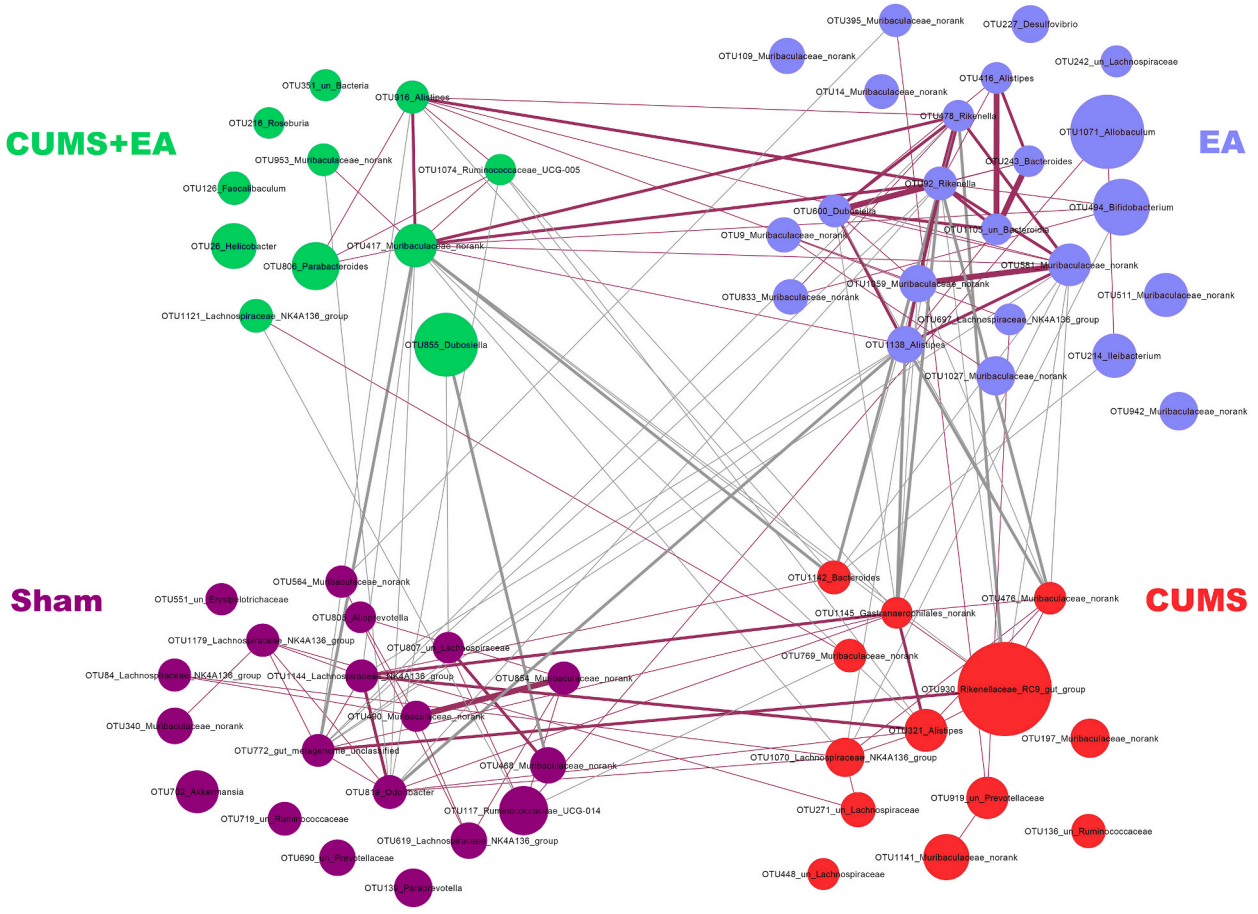

Fig. 6. The network of OTUs is differentially enriched in the four groups. Nodes indicate OTUs enriched in the Sham (purple), EA (blue), CUMS (red), and CUMS + EA group (green), and node sizes reflect the mean abundance of significant OTUs. The width of the line represents the absolute value of the correlation coefficient, and only the line with the absolute value of the correlation coefficient greater than 0.7 is shown. The color of the line represents positive and negative correlation: pink is a positive correlation and gray is a negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

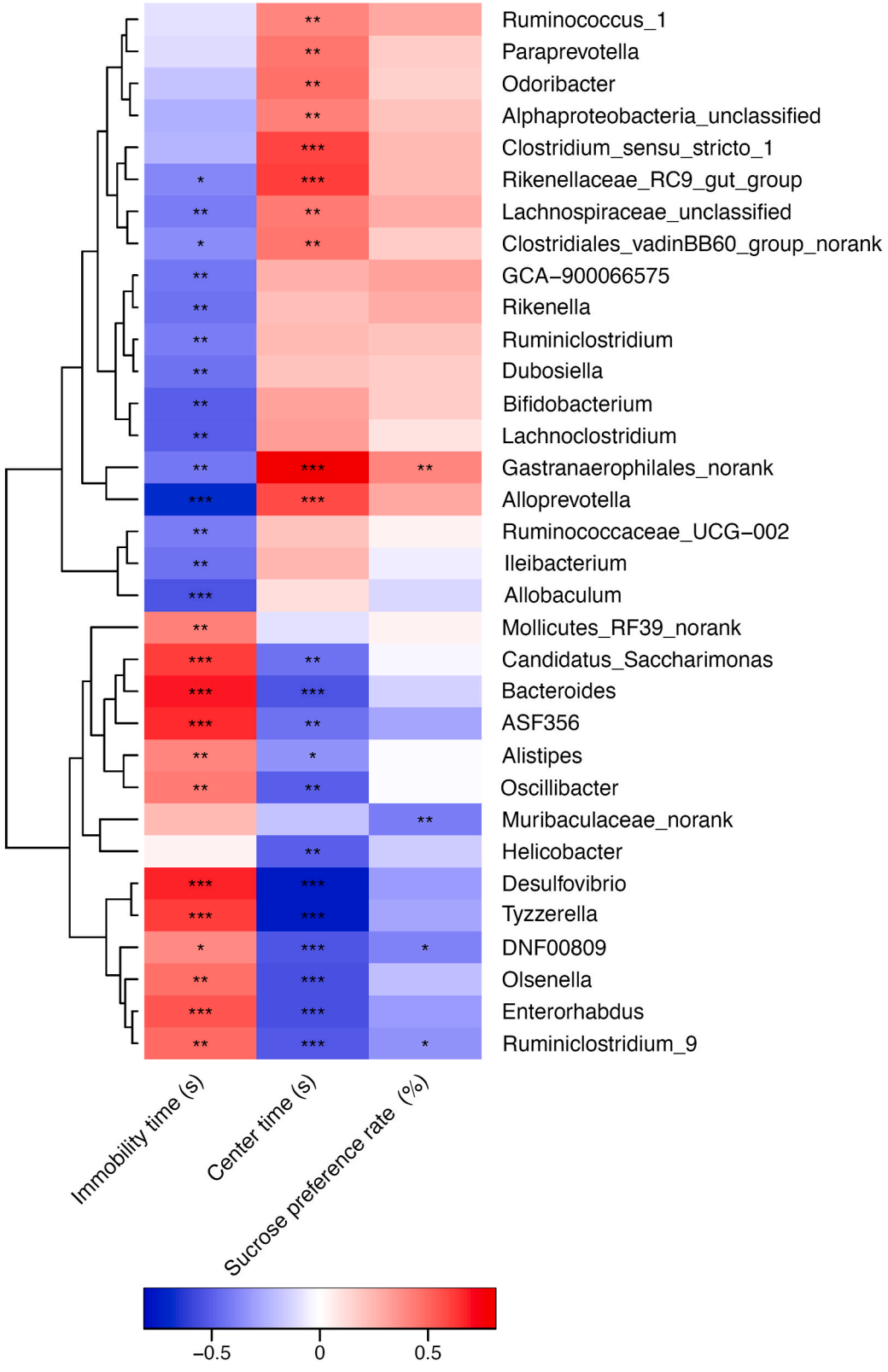

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Fig. 7. Associations of behavioral parameters and gut microbiota. The heat map shows the correlation coefficients between depressive-like behaviors and bacterial taxa at the genus level. Blue and red squares indicate negative and positive correlations, and the intensities of the colors are proportional to the degree of correlation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

DNF00809, *Tyzzerella*, *Desulfovibrio*, *Oscillibacter*, *Alistipes*, *ASF356*, *Bacteroides*, *Candidatus_Saccharimonas,* and *Mollicutes_RF39_norank* was positively correlated with IT in the TST. Meanwhile, the abundance of *Ruminococcus_1*, *Paraprevotella*, *Odoribacter*, *Alphaproteobacteria_unclassified*, *Clostridium_sensu_stricto_1*, *Clostridiales_vadinBB60_group_norank*, *Lachnospiraceae_unclassified*, *Rikenellaceae_RC9_gut_group*, *Alloprevotella,* and *Gastranaerophilales_norank* was positively correlated; while the abundance of *Candidatus_Saccharimonas*, *Bacteroides*, *ASF356*, *Alistipes*, *Oscillibacter*, *Helicobacter*, *Desulfovibrio*, *Tyzzerella*, *DNF00809*, *Olsenella*, *Enterorhabdus,* and *Ruminiclostridium_9* was negatively correlated with the TSC in the OFT. Moreover, the abundance of *Gastranaerophilales_norank* was positively correlated, while *Muribaculaceae_norank*, *DNF00809,* and *Ruminiclostridium_9* was negatively correlated with SP rate. Taken together, the abundance of these bacteria was correlated with depressive-like behaviors, and the regulation of these bacteria might be a potential target for antidepressant strategy.

4. Discussion

The current study elucidates that EA alleviates depressive-like behaviours in a mouse model of CUMS. Moreover, we characterized the gut microbial signatures specific to EA treatment for the first time by using 16S rRNA microbiome sequencing. Notably, we explored the correlations between depressive-like behaviors and changed microbiota, as well as the interaction between characteristic bacteria. Aside from the potential explanation of the pathogenesis of depression, these results may also help to reveal the new target of the antidepressant effect of EA.

Bidirectional communication of microbiome-gut-brain axis (MGBA) has been widely elucidated, and the influence of stress, mode of delivery, probiotics, diet, and environmental exposure on brain function through the influence of gut microbiota is underlined. Dysfunction of gut microbiota was not only associated with digestive system diseases but also neuropsychiatric disorders [44,45]. The relationship between dysfunction of gut microbiota and depression has been largely revealed [23,24,26,46]. It is well known that the brain can modulate the enteric nervous system and gastrointestinal tract via the HPA axis and autonomic nervous system [47], and thus influence the gut microbiota by affecting the microenvironment [48]. Therefore, neuromodulation technology such as acupuncture could affect the composition of gut microbiota. Consistent with these observations, we identified that EA regulates the gut microbiome in both depressed and normal mice, indicating that the regulation of gut microbiota might be involved in the antidepressive-like effects of EA. We speculate that similar to other neuroregulatory techniques, the influence of EA on microbiota was also indirectly via regulating the activity of the brain-gut axis. Previous works already found that EA could regulate the function of the vagus nerve [49], and the immune and endocrine systems such as systemic inflammation [50], oxidative stress [51] and sex hormones [52], all of which can affect the microbial structure and play a role in the pathogenesis of depression [53]. On the other hand, the overall efficacy of EA combined with probiotics was superior to that of the conventional drugs [54], suggesting that the microbial function may also have a synergistic effect on the antidepressant effect of EA. However, the comparison of the antidepressant effects of EA combined with probiotics and EA alone, as well as their impact on the microbiota, needs further exploration. Moreover, the present study only stimulated the acupoint Baihui, stimulated other acupoints related to intestinal function, such as Zusanli (ST 36) and Zhongwan (CV 12), and observed their effects on gut microbiota, which may further verify the regulatory effect of EA on microbiota [55].

The association between the diversity of gut microbiota and depressive-like behaviors is not consistent. For example, a recent work showed that the diversity but not the richness was reduced [56], while another work found that the Chao1 and ACE indices were decreased while there is no significant difference in the Simpson and Shannon indices in CUMS-treated rats [57]. Similarly, a previous work elucidated that the diversity and richness were reduced in CUMS-treated mice [58], while another study found that these indices were not changed in the same model [32]. The present study also showed that only the richness indices (Ace and Chao) decreased in the CUMS group. However, there is no significant difference between CUMS and CUMS + EA in the α-diversity values. Meanwhile, the β-diversity was different among the four groups and the EA group is distinguished from other groups, indicating the effect of EA on the diversity of gut microbiota is not obvious.

Although phylum *Actinobacteria* only represent a small percentage of the human microbiota, they are crucial for maintaining intestinal homeostasis [59]. Nowadays, they produce two-thirds of antibiotics and a vast array of immunosuppressants and antiviral compounds [60,61]. Notably, *Actinobacteria* might be a potential biomarker for the antidepressant efficacy of ketamine [62] and the alleviating effect of *Actinobacteria* on depression also has been reported [63]. In our study, the abundance of *Actinobacteria* was increased in the EA group (EA *vs.* sham) and CUMS + EA group (CUMS + EA *vs.* CUMS), suggesting *Actinobacteria* might also be a potential target for the antidepressive-like effect of EA. On the contrary, previous studies found that the relative abundance of *Actinobacteria* was increased in patients with major depressive disorder (MDD) [23,64]. The reasons for the above differences may be related to factors such as the disease phenotype, severity of symptoms of the enrolled patients, and microbial detection methods. It also strongly suggests that the effect of EA on *Actinobacteria* needs to be confirmed in clinical research.

Moreover, at the genus level, EA treatment also increased *Rikenella*, *Dubosiella*, *Ileibacterium*, *Bifidobacterium*, and *Allobaculum* in both normal and depressed mice, all of which were positively correlated with the time spent in the central area of the OFT and negatively correlated with the immobility time displayed in the TST, suggesting these genera were involved in the antidepressant

effects of EA. However, a previous clinical study found *Actinobacteria* was increased while the *Bacteroidetes* was decreased in patients with depression [23]. Moreover, the family *Prevotellaceae* and genera *Coprococcus* and *Faecalibacterium* were decreased in depression subjects when compared to controls [65]. A preclinical study also found that *Bacteroides*, *Alloprevotella,* and *Rikenella* were increased, whereas *Allobaculum* was decreased in CD36$^{-/-}$ mice, which could alleviate depressive-like behaviors [66]. These discrepancies may be related to the inconsistency of animal models or the sources of clinical samples. Moreover, we also found that the genus *Ruminococcaceae_UCG-002* and *Lachnoclostridium* were abundant in the CUMS group, which were negatively correlated with time spent in the central area of the OFT. Meanwhile, *Rikenellaceae_RC9_gut_group* was also abundant in the CUMS group, which was negatively correlated with time spent in the central area of the OFT, but positively correlated with immobility time displayed in the TST, indicating that these three kinds of bacteria are related to depressive-like behavior and might be target bacteria for antidepressant effects.

It should be noted that this study did not explore the potential mechanism of EA regulating the microbiota. Previous works already found that EA could regulate the immune and endocrine systems such as systemic inflammation [50] and oxidative stress [51], all of which can affect the microbial structure and play a role in the pathogenesis of depression [53]. On the other hand, EA also regulates the function of the vagus nerve [49], observing the effect of EA on microbiota after subdiaphragmatic vagotomy may help understand the neural mechanism of EA. Moreover, recent evidence suggests that long noncoding RNAs (lncRNAs) and exosomal microRNAs (miRNAs), especially miR-139-5p, play nonnegligible roles in the pathogenesis of depression [67,68]. The effects of EA on lncRNAs and miRNAs may also partially explain its antidepressant effect. However, we just identified the microbiota that is associated with the anti-depressive effect of EA, the impact and mechanism of these microbiotas on depressive-like behavior should be further verified through the combination of germ-free mice and fecal bacterial transplantation. In addition, a previous study found that 2 Hz EA treatment for 14 d can effectively improve depressive-like behavior in rats with CUMS, and its mechanism may be related to its regulation of hippocampal basic fibroblast growth factor [69]. A recent clinical study also found that continuous intervention with EA for 8 weeks has an improvement effect on the mental symptoms in patients with insomnia and depression, and the therapeutic effect can last up to 32 weeks [70]. We only observed the antidepressant effect of EA for one week, the effect of longer-duration EA on CUMS mice is still unknown. Moreover, the present study only observed the differences in microbiota between different treatment groups after EA intervention and did not compare the changes in microbiota before and after EA intervention within the same group. Furthermore, we did not explore the sustained impact of EA. Further observation of the sustained effects of EA on mouse microbiota at different time stages may help explain the sustained effects of EA.

In general, the neuromodulation effect of EA is related to its acting site and parameters. For example, EA applied at the governing vessel 20 (GV20, Bai hui) and governing vessel 29 (GV29, Yintang) improved the quality of sleep for insomnia in patients with depression [70]. Preclinical studies found that EA applied at GV20 and GV29 (continuous wave with 2 Hz frequency and 0.6 mA intensity) could alleviate depressive-like behaviors in CUMS-treated rats [69], and EA applied at GV20 (100 Hz, 2 mA) could alleviate sleep deprivation-induced depressive-like behavior [71]. Interestingly, a large number of studies elucidate that EA (2/15 Hz) applied at the GV20 conferred neuroprotective effects [72,73]. Based on those observations, the parameter of EA treatment in the present study was 2/15 Hz with an intensity of 1 mA, and it was applied at the GV20, which may provide data for explaining the mechanism of acupuncture at potential acupoints in Chinese medicine. However, the effect of different EA parameters, acupuncture sites, and time course, especially the long-time effects on gut microbiota and depressive-like behavior needs further investigation.

In addition, previous studies found that inhalation anesthesia can directly affect the gut microbiota in rodents [74,75], we cannot rule out the potential effect of inhaling anesthetics on gut microbiota due to mice being anesthetized during EA stimulation. Moreover, other factors such as gender can also lead to differences in microbial composition [76]. Although male mice can partially avoid the effects of physiological cycle hormone levels on the microbiota, female mice exposed to CUMS also induced depressive-like behaviors and the effect of EA on female CUMS-treated mice and its regulatory effect on gut microbiota should be elucidated in the future. Finally, the symptoms of depressive patients are more complex, and the improvement effect of EA on depressive symptoms and its impact on intestinal flora in depressive patients need to be verified through clinical research.

In summary, our results showed that EA treatment alleviated depressive-like behavior and regulated the gut microbiome in a mouse model of CUMS. Meanwhile, we assessed the relationship between depressive-like behavior and microbiota characteristics. These findings provide evidence regarding the potential possibility of using EA in the treatment of depression and new insights into its mechanisms. It also provides a potential theoretical basis for the application of EA monotherapy and EA combined with other novel treatment strategies, such as music therapy and effective extraction of traditional Chinese medicine [77,78]. Moreover, this study may further provide theoretical support for the treatment of depression with probiotics and the explanation of the mechanism of neural regulation technology from regulating brain-gut axis activity. However, the effectiveness and potential side effects of EA with different parameters and its molecular mechanisms of action, as well as the characteristics and function of microbiota at the species level should be investigated through basic and clinical research in the future.

Ethics statement

The experimental procedures were under the guidelines and approved by the Animal Use and Protection Committee of the Xi'an Gaoxin Hospital (No. 2023KY011).

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Data availability statement

The data will be made available on request.

CRediT authorship contribution statement

Jia-quan Wei: Writing – original draft, Investigation. **Jie Bai:** Writing – original draft, Funding acquisition, Data curation. **Cuihong Zhou:** Methodology. **Huan Yu:** Formal analysis. **Wen Zhang:** Formal analysis. **Fen Xue:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Hong He:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Fen Xue reports financial support was provided by National Natural Science Foundation of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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