Alpha Psychiatry

ORIGINAL ARTICLE
Alpha Psychiatry 2024;25(4):493-501
DOI: 10.5152/alphapsychiatry.2024.241561

Low-frequency Transcranial Magnetic Stimulation Ameliorates Anhedonic Behaviors and Regulates the Gut Microbiome in Mice Exposed to Chronic Unpredictable Mild Stress

ABSTRACT

Objective: This paper presents a preliminary study on whether low-frequency transcranial magnetic stimulation (LF-TMS) can modulate the gut microbiota in mice with chronic unpredictable mild stress (CUMS).

Methods: Mice received LF-TMS (1 Hz, 20 mT) for 28 consecutive days under chronic unpredictable mild stress (CUMS). The composition of gut microbiota of stool samples were tested.

Results: CUMS caused significant changes in gut microbiotas, specifically in community diversity of gut microbiotas ($P < .05$). Compared with the stressed group mice, the Chao1 index ($P < .05$), Observed species index ($P < .05$), Faith's PD index ($P < .05$) and Shannon index ($P < .05$) of the LF-TMS treatment group were significantly increased. Furthermore, 1 Hz LF-TMS-treatment partially recovered chronic stress induced changes of microbiotas, such as the abundance of *Chloroflexi*, *Actinobacteria*.

Conclusion: These results manifested that LF-TMS treatment can improve the anhedonic behaviors caused by CUMS in mice, which are connected with regulating the related intestinal microbial community disturbance, including species diversity, structure of gut microbiota, and species composition.

Keywords: Low-frequency transcranial magnetic stimulation, Chronic unpredictable mild stress, Gut microbiota

Introduction

Major depressive disorder (MDD) is a prevalent chronic medical condition defined by low mood, cognitive disorder, insomnia, and depression.[1] The World Health Organization (2019) evaluates that the global productivity loss due to its high prevalence, recurrence, and mortality rates amounts to $1 trillion USD annually.[2,3] However, the current pathophysiological explanations for major depression still mainly focus on the paranormal anomalies of monoamine neurotransmitters.[4] Antidepressants generated under these therapies only alleviate impairments in about 40% to -50% of patients with major depression and vary widely in their response to existing treatments, being effective in less than half of diagnosed patients and even leading to irretrievable side effects, drug withdrawal, and high relapse rates.[5-7] Therefore, it is urgent to further study the potential pathophysiological mechanism of major depression from a new perspective. Studies on the human microbiome suggest that the regulation of the microbiota-gut-brain axis generates an effect on mental diseases like depression.[8] Therefore, the gut microbiota and 'its metabolites play a vital role in depression. Dysbiosis is involved in the occurrence of depression, meanwhile providing a new therapeutic approach.[9]

The microbial-gut-brain axis is a two-way functional system. Microbiome changes can emerge early in MDD and may result in the generation of MDD, but lasting abnormal processes in

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Received: February 13, 2024
Revision Requested: March 18, 2024
Last Revision Received: April 10, 2024
Accepted: April 25, 2024
Publication Date: July 1, 2024

Cite this article as: Gao L, Zhao X, Wu L, et al. Low-frequency transcranial magnetic stimulation ameliorates anhedonic behaviors and regulates the gut microbiome in mice exposed to chronic unpredictable mild stress. *Alpha Psychiatry.* 2024;25(4):493-501.

MDD further lead to dysbiosis by changing the gut environment.[9] Stress plays a significant role in the organism and brings about disorder of the intestinal microbiota. The study manifested that exposure to 2 hours of social disruption, can influence microbial populations in the colon, that increased plasma biomarkers of intestinal barrier permeability in depressed patients were associated with changes in gut microbes.[10-12]

Recent studies have shown that the US Food and Drug Administration supported the therapy of repeated transcranial magnetic stimulation (rTMS) for remedying major depression in 2008.[13] There are reports that have revealed that rTMS relieves depressive-like symptoms and modulates the gut flora and medium-to-long-chain fatty acids in chronic unpredictable mild stress (CUMS) induced mice.[14] In addition, low-frequency transcranial magnetic stimulation (LF-TMS; 1 Hz) can improve depressive-like symptoms and learning and memory disorders in animal models by regulating synaptic function, and can also treat depressive symptoms in depressed patients.[15,16]

However, currently very few studies have been carried out on the ameliorative function of LF-TMS on depressed mice focusing on the intestinal flora, and their potential mechanism of action has not been elucidated deeply.[17] Thus, this study exposed mice to CUMS for 4 weeks to induce depression-like symptoms. Intestinal microbial sequencing was used to investigate the mechanism of LF-TMS on depression through its effects on intestinal flora regulation.

Methods

Mice

Thirty male C57BL/6 mice of 6 to 8 weeks (18 to 25 g) used in present experiment were acquired from the Animal Laboratory of the North China University of Technology. The mice were maintained under a 12-hour light-dark cycle at ambient temperature (23 to 25°C) and constant humidity (40% to 50%) with free access to food and water. All experiments were performed in accordance with the Animal Management Regulations of the Ministry of Health of the People's Republic of China and were approved by the Animal Research Ethics Committee of North China University of Science and Technology (LX2021 071). All mice were divided into three groups: wild group (Control [CON], n = 10), stressed group (CUMS, n = 10), and intervention group (CUMS+LF-TMS, n = 10). The stressed group mice were subjected to chronic stress treatment for 4 weeks, while wild-type mice were maintained under normal conditions.

Chronic Unpredictable Mild Stress

The CUMS schedule was adopted from a previous report.[18] The animals were exposed to a series of mild stressors over 28 days. The stressors included dark/light cycle reversal, ice water swimming (4°C, 1 minute), bondage (2 hours), white noise (85 dB, 5 minutes), cage tilting (45° tilting), food and water deprivation, and tail-clipping (1 minute). One stressor was applied to each mouse per day. The order of stressors was varied every week to ensure unpredictability. All animals were fed without apparent damage.

Low-frequency Transcranial Magnetic Stimulation (LF-TMS)

The LF-TMS protocol was based on our previous study.[13] Mice maintained normal respiration during rTMS cure. The rTMS consists of a magnetic stimulator and a circular coil with a diameter of 6.5 cm connected to the magnetic stimulator. The center of the coil was pressed against the mouse's head 5 mm from the skin. Each animal received 28 days of pulse training with a pulse intensity of 1 Hz, 20 mT, and an action time of 60 seconds. LF-TMS was conducted at 10 AM every day. The wild group mice were afforded the same restraint procedure and exposed to noisy magnetic stimulation. The animals were maintained in restraints during individual treatment.

Sucrose Preference Test (SPT)

The SPT method is consistent with our previous reports.[13] Sucrose preference was calculated by the following formula: Sucrose preference (%) = Sucrose intake (g) / [sucrose intake (g) + water intake (g)] × 100 %.[19] The test was conducted before and after the 4 weeks of modeling.

Sample Collection

After modeling and treatment, mouse fecal samples were collected in sterile freezing tubes from 8 AM to 10 AM, and immediately frozen in liquid nitrogen for 16S rRNA gene sequencing. All mice were terminally anesthetized with isoflurane (G45 991, EZVET, Beijing ZS Dichuang Technology Development Co., Ltd., Beijing, China) after the animal behavioral experiment, and the hippocampal tissues were extracted.

16S rRNA Gene Sequencing

V4-V5 hypervariable regions of the bacterial 16S rRNA gene were amplified through polymerase chain reaction with primers F: GTGCCAGCMGCCGCGGTAA and R: CCGTCAATTCCTTTGAGTTT. Each sample was repeated three times. Amplicons were pooled equimolarly and paired-end sequenced using an Illumina MiSeq platform after being extracted and quantified. DADA2[20] was adopted to perform quality filtering, splicing, denoising, and dechimerization using qiime dada2 denoise-paired. After dereplication by DADA2, we acquired a feature table and denoised feature sequences, which are named amplicon sequence variants (ASVs) (corresponding to ASV representing sequence). Clustering was performed using Vsearch. Firstly, cutadapt to excise primer fragments of sequences, setting -O to ten and discarding sequences that did not match the primers; Vsearch's fastq_mergepairs module was employed to splice sequences; quality control of spliced sequences was performed using the fastq_filter module; the derep_fulllength module was used to remove duplicate sequences; the cluster_size module was conducted to cluster the de-duplicated sequences at the 98% similarity level, and the uchime_denovo module was employed to remove chimeras. The chimeras were filtered by a perl script (https://github.com/torognes/vsearch/wiki/VSEARCH-pipeline) in the set of sequences after Qualtiy Control (QC) to obtain high-quality sequences. Using the cluster_size module, high-quality sequences were clustered at the 97% sequence similarity level, and operational taxonomic unit (OTU) tables and representative sequences were produced for subsequent microbiomics analysis, respectively. Linear discriminant analysis Effect Size (LEfSe) analysis screened different strains based on a linear discriminant

MAIN POINTS

- *CUMS caused changes in gut microbiota.*
- *LF-TMS improves the anhedonic behaviors in CUMS mice.*
- *LF-TMS recovers the intestinal microbial community disturbance in CUMS mice.*

analysis score (LDA score) greater than 2 and the inter-group non-parametric factor Kruskal-Wallis test.

Statistical Analysis

Behavioral data are shown as mean ± standard error of mean (SEM). Statistical analyses were conducted using GraphPad Prism 8.0 (San Diego, California, USA). Differences in means among groups were analyzed using one-way Analysis of Variance (ANOVAs), followed by Bonferroni post hoc test. Alpha diversity data were compared by Kruskal-Wallis test with Dunn's post hoc test. $P < .05$ was considered significantly different.

Results

The hypothesis was confirmed by the following results, which were acquired from behavioral experiments and 16S rRNA Gene Sequencing.

Influence of LF-TMS on Body Weights and Sucrose Preference in Stressed Mice

Body weights and sucrose preferences were measured before and after modeling. Nutritional and anhedonia statuses were evaluated by body weight tests and sucrose preference measurements, respectively.

The functions of LF-TMS on body weights are shown in Figure 1A. One-way ANOVA indicated that there were statistical differences in body weights among the three groups (Figure 1A, $F_{(2, 27)} = 107.8$, $P < .001$). Post-hoc tests showed that body weights were distinctly lower in the model group than in the Control group ($P < .001$, at the 28th day). However, it was considerably elevated by LF-TMS in the intervention group mice compared to the model group ($P < .001$, at the 28th day). These results indicated that LF-TMS can significantly reverse CUMS-induced weight loss.

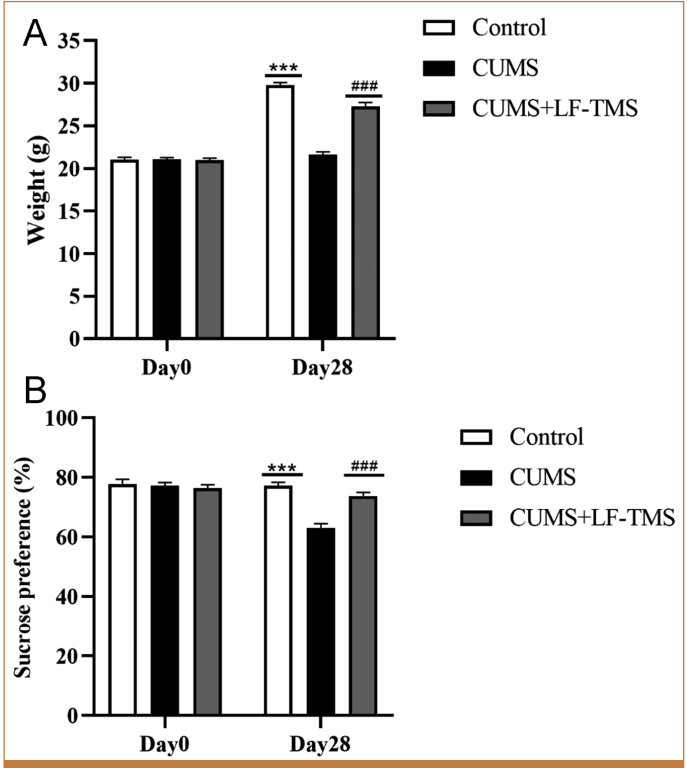

Figure 1. The effects of chronic unpredictable mild stress (CUMS) and low-frequency transcranial magnetic stimulation (LF-TMS) treatment on body weight (A) and sucrose preference (B). Data are presented as mean ± standard error of mean (SEM). ***$P<.001$ denote significant difference between the Control group and the CUMS group, ###$P<.001$ denote significant difference between the CUMS group and the CUMS+LF-TMS group, n = 10 in each group.

The functions of LF-TMS on sucrose preference are shown in Figure 1B. One-way ANOVA illustrated that there were statistical differences in sucrose preference among the three groups ($F_{(2, 27)} = 32.95$, $P < .001$). Post-hoc tests revealed that the sucrose preference was distinctly decreased in the model group compared to the wild group ($P < .001$, at the 28th day). However, it was dramatically elevated by LF-TMS in the treatment group compared to the model group ($P < .001$, at the 28th day). This suggests that LF-TMS effectively alleviates the development of anhedonic symptoms in a mouse model of CUMS.

Influence of LF-TMS on Gut Microbiota in Stressed Mice

Species Diversity Analysis of Gut Microbiota: Following this, we researched the impact of LF-TMS on the intestinal flora composition of stressed mice using 16S rRNA gene sequencing. Both the Rarefaction curve (Figure 2A) and the Cumulative species curve (Figure 2B) tended to be flat, indicating that the richness, diversity, and uniformity of all sample communities were good and that the sequencing data were large enough to fully reflect most of the intestinal microbial diversity information in the stool samples of each group of mice, which could be used for subsequent microbiome analysis. In order to evaluate the Alpha diversity of microbial communities more comprehensively, the Chao1 index and Observed species index were used to characterize the richness, Faith's PD index was used to characterize the evolution-based diversity, and Shannon index and Simpson index were used to characterize the diversity. Post-hoc analysis indicated that compared with the wild group, the Chao1 index (Figure 2C, $P < .05$), Observed species index (Figure 2D, $P < .05$), Faith's PD index (Figure 2E, $P < .05$) and Shannon index (Figure 2F, $P < .05$) of the stressed group were distinctly decreased. There was a similar result in the Simpson index, although the difference was not significant (Figure 2G, $P > .05$). Compared with the CUMS group, the Chao1 index (Figure 2C, $P < .05$), Observed species index (Figure 2D, $P < .05$), Faith' s PD index (Figure 2E, $P < .05$) and Shannon index (Figure 2F, $P < .05$) of the CUMS+LF-TMS group were distinctly elevated. In addition, compared to the wild group, model group mice had a relatively slow downtrend in the OTU rank abundance curve (Figure 2H) at 0 to 200 species ranks and a relatively sharp drop in the curve at 400 to 600 species ranks, but the curve was narrower in the horizontal direction. This result may be related to the decrease of beneficial bacteria in the CUMS group. The curve of the CUMS+LF-TMS group was flat, while in the horizontal direction, the curve was wider than that of the Control group, compared to the CUMS group. This data illustrates that species abundance and homogeneity in stressed mice can be elevated by LF-TMS. The above results showed that CUMS-induced depression significantly decreased the species richness and diversity of intestinal microflora in mice, while LF-TMS treatment significantly elevated the species richness and diversity of CUMS-induced intestinal microflora in mice.

Analysis of the Structure of Gut Microbiota: Non-metric multidimensional scaling analysis (NMDS, Figure 3A), principal

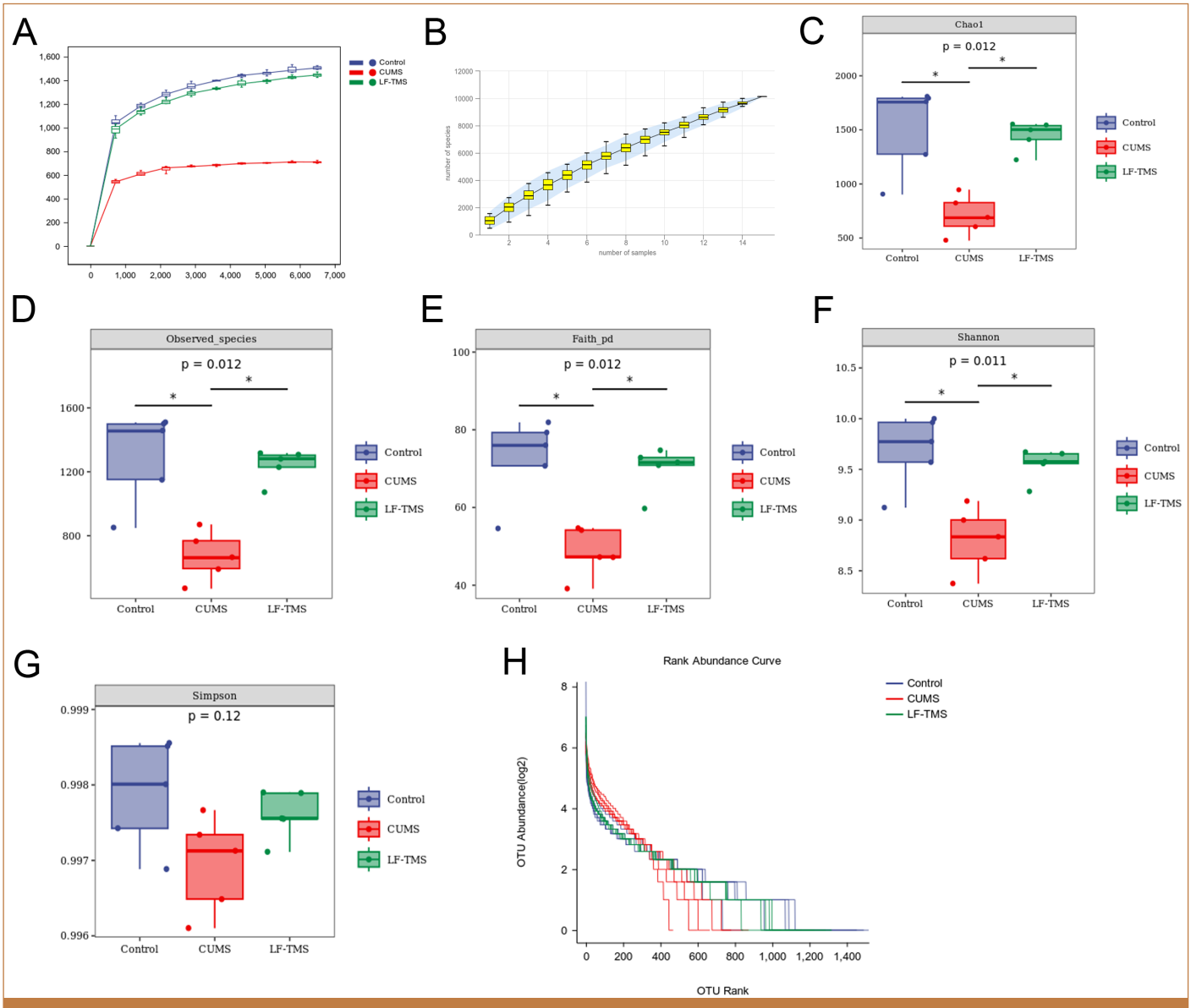

Figure 2. Species diversity analysis of gut microbiota in mice in all groups. (A). Rarefaction curve. (B). Cumulative species curve. (C). Chao1 index. (D). Observed species index. (E). Faith' s PD index. (F). Shannon index. (G). Simpson index. (H). Rank abundance curve. n = 5 in each group.

coordinates analysis (PCoA, Figure 3B) and PCA (Figure 3C-G) analysis based on different taxa were performed to evaluate the differences in intestinal microbial communities and beta diversity in each group. NMDS and PCoA analysis based on the weighted Unifrac distance algorithm were conducted to check the effect of LF-TMS on the composition of the intestinal flora of stressed mice. The NMDS and PCoA plots revealed no significant separate clustering in microbiota structure among the three groups. However, the distance between the model group and the wild group was longer than that between the LF-TMS treatment group. The β-diversity was shown in principal component analysis (PCA) among samples at 5 different taxonomic levels (phylum, Figure 3C; class, Figure 3D; order, Figure 3E; family, Figure 3F; and genus, Figure 3G). The three groups had clusters at five levels, but there was no statistical difference. There was no clustering of microbial communities in the Control group and CUMS group at the class, order and genus levels, which proves that there were differences in the composition of the intestinal flora between the Control group and CUMS group. CUMS induced intestinal flora structure disturbance in mice with depression. However, there was clustering of microbial communities between the Control group and the LF-TMS treatment group at the class and order levels, but no statistical difference, which indicates that there is no obvious difference in the composition of the intestinal flora between the Control group and LF-TMS treatment group. The intestinal flora structure of LF-TMS treatment group deviated from the CUMS group and tended towards the Control group, indicating that LF-TMS treatment could make the intestinal flora structure of depressed mice return to a normal state.

Analysis of Species Composition at Different Taxonomic Levels: Hierarchical clustering analysis was carried out on animal intestinal microorganisms, and the Unweighted pair-group method with

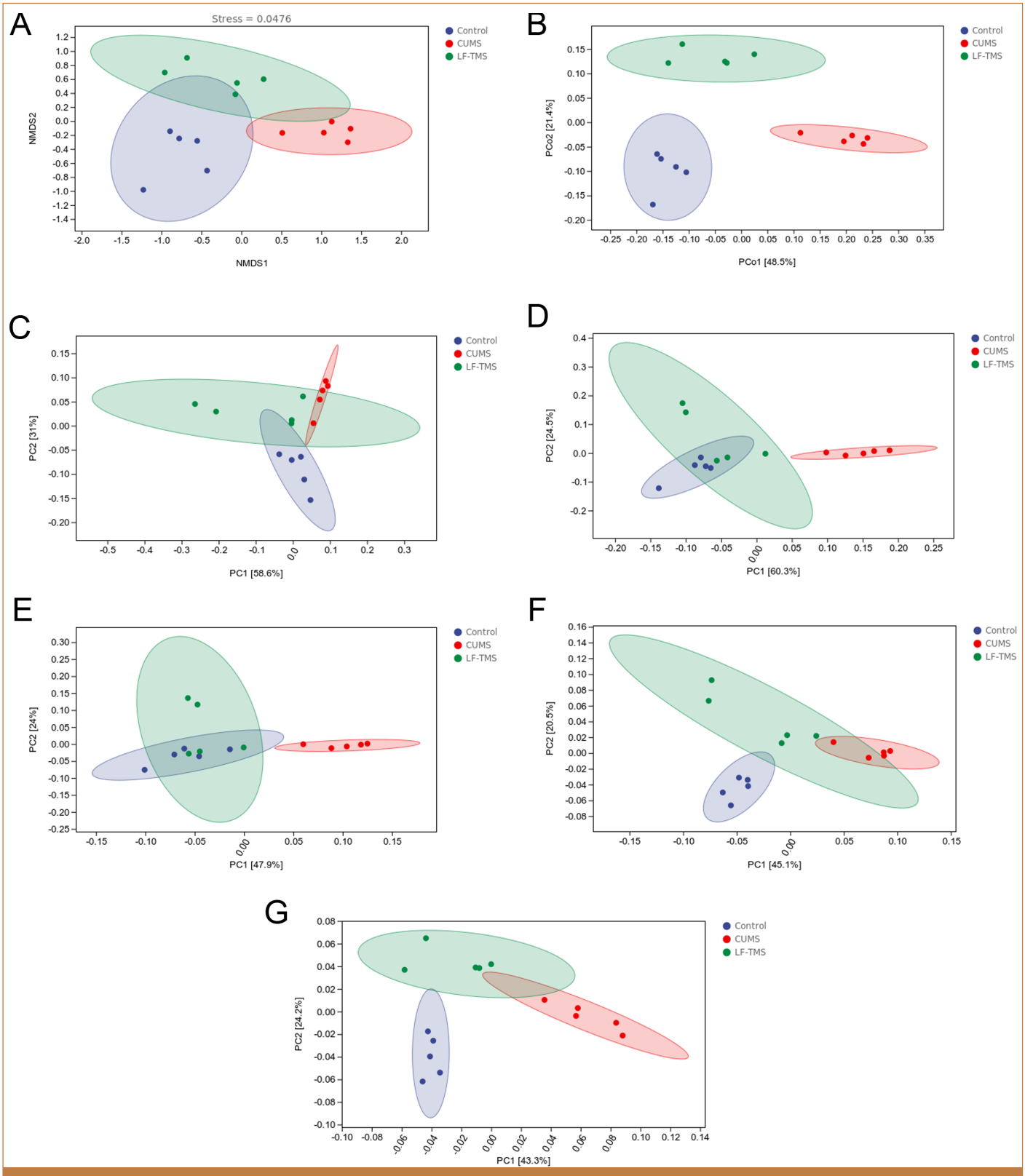

Figure 3. Analysis of gut microbiota structure of mice in all groups. (A). Non-metric multidimensional scaling (NMDS) analysis. (B). Principal coordinates (PCoA) analysis. C-G. The β-diversity was shown in principal component analysis (PCA), including the (C) PCA analysis of phylum level, (D) PCA analysis of class level, (E) PCA analysis of order level, (F) PCA analysis of family level, (G) PCA analysis of genus level. n = 5 in each group.

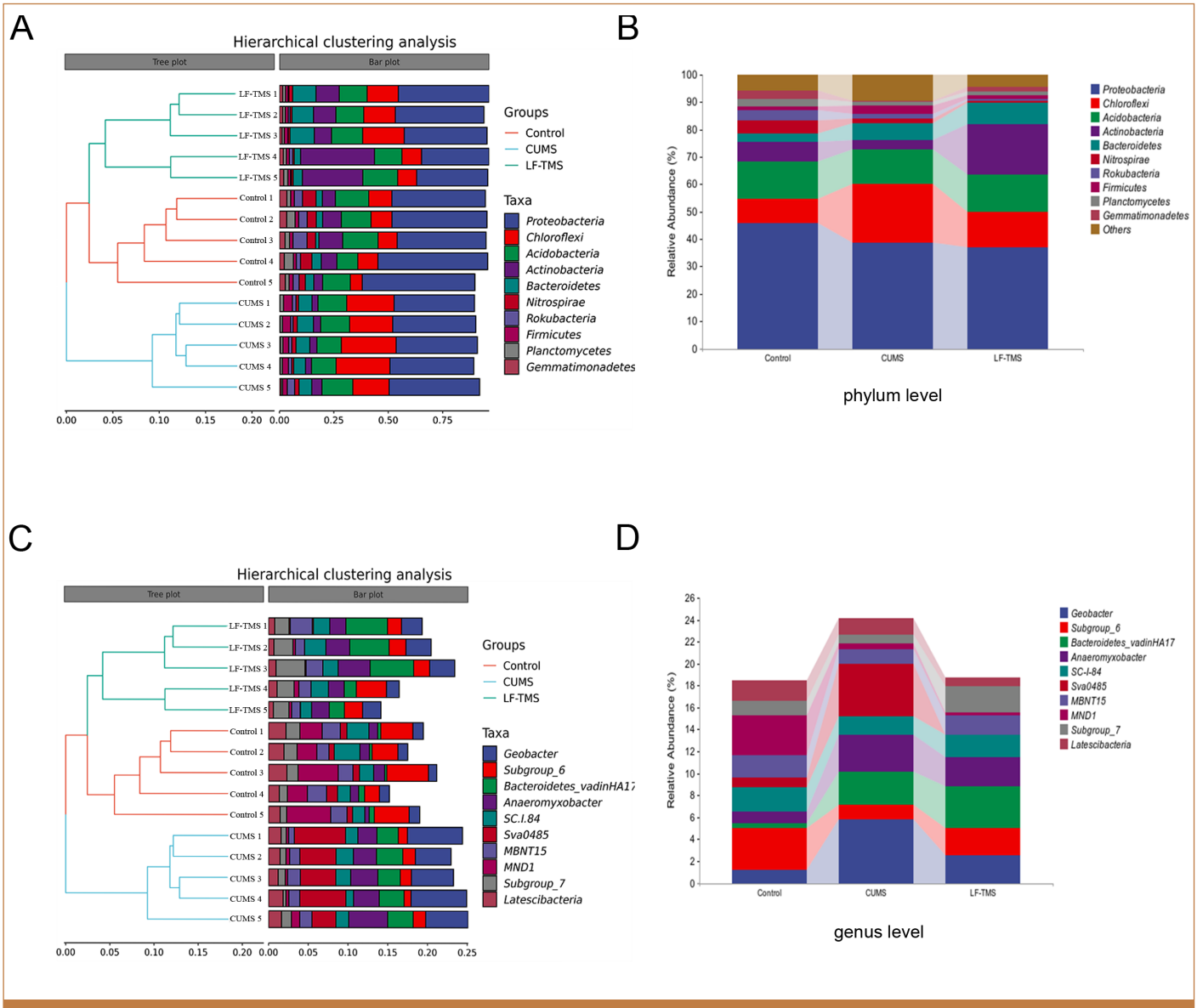

Figure 4. Relative abundance analysis of gut microbiota in all groups. Hierarchical cluster analysis at the phylum level, relative abundance at the phylum level, hierarchical cluster analysis at the genus level, and relative abundance at the genus level (A-D). n = 5 in each group.

arithmetic mean (UPGMA) algorithm was employed to analyze the selected Top 10 dominant species. At the phylum level (Figure 4A and B), CUMS shifted the microbiota profile towards a higher relative abundance of *Chloroflexi* and *Bacteroides*, and a lower relative abundance of *Proteobacteria* and *Actinobacteria*. Compared to CUMS, the relative abundance of *Chloroflexi was* reduced and relative abundance of *Actinobacteria* increased in the LF-TMS treatment, the relative abundance of *Bacteroides* and *Proteobacteria* did not change. At the genus level (Figure 4C and D), CUMS shifted the profile toward a higher relative abundance of *Geobacter*, *Bacteroidetes_vadinH17*, *Anaeromyxobacter,* and *Sva0485*, and a lower relative abundance of *Subgroup_6*.Compared with the model group, the LF-TMS treatment group reduced the relative abundance of *Geobacter* and *Sva0485* and elevated the relative abundance of *Subgroup_6*, but there were no significant changes in *Bacteroidetes_vadinH17* and *Anaeromyxter*. These results indicate that there is a high degree of specificity in the composition of the intestinal microbiota of mice among groups, and that both CUMS and LF-TMS treatments alter the gut microbiota of mice.

Analysis of the Key Differential Genera: We verified differences in taxonomic composition among the three groups by linear discriminant analysis (LDA) and effect size (LEfSe) analysis (Figure 5A and B). A cladogram representative of the colonic microbiota structure represented the predominant bacteria and the greatest differences in taxa among the three communities. Differential genera enriched in each group are indicated by different colors. We found that the relative abundance of phyla *Proteobacteria, Nitrospirae,Rok ubacteria, andGemmatimonadetes*; classes *Gammaproteobacteria, Alphaproteobacteria, NC10,Gemmatimonadetes, and Sub group 6*; orders *Betaproteobacteriales, Subgroup6, Gemmatimonadales, Rho dospirillales, andRokubacteriales*; families *Nitrosomonadaceae,*

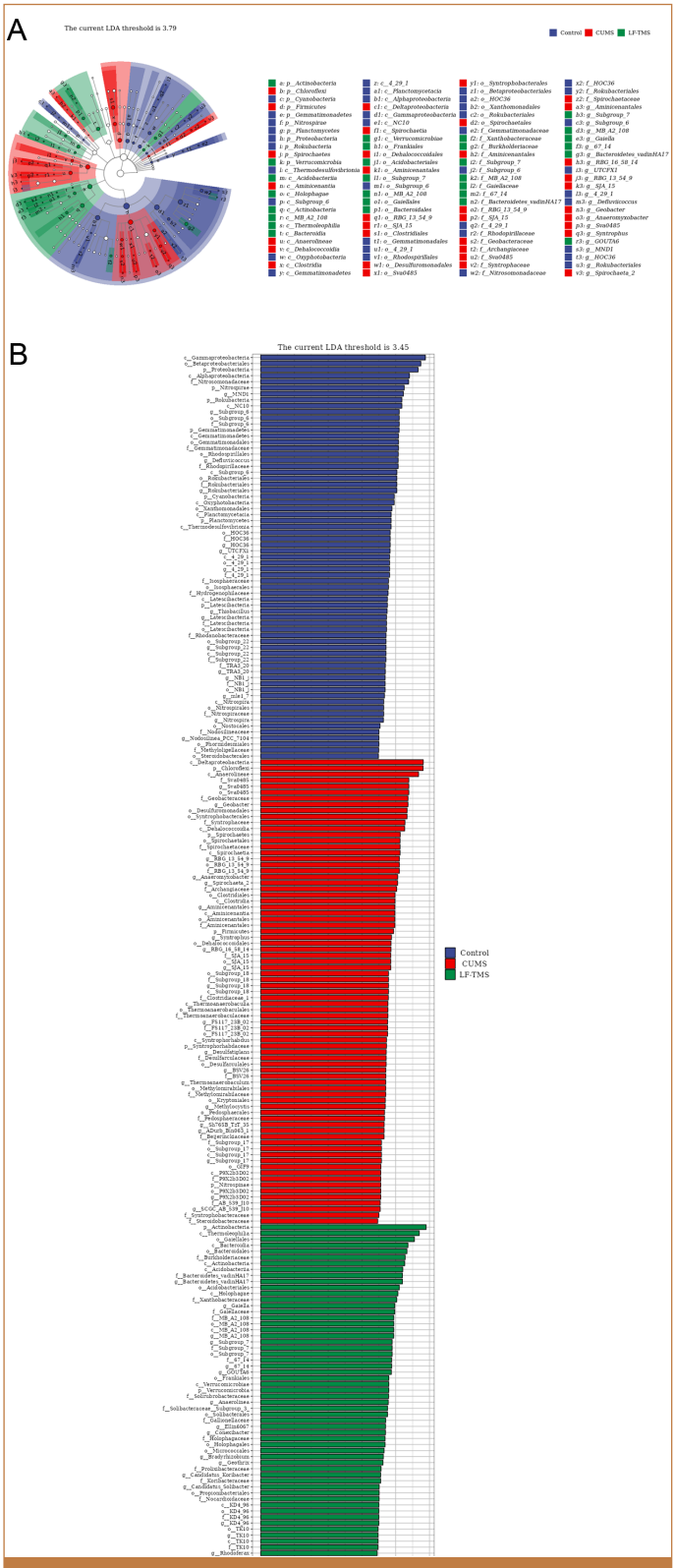

Figure 5. Linear effect size (LEfSe) analysis of gut microbiota in all groups. A. Evolutionary branching diagram. B. Linear discriminant analysis (LDA) scores, the vertical coordinates are the taxonomic units with significant differences among groups, and the horizontal coordinates are bar graphs to visualize the logarithmic scores of LDA analysis for each taxonomic unit. n = 5 in each group.

Subgroup6, Gemmatimonadaceae, Rhodopirillaceae, Rokubacteriales, and genus *MND1, Sub group six6, Defluviicoccus, Rokubacteriales* were enriched in the Control group. The abundance of phylum *Chloroflexi, Spirochaetes*; classes *Deltaproteobacteria, Anaerolineae, Dehalococcoidia, Spirochaetia*, order *Sva0485, Desulfuromonadales, Syntrophobacterales, Spirochaetales, RBG_13_54_9*, family *Sva0485, Geobacteraceae, Syntrophaceae, Spirochaetaceae, RBG_13_54_9, Archangiaceaea,* and genus *Sva0485, Geobacter, RBG_13_54_9, Anaeromyxobacter, and Spirochaeta_2* were enriched in the CUMS group. The abundance of phylum *Actinobacteria*; classes *Thermoleophilia, Bacteroidia, Actinobacteria,Acidobacteriia, andHolophagae*; orders *Gaiel lales,Bacteroidales, andAcidobacteriales*; families *Burkholderiaceae, Bacte roidetesvadinHA17, andXanthobacteraceae*; genus *Bacteroidetes_vadinH A17* were enriched in the CUMS + LF-TMS group (Table 1). The dominant bacteria in the wild mice group were *Proteobacteria*, the dominant bacteria in the Control group were *Chloroflexi*, but *Proteobacteria* decreased, and the dominant bacteria in the LF-TMS group were *Bacteroides* and *Actinobacteria* (Table 1).

Discussion

Many researchers have reported that low-frequency TMS can improve depressive symptoms in stressed mice.[16] However, the potential mechanism of LF-TMS in the treatment of depression is still unclear in detail. This study verified that LF-TMS can reduce depression induced by CUMS by modulating intestinal flora disorders.

CUMS protocol is a widely adopted model of depression.[21] Therefore, CUMS was chosen to induce depression in our study. Firstly, the weight of each group of animals was analyzed. The results showed that the weight base of each group was the same before the depression model was built, and there was no significant difference. After modeling, the weight of animals in the model group decreased significantly, while the weight of mice in the LF-TMS treatment group increased significantly. Depression is a common neuropsychiatric disorder that may cause anhedonia and behavioral despair.[22] In this study, the sucrose preference test (SPT) was performed to measure anhedonia, the core symptom of depression. The results indicated that the sucrose preference rate of mice in three groups was the same before modeling without significant difference. After modeling, the sucrose preference rate of animals in the model group decreased significantly, while the sucrose preference rate of mice in the LF-TMS treatment group increased markedly.

Recent investigations suggested that depression can influence the stability of the gut microbiota in people with major depression, using the 16S rRNA gene sequencing approach.[23] Presently, the effect of LF-TMS therapy on intestinal microbiota in mice with CUMS-induced depression was detected by means of 16S rRNA gene sequencing analysis. The results implied that LF-TMS treatment significantly elevated the CUMS-induced reduction in species richness and diversity of mice intestinal microbiota. NMDS, PCoA, and PCA analysis revealed that the intestinal microbiota composition of depressed mice tended to be similar to that of the wild group after treatment with LF-TMS, indicating that the treatment with LF-TMS could change the intestinal microbiota structure distribution of depressed mice to some extent and ameliorate the intestinal microbiota disorder induced by depression. Furthermore, the abundance of different levels of intestinal

Table 1. Key Differentiating Bacteria Genera in Each Group

	Phylum	Class	Order	Family	Genus
Control	*Proteobacteria* *Nitrospirae* *Rokubacteria* *Gemmatimonadetes*	*Gammaproteobacteria* *Alphaproteobacteria* *NC10* *Gemmatimonadetes* *Sub group 6*	*Betaproteobacteriales* *Sub group _ 6* *Gemmatimonadales* *Rhodospirillales* *Rokubacteriales*	*Nitrosomonadaceae* *Sub group _ 6* *Gemmatimonadaceae* *Rhodopirillaceae* *Rokubacteriales*	*MND1* *Sub group _ 6* *Defluviicoccus* *Rokubacteriales*
CUMS	*Chloroflexi* *Spirochaetes*	*Deltaproteobacteria* *Anaerolineae* *Dehalococcoidia* *Spirochaetia*	*Sva0485* *Desulfuromonadales* *Syntrophobacterales* *Spirochaetales* *RBG_13_54_9*	*Sva0485* *Geobacteraceae* *Syntrophaceae* *Spirochaetaceae* *RBG_13_54_9* *Archangiaceae*	*Sva0485* *Geobacter* *RBG_13_54_9* *Anaeromyxobacter* *Spirochaeta_2*
CUMS+ LF-TMS	*Actinobacteria*	*Thermoleophilia* *Bacteroidia* *Actinobacteria* *Acidobacteriia* *Holophagae*	*Gaiellales* *Bacteroidales* *Acidobacteriales*	*Burkholderiaceae* *Bacteroidetes vadinHA17* *Xanthobacteraceae*	*Bacteroidetes _vadinHA17*

CUMS, Chronic Unpredictable Mild Stress; LF-TMS, Low-frequency Transcranial Magnetic Stimulation.

microbiome was different among the three groups. The abundance of *Chloroflexi* and *Bacteroides* increased in the CUMS group, while the abundance of *Proteobacteria* and *Actinobacteria* decreased. LF-TMS treatment can regulate the intestinal microbiome, reduce the abundance of *Chloroflexi* in the intestinal microbiome of depressed mice, increase the abundance of *Actinobacteria*, and then improve depression. This is consistent with the findings of increased *Bacteroidetes* abundance in the model group of rats.[24] However, in this study, elevated *Bacteroides* levels were not evident after LF-TMS treatment. It has also been found that *Bacteroides* are the most abundant bacteria in the human gut microbiota, and these bacteria exhibit prominent stability and remarkable resilience to temporary disturbances.[25] Just as an increase in *Bacteroidales* may be the key to Paeoniflorin alleviating depressive symptoms.[26] The results of this study indicate that the intestinal microbiome is an important target for LF-TMS to relieve depressive symptoms, and regulating the composition and function of the intestinal microbial community is helpful for the clinical treatment of depressed patients.

The gut and brain communicate via a bidirectional system regulated through the interplay of neural, hormonal, metabolic, and immune pathways.[27] Researches have illustrated that depression is closely connected to inflammation, and many anti-inflammatory drugs have been shown to improve depression-like symptoms, indicating that inflammation participates in the pathogenesis of depression.[28] Therefore, we hypothesized that CUMS-induced anhedonia and intestinal flora dysregulation may be relevant to inflammation, but the specific mechanism of influence needs to be further explored.

In conclusion, LF-TMS treatment can improve the depressive symptoms caused by CUMS in mice and play an antidepressant role by improving the related intestinal microbial community disturbance. The antidepressant mechanism of LF-TMS is multi-link and multi-target. However, in this study, it was only discussed at the level of intestinal flora, and its positive effect on emotion and its potential molecular mechanisms need to be further studied.

Availability of Data and Materials: *The authors confirm that the data supporting the findings of this study are available within the article.*

Ethics Committee Approval: *This study was approved by the Animal Research Ethics Committee of North China University of Science and Technology (approval number: LX2021071, date: 2021.03.01).*

Informed Consent: *Not applicable.*

Peer-review: *Externally peer-reviewed.*

Author Contributions: *Concept – L.G., X.Z., H.W., X.S.; Design – X.S.; Supervision – H.W.; Resources – X.S.; Materials – L.W.; Data Collection and/or Processing – L.W., C.L., R.D.; Analysis and/or Interpretation – L.G., X.Z., L.W.; Literature Search – R.D.; Writing – L.G., X.Z., L.W., C.L., R.D., H.W., X.S.; Critical Review – H.W.*

Acknowledge: *Not applicable.*

Declaration of Interests: *The authors have no conflict of interest to declare.*

Funding: *This work was supported by grants from the key project of Hebei Natural Science Foundation Chinese Medicine Joint Fund (H2022 209080), the key research project of North China University of Science and Technology in 2023 202 (ZD-YG-202 314-23), and the research project of basic scientific research service fees in provincial universities of North China University of Technology (JQN2023 039).*

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