The role of microbiota - oral - brain axis in anxiety and depression

Fangzhi Lou  
Stomatological Hospital of Chongqing Medical University

Shihong Luo  
Stomatological Hospital of Chongqing Medical University

Huiqing Long  
Stomatological Hospital of Chongqing Medical University

Lu Yang  
Stomatological Hospital of Chongqing Medical University

Haiyang Wang  
The First Affiliated Hospital of Chongqing Medical University

Yiyun Liu  
The First Affiliated Hospital of Chongqing Medical University

Juncai Pu  
The First Affiliated Hospital of Chongqing Medical University

Li Yan  
Chongqing Medical University

Ping Ji  
Stomatological Hospital of Chongqing Medical University

Xin Jin (500934@hospital.cqmu.edu.cn)  
Stomatological Hospital of Chongqing Medical University

Research Article

Keywords: Chronic restraint stress, Depression, Microbiota - oral - brain axis, 16S rRNA sequencing, Liquid chromatography/mass spectrometry

Posted Date: March 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2633534/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** The gut microbiota plays a vital role in mental disorders, and the gut-brain axis has also been studied in the pathogenesis of depression. Furthermore, studies show the presence of the oral-gut microbiome axis. Oral microbiota dysbiosis is linked with mental disorders, suggesting that pathologic bacteria from the oral cavity may contribute to the development of mental disorders. However, whether independent oral microbiome that do not depend on gut microbes impacts the progression of depression, and the presence and mechanism underlying about the microbiota-oral-brain axis in depression remains unclear. Here, saliva microbiota transplantation in germ-free mice used to study the impacts and mechanism of oral microbes on depression. 16S rRNA sequencing and liquid chromatography/mass spectrometry were performed on saliva, stool and serum to investigate alterations in microbiota and metabolites.

**Results:** It was observed that differential shaping effect of oral and intestinal microbiota on depression. Moreover, germ-free mice transplanted with saliva from chronic restraint stress (CRS)-exposed mice displayed emotional impairment and oral microbial dysbiosis with significant differential abundance of bacterial species including the enrichment of Pseudomonas, Pasteurellaceae and Muribacter and depletion of Streptococcus. Metabolomic analysis showed the alternation of metabolites in the serum of CRS-exposed germ-free mice, especially Eicosapentaenoic Acid. Furthermore, ectopic colonization of oral microbiome induced by CRS in the intestine impaired gut barrier function, which is associated with increased blood-brain barrier permeability. Pseudomonas supplementation exacerbated depression-like behavior, while Eicosapentaenoic Acid treatment conferred protection against depression-like states in mice.

**Conclusions:** These results suggest the external shaping process of gut microbiota by oral microbiome and indicate the great value of oral saliva samples in monitoring and assessing depression progression in clinical settings.

Introduction

In recent years, depression has been widely recognized as one of the most prevalent mental health problems and harms more than 300 million people worldwide that has attracted great attention [1, 2], and the outbreak of the coronavirus disease (COVID-19) has exacerbated the development of public mental disorder [3]. The underlying mechanisms of depression remain unclear. Studies have reported that very few patients with severe major depression could receive effective treatment for depression, most patients with anxiety and depression have not been diagnosed and received a quate treatment in time [4]. Therefore, a more detailed understanding of mechanisms of the development of depression is needed for the development of treatment strategies to ameliorate depression.

The connection between gut microbiome and depression has been reported. The bidirectional communication of gut-brain has been suggested as a possible basis for their interconnectedness [5].
gut microbiome interacts with the central nervous system (CNS) through microbial-derived metabolites and neurotransmitters such as γ-aminobutyric acid (GABA) and serotonin (5-hydroxytryptamine, 5-HT) [6]. Accumulating evidence supports an important role of microbiota–gut–brain axis in depression [2, 7, 8]. Oral microbiome, as the second-largest microbiota in human, is closely related to the development of various pathological conditions. The gut is linked to the oral cavity, and several studies have reported that microbes in the oral cavities are involved in the formation of the gut microbiota [9]. Previous studies suggested that the oral cavity may act as a reservoir for potential intestinal pathobionts that can exacerbate inflammatory bowel disease [10]. However, this process of ectopic colonization of oral microbiome remains unclear due of the lack of quantitative data. Moreover, there are studies have shown that the oral microbiota may be related to the progression of Alzheimer’s disease (AD) [11]. Recently, studies link oral microbiome composition to anxiety and depression [12]. However, whether independent oral microbiome that do not depend on gut microbes impacts the progression of depression, and the presence and mechanism underlying about the microbiota-oral-brain axis in depression remains unclear. In this study, we applied a multi-omics approach that integrates the oral and gut microbiome and serum metabolome, and conventional and germ-free CRS mice model was established to explore potential influence of oral microbiota on the gut and brain in depression.

**Materials And Methods**

**Study cohorts**

Participants (n = 157) aged 18–60 years were recruited from the medical examination center of the Stomatological Hospital of Chongqing Medical University. This study was approved by the Ethics Committee of Stomatological Hospital of Chongqing Medical University (Approval No. 2021-5), with informed written consent was obtained from all participants. The Hamilton Anxiety Scale (HAMA) and seventeen items version of Hamilton Depression Scale (HAMD-17) were used to evaluate their anxiety and depression levels, respectively. HAMA and HAMD-17 scores over seven points were defined as anxiety and depression, respectively [13, 14]. Patients with anxiety and depression were required to meet the Diagnostic and Statistical Manual of Mental Disorders. This is a cross-sectional study consisting of 87 patients with anxiety and depression (ANDP) and 70 healthy subjects (healthy controls). Healthy subjects without any mental disorders, and only those with both HAMA and HAMD-17 scores less than seven were included. Eligible patients were drug-naive. Exclusion criteria included a history of schizophrenia, schizoaffective or bipolar disorder; history of diabetes, cardiovascular disease, chronic inflammatory disease, thyroid disease, or cancer; currently pregnant or breastfeeding; substance abuse or acute intoxication; history of antibiotic use within 1 month prior to sampling. Then, saliva samples were collected from the all participants and were quick frozen in liquid nitrogen and then stored at −80 °C for further microbiome and metabolomics analysis.

**Animals**

Female Kunming mice, 4–5 weeks old, were purchased from the Cavensbiogle Model Animal
Research Co. LTD (Jiangsu, China), and germ-free Kunming female mice, 4–5 weeks old, were obtained from the Experimental Animal Research Center of the Third Military Medical University (Chongqing, China). The germ-free (GF) mice were kept in flexible film gnotobiotic isolators in the NHC Key Laboratory of Diagnosis and Treatment on Brain Functional Diseases, The First Affiliated Hospital of Chongqing Medical University (Chongqing, China) until the beginning of the behavioral tests. All animal procedures followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University and were approved by the Laboratory Animal Ethics Committee of Chongqing Medical University (Approval No. 2021063).

**Chronic restraint stress (CRS) mice model and treatment**

After a week of acclimatization, mice were divided into the control group and the CRS group where mice were constrained and placed for 2~5 hours daily for 4 weeks in a well-ventilated 50ml conical tube. For the intervention experiment, all mice received a cocktail of antibiotics with 1 g/L metronidazole (MedChemExpress, HY-B0318, China), 1 g/L Ampicillin (Beyotime, SY007, China), 1 g/L neomycin (Beyotime, ST2533, China), 0.5 g/L vancomycin (Beyotime, ST2807, China) in drinking water starting 2 weeks before administered treatment and continuing until the end of the experiment [15]. Concretely, the antibiotic-treated mice were divided into three groups: the control group, the group treated with Eicosapentaenoic Acid (EPA, 150mg/kg/day, MedChemExpress, China), and the group treated with Pseudomonas aeruginosa (PA, CMCC10104). PA strain was cultured in Luria-Bertani liquid medium at 37°C under aerobiotic conditions. Then, the bacterium suspension for oral administration was prepared with a final density of $1 \times 10^9$ CFU/mL. All experimental groups were given 200 μL/mouse suspension solution continuously for 3 weeks, while the control group received an equivalent volume of sterile PBS by gavage. Meanwhile, all antibiotic-treated mice were performed CRS when administered treatment.

**Oral microbiome transplantation experiments**

The sources for the samples were healthy control mice and CRS mice. Briefly, the oral microbiome used as the transplantation was sampled using cotton swabs that were rubbed against the surface of the oral mucosa of mice and resuspended in artificial saliva medium [16, 17]. Mice were inoculated with the oral microbiomes by oral gavage and swabbing on the paws for 3 days and then placed in a gnotobiotic environment under the same feeding conditions. Mice performed behavioral tests 4 weeks after oral microbiota transplantation.

**Behavioral tests**

The behavioral tests include the open-field test (OFT), forced swim test (FST) and tail suspension test (TST), and quantified using video-tracking apparatus (SMART, Barcelona, Spain) and analyzed using EthoVision XT 13.0 software. Detailed procedures are described in our previous experiment [18].

**16S rRNA gene-sequencing**
DNA of oral and fecal microbiota was separated and then sent to the Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) for sequencing. Amplified reactions were performed using GeneAmp PCR System 9700 (ABI, USA) and quantified using QuantiFluor-ST (Promega, USA). The constructed library was analyzed using the Illumina MiSeq platform (Illumina, CA, USA). Then, the optimized data is processed by sequential noise reduction method (DADA2/Deblur, etc.), and sequence and abundance information are obtained by ASV (Amplicon Sequence Variant). The data were analyzed on the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/).

**Liquid chromatography tandem mass spectrometry (LC-MS/MS) and data analysis**

Serum samples from patients and mice were collected and preprocessed. Detailed procedures are described in our previous experiment [18]. The data were analyzed on the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/). Differential metabolites among groups were screened out via a value of p < 0.05 and VIP (variable importance value) >1 based on Student's t-test. KEGG pathway enrichment analysis of the identified differential metabolites performed by MetaboAnalyst 4.0 (http://www.MetaboAnalyst.ca/).

**Western blot analysis**

The total protein from tissue sample was extracted by using total Protein Extraction Kit (Beyotime, China). The following primary antibodies were applied: rabbit anti-ZO-1 (1:5000, 21773-1-AP, Proteinetch Group) and anti- β-actin (1:1000; 4970s, Cell Signaling).

**Immunofluorescent staining**

The tissue samples were fixed with 4% paraformaldehyde, and paraffin-embedded. Next dewaxing, rehydration, antigen retrieval, blocking, and then incubated with primary antibodies (ZO-1, 1:1000, 21773-1-AP, Proteinetch Group) for overnight at 4°C. The tissue was stained with a goat anti-rabbit DyLight 594 secondary antibody (1:500; Beyotime, China) for 1 hour at room temperature. Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI). The tissues were analyzed using a confocal laser scanning microscopy (Nikon, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction (RT-PCR)**

RT-PCR was performed of the following genes and primer sequences of corresponding genes: ZO-1 (forward 5′- GCTGTGGGTAACTCCATCCT -3’ and reverse 5′- GGCTGACAGTGGAAGTAGCA -3’), Occludin (forward 5′- GGAGTTTCAGGTGAATGGGTCA-3′ and reverse 5′- AAATGTCCAGGCTCCCAAGA-3’), Claudin-1 (forward 5′- CCCTTCAGCAGGAAGAGT -3’ and reverse 5′- CCCTTCAGCAGGAAGAGT -3’). The results were normalized against β-actin and were calculated with the 2^−ΔΔCT method.

**Histological analysis**
Colon tissue samples from the mice were embedded in paraffin and cut into 7μm thick sections. Then, the sections were stained with hematoxylin and eosin (H&E). The sections were scanned and viewed using Pannoramic scanner (3DHISTECH, Germany).

**Enzyme linked immunosorbent assay (ELISA) analysis**

ELISA kit (mlbio company, Shanghai, China) were used to detect the 5-HT and norepinephrine levels according to the manufacturer’s instructions. The absorbance values were measured at 450 nm using a microplate reader (SpectraMAX iD5, USA).

**Statistical analysis**

The analyses of microbiota and metabolomics data were performed on the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/). The data were expressed as the mean ± standard deviation (SD) and analyzed using Student’s t-test or one-way ANOVA. Significance level was set at $p$-value < 0.05 and 95% confidence intervals. Correlations analysis were performed using Spearman's rank correlation on the online tool of Majorbio Cloud Platform, and the visual presentation of correlations was shown by a heat map. Statistical analysis was performed using the GraphPad InStat version 8.0 (GraphPad Software Inc., San Diego, California, USA) and Statistical Package for Social Sciences (SPSS) version 25.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Scores of anxiety and depression are associated with changes in the composition of the oral microbiota and related metabolites**

We applied a multi-omics approach to reveal molecular mechanisms underlying the microbiome-oral-brain axis interplay in anxiety and depression. We first assessed the relationships of oral bacterial composition and anxiety and depression using the Hamilton Anxiety Scale (HAMA) [13] and seventeen items version of Hamilton Depression Scale (HAMD-17) [14] in patients with health (n=70, HAMD: 0-7, HAMA < 7) and anxiety and depression (ANDP, n=87, HAMD > 7, HAMA > 20). Pan/Core species analysis is used to describe the changes in total species and core species with the increase of sample size. The results showed that the number of total ASV increased and then gradually flattened out and the number of shared ASV decreased and then gradually flattened out, suggesting that the sequencing sample size is sufficient (Figure S1A). There were no significant differences in the oral microbial community richness (Sobs, Chaos and Ace index) and diversity (Shannon index) between the two groups (Figure S1B). Then, the principal co-ordinates analysis (PCoA) was applied to reveal the overall bacterial phenotypes of beta-diversity. PCoA showed oral microbiota structure between health and anxiety and depression patients were distinct (Fig. 1A). Typing analysis on genus level showed that two distinct clusters: type I (g_Streptococcus) and type II (g_Pseudomonas), in which type II focus on the oral microbiomes sample of anxiety and depression patients (Fig. 1B). The composition of oral microbiota differed between the two groups (Fig. 1C, Figure S1C), Pseudomonas was significantly increased, while Leptotrichia and
Solobacterium were significantly decreased in ANDP group compared with health group (Fig. 1D). Circos maps of communities show the relationship between samples and species, reflecting the proportion of dominant species in each group. Largely, Circos figures demonstrated that the dominant species in ANDP group is genus Pseudomonas (Fig. 1E). Compositional analysis by LefSe revealed that oral swabs of healthy controls were enriched with the genus Leptotrichia and Solobacterium, family Erysipelotrichaceae and ASV130 and so on, and ASV384 was predominant microbiota in ANDP group (Fig. 1F).

To reveal metabolic phenotypes related to the oral microbiome in anxiety and depression, we performed metabolic profiling of the saliva from health and anxiety and depression patients. The Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) score plot showed distinct clusters of the saliva metabolites between the ANDP and health control groups (Fig. 1G), suggesting that the metabolic patterns were reprogrammed in ANDP patients. The Venn diagrams displayed the difference between each group, exhibiting specific 17 metabolites in ANDP and 21 metabolites in the health control group, respectively (Fig. 1H). A total of 87 significantly changed metabolites between the two groups samples were identified of which 24 showed a downward trend, and 63 showed an increasing trend (Fig. 1I). Briefly, top 32 metabolites (VIP > 2, p < 0.05) were exhibited (Figure S1D), including Eicosapentaenoic Acid, amino acids, bile acid, steroids, fatty acids and other classified metabolites. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that 20 altered metabolites-associated metabolic pathways, including primary bile acid biosynthesis, glycine, serine and threonine metabolism, fatty acid biosynthesis and tryptophan metabolism (Fig. 1J) were associated with mental disorders [7, 19-22].

Next, we explored the potential correlations of these differential oral microbiota and differential saliva metabolites. Overall, the results showed that there is a correlation with differential oral microbiota and metabolites of which Pseudomonas was positively correlated with 12-Ketodeoxycholic acid and negatively correlated with Eicosapentaenoic Acid (Fig. 1K), implying that the pathogenic oral microbial-derived metabolites induced by anxiety and depression may affect the occurrence and development of anxiety and depression. Taken together, these data suggest that mental disorders were accompanied by oral microbial dysbiosis and altered metabolites.

**Differential oral and intestinal microbiota disorders in CRS mice**

Behavioral tests were performed after 4 weeks of CRS model establishment. The immobility time for the FST and TST was remarkably lengthened and the center motion distance for the OFT significantly decreased in CRS mice as compared with control mice, suggesting obvious anxiety- and depression-like behaviors (Fig. 2A). Next, we analyzed the microbiota of oral swab and stool samples from mice with CRS and controls using 16S rRNA sequencing. The α-diversity values including species richness (Sobs, Chaos and Ace index) and species diversity (Shannon index) were compared between the CRS and control groups. There were no significant differences in the oral and gut microbial community richness and diversity between the two groups (Figure S2A, B). PCoA showed that oral and gut microbiota structure the two groups all could be distinguished at the ASV level (Fig. 2B, C). The composition of oral
and gut microbiota differed between the CRS and control groups (Fig. 2D, E). We found that Pseudomonas, Pasteurellaceae and Muribacter was higher, while Streptococcus was reduced in CRS group compared with control group at the genus level for oral microbiota (Fig. 2F). For gut microbiota, the relative abundance of Muribaculaceae and Saccharimonas was enriched in the CRS group relative to the control groups, whereas Lachnospiraceae and Desulfovibrionaceae was lower than control groups (Fig. 2G). LEfSe analysis showed that, compared to control groups, CRS group was characterized by enriched ASV belonging to the Phylum Proteobacteria and Actinobacteriota and the families Pasteurellaceae and Nocardiaceae for oral microbiota (Fig. 2H, I). For gut microbiota, LEfSe analysis showed that CRS group was characterized by enriched ASV belonging to the Phylum Bacteroidota and the families Muribaculaceae compared to control groups, and depleted ASV belonging to the Phylum Firmicutes and Desulfobacterota and the family Desulfovibrionaceae and Lachnospiraceae (Fig. 2J, K). Together, these results indicate that CRS induced oral and gut microbiota disorders, and have significantly different microbial signatures in oral and gut, suggesting that differential shaping effect of oral and intestinal microbiota on depression.

Saliva microbiota transplantation in germ-free mice recapitulate the alteration of oral microbiota in CRS exposed conventional mice

To confirm the direct role of altered oral microbiota induced by CRS on depression, we transferred oral swabs from CRS mice and controls to GF mice, referred to as “GF-Stress group” and “GF-control group” respectively. Then, we performed 16S rRNA sequencing on oral swabs in GF mice. Similar to the conventional mice model, there were no found significant differences in the oral microbial community richness (Sobs, Chaos and Ace index) and diversity (Shannon index) between the GF-Stress group and GF-control group (Figure S3A). PCoA showed that oral microbiota structure the two groups could be separated completely at the ASV level (Fig. 3A). At the genus level, the Venn diagrams displayed that specific 183 genera in GF-Stress group and 25 genera in GF-control group, and shared 45 genera (Fig. 3B). The composition of oral microbiota differed between the two groups (Fig. 3C, Figure S3B). Clearly, the dominant species in GF-Stress group is genus Pseudomonas and Lactobacillus (Fig. 3D). We further tested consistence of microbiota alterations between the two mice models. And we found that bacteria with increased abundance in CRS-exposed mice compared with control mice were consistently increased in GF-Stress mice compared with GF-Control mice, whereas bacteria with decreased abundance in CRS-exposed mice were consistently reduced in GF-Stress mice compared with GF-Control mice (Fig. 3E). Together, these results indicate that the transplantation of oral swabs from CRS mice could induces oral microbiota alterations, and oral swabs microbiota transplantation in GF mice recapitulate the alteration of oral microbiota in CRS exposed conventional mice.

An emotional impairment is transferred to germ-free mice through the oral microbiota

To investigate whether oral microbiome works in the progression of depression independent of gut microbes, we inoculated respectively oral microbiota from mice with health and well-established CRS model into GF mice (Fig. 3F). GF mice colonized with oral microbiome from CRS model developed more
obvious anxiety- and depression-like behaviors than GF mice that inoculated with oral microbiome from control mice (Fig. 3G-I). Specifically, the immobility time for the FST (Fig. 3G) and TST (Fig. 3H) was obviously increased and the center motion distance for the OFT (Fig. 3I) markedly shortened in CRS mice as compared with control mice. These results indicate that the presence of oral microbiome contributes to the development of emotional impairment.

**CRS-altered oral microbiota induces microbial-derived metabolites alteration in germ-free mice**

To determine alterations in serum metabolites after oral microbiota transplantation from CRS mice, we performed liquid chromatography tandem mass spectrometry (LC-MS/MS) on serum in GF mice from GF-Stress group and GF-control group. The Principal Component Analysis (PCA) and OPLS-DA score plot showed a complete separation of the serum metabolites between the GF-Stress group and GF-control group (Fig. 4A). The Venn diagrams displayed the difference between each group, exhibiting specific 7 metabolites in GF-Stress group (Fig. 4B). A total of 201 significantly changed metabolites between the two groups samples were identified of which 68 showed a downward trend, and 133 showed an increasing trend (Fig. 4C). Top 30 metabolites were exhibited (Fig. 6D), including Eicosapentaenoic Acid, amino acids, bile acid, steroids, fatty acids and other classified metabolites. Importantly, Eicosapentaenoic Acid was significantly decreased in GF-Stress group compared with GF-control group (Fig. 4D). KEGG enrichment analysis showed that 20 altered metabolites-associated metabolic pathways, including Glycine, serine and threonine metabolism, Steroid hormone biosynthesis, Butanoate metabolism, Fatty acid biosynthesis, primary bile acid biosynthesis, Alanine, aspartate and glutamate metabolism and GABAergic synapse (Fig. 4E). These data indicate that oral microbiota induced by CRS promotes the alteration of serum metabolites in GF mice.

The potential relationships between serum metabolites and differential oral microbiota induced by CRS were evaluated. In general, Procrustes analysis showed significant correlation between differential oral microbiota and serum metabolites (Fig. 4F). In particular, Eicosapentaenoic Acid was negatively correlated with Pseudomonas, Romboutsia, Pasteurellaceae and Muribacter, but positively correlated with Streptococcus, Turicibacter and Rodentibacter. 4-Phenyl-3-buten-2-ol, 7-Ketcholesterol and 12-Ketodeoxycholic acid was positively correlated with Pseudomonas, Rhodococcus and Muribacter, but negatively correlated with Turicibacter and Streptococcus (Fig. 4G). Together, these results indicate that oral microbiota from CRS models causes oral microbial-derived metabolites alteration in GF mice, implying that the pathogenic oral microbial-derived metabolites could enter the blood circulation system and play an important role in psychiatric illnesses.

**Oral microbiota and microbial-derived metabolites induced by CRS could directly impact brain function in germ-free mice**

The blood-brain barrier controls substance transport between the blood and the brain, and its permeability is reflected tight junction proteins [23]. We assessed the mRNA expression of ZO-1, occludin, and claudin-1 by RT-PCR in hypothalamus and hippocampus regions of GF-Stress group and GF-control group. Significantly lower expression of ZO-1, occludin, and claudin-1 was observed in GF-Stress group
compared with GF-control group (Fig. 5A). Western blot and immunofluorescence staining used to detect the protein expression of ZO-1 in the frontal cortex, and the results were consistent with RT-PCR (Fig. 5B, C). Moreover, the frontal cortex in the GF-Stress group displayed lower levels of 5-hydroxytryptamine (5-HT) and higher levels of norepinephrine (Fig. 5D), implying the alteration of neurotransmitter. These results indicate that oral microbiota from CRS models causes the increasing of blood-brain barrier permeability and neurotransmitter imbalance in GF mice, which shows the presence of oral-brain axis in depression.

To further clarify the potential roles of the microbiota-oral-brain axis, correlations between the representative values of behavioral changes, levels of neurotransmitter in the frontal cortex and changes in oral microbial-derived metabolites were assessed. As depicted in Fig. 4F and G, the oral microbiota that significantly differed between groups were highly correlated with the expression of serum metabolites. Additionally, microbial-derived metabolites were significantly correlated with neurotransmitter in the frontal cortex and depression-like behavioral changes (Fig. 5E). Together, these results indicate that oral microbiota can exert significant roles in mental disorders by oral-brain axis.

**Ectopic colonization of oral microbiome induced by CRS in the intestine impaired gut barrier function in germ-free mice**

Studies showed that oral bacteria do not colonize the distal gut in a healthy state [24]. In this study, PCoA showed that the microbiota compositions of the saliva were clearly separated from that of the stool in the CRS groups (Fig. 6A). However, we also found the same bacteria in the saliva and stool samples in CRS states (Fig. 6B), suggesting that we may warrant further investigation for an underlying pathology. To gain the underlying mechanisms of mental disorders induced by oral microflora from CRS mice, we performed 16S rRNA sequencing on stool in GF mice from GF-Stress group and GF-control group. The data showed that the α-diversity values including gut microbial species richness (Sobs, Chaos and Ace index) and species diversity (Shannon index) were significant differences between the GF-Stress group and GF-control group (Fig. 6C). PCoA showed that gut microbiota structure the two groups could be separated completely at the ASV level (Fig. 6D). The composition of gut microbiota differed between the two groups (Fig. 6E). From the gut community Circos diagram, we observed that the dominant genera in GF-Stress group were Muribaculaceae, Lactobacillus, Alistipes, Bacteroides, Erysipelatoclostridium, Helicobacter, Turicibacter and Monoglobus (Fig. 6F). Among them, the abundance of Alistipes, Erysipelatoclostridium, Helicobacter, Turicibacter and Monoglobus was significantly increased in GF-Stress group (Fig. 6G). LEfSe analysis showed that GF-Stress group was characterized by enriched ASV belonging to the Phylum Bacteroidota and Campilobacterota and the families Rikenellaceae and Helicobacteraceae, compared to GF-control group, and depleted ASV belonging to the Phylum Firmicutes, Bacteroidota and Desulfobacterota and the family Erysipelotrichaceae, Prevotellaceae and Desulfovibrionaceae (Fig. 6H). These data suggest that oral microbiota induced by CRS colonized the intestinal tract and induced gut microbiota dysbiosis.
Hematoxylin and eosin (HE) staining from colon revealed that the epithelium layer was loose, the structure of intestinal crypts was abnormal, the goblet cells were reduced and infiltration of inflammatory cells in GF-Stress group compared with the GF-control group (Fig. 6I). This data indicates that the intestinal morphological characteristics were altered in GF-Stress group, and thus the intestinal function may also be altered by oral microbiota. Next, we studied the effects of oral microbiota on the mRNA and protein levels of intestinal mucosa tight binding protein genes (ZO-1, occludin and claudin-1). The RT-PCR results demonstrated that, compared to GF-control group, the mRNA expression levels of ZO-1, occludin and claudin-1 were significantly downregulated in GF-Stress group (Fig. 6J). The colon tissue immunofluorescence results demonstrated that the protein expression of ZO-1 was markedly reduced in GF-Stress group compared to GF-control group (Fig. 6K). These data indicate that oral microbiota induced by CRS harm the gut barrier function and increase intestinal permeability in GF mice.

**Pseudomonas aeruginosa intervention and Eicosapentaenoic Acid supplementation change depression-like behavior association with microbial translocation in antibiotic-treated mice**

To strengthen the effect of the microbiota and microbial-derived metabolites that significantly differed between groups on anxiety and depression, we also used antibiotic-treated mice that were orally treated with a neomycin, metronidazole, vancomycin and ampicillin in the drinking water. The Pseudomonas aeruginosa (PA) and Eicosapentaenoic Acid (EPA) was administrated to antibiotic-treated mice by oral gavage lasting 3 weeks. Then, the behavioral phenotypes were evaluated (Fig. 7A). Compared with CRS controls, the immobility time for the FST and TST was obviously reduced and the center motion distance for the OFT markedly extended in the mice treated with EPA (Fig. 7B-D), suggesting EPA supplementation confers protection against depression-like states in mice. Conversely, PA intervention exacerbated anxiety- and depression-like states (Fig. 7E-G). Overall, these results suggest that oral microbiota and microbial-derived metabolites affect anxiety- and depression-like states in antibiotic-treated mice.

**Discussion**

Recently, the specific role and mechanism of microbiota in CNS disorders have attracted increasing attention. Despite the effect of gut microbiota on the depression is well-studied [2, 6–8, 25], we know less about whether the altered oral microbiota by exposure to stress state plays an important role in the progression of depression. Here, we studied the processes by which oral microbes shape the gut microbiota, focusing on how this shaping process is associated with mental disorders. We observed concordance between the change of oral microbiota transplantation in GF mice and the alteration of oral microbiota in CRS exposed conventional mice, characterized by overall synergy of the bacterial interaction and niche differences represented by Pseudomonas. We identified an emotional impairment is transferred to GF mice through the oral microbiota. Our data also indicate that oral microbiome and microbial-derived metabolites induced by CRS impaired gut barrier and the blood-brain barrier function in GF mice. These findings enabled us to better understand how oral microbes affect the gut microbiota and increase the potential to develop saliva-based microbiome approaches to evaluate and regulate mental health.
The gut microbiota is known as the “second human genome”, and the oral microbiota is the second-largest microbiota in human after intestinal microbiota that occupies an extremely important position in some diseases [26, 27]. Moreover, previous studies have reported dysbiosis of the oral microbiota can alter the gut microbial composition, leading to the progression of cancers and other diseases [28, 29]. The alterations of oral microbiota ecosystem can influence all kinds of systemic disorders including diabetes, cancer, rheumatoid arthritis, cardiovascular disease and AD [11, 30–32]. Interestingly, a finding link oral microbiota to panic disorder (PD). The studies indicated that the alpha diversity and beta diversity of oral microbiota were different between the two groups by performing 16S rRNA sequencing to patients with PD and healthy controls, suggesting oral microbiota disorders in patients with PD [33]. However, a limitation of this previous study is lack of convincing and strong evidence. In this study, we systematically investigated the oral microbiota profiles and serum-based metabolomics in patients with abnormal depression scores using 16S rRNA sequencing and LC–MS approach. In concordance with previous finding, we found that oral microbiota differed between patients with abnormal depression scores and healthy controls, but we did not observe significant differences in the oral microbial alpha diversity between the two groups. The composition of oral microbiota differed between the two groups, including increased Pseudomonas and decreased Leptotrichia and Solobacterium in patients with abnormal depression scores. Besides, we identified multiple differential metabolites in patients with abnormal depression scores. These findings suggest that depression disorders pathogenesis may involve an extensive oral microbiota and metabolic disturbance. However, to further investigate the possible mechanisms of oral microbiota in depression, experiments in animal models will be necessarily performed. Consistent with our previous study [18], CRS induced anxiety- and depression-like behavior in mice. Additionally, we found that CRS induced oral and gut microbiota disorders in mice, and we observed the differential oral and intestinal microbiota disorders in CRS mice. Therefore, it is of great significance to further study whether independent oral microbiome impacts the progression and mechanism of depression.

Previous studies revealed that fecal microbiota transplantation from patients with mental disorders induces relevant behaviors in germ-free recipient mice, and the oral microbiome transplantation from oral squamous cell carcinoma mice model increases tumorigenesis in germ-free recipient mice [17, 34]. The direct role of the CRS-altered oral microbiota in depression was further investigated in GF mice with saliva microbiota transplantation. In this study, we first demonstrated that the oral microbiome transplantation from CRS mice induces relevant anxiety- and depression-like behaviors, suggesting that an emotional impairment is transferred to GF mice through the oral microbiota. In addition, our study found that the alteration of oral microbiota transplantation in GF mice was consistent with the alteration of oral microbiota in exposed-CRS conventional mice. In particular, Pseudomonas was significantly enriched in both CRS-exposed mice and in GF-Stress mice. The blood-brain barrier is a physiological barrier that controls the passage of molecules between the blood and the brain parenchyma, and its impairment could impact functioning of neurons [35, 36]. Studies have reported that the gut microbiota accommodate the integrity of blood-brain barrier as a potential regulator [36]. We demonstrated that oral microbiota from CRS mice could increase the blood-brain barrier permeability as evidenced by the lower
expression of tight junction-related proteins and alter neurotransmitter such as 5-HT and norepinephrine levels in GF mice, which shows the presence of microbiome-oral-brain axis in depression.

We also identified that CRS-stimulated oral microbiota induced serum metabolites alteration in GF mice. Integrated microbiome and metabolomics analysis revealed that interactions among depression disorders associated microbiota and metabolites are altered, implying that microbiota and microbial-derived metabolites may affect the development of neuroinflammation within the brain by the blood circulation system that in turn can reflect the depression pathogenesis [37]. We further found that changes in serum metabolites were significantly correlated with alteration in abundance of oral microbes, in which EPA had the most negatively correlation with Pseudomonas. EPA is a polyunsaturated fatty acid and is reported to prevent cardiovascular disease [38, 39]. Pathway enrichment analysis showed that fatty acid biosynthesis and primary bile acid biosynthesis were altered in germ-free recipient mice. The alteration of this metabolite at CRS state hints at their potential roles in depression and warrants further investigation. It has been demonstrated that Pseudomonas intervention and EPA supplementation change depression phenotypes, further supporting our discovery in this study. Integrated correlations between the representative values of behavioral changes, levels of neurotransmitter in the frontal cortex and changes in oral microbial-derived metabolites indicated that oral microbial can exert significant roles in anxiety and depression by oral-brain axis.

There are studies supported the importance of oral-gut microbiome axis in the development of diseases [31, 40, 41]. The impairment of the oral barrier can cause interorgan translocation, including the oral microbiota can translocate to the gut, which can alter the microbial ecosystems in gut and influence the pathophysiological processes of intestinal tract [9]. In our study, we also found that some bacteria from the saliva were ectopic colonized in intestinal tract in stress states. Next, we further evaluated gut barrier function in germ-free recipient mice and identified that the oral microbiota induced by CRS colonized the intestinal tract and induced gut microbiota dysbiosis, and harms intestinal morphological characteristics and permeability. Oral and gut microbiota dysbiosis affects not only local areas, but also distant tissues and organs. Oral microbiota that enters the intestinal tract can produce some metabolic endotoxins which further induce inflammatory related changes in various tissues [42–44]. The low levels of 5-HT and high levels of norepinephrine are strongly associated with the pathophysiology of depression [7, 45], consistent with our findings.

Together, our data revealed the differential shaping effect of oral and intestinal microbiota on depression. This study also indicated that the microbial communication between the oral cavity and gut, and identified the association between oral microbiota and derived metabolites and mental disorders. Our work demonstrated a promising potential of oral microbes and derived metabolites for noninvasive diagnosis and interventions of depression disorders.

Declarations

Acknowledgements
Authors' contributions

Fangzhi Lou and Shihong Luo contributed to conception and data analysis, and drafted the manuscript; Huiqing Long and Li Yan contributed to data analysis of the manuscript; Yiyun Liu and Juncai Pu contributed to conception and design, and critically revised the manuscript; Haiyang Wang and Ping Ji revised the manuscript; Xin Jin contributed to conception, design and data analysis, and drafted the manuscript. All authors had the opportunity to discuss the results and comment on the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Funding

This work was supported by grants from the National Natural Science Foundations of China (No. 81870775) and the Natural Science Foundation of Chongqing (No. CSTB2022NSCQ-MSX1148).

Availability of data and materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Stomatological Hospital of Chongqing Medical University (Approval No. 2021-5) and the Laboratory Animal Ethics Committee of Chongqing Medical University (Approval No. 2021063). All participants provided written informed consent.

Consent for publication

Not required.

Competing interests

The authors declare that they have no competing interests.

Author details

1Key Laboratory of Psychoseomadsy, Stomatological Hospital of Chongqing Medical University, Chongqing, China. 2Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, Chongqing, China. 3NHC Key Laboratory of Diagnosis and Treatment on Brain Functional Diseases, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China. 4School of Public Health and Management, Chongqing Medical University, Chongqing, China.
References


Figures

Figure 1
Associations of the oral microbiota composition and related metabolites with depression scores. **A** PCoA analyses based on the Bray–Curtis distances at the ASV level between oral bacterial communities of health (n = 70) and depression patients (n = 87). **B** Oral saliva samples are clustered into type 1 and type 2 by using PCoA. **C** Community bar plot analysis at the genus level. **D** Box plots showing the relative abundance of the Pseudomonas, Leptotrichia and Solobacterium. **E** Circos plot of communities showing the relationship between saliva samples and species in each group. **F** Compositional analysis by LefSe showing the predominant microbiota between two groups. **G** OPLS-DA score plot of saliva metabolite profiling between the two groups. **H** The Venn plot showing the difference of metabolites between the two groups. **I** Volcano plot showing significantly changed metabolites between the two groups samples. **J** KEGG enrichment analysis showing metabolites-associated metabolic pathways. **K** Spearman's rank correlation between differential oral microbiota at the genus and metabolites. *P* < 0.05, **P** < 0.01, ***P*** < 0.001.
Figure 2

CRS-induced oral microbiota disorders are significantly different from microbiota alterations in gut. A Behavioral testing. B PCoA analyses based on the Bray–Curtis distances at the ASV level between oral bacterial communities of the stress groups (CRS, n = 10) and control groups (n = 6). C PCoA analyses based on the Bray–Curtis distances at the ASV level between gut bacterial communities of the stress groups (CRS, n = 10) and control groups (n = 6). D, E Community barplot analysis of oral and gut
microbiota at the genus level between the CRS and control groups. F Box plots showing the relative abundance of the Pseudomonas, Muribacter and Pasteurellaceae for oral microbiota. G Bar plot showing the relative abundance of the Muribaculaceae, Saccharimonas, Lachnospiraceae and Desulfovibrionaceae for gut microbiota. H, I Compositional analysis by LefSe showing the predominant microbiota for oral microbiota between two groups. J, K Compositional analysis by LefSe showing the predominant microbiota for gut microbiota between two groups. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 3
Saliva microbiota transplantation in GF mice recapitulate the alteration of oral microbiota in CRS exposed conventional mice. **A** PCoA analyses based on the Bray–Curtis distances at the ASV level between oral bacterial communities of the GF-Stress groups (n = 8) and GF-control groups (n = 8). **B** Venn plot showing the difference of bacterial genera between the two groups. **C** Community bar plot analysis of oral microbiota at the genus level between the two groups. **D** Circos plot of communities showing the relationship between saliva samples and species in each group. **E** Consistent alteration in bacteria abundance (p<0.05, Stress-exposed mice vs Stress-free mice; GF-Stress mice vs GF-Control mice) in two mice model (GF mice and conventional KM mice). The FC in abundance between Stress and non-Stress was calculated. **F** Experimental workflow. **G–I** Behavioral tests for the FST, TST and OFT. *P < 0.05.
Figure 4

CRS-stimulated oral microbiota induces microbial-derived metabolites alteration in GF mice. A PCA and OPLS-DA score plot of serum metabolite profiling between the two groups (n = 6, respectively). B Venn plot showing the difference of metabolites between the two groups. C Volcano plot showing significantly changed metabolites between the two groups samples. D Heatmap of top 30 metabolites (VIP > 2, P < 0.05). E KEGG enrichment analysis showing metabolites-associated metabolic pathways. F Procrustes Analysis showing associations between differential oral microbiota at the genus and metabolites. G
Correlation heatmap showing associations between differential oral microbiota at the genus and metabolites. * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 5

Oral microbiota induced by CRS increases the blood-brain barrier permeability and neurotransmitter imbalance in GF mice. **A** RT-PCR showing the mRNA expression levels of ZO-1, occludin and claudin-1 from hypothalamus and hippocampus tissue. **B** Western blot showing the protein expression of ZO-1 in the frontal cortex. **C** Immunofluorescence staining showing the protein expression of ZO-1 in the frontal cortex. Scale bars, 50 μm. **D** ELISA showing 5-HT and norepinephrine level. * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **E** Pearson correlation heatmap focusing on the correlations between microbial-derived metabolites, behavioral and neurotransmitter values in the frontal cortex significantly influenced by CRS.* $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.  

---

**A**

**B**

**C**

**D**

**E**
Figure 6

Ectopic colonization of oral microbiome induced by CRS in the intestine impaired gut barrier function in GF mice. **A** PCoA analyses based on the Bray–Curtis distances at the ASV level between saliva and stool bacterial communities of the stress groups (n = 10). **B** Heatmap of showing the same bacteria in the saliva and stool samples in CRS states. **C** Alpha diversity of the gut microbiota involving Sobs, Chaos, Ace and Shannon index. **D** PCoA analyses based on the Bray–Curtis distances at the ASV level between...
gut bacterial communities of the GF-Stress groups (n = 6) and GF-control groups (n = 6). E Community bar plot analysis of gut microbiota at the genus level between the two groups. F Circos plot of communities showing the relationship between stool samples and species in each group. G Bar plot showing the relative abundance of the significant differences among the gut microbiota. H Compositional analysis by LefSe showing the predominant microbiota for gut microbiota between two groups. I Hematoxylin and eosin (HE) staining from colon tissue. The black arrow represents the goblet cells, the red arrow represents the abnormal structure of intestinal crypts, the blue arrow represents the inflammatory cells. Scale bars, 200 μm. J RT-PCR showing the mRNA expression levels of ZO-1, occludin and claudin-1. K Immunofluorescence staining from colon tissue showing the protein expression of ZO-1. Scale bars, 50 μm. * P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

Pseudomonas aeruginosa intervention and Eicosapentaenoic Acid supplementation change depression-like behavior. A Experimental workflow. B-G Behavioral tests for the FST, TST and OFT (n = 5). * P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

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