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The role of gut microbiota in chronic restraint stress-induced cognitive deficits in mice

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Abstract

Background Chronic stress induces cognitive deficits. There is a well-established connection between the enteric and central nervous systems through the microbiota-gut-brain (MGB) axis. However, the effects of the gut microbiota on cognitive deficits remain unclear. The present study aimed to elucidate the microbiota composition in cognitive deficits and explore its potential in predicting chronic stress-induced cognitive deficits.

Methods Mice were randomly divided into control and chronic restraint stress (CRS) groups. The mice subjected to CRS were further divided into cognitive deficit (CRS-CD) and non-cognitive deficit (CRS-NCD) groups using hierarchical cluster analysis of novel object recognition test results. The composition and diversity of the gut microbiota were analyzed.

Results After being subjected to chronic restraint distress, the CRS-CD mice travelled shorter movement distances ($p = 0.034$ vs. CRS-NCD; $p < 0.001$ vs. control) and had a lower recognition index than the CRS-NCD ($p < 0.0001$ vs. CRS-NCD; $p < 0.0001$ vs. control) and control mice. The results revealed that 5 gut bacteria at genus levels were significantly different in the fecal samples of mice in the three groups. Further analyses demonstrated that *Muricomes* were not only significantly enriched in the CRS-CD group but also correlated with a decreased cognitive index. The area under the receiver operating curve of *Muricomes* for CRS-induced cognitive deficits was 0.96.

Conclusions Our study indicates that the composition of the gut microbiota is involved in the development of cognitive deficits induced by chronic restraint stress. Further analysis revealed that *Muricomes* have the potential to predict the development of chronic stress-induced cognitive deficits in mice.

Keywords Gut microbiota, Chronic restraint stress, Cognitive deficits, *Muricomes*, Mice

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Introduction

Cognitive deficits or dysfunctions are core features of mental disorders, including psychotic symptoms [1]. Treatments targeting cognitive deficits can improve the quality of life of individuals [1, 2]. However, drugs that are currently available have limited effects on cognitive deficits [2, 3]. Therefore, it is important to explore the mechanisms underlying cognitive deficits and develop new treatment strategies.

Research using animal models, suggest that cognitive deficits could be induced by chronic stress [4]. Although essential for active physiological and behavioral responses, chronic stress is detrimental to individuals [5] and can induce cognitive deficits as well as alter immune responses [5, 6]. Additionally, the gut barrier is known to be damaged by chronic stress [7]. There is also evidence of a well-established association between the central and enteric nervous systems through the microbiota-gut-brain (MGB) axis [8] which plays an important role in maintaining homeostasis. Several studies have reported that stress significantly affects the MGB axis [9]. In addition, animal models used in studies have often revealed the involvement of the MGB axis in cognitive deficits such as Parkinson's disease [10, 11]. There is further corroboration, that the gut microbiota may be related to gut barrier dysfunction and motor deficits in Parkinson's disease [10]. Research targeting the gut microbiota has demonstrated potential efficacy in the treatment of psychiatric disorders [12]. Hence, fecal microbiota transplantation protects against rotenone-induced Parkinson's disease [11].

CRS was shown to be linked with imbalances in the gut microbiota [13]. Modulating the composition of the gut microbiome was found to alleviate symptoms of depression and anxiety [14]. Treatment with prebiotics was found to be beneficial for stress-related behaviors [15]. These findings suggest that the microbiota-gut-brain axis is involved in chronic stress-related behaviors. However, the exact gut microbiota on cognitive deficits induced by chronic stress remains unclear. The present study aimed to elucidate the microbiota composition of cognitive deficits and explore the potential of the microbiota in predicting chronic stress-induced cognitive deficits considering the relationship between the MGB axis, cognitive deficits, and stress.

Materials and methods

Animals

A total of 21 male C57BL/6J mice (8 w, No. 44824700001143) were obtained from Gempharmatech Biotechnology Co., LTD (Guangdong, China). The mice were provided with a consistent diet and housing environment since birth. Prior to the start of the experiment, all mice were allowed to acclimate to their surroundings for one week without any interventions. During this time, the mice were given unrestricted access to food and water, and housed under conditions with a natural alternating light-dark cycle, and temperatures of 22 °C±2 °C and 60% ± 5% of humidity. During the acclimation period, three to five mice were housed together in each cage. Following the acclimation period, the mice were randomly assigned to either the CRS group or the control group. All animal care and research protocols were approved by the Ethical Approval for Research Involving Animals of Guangdong Province Hospital (No.2021005).

Open field test (OFT)

The OFT has been utilized as a reliable tool in previous studies to evaluate exploratory activity and anxiety-like behavior. It provides a comprehensive assessment of cognitive function [16–18]. The mice were placed in a 50 cm × 50 cm × 50 cm open field chamber. The floor was divided into six lines and sixteen squares. Each mouse was placed in the center of an open-field chamber for 5 min of free movement. The movement parameters of the mice were recorded using an automatic tracking system (Smart3.0; Harvard PanLab). The total distance moved was then analyzed.

Novel object recognition test (NORT)

NORT is commonly used to assess learning and memory in mice [19]. It is widely utilized in studies investigating cognitive deficits [19, 20]. On the adaptation day, the mice were placed in a 40 cm × 40 cm × 40 cm open-field chamber for 10 min to move freely. On the first test day, each mouse was placed in the same field chamber which contained two identical objects in opposite corners, 6 cm away from the box walls. On the second test day, the mice were placed again in the same field chamber with one original familiar object and one new object of different shape, size, and material. The time expended exploring the familiar and novel objects was recorded.

Moreover, the cognitive index was calculated using the following formula: novel object exploration time / (novel object exploration time+familiar object exploration time).

Fecal samples were collected immediately after NORT after CRS and stored at −80 °C for further use.

Procedure

Twenty one mice were randomized into two groups. The control group consisted of 8 mice, and the chronic restraint stress (CRS) group consisted of 13 mice. All mice underwent the baseline OFT and NORT. The mice in the CRS group were fasted and subjected to 50 ml conical tubes with holes and airflow for 6 h per day for 7 days. Control mice were fasted and secured simultaneously without restraint. All mice in the control and CRS groups

underwent the OFT and NORT. The cognitive index of mice subjected to CRS was entered into IBM SPSS Statistics version 22.0 (IBM, NY, USA) and analyzed using hierarchical cluster analysis. The results were displayed in a dendrogram generated from the agglomeration schedule. After cluster analysis of the NORT results, the mice in the CRS group were divided into CRS-induced cognitive deficit (CRS-CD) and non-cognitive deficit (CRS-NCD) groups. The details of the procedure are presented in a flowchart (Fig. 1A).

All mice were euthanized by cervical dislocation five minutes after intraperitoneal injection of 0.3% sodium pentobarbital (0.1 ml/10 g) following the completion of the experiments.

DNA extraction and polymerase chain reaction amplification

Microbial DNA was extracted from fecal samples using E. Z. N. A. DNA Kit (Omega Bio-tek, Norcross, GA, U.S.), according to the manufacturer's instructions. The V1-V9 region of the bacteria 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) using primers 27F 5'-AGRGTTYGATYMTGGCTCAG-3' and 1492R 5'-RGYTACCTTGTTACGACTT-3', where a barcode is an eight-base sequence unique to each sample. According to the manufacturer's instructions of AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.), amplicons were extracted from 2% agarose gels and purified.

Library Construction and sequencing

According to the manufacturer's instructions of Pacific Biosciences, SMRTbell libraries were prepared from amplified DNA using blunt ligation. Sequencing Kit 2.0 was used to purify SMRTbell libraries from the Zymo and HMP mock communities which were sequenced on dedicated PacBio Sequel II 8 M cells. Purified SMRTbell libraries from pooled and barcoded samples were then sequenced on a single PacBio Sequel II cell. Amplicon sequencing was performed by Shanghai Biozero Biotechnology Co. Ltd. (Shanghai, China).

Processing of sequencing data

PacBio raw reads were processed using the SMRT Link Analysis software version 9.0 (https://www.pacb.com/support/software-downloads/) to obtain demultiplexed circular consensus sequence (CCS) readings with the following settings: minimum number of passes=3 and minimum predicted accuracy=0.99. Raw reads were processed using the SMRT Portal to filter the sequences for length (<800 or >2500 bp) and quality. Sequences were further filtered by removing barcodes, primer sequences, chirmas, and those containing ten consecutive identical bases.

Operational taxonomic units (OTUs) were clustered with a 98.65% similarity cutoff using UPARSE (version 7.1; http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME.

The sequences were dereplicated and subjected to the DADA2 algorithm (recommended by QIIME 2) to identify indel-mutations and substitutions [21]. Additionally, trimming and filtering were performed on paired reads with a maximum of two expected errors per read (maxEE=1). After merging paired reads and chimera filtering, the phylogenetic affiliation of each 16 S rRNA gene sequence (herein called RSVs) was analyzed using the RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU132)16 S rRNA database using a confidence threshold of 70% [22].

Statistical analysis

Alpha- and beta-diversity

Rarefaction analysis based on mothur-version v.1.21.1 [23] was conducted to reveal diversity indices, including the Chao, Simpson, and Shannon diversity indices. The rarefaction curve was submitted as supplementary file 1. The Beta diversity was performed using UniFrac [24] to compare the results of the principal component analysis (PCA) utilizing the community ecology package R-forge (Package vegan 2.0 was used to generate a PCA figure).

Mantel tests were used to examine the Spearman's rank correlation between the cognitive index, movement distances, and the bacterial community similarity using Bray-Curtis distance matrices with 999 permutations, and the vegan package in R. To further confirm the observed differences, multivariate analysis of variance (MANOVA) was conducted. In order to determine the relationship between the cognitive index and microbiota, Spearman's correlation coefficients were assessed (Package psych v2.2.5). A correlation was considered statistically significant when the absolute value of the Spearman's rank correlation coefficient (Spearman's r) was more than 0.6 and p values less than 0.05. All statistical analyses were performed using the R statistics package. R (heat map package) and cytoscape version 3.9.1 (http://www.cytoscape.org) were used to visualize the relationships using correlation heat maps and network diagrams, respectively. A one-way analysis of variance was performed to assess statistically significant differences in diversity indices between samples. Venn diagrams were drawn using the online tool "Draw Venn Diagram" (http://bioinformatics.psb.ugent.be/webtools/Venn) to analyze overlapped and unique OTUs during the treatment processes. Moreover, a one-way permutational analysis of variance was performed using the R vegan package to assess statistically significant effects of the treatment processes on bacterial communities. It

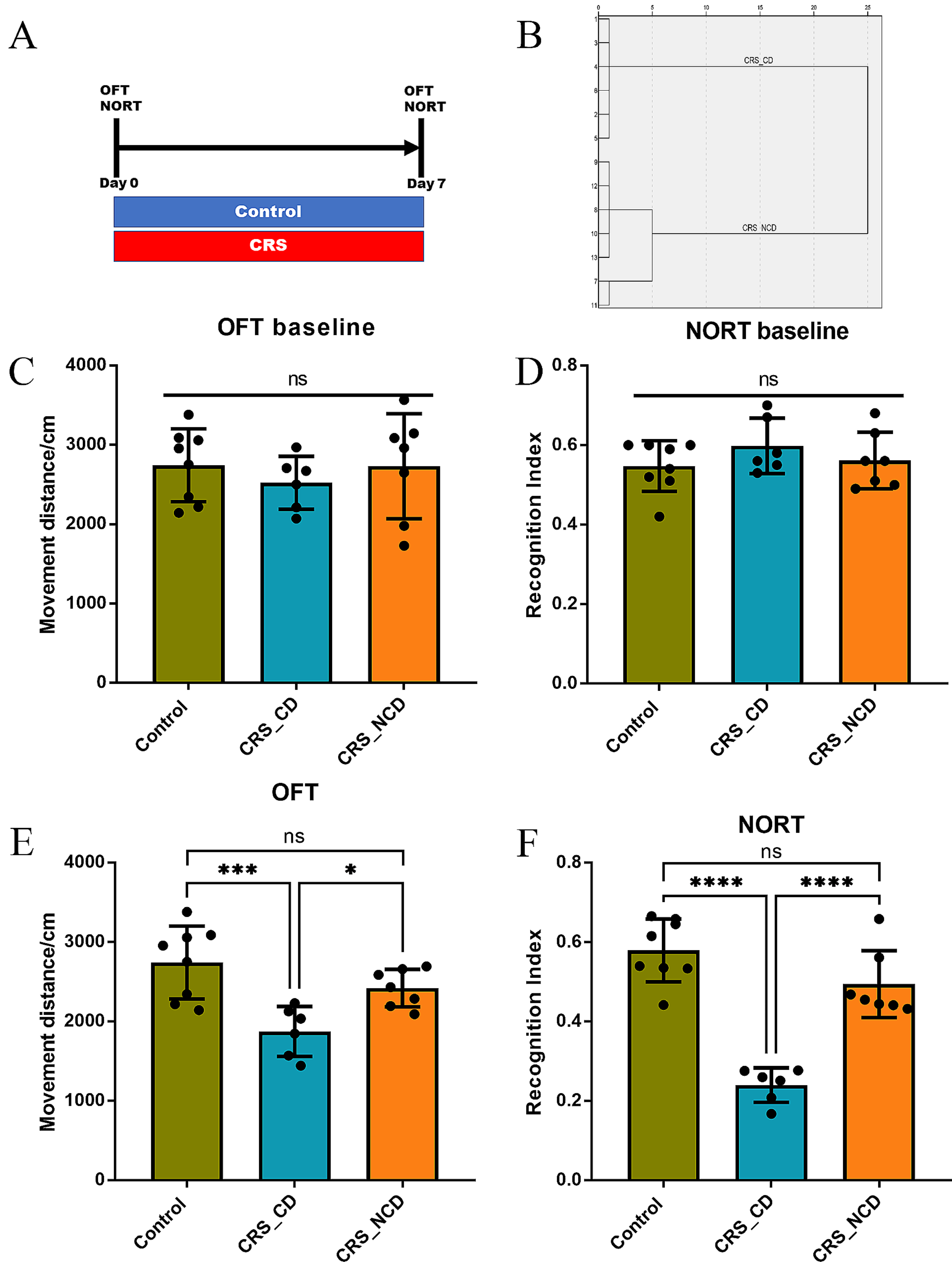

Fig. 1 The procedure, the OFT, and NORT results. (**a**) The procedure of the study. (**b**) The hierarchical cluster analysis of the cognitive index of NORT. The y axis (longitudinal axis) is the mice's code in present study. The x axis (transverse axis) means the relative distance of the clustering category. (**c**) The results of OFT at baseline. (**d**) The results of NORT at baseline. (**e**)The results of OFT on day 7. (**f**) The results of NORT on day 7. Data are presented as mean ± SEM. ns: $p > 0.05$, *$p < 0.05$, ***$p < 0.001$

was considered statistically significant when p values less than 0.05.

Linear discriminant analysis effect size (LEfSe) analyses

LEfSe was performed to identify biomarkers for high-dimensional colonic bacteria. The Kruskal–Wallis sum-rank test was used to examine changes and dissimilarities among classes, an then linear discriminant analysis (LDA) to assess the effect size of each distinctively abundant taxa [25].

Receiver operating characteristic (ROC) curve

ROC curves were used to detect the recognition of gut bacteria in CRS-induced cognitive deficits. The mice in CRS-CD group were considered subjects with disease, and mice in CRS-NCD and control groups were considered without disease. The area under the curve (AUC) represents the diagnostic accuracy. An AUC of 0.7 to 0.8 suggests acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding [26].

Results

OFT and NORT results between CRS-CD and CRS-NCD groups

CRS-CD and CRS-NCD mice were categorized using a hierarchical cluster analysis of the NORT index (Fig. 1B).

The movement distances ($p=0.686$, Fig. 1C) and cognitive index at baseline ($p=0.391$, Fig. 1D) exhibited no significant differences among the three groups. After being subjected to chronic restraint distress, the CRS-CD mice travelled shorter movement distances (MD=-577.9 cm, 95%CI -1052 to -37.93 cm, $p=0.034$ vs. CRS-NCD; MD=-866.3cm, 95%CI -1358 to -374.2cm, $p<0.001$ vs. control, Fig. 1E) and had a lower recognition index than the CRS-NCD (MD=-0.25, 95%CI -0.36 to -0.15, $p<0.0001$ vs. CRS-NCD; MD=-0.34, 95%CI -0.44 to -0.24, $p<0.0001$ vs. control, Fig. 1F) and control mice. Hence, this suggests that chronic stress can induce cognitive deficits.

Richness and diversity of the bacterial community

Previous studies have used diversity to refer to the variety of species and bacteria within a habitat, and the Chao 1, Shannon, and Simpson indices are commonly used to evaluate the diversity of the microbiota [27, 28]. The Chao 1 ($p=0.590$, Fig. 2A), Shannon ($p=0.855$, Fig. 2B), and Simpson ($p=0.722$, Fig. 2C) indices were not significantly different among fecal samples from the CRS-CD, CRS-NCD, and control groups. Regarding diversity, which was used to represent differentiation among habitats [27], PCA demonstrated that the dots among the three groups were not significantly different (Fig. 2D).

Gut microbiota composition in CRS-CD, CRS-NCD, and control groups

Fecal samples from the CRS-CD mice had the lowest operational taxonomic unit (OTU) richness (142 OTUs) based on the number of OTUs at the genus level (Fig. 3A). Fecal samples from the CRS-NCD and control mice had similar OTU richness, with 162 and 191 OTUs at the genus level, respectively (Fig. 3A). The three groups shared 108 OTUs. Although the dominant genera were shared among the three groups, their relative abundances varied (Fig. 3B).

Specific microbial taxa and their association with CRS-CD

LEfSe analysis was conducted to further explore the relevant potential taxa for CRS-CD [29]. The results showed that the family *Tannerellanceae* was enriched in CRS-CD mice (Fig. 4A). Further analyses indicate that 38 taxonomic clades (a=0.01) with an LDA score higher than 2.0 were significantly different among the three groups (Fig. 4B). *Tannerellaceae*, *Parabacteoides*, *Parabacteoides_massiliensis*, *Duncaniella_freteri*, *Erysipelatoclostridium*, *Erysipelatoclostridium_coleatum*, *Muricomes*, *Muricomes_contortus_B*, and *Muricomes_sp00315025* were significantly enriched in the CRS-CD group (Fig. 4B). This implies that this microbiota may be associated with CRS-CD.

Differences in the gut microbiota composition among the three groups

The results revealed that 5 gut bacteria at genus levels were significantly different in the fecal samples of mice in the three groups. The relative abundances of *Muricomes* at genus level (Fig. 5A), was significantly increased in the CRS-CD group compared to the other two groups. The relative abundances of the remained four gut bacteria at genus level (Fig. 5B-E) in the CRS-CD group were not significantly increased or decreased compared to the other two groups. The supplementary file 2 contains information about the other 9 gut bacteria at the class, order, family, and species levels, which were found to be significantly different in the fecal samples of mice in the three groups.

Correlation analysis between NORT and gut bacteria levels

The correlation between the recognition index and bacteria was analyzed. The results confirmed that the abundance of *Muricomes*, *Parabacteoides*, and *Erysipelatoclostridium coleatum* was negatively correlated with both CRS-induced cognitive and motor deficits (Fig. 6A). The detailed r and p valus were shown in supplementary file 3.

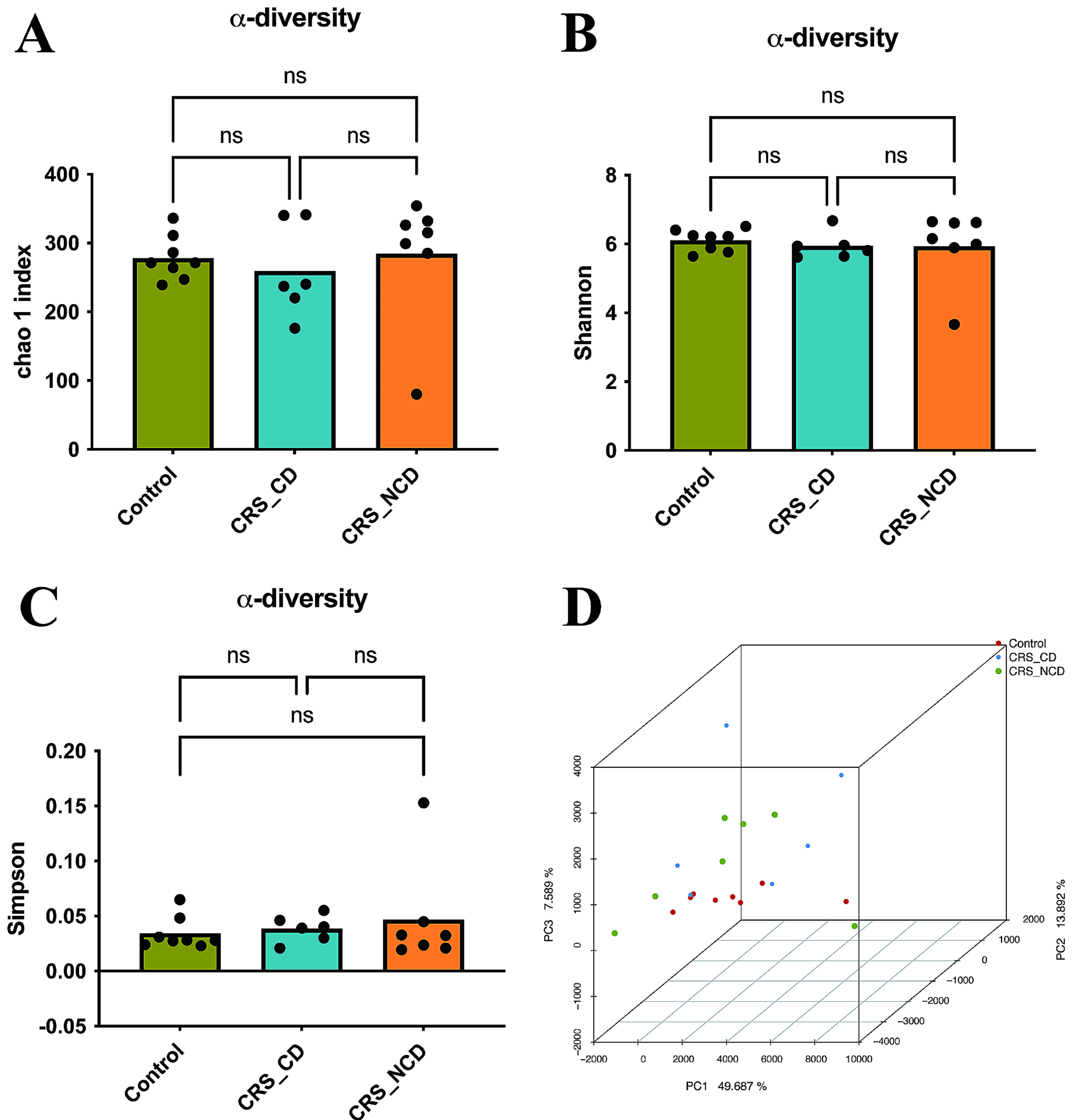

Fig. 2 Alpha diversity and beta diversity in gut microbiota. (**a**) Chao 1 index of diversity. (**b**) Shannon Index of diversity. (**c**) Simpson Index of diversity. (**d**) The PCA of beta diversity. Red dots indicate the control group, blue dots the CRS-CD group and green dots the CRS-NCD group. Data are presented as mean ± SEM. ns: $p > 0.05$

Gut bacteria for the diagnosis of CRS-CD using ROC curve analysis

The aforementioned results showed that *Muricomes* were not only significantly enriched in the CRS-CD group but also correlated with a decreased cognitive index. This means that *Muricomes* may be the key gut bacteria involved in CRS-induced cognitive deficits. The ROC curve was used to indicate the diagnostic ability of gut bacteria for CRS-CD. The AUC under ROC of *Muricomes* for CRS-induced cognitive deficits was 0.9556 (95% confidence interval, 0.8724–1.000, $p = 0.0014$). (Fig. 6B).

Fig. 3 Gut microbiota composition at the genus level. (**a**)The Venn diagram exhibits the number of shared and unique OTUs between the three groups at the genus level. (**b**) Composition of bacteria in the three groups at the genus level

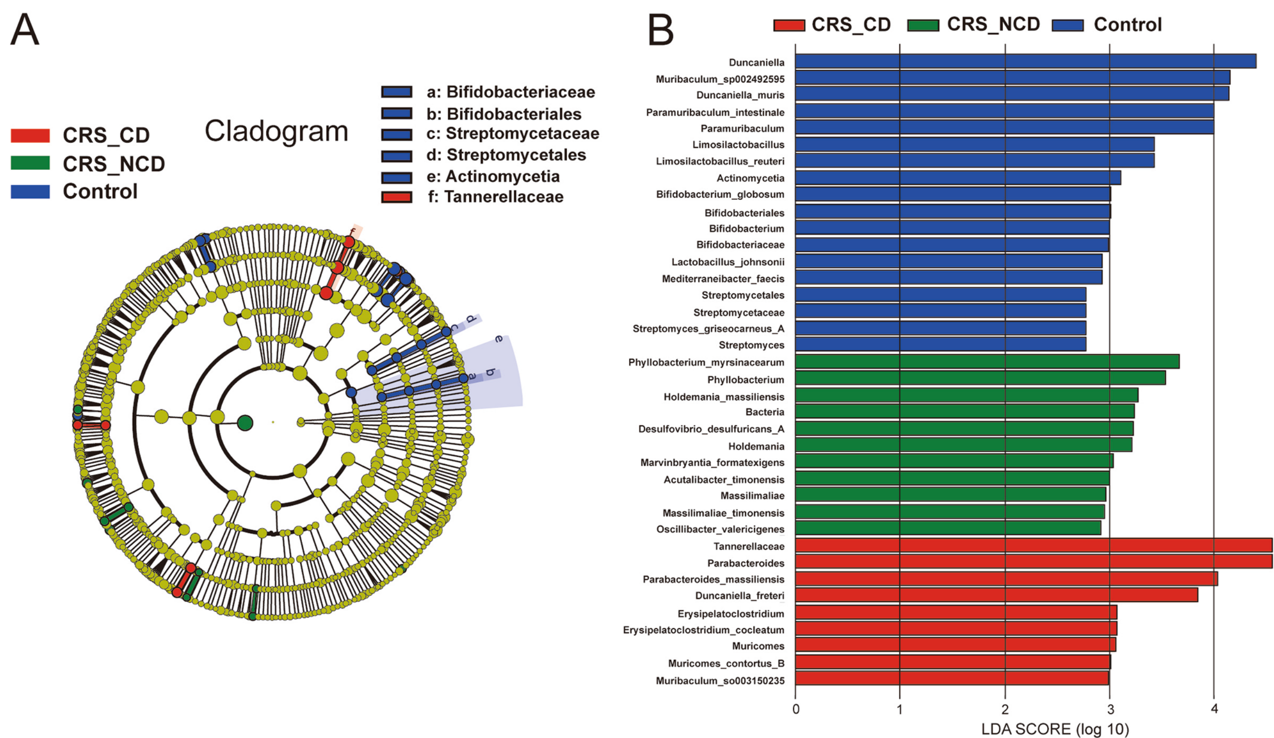

Fig. 4 LEfSe results. (**a**) Cladogram representation of the gut microbiota taxa associated with the CRS-CD. (**b**) Association of specific microbiota taxa with the CRS-CD group using LDA effect size (LEfSe). Red indicates taxa enriched in the CRS-CD group, green indicates taxa enriched in the CRS-NCD group, and blue indicates taxa enriched in the control group

Discussion

The results of the present study suggest that CRS could induce cognitive deficits in mice, as indicated by the decrease in movement distances and recognition indices. The fecal microbiota composition differed among CRS-CD, CRS-NCD, and Control mice. Further analyses showed that *Muricomes* were significantly enriched in CRS-CD mice and were negatively correlated with the cognitive index. Moreover, *Muricomes* were able to predict cognitive deficits induced by CRS in mice.

Growing evidence has confirmed an association between chronic stress and cognitive deficits [30, 31]. Chronic stress is also a risk factor for depression, anxiety, and Alzheimer's disease [32, 33]. In humans, cognitive deficits, particularly in the memory domain, are susceptible to perceived stress [31]. The results of the present study illustrated that the movement distance and NORT index of CRS-CD mice were significantly lower than those of CRS-NCD and control mice. This indicates that CRS can induce cognitive deficits.

Nonetheless, the mechanisms underlying the chronic stress-induced cognitive deficits remain unclear. Animal studies have provided evidence that the MGB axis plays an important role in cognitive deficits [34, 35]. In mice with CRS-induced depression, there is evidence of alterations in the composition of the gut microbiota [36]. Certain key microbes have been found to potentially affect brain function by participating in metabolomic pathways and influencing the host's immune activity [7]. It is suggested that in CRS mice, the gut microbiota may enter the bloodstream through a compromised intestinal barrier, leading to behavioral disturbances through cytokine activation and inflammation [37]. Additionally, the gut microbiota is known to play a role in the breakdown of short-chain fatty acids (SCFAs), which are important for the development and maintenance of the central nervous system [38]. Furthermore, it has been observed that antidepressant treatment in mice exposed to CRS results in an increase in the abundance of the *Lactobacillus acidophilus* genus [39]. Similarly, our results have also demonstrated that chronic stress was associated with gut microbial composition.

As evidence has supported the role of gut microbiota dysbiosis in cognitive deficits, it may be possible to explore their diagnostic ability [40]. The gut microbiota has manifested potential in predicting anesthesia- and surgery-related cognitive dysfunction in mice [41]. The results of the present study consistently support the association between *Muricomes* and cognitive indices. Furthermore, *Muricomes* confirmed the ability to predict cognitive deficits in mice subjected to chronic restraint stress. In elderly patients undergoing orthopedic or abdominal surgery, specific bacterial species have shown promise in predicting the onset of postoperative

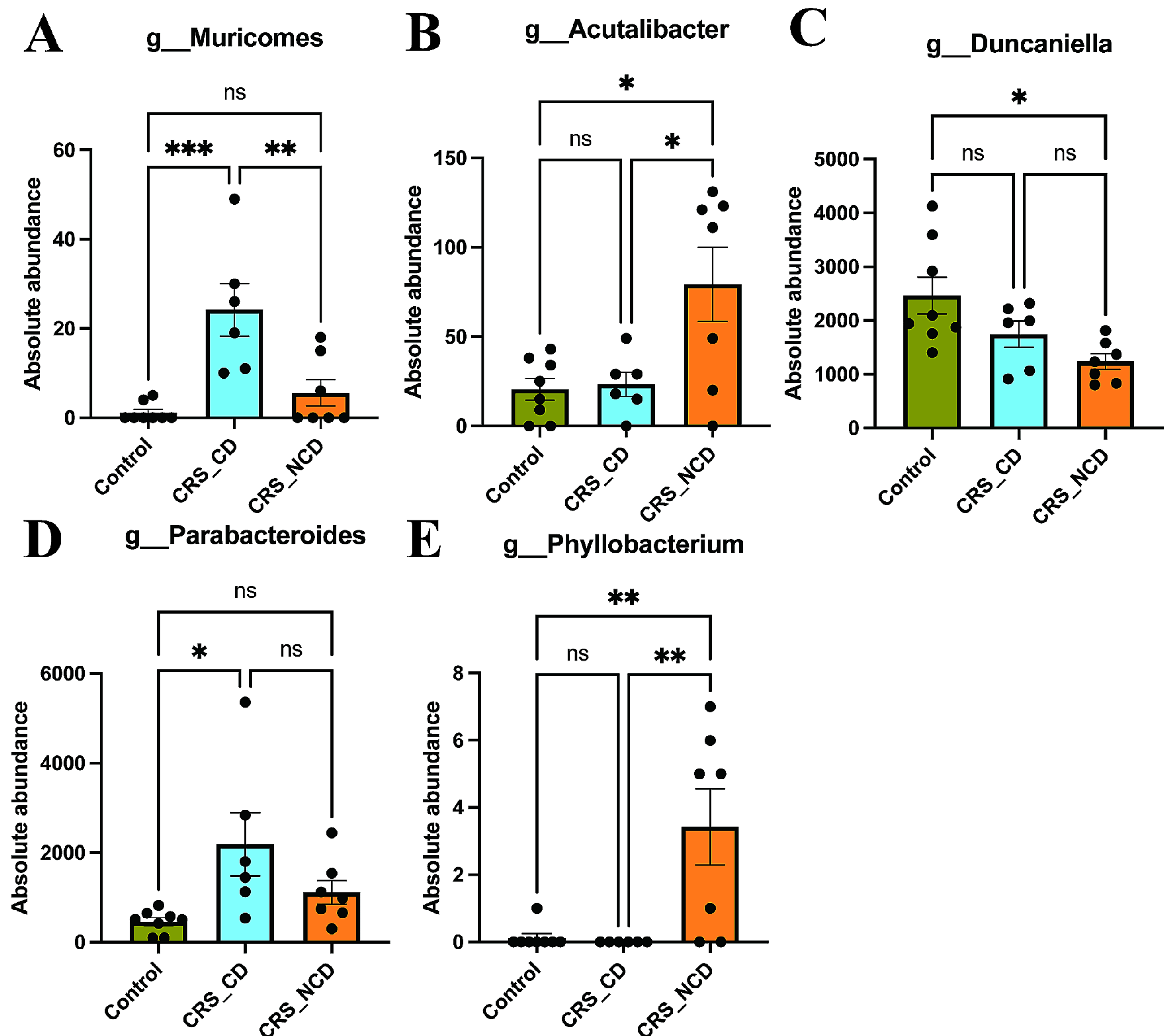

Fig. 5 Differential of the gut bacterium at genus levels. (**a**) Relative abundance of Genus *Muricomes*. (**b**) Relative abundance of Genus *Acutalibacter*. (**c**) Relative abundance of Genus *Duncaniella*. (**d**) Relative abundance of Genus *Phyllobacterium*. (**e**) Relative abundance of Genus *Parabacteroides*. Data are presented as mean ± SEM. ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

cognitive dysfunction or delirium [42, 43]. However, the α-diversity of gut composition is not considered useful in predicting mental disorders [44]. Therefore, it is worthwhile to further investigate the potential use of these specific gut microbiota in predicting cognitive deficits in a clinical setting in the future.

Several strategies have been explored to modify or restore the gut microbiota. Probiotics have been found to benefit the gut microbiota and stress-related cognitive deficits [15, 45]. Furthermore, fecal bacterial transfer from NIH Swiss mice to germ-free BALB/c mice were shown to induce exploratory behavior, whereas the exploratory behavior of germ-free NIH Swiss mice reduced in those that received BALB/c microbiota [46]. Fecal bacterial transfer from depressed patients to microbiota-deficient rats also induced depression-like behavior [47]. Inducing microbiota from healthy individuals to a patient through fecal bacterial transfer has been shown to improve the symptoms of cognitive deficits [48, 49]. Recent evidence also indicates that appropriate exercise attenuated gut dysbiosis and improved cognitive function after surgery in mice [50]. The findings of previous studies suggest that gut microbiota dysbiosis can induce cognitive deficits, and that re-establishment of the gut microbiota can improve the symptoms of cognitive deficits [47–50].

The study has some limitations. Firstly, this study only focused on male mice. However, previous research has

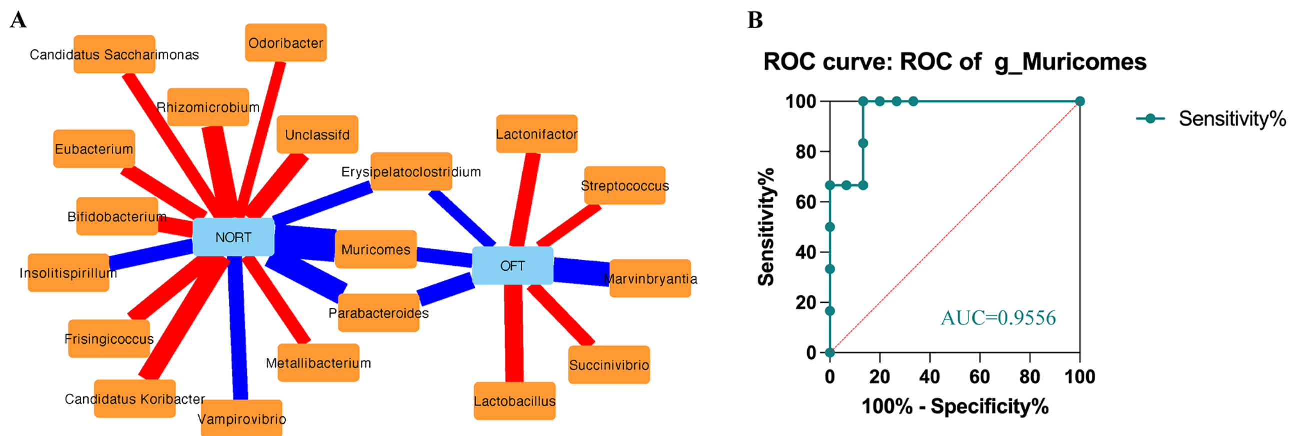

Fig. 6 Correlations between cognitive index, movement distance and the composition of gut bacterium, and ROC curves of the gut bacterium count for the diagnosis of CRS-CD (**a**) The abundance of Muricomes, Parabacteoides, and Erysipelatoclostridium collated arenegatively correlated to both CRS induced cognitive and motor deficits. Red indicates a positive correlation and blue indicates a negative correlation (**b**) AUC under ROC for CRS-induced cognitive deficits is 0.96 (95% confidence interval, 0.87-1.00)

indicated a potential correlation between gender and cognition [51, 52]. Second, the detailed mechanism of the effect of microbiota on cognitive deficits, especially in the functional areas of the brain, has not been explored. *Muricomes* are abundant in mice with cognitive deficits. However, whether treatment targeting *Muricomes* can improve the cognitive index has not been explored. Third, the number of mice were limited. Last but not least, there are various tests were used to measure memory and learning [16, 53], and only two of them used in present study. Therefore, the results of the study must be confirmed and further elucidated.

In conclusion, the present study supports the idea that the composition of gut microbiota is involved in the development of cognitive deficits induced by chronic stress. Our results revealed that *Muricomes* are significantly related to cognitive deficits induced by chronic stress. Therefore, *Muricomes* might be able to predict cognitive deficits induced by chronic restraint stress in mice.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03435-w.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

QL, and JZ acquired data, analyzed the data and prepared Figs. 1, 2, 3, 4, 5 and 6. LZ, XL, TS, HX, and AM analyzed the data. GZ, and YS designed the study and drafted the manuscript. QZ designed the study, analyzed the data, prepared Figs. 1, 2, 3, 4, 5 and 6 and drafted the manuscript. All authors approved the final version of the manuscript for publication.

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Data availability

Sequence data that support the findings of this study have been deposited in National Center for Biotechnology Information with the primary accession code PRJNA1030789.

Declarations

Ethics approval and consent to participate

All animal care and research protocols were approved by the Ethical Approval for Research Involving Animals of Guangdong Province Hospital (No.2021005).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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