Food & Function

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Journal:	*Food & Function*
Manuscript ID	FO-ART-02-2019-000399.R2
Article Type:	Paper
Date Submitted by the Author:	25-Jul-2019
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Impact of Traditional Chinese Medicine Treatment on Chronic Unpredictable Mild Stress-induced Depression-like Behaviors: Intestinal Microbiota and Gut Microbiome Function

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Abstract Gut microbiota dysbiosis is a recognized contributing factor to many noncommunicable diseases, but more evidences are still needed to illustrate its causative impact on mental and brain health disorders and mechanism(s) for targeted mitigation. Traditional Chinese Medicine (TCM) has been used in the management of neuropsychiatric diseases for many years in China. In this study, a randomized, controlled trial was conducted to examine the impact of stress on gut microbiota dysbiosis and depression, and TCM in alleviating the damages using Chronic Unpredictable Mild Stress (CUMS) rats, a well-established rodent model for depression. The behaviors of rats and the profiles of the fecal microbiota were assessed by an array of behavioral tests and 16S rRNA gene sequencing, and intestinal microbial function was assessed by shotgun sequencing-based metagenomic analysis of microbial DNA from fecal samples. Data on brain targeted metabolites by liquid chromatography-mass spectrometry (LC-MS) were also discussed.

Depressive and anxiety-like behaviors and changes in fecal microbiota profile were observed in CUMS rats, which were then significantly reversed in CUMS rats received TCM. Specifically, TCM treatment reduced the levels of *Firmicutes*, and *Ruminococcus*, and increased the abundance of *Bacteroidetes* and *Roseburia*, reportedly being associated with relieving psychiatric disorders. Furthermore, the levels of brain metabolites perturbed by CUMS were reversed by TCM treatment, and Spearman's correlation analysis illustrated strong correlation between brain metabolites and perturbed fecal

microbiota genera. Finally, the fecal microbiome of CUMS rats was characterized by alterations in amino acid metabolism and evaluation of bile acid biosynthesis, and TCM-treated rats showed elevation of cysteine and methionine metabolism. Overall, these results indicated that administration of the TCM may mitigate CUMS-induced depression-behaviors, and it is correlated to reversing CUMS-induced intestinal microbiota dysbiosis; and evidences also supported related changes in brain metabolites. These findings set up the foundation to further reveal the exact causal relationship among the TCM formula, host responses, gut microbiota dysbiosis and the levels of brain metabolites, and enabled scientific interpretation of the therapeutic function of the TCM.

Introduction

Depression, a widespread and debilitating mental disorder, is characterized by low mood, sensitivity, sleeplessness, and loss of interest in enjoyable activities.[1, 2] As a multi-factorial disease influenced by internal and external factors, depression affects more than 300 million people of all ages worldwide [3] and has become one of the most attention-worthy disorders due to the high rates of morbidity and mortality.[4-6] So far, as the etiology of depression is still unclear,[2] there is a lack of effective approaches to cure the disease.

Increasing data in the past few years have illustrated that host intestinal microbiota dysbiosis is an important risk factor for many modern diseases, such as diabetes,[7] obesity,[8, 9] and gastrointestinal disorders,[10-12] etc. Particularly gut microbiota dysbiosis is

also correlated to the pathology of several mental disorders, including depression and anxiety.[13-17] For example, a clinical study on the profiles of gut microbiota involving 37 depressed patients and 18 non-depressed controls illustrated that the operational taxonomic units (OTUs) of the order *Bacteroidales* and the family *Lachnospiraceae* were under-represented in patients suffered from depression.[18] Another study found that the abundance of *Clostridium* was significantly elevated in the gut of children with autism spectrum disorder compared with that in healthy children.[19] Moreover, data from preclinical studies emerged in the past two decades have also suggested the association between psychological stress and gut microbiota dysbiosis. Using a rhesus monkey model, Bailey and Coe [20] illustrated that alterations in the composition of the gut microbiota were correlated with anxiety-related behaviors and serum stress hormones levels by early-life stress. Other studies using mouse models have reported that *Lactobacillus rhamnosus* affected the emotions potentially via the vagus nerve; and supplementation of *Bifidobacterium* prevented the onset of depression from stress.[21, 22] Moreover, germ-free mice inoculated with microbiota derived from patients with major depressive disorder or from healthy control individuals exhibited depression-like or normal behaviors accordingly.[23] Depression-associated microbiota exhibited dysbiosis in microbial genes and host metabolites involved in carbohydrate and amino acid metabolism.[23] These data illustrated the causative and transmissible nature of the gut microbiota associated with depression. Taken together, these studies indicated that gut microbiota dysbiosis likely plays a causal role in the development of characteristics of

depression. Thus, modulating the gut microbiota with prebiotics, probiotics, dietary changes, and drugs may effectively mitigate such mental disorders.

The gut microbiota-brain axis is a complex multiorgan bidirectional signaling system between the gut microbiota and brain that plays a crucial role in the pathogenesis of depression.[23, 24] Within the framework of classical monoamine hypothesis of depressive disorder pathogenesis, studies have focused on levels of brain metabolites, including 5-hydroxytryptamine (serotonin, 5-HT), 5-hydroxyindoleacetic acid (5-HIAA), gamma-aminobutyric acid (GABA) and acetylcholine.[25, 26] Several studies have also indicated that the levels of brain metabolites can be rebalanced by the treatment with antidepressants.[27, 28] Therefore, it is essential to assess the alterations of brain metabolites, which would help for understanding the effects of TCM and explore the role of gut microbiota-brain axis in the development of depression.

Traditional Chinese medicine (TCM), which is a form of polypharmacy, has been developed and applied in treating many diseases for over 2,500 years in China.[29, 30] However, the modes of action of many types of TCM are still unclear. Data from multiple reports suggested that gut microbiota mediated the effects of Gegen Qinlian decoction and a specifically designed herbal formula (AMC), indicating that the herbal medicine alleviated diabetes potentially by modulating gut microbiota.[29, 31] Therefore, we hypothesized that the gut microbiota might also be involved in the therapeutic function of TCM. To test the hypothesis, a chronic unpredictable mild stress (CUMS) depression rat

model was established and treated with a formulated TCM. After 6 weeks of treatment, the effects of the TCM on behaviors and intestinal microbiota of the rats were investigated.

Materials and methods

Preparation of TCM

The TCM formula used in the study mainly composed of Component Ⅰ with 8 herbs (*Bupleurum chinense* DC., *Angelica sinensis (Olive.)* Diel, *Paeonia lactiflora* Pall, *Atractylodes macrocephala* Koidz., *Poria cocos* (*Schw.*) Wolf, *Glycyrrhiza uralensis* Fisch., *Zingiber officinale* Rosc., *Mentha haplocalyx* Briq.) (see Table S1 for details) and Component Ⅱ (tea polyphenol). The key active ingredients of this formula are ferulic acid, paeoniflorin, aurantiamarin, and polyphenols, and the amounts of key fractions were presented in Table S2. The ready-to-feed granules of Component I were prepared by soaking the herbs in water for 2 h at the ratio of 1:10 (W: V), and boiling the herbs for 2 h to extract the drug ingredients. The decoction was collected, and the herbs were subjected to extraction with water two more times, at the ratio of 1:8 (W:V), for 1 h each. The liquid extracts were combined, concentrated at 50 ℃, spray dried to granules and packaged.

The extracts of Component Ⅱ were prepared as described previously with slight modification.[32] The material of Component II was *Camellia sinensis*, and it was provided by Kemin Industries, Inc, U.S.A. The sample was grounded into a fine powder, and

stored at −20 °C until use. Briefly, 10 kg of tea powder was extracted with 150 L of distilled water at 100 °C for 30 min. Then, the extract was centrifuged at 4500 g for 15 min, and the resulting insoluble residue was subjected to extraction with water for two more times as described above. The supernatants were combined, and concentrated at 50 °C. The resulting precipitate was dissolved, filtered and purified. Finally, the active ingredients were analyzed by high-performance liquid chromatography (HPLC), and the extraction of *Camellia sinensis* was concentrated and lyophilized.

The granules were stored in a cool and dry place without light, with a shelf life of one year. The granules of Component Ⅰ (41.15 mg/ml) and Component Ⅱ (7.82 mg/ml) were resuspended in sterile water and administered to the experimental mice by oral gavage.

Animal Model and Experimental Schedule

The animal protocol of the study was approved by Institutional Animal Care and the Committee on the Ethics of Animal Experiments of South China Agricultural University (Permit Number 2017-B017). Pathogen-free adult male Sprague-Dawley rats (180-220 g) were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Overall, the animal study lasted for 15 weeks. All rats were given one week to adjust to the laboratory environment (12 h light/dark cycle, at 25±1℃, and 55-65% relative humidity), with free access to food and water, and five rats per cage. After 7 days of acclimatization to the environment, sixty-six rats were randomly divided into two groups:

healthy control group (HC, n=12), chronic unpredictable mild stress group (CUMS, n=54), and all rats were housed independently. With the exception of HC rats, all depressed rats were subjected to eight different chronic unpredictable mild stimuli for 14 weeks, including 8-week depression model development and 6-week TCM treatment period. The CUMS-resistant rats were screened out using behavior tests (sucrose preference test (SPT), open filed test (OPT) and light/dark test (LDT)) after eight weeks of exposure to CUMS. The rest of the rats were randomly divided into two groups as follows: the model group (CUMS, n=6) and the traditional Chinese medicine treatment group (TCM, n=19). According to the previous research and the result of our behavior tests, the TCM group was administered with a mixture of Component Ⅰ (164.6 mg/kg/d) and Component Ⅱ (31.25 mg/kg/d) for six weeks by oral gavage, while the HC and CUMS rats were administered with equal volume of sterile water. SPT was performed in Week 0, 9 and 15. Behavior tests (OPT, LDT) were performed pre- and post-TCM treatment. At the end of the experiment, the rats were fasted for 12 h, anesthetized with 6% (v/v) chloral hydrate and euthanized.

Chronic unpredictable mild stress

The CUMS procedures were performed as previously described by Li et al. (2013)[33] and Liu et al. (2015)[34] with slight modification. Eight different chronic stressors were randomly arranged across 14 weeks: 1. Food and water deprivation for 24 h; 2. Flash stimulation (150 flashes/min, 5 min); 3. 24 h 45° cage tilt; 4. Overnight illumination; 5.

Wet caging (200 ml of water was putted into the sawdust bedding, 24 h); 6. Tail suspension for 5 min; 7. 60 ℃ heat stimulation for 6 min; 8. Nip tail for 5 min. All stressors were arranged at least 13 times and without repetitive stressors in two consecutive days. To prevent affected by the CUMS rats, the HC rats were housed in adjacent room had no contact with the model animals. The model rats were authenticated by behavior tests when finished the period of model development.

Sample collection

At least 2 fecal pellets were obtained from each rat in Week 9 and 15. The rats were placed in a metabolizable cage, and the fecal samples were collected 5 min later using a sterile conical tube. Fecal samples were collected during the metabolic bloom period (9:00-10:00 am), and all samples subjected to DNA extraction and then immediately stored at -80℃ for 16S rRNA V4-V5 amplicon sequencing and metagenome analysis.

Then, the brains of the rats were collected and snap frozen into liquid nitrogen for further assessment of the brain metabolites serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), gamma-aminobutyric acid (GABA) and acetylcholine.

Behavioral testing

The body weights of all rats were recorded every week. SPT, OPT and LDT were carried out as previously described by Bravo et al,[21] Liu et al[35] and De et al.[36]

The source preference test

Anhedonia was assessed using the SPT, as described in previous study with minor modifications.[35] Prior to initiation of the CUMS procedure, an SPT training was conducted. On the first training day, rats were given two bottles of sucrose solution to accommodate. Followed by 24 hours, one of the sucrose bottles was replaced with sterile water. After the adaptation phase, the rats were deprived of water and food for 24 h. Then, the rats were given free access to both water and sucrose solution for 4 h, after which, the remaining amount of water and the sucrose solution were measured. The sucrose preference ratio was determined using the follows formula: The sucrose preference ratio (%) = sucrose intake (ml) × 100% / [sucrose intake (ml) + water intake (ml)].

The depression and anxiety-like behaviors test

The OPT and LDT are commonly used to assess depression and anxiety-like behavior.[21, 36] The former is widely used to measure the activity and the exploration of rats, while the latter is performed to test the preference for darkness. An open field consisting of a black square (80 cm × 80 cm × 60 cm) was divided into 16 equal squares. Each rat was placed in the center of an open arena and rat behavior was recorded for 5 min. Prior to the start of the tests, the rats were given 30 s to get adapted. The total numbers of crossing and rearing were recorded. In LDT, each rat was placed in the center of an apparatus (40 cm × 30 cm × 35 cm), which contained two chambers of equal size, one bright and the other dark. The total time spent in the dark zone was recorded for 5

min. In order to minimize cross-contamination, the experiment area was cleaned using 70% ethanol after each rat completed the test. To avoid subjective errors, both anxiety-like behavior tests were evaluated by two trained observers independently.

Intestinal microbiota analysis by 16S rRNA gene sequencing

To profile the microbial composition, fecal samples were subjected to total genome DNA extraction using the QIAamp DNA Stool Mini Kit following the manufacturer's instruction (Qiagen, USA). The V4-V5 region of 16S rRNA genes of the samples was amplified by PCR (98 °C for 60 s, followed by 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 60 s, and 72 °C for 5 min), using primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 907R 5'-CCGTCAATTCCTTTGAGTTT-3'. The sequencing libraries of the V4-V5 region of the 16S rRNA genes were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's instruction, and index codes were added. The libraries were sequenced using an Illumina HiSeq 2500 platform (manufacturer). QIIME software (Version 1.7.0) was used to analyze alpha-(within samples) and beta-(among samples) diversity. Reads were first filtered by QIIME quality filters and chimera sequences were removed using UCHIME algorithm. The filtered sequences were then clustered into OTUs according to representative sequence using Uparse software (Version 7.0.1001) and classified against the Green genes Database with a threshold of 97% sequence similarity. Alpha diversity is applied in analyzing complexity of species diversity for a sample through 5 indices,

including Observed-species, Chao 1, ACE, Simpson, and Shannon. Principal-coordinate analysis (PCoA) based on bray_curtis algorithm was used to assess the variation of bacterial composition among different groups and different phases. These analyses were performed using the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com). The 16S rRNA sequence data of representative samples were deposited in Sequence Read Archive (SRA) database (Accession number: SUB4976732).

Assessment of brain metabolites

Chemicals and reagents

The reference standards of 3-(2-Aminoethyl)-5-hydroxyindole (HPLC, ≥98.0%), 5-hydroxyindoleacetic acid (HPLC, ≥98.0%), gamma-aminobutyric acid (HPLC, ≥97.0%) and acetylcholine (TLC, ≥99.0%) used as the external standards (ESs) were purchased form Sigma-Aldrich Company (American). Deionized water was prepared using a Super-Q Plus water purification system (Millipore, Bedford, MA, USA). All of the other chemicals used were analytical grade.

Preparation of calibration standards

Stock solutions of IS (1.00 mg/mL) were prepared by dissolving suitable amounts of solutes in methanol. Each stock solution was serially diluted with methanol to prepare working standard solutions with the desired concentrations and stored at 4 °C.

Sample preparation

Representative brain samples were weighed and homogenized on ice. For LC-MS

analysis, brain samples (30 mg) were dissolved in 800 μL of methanol/acetonitrile (1:1, V/V), vortexed 30 s and centrifuged for 15 min at 13000 rpm. Then, 600 μL supernatant of each sample was transfer to an EP tube and vacuum dried; the pellet was resuspended in 400 μL of acetonitrile aqueous solution (with a volume concentration of 50%) and vortexed for 30 s; the brain solution was incubated at 4 °C water bath for 10 min, followed by centrifuged at 13000 rpm for 15 min. The resulting supernatant was stored at 4 °C, and 5 μL of the supernatant was used for LC-MS analysis.

LC-MS analysis

Chromatographic analysis was conducted using an AB Sciex Ultra high Performance LC system equipped with an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm, Waters Corp., Milford, USA). The autosampler and column compartment were maintained at 4 °C. The mobile phase consisted of solvents A (99.9% H_2O + 0.1% formic acid) and B (99.9% acetonitrile + 0.1% formic acid). The elution gradient program for samples was: 5% B for 0 min; 20% B from 3 min; 60% B from 4 min and 5% B from 5 min; 5% B for 9 min the flow rate was 300 μL/min.

Mass spectra (MS) were conducted using an AB Sciex Triple TOF 5600+ (Analyst TF 1.7, AB Sciex) TOF mass spectrometer combined with an electrospray ionization source in positive and negative ion scan mode. TOF parameters were: positive ion and negative ion capillary voltage, 5.5 KV and 4.5 KV; desolvation temperature, 550 °C; atomization pressure, 55 Pa; source temperature, 100 °C; scan time and inter scan delay,

0.15 and 0.02 s, respectively. The lock mass in all analyses were 5-HT (Mr=176.22), 5-HIAA (Mr=191.18), GABA (Mr=103.12) and Acetylcholine (Mr=181.66), used at a concentration of 0.5 μg/mL and infused at a flow rate of 10 μL/min. Raw data were acquired using the centroid mode; the mass range was set from m/z 150 to m/z 400. The MS data from brain samples were first processed by MultiQuant (version 3.0, SCIEX).

Correlation analysis

To explore the functional relationship between the gut microbiota and brain metabolites (5-HT, 5-HIAA, GABA, and Acetylcholine), we formulated one correlation matrix based on Spearman's correlation coefficient ($|r| \geq 0.5$, $p < 0.05$). Spearman correlation was calculated and plotted in R (version 3.5.0, package corrplot).

Shotgun metagenomic analysis of fecal samples

To explore the microbial function of the rat fecal microbiota, the DNA extracts of the representative samples were further subjected to shotgun metagenomic sequencing analysis on an Illumina HiSeq 4000 platform (Illumina) using HiSeq 4000 PE Cluster Kit and HiSeq 4000 SBS Kits.

Open reading frames (ORFs) predicted from all samples were merged and aligned to each other. Gene pairs with greater than 95% identity (no gap allowed) and aligned reads covering over 90% of the shorter reads were grouped together. The longest ORF in each group was used to represent the group, and the other ORFs of the group were regarded as

redundant sequences. ORFs with a length less than 100 bp were subsequently filtered out. Based on this reference gene set, a taxonomic assignment and functional annotation were further conducted using the latest version (Version 2.2.28+) of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The metagenomics datasets were deposited into the Sequence Read Archive (SRA) database (accession number: SUB4976770).

Statistical analysis

Results on behavior assessments (body weight, sucrose preference ratio, numbers of crossing and rearing, time spent in the dark zone), alpha diversity index value, the relative abundance of fecal microbiota and the levels of brain metabolites were compared among groups using ANOVA followed by the LSD test using Statistical Package for Social Science program (SPSS 22.0, Chicago, USA). Significance was accepted at the level of $p < 0.05$, $p < 0.01$ or $p < 0.001$. Moreover, Linear discriminant analysis effect size (LEfSe) analysis was used to identify the differential KEGG pathway and KEGG Orthology (KO) representation between fecal microbiomes of the two groups, and the LDA threshold value greater than 2 was used as the cutoff value for statistical significance based on a *p*-value of 0.05.

Results

TCM reversed CUMS-induced depressive and anxiety-like behaviors

As illustrated in Table 1, there were no statistically significant differences in sucrose preference ratio among the rats during the adaption period. After 9 weeks, one-way

ANOVA revealed that SPT and anxiety-like behavior tests (e.g., sucrose preference ratio, open field rearing and crossing numbers, and total time spent in the dark zone) of the CUMS-induced rats were significantly different compared with those in the HC group (Table 1). The data suggested that the CUMS-induced depressive model had been successfully established. After 6 weeks of TCM treatment, one-way ANOVA suggested that TCM reversed the behaviors of depression and anxiety, including recovering sucrose preference ratio, improving the activity rates, and significantly decreasing the time spent in the dark zone of the CUMS-induced rats (Table 2). Furthermore, as illustrated in Figure S1, CUMS led to the decrease in body weight. But after 6 weeks of TCM treatment, the body weights bounced back. These data suggested that TCM could relieve CUMS-induced behavioral changes.

TCM treatment modulated fecal microbiota of CUMS rats

The consistency of the 16S rRNA gene sequencing data of the intestinal microbiota in the experimental and control rats was illustrated by Sobs index and Shannon index. (Figure S2a and Figure S2b). Analysis of alpha diversity based on the OTU level revealed that there were no significant differences among experimental and control groups (Table S3).

As presented in Figure S3, hierarchical clustering tree analysis based on OTU level revealed that the fecal microbiota of the SEN group (rats are sensitive to CUMS) strikingly deviated from the RES group (rats are resistant to CUMS) in Week 9. PCoA

analysis revealed that the fecal microbiota of the CUMS rats significantly deviated from the HC group in Week 9 (Figure S4a). Figure S4c depicted a clear separation between the TCM (Week 15) and CUMS (Week 9) mice, however, the profiles of fecal microbiota of the HC (Week 9) rat was similar to those of the HC (Week 15) group (Figure S4b). Moreover, after 6 weeks of TCM treatment, the fecal bacterial profiles of the TCM group deviated from those of the CUMS group, and the microbial communities of TCM group were closely related to those of the HC group (Figure S4d).

Figure S5 illustrated the detailed overview of the intestinal bacterial composition of each group at the phylum and family levels. *Firmicutes* and *Bacteroidetes* were the most abundant phyla in all samples, accounting for 95% (in the HC and CUMS groups) and 96% (in the TCM group) of the total bacterial sequences (Figure S5a). At the family level, *Bacteroidales_S24-7_group*, *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Bacteroidaceae*, *Coriobacteriaceae*, *Erysipelotrichaceae*, *Prevotellaceae*, and other families dominated in all three groups of rats (Figure S5b-d). These results indicated that the predominant taxa were similar in the three experimental groups.

Based on results of the behavior tests, the corresponding samples of representative rats from each group were further assessed. As illustrated in Table S4, at the phylum level, the relative abundance of *Firmicutes* ($p < 0.05$) was significantly increased (Fig. 1a), whereas that of *Bacteroidetes* ($p < 0.01$) was significantly decreased in the CUMS

group compared with those in the HC group (Fig. 1b). Relative to the CUMS group, the levels of *Firmicutes* and *Bacteroidetes* were reversed in the TCM group; however, there were no significant difference comparing with CUMS group (Fig. 1a and 1b). At the genus level, the CUMS group exhibited significant increases in the abundance of *Lactobacillus* ($p < 0.05$), *Ruminococcus_2* ($p < 0.05$), and *Clostridium sensu_stricto_1* ($p < 0.01$) compared with those in the HC group (Fig. 1c-e). In contrast, *Enterorhabdus* ($p < 0.05$), *Roseburia* ($p < 0.01$), and *Lachnospiraceae_UCG-001* ($p < 0.05$) were significantly less abundant in the CUMS group than in the HC group (Fig. 1f-h). Furthermore, TCM treatment dramatically reversed the changes of *Ruminococcus_2* and *Roseburia*.

Influence of TCM on the function of the fecal microbiome

As illustrated in Fig. 2a and Fig. 2c, 12 KEGG pathways and 20 KOs were characterized in differentiating the fecal microbiomes of CUMS and HC rats, whereas 4 KEGG pathways and 7 KOs discriminated the fecal microbiomes of CUMS and TCM rats (Fig. 2b and Fig. 2d). KEGG pathway analysis showed that components of the bacterial secretion system, nucleotide excision repair, microRNAs in cancer, sulfur relay system, NOD-like receptor signaling pathway, propionate metabolism, biosynthesis of ansamycins, and nitrotoluene degradation were enriched in the HC group, whereas components related to protein export, cationic antimicrobial peptide resistance, streptomycin biosynthesis, and acarbose and validamycin biosynthesis were enriched in

the CUMS group. After TCM treatment, pathways of lysine biosynthesis, protein export, microRNAs in cancer, and bacterial secretion system were enriched in the TCM group. These were in agreement with the enriched functions in the HC group.

The description of KOs and KEGG pathways for all these KOs were presented in Table S5. In analysis of KO categories, the changes were relatively diverse; therefore, the changes were mapped to KEGG pathways. As an outcome, 24 differentially expressed KOs were primarily associated with amino acid metabolism, nucleotide metabolism, bile acid biosynthesis, glycan degradation, bacterial secretion system, quorum sensing, ATP-binding cassette transporters, and antimicrobial biosynthesis. The abundant KO genes in the CUMS mice were involved in ko00120 (primary bile acid biosynthesis), ko00121 (secondary bile acid biosynthesis), ko00240 (pyrimidine metabolism), ko00250 (alanine, aspartate; and glutamate metabolism), and ko00511 (other glycan degradation) increased, while KO gene copies involved in ko03070 (bacterial secretion system), ko00270 (cysteine and methionine metabolism), ko05206 (microRNAs in cancer), and ko03430 (mismatch repair) increased after TCM intervention for 6 weeks. Overall, these data suggested that TCM may act as prebiotics by modulating the intestinal microbiota to affect certain metabolic pathways, thereby impacting host health.

Discussion

CUMS rats are recognized animal model to study depression.[37-39] In this study, TCM improved the mood of CUMS rats, including increased sucrose preference ratio,

improved activity and exploration, and decreased the time spent in the dark zone, which was consistent with the outcomes of sesamol, resveratrol and Chaihu-Shugan-San treatments from published studies.[1, 40, 41]

To investigate the potential correlation between gut microbiota and brain metabolites, we have also assessed the impact of TCM on brain metabolites**.** As illustrated in Fig. 3, TCM treatment significantly increased the production of brain 5-HT, 5-HIAA, GABA, and Acetylcholine. Significant differences were identified among the three groups of rats by One-way ANOVA assessment. The standard curves of each external standard were presented in Figure S6. Furthermore, brain metabolites were altered in the TCM treatment group to a level close to that in the HC group.

As illustrated in Fig. 4, ($|r| > 0.5$, $p<0.05$),[37] strong correlations were found between brain metabolites and specific gut microbiota genera. For example, 5-HIAA showed a strong positive correlation with the genera *Lachnospiraceae_UCG-006*, *norank_o_Mollicutes_RF9*, and *Ruminococcaceae_UCG-004*, while the genera *Lactobacillus*, *Blautia*, and *Ruminococcus_2* were negatively correlated with 5-HIAA. Meanwhile, *Lachnospiraceae_UCG-006* and *[Eubacterium]_coprostanoligenes_group* were positively correlated with GABA. Moreover, *Blautia* was negatively correlated with acetylcholine, while *Lachnospiraceae_UCG-006* was positively correlated with acetylcholine. However, the above data were based on assessment of brain samples in comparing to an external standard curve. In future studies we will further consider to use

an internal standard to quantify the brain substances for comparison and potentially improve data quality.

In a previous study, Liu and his colleagues suggested that ferulic acid, an active constituent of component Ⅰ, has profound effect on suppressing CUMS-induced inflammation.[35] Moreover, polyphenols are also implicated in preventing chronic diseases, such as neuropsychiatric diseases, because of their antimicrobial and anti-inflammatory properties.[42, 43] Furthermore, in recent years, growing evidence has demonstrated that gut microbiota plays a critical role in the development or progression of depression.[13-15, 44, 45] Therefore, we hypothesized that the antidepressant effect of TCM was closely associated with gut microbiota and intestinal microbial functions. Our data on alpha diversity revealed that there were no significant differences in species abundance and diversity of the fecal microbiota among the three groups, while the CUMS group showed a decreasing tendency in alpha diversity. Consistently, previous studies have also demonstrated that there were no significant differences between depressed and normal individuals in alpha diversity.[14, 46] However, some studies have also reported that the microbial diversity in the disease group is lower than that in healthy controls.[8, 13, 47, 48] Although greater bacterial diversity is potentially beneficial to human health, its role in the function of the central nervous system remains unclear. Our PCoA results demonstrated that the microbial communities in the CUMS group had a distinct composition compared with those in the HC rats, despite of their access to similar food

and water, supporting the potential impact of chronic stressors on the profile of gut microbiota. However, the gut microbiota profiles of the TCM rats exhibited a separation from those of CUMS rats but similarity to the profiles of the HC group. The data suggested that exposure of CUMS rats to TCM modulated the profiles of the intestinal microbial population. These findings were in agreement with a recent human study, revealing significant microbiome alterations in patients with depression in response to treatment with ginseng decoction.[31] Our results were also consistent with a previous study demonstrating that exposure of high fat diet-fed animals to resveratrol and quercetin resulted in significant alterations in the composition of the intestinal flora.[8] These results indicated that the antidepressant effects of TCM may be at least partially due to its effects on modulating microbiota dysbiosis.

Furthermore, we also found that the relative abundances of the genera *Lactobacillus*, *Ruminococcus_2*, and *Clostridium sensu_stricto_1* were significantly increased in the fecal microbiota of CUMS rats with respect to those in the HC rats, whereas the relative abundances of the genera *Enterorhabdus*, *Roseburia*, and *Lachnospiraceae_UCG-001* were significantly reduced in the CUMS rats. However, only the levels of the genera *Ruminococcus_2* and *Roseburia* were reversed by the TCM.

Lactobacilli belong to lactic acid bacteria and colonize several sites of the body, including the skin, vagina, and entire gastrointestinal tract, starting with the oral cavity. Surprisingly, *Lactobacilli* have been indicated in over 200 cases of

Lactobacillus-associated infections.[49] Within the genus of *Lactobacillus*, there are a broad spectrum of species with diversified physiological properties. However, certain species have clear predominance in the intestine.[50, 51] Thus, while most *Lactobacillus* strains have beneficial applications, some may have different roles in host neurological functions. In fact data from a recent clinical study demonstrated that *Lactobacillus sp.* were enriched in the fecal microbiota of autistic individuals,[14] and another study discovered a significant increase in the absolute amount of *Lactobacillus* in the dysbacteriosis gut of children with autism,[52] in accordance with our findings. Members of *Ruminococcus*, within the family *Ruminococcaceae*, have been reported to be enriched in diseased individuals suffering from multiple sclerosis [53] and arthritis.[54] Consistent with findings from this CUMS rat study, an increased level of *Ruminococcus* has also been reported in subjects with psychiatric disease.[55, 56] *Rosebuia*, being part of the *Lachnospiraceae* family, is a butyrate-producing bacterium characterized by alleviation of metabolic diseases, such as obesity and diabetes.[31, 57] In agreement with our results, previous studies have shown that the levels of *Roseburia* were consistently higher in healthy controls than that in patients with depression.[23, 58]. Although the precise physiological implications of *Enterorhabdus* in depression are unknown, it is likely that changes in the levels of these bacteria induced by CUMS partially contributed to the depressant behavior in rats. Recent studies have also shown that the levels of *Clostridium spp*. were significantly evaluated in the gut of patients with mental diseases compared with those in healthy controls.[19, 37, 59-61] However, a clinical study on patients with major

depressive disorder demonstrated that *Clostridium_sensu_stricto* was abundant in healthy controls and was the key phylotype differentiated patients and healthy controls, inconsistent with our results.[62] In agreement with our data, *Lachnospiraceae_UCG-001* in the *Lachnospiraceae* family was reported with decreased abundance in patients with psychiatric comorbidities by others [46, 56]. Taken together, data from our study, supported by evidences from the literatures, suggested a correlation between alterations in the gut microbiota and depression-like symptoms, and the effectiveness of the TCM treatment in reversing some of the microbial changes, along with the improvement in behaviors.

The metagenomic data also suggested systemic metabolic alterations by the TCM treatment. In total, 12 pathways and 20 KOs were discriminated between HC and CUMS rats, while 4 pathways and 7KOs in CUMS rats were differed from TCM rats. While the metabolic functions of the microbiota associated with depression remain largely unknown, a preclinical study by Zheng et al reported that humanized depressed mice were characterized by disturbances in microbial functions involved in amino acid metabolism.[63] Our data also illustrated an elevation in the gut microbial function of alanine, aspartate, and glutamate metabolism and lysine biosynthesis in CUMS rats. In addition, we found that stress-induced rats were characterized by significant enrichment of genes involved in bile acid biosynthesis, whereas the TCM-treated rats were characterized by elevations in cysteine and methionine metabolism. In agreement with these findings, alterations in gut microbial bile acids were previously reported in

depressed patients and animal models of depression.[37, 64, 65] Moreover, cysteine is a precursor of glutathione, and a review supported our finding by showing that glutathione allows bacteria to maintain homeostasis under oxidative stress.[66] According to Spearman's correlation analysis, the brain metabolites exhibited correlations with certain bacterial genera of fecal microbiota. Despite there is no previous report on the direct association between specific gut bacteria and the production of brain metabolites, several studies did show that the short-chain fatty acid (SCFA)-producing bacteria *Lachnospiraceae* and *Ruminococcaceae* were highly abundant in healthy controls.[8, 15, 67] Additionally, valproic aicd, an SCFA, can increase the production of GABA.[68, 69] Therefore, we speculated that *Lachnospiraceae* and *Ruminococcaceae* may increase the content of brain metabolites by producing SCFAs.

It is important to recognize that gut microbiota functional analysis is based on shotgun sequencing data of very short DNA fragments from the fecal microbiota DNA extracts, and further uses mathematic calculations to predict the metabolic pathways. Therefore the data should only be used as indicative reference, instead of solid evidences. The availability of an enriched database of whole genome sequences of related gut bacteria and full annotation of their metabolic pathways and related genes will enable proper interpretation of the gut microbiota functions. Based on results from this study indicating the potential involvement of cysteine and methionine, we plan to adopt non-targeted metabolomics to identify and quantify more related metabolomics in fecal

samples, including cysteine and methionine, in follow-up studies.

Overall, our findings were mostly in accordance with previous reports demonstrating that gut microbiota dysbiosis is induced by CUMS and that the TCM could alleviate the symptoms of depression, likely attributed to intestinal microbiota modulation. Further studies are needed to fully elucidate the mechanisms of theTCM therapy in alleviating depression and other neuropsychiatric diseases.

Conclusions

In this study, we have illustrated the correlation between gut microbiota dysbiosis and depressive and anxiety-like symptoms using the CUMS rat model. Data from the study supported the notion that gut microbiota has critical roles in host brain health, and the antidepressant effect of the TCM potentially is delivered through the gut microbiota-brain axis. Particularly, the TCM treatment led to altered abundance of *Ruminococcus* and *Roseburia*, and potentially elevated cysteine and methionine metabolism after CUMS, which provided an interpretation of the antidepressant effect of the TCM. Data from our study further illustrated the rationale behind the ancient Chinese medicine: the cause of depression, and therefore the target for therapy may not be limited to the brain but the whole body. In this case, gut microbiota dysbiosis may have major impact on brain health.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially supported by the Fundamental Research Funds for the Central Universities, SCUT (D2170320), the Construction of the First Class Universities (Subject) and Special Development Guidance Special Fund (K5174960), and the Innovation Project of Infinitus (China) Research & Development Center Co., Ltd (D9163940).

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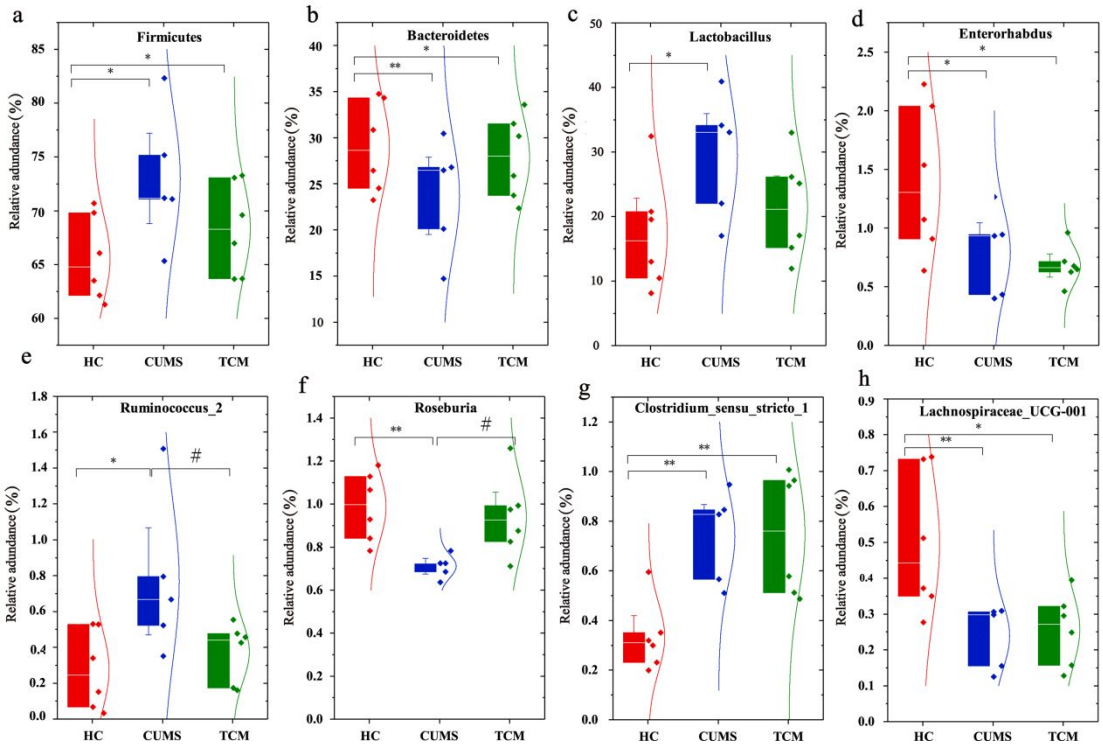

Figure 1. TCM modulated the composition of gut microbiota at the phylum and genus levels. (HC: n=6; CUMS: n=5; TCM: n=6; representative) **(a)** The levels of *Firmicutes*. **(b)** The relative abundance of *Bacteroidetes*. **(c)** The genus levels of *Lactobacillus*. **(d)** The genus levels of *Enterorhabdus*. **(e)** The genus levels of *Ruminococcus_2*. **(f)** The genus levels of *Roseburia*. **(g)** The genus levels of *Clostridium sensu_stricto_1*. **(h)** The genus levels of *Lachnospiraceae_UCG-001*. Data are expressed as mean ± SEM. Significant different with HC are indicated: $^{*}p < 0.05$; $^{**}p < 0.01$. Significant different with CUMS are indicated: $^{\#}p < 0.05$.

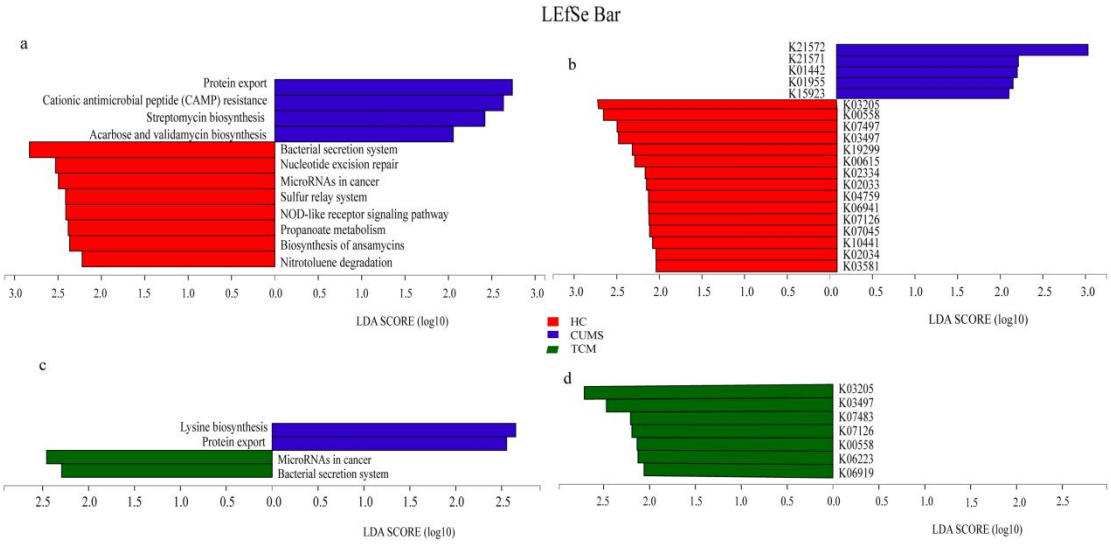

Figure 2. LEfSe analysis on the KEGG pathways and KOs categories (n=3 per group, representative). **(a)** Key KEGG pathways for discriminating the fecal microbiomes of the CUMS and HC group. **(b)** KOs categories for discriminating the fecal microbiomes of the CUMS and HC group. **(c)** Key KEGG pathways for discriminating the fecal microbiomes of the CUMS and TCM rats. **(d)** KOs categories for discriminating the fecal microbiomes of the CUMS and TCM rats. The LDA threshold value greater than 2 was used as the cutoff value for statistical significance based on a *p*-value < 0.05.

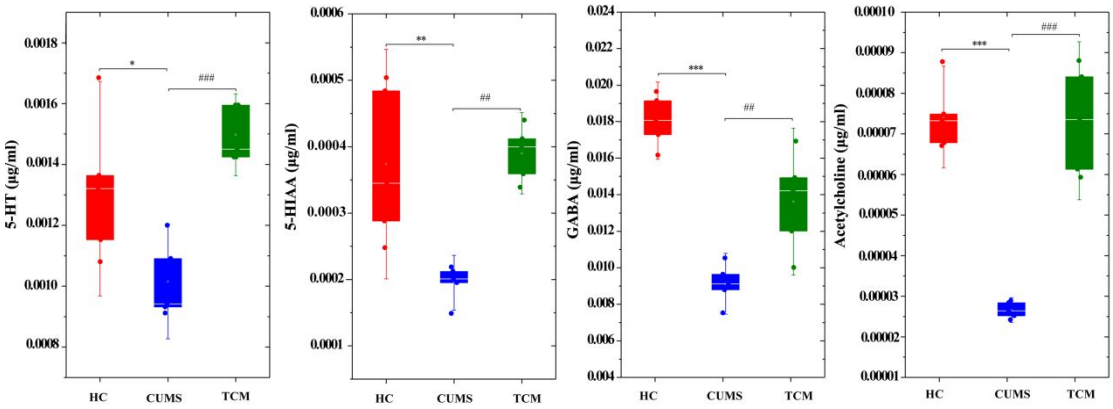

Figure 3. Brain neurotransmitters level in three experiment groups (n=5 per group, representative). (**a**) 5-HT; (**b**) 5-HIAA; (**c**) GABA; (**d**) Acetylcholine. Data are expressed as mean ± SEM. Significant different with HC are indicated: $^{*}p< 0.01$; $^{**}p< 0.01$; $^{***}p < 0.001$. Significant different with CUMS are indicated: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$.

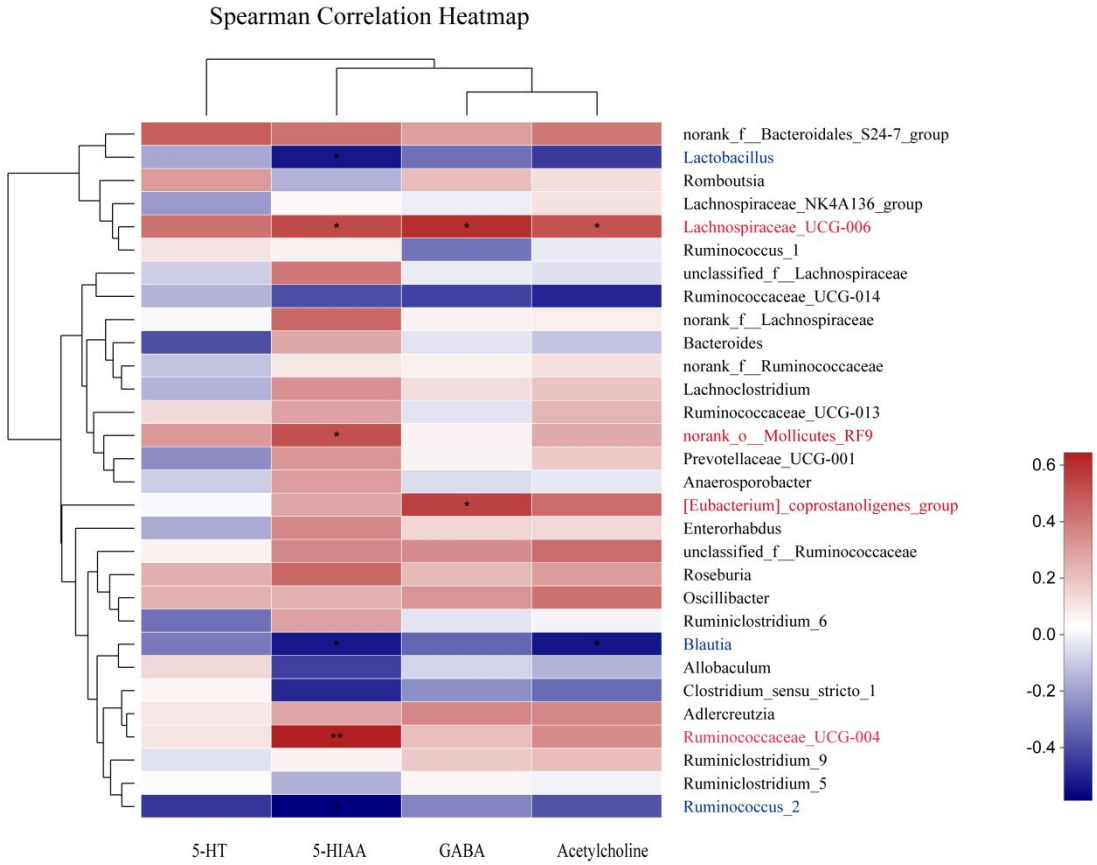

Figure 4. Spearman's correlation matrix between gut microbiota and altered brain neurotransmitters. Red indicates altered brain neurotransmitters were positively correlated with perturbed gut microbiota. Blue indicates altered brain neurotransmitters were negatively correlated with perturbed gut microbiota. $|r| \geq 0.5$, $^{*}p < 0.05$, $^{**}p < 0.01$.