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ORIGINAL ARTICLE

Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism

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Major depressive disorder (MDD) is the result of complex gene–environment interactions. According to the World Health Organization, MDD is the leading cause of disability worldwide, and it is a major contributor to the overall global burden of disease. However, the definitive environmental mechanisms underlying the pathophysiology of MDD remain elusive. The gut microbiome is an increasingly recognized environmental factor that can shape the brain through the microbiota-gut-brain axis. We show here that the absence of gut microbiota in germ-free (GF) mice resulted in decreased immobility time in the forced swimming test relative to conventionally raised healthy control mice. Moreover, from clinical sampling, the gut microbiotic compositions of MDD patients and healthy controls were significantly different with MDD patients characterized by significant changes in the relative abundance of Firmicutes, Actinobacteria and Bacteroidetes. Fecal microbiota transplantation of GF mice with 'depression microbiota' derived from MDD patients resulted in depression-like behaviors compared with colonization with 'healthy microbiota' derived from healthy control individuals. Mice harboring 'depression microbiota' primarily exhibited disturbances of microbial genes and host metabolites involved in carbohydrate and amino acid metabolism. This study demonstrates that dysbiosis of the gut microbiome may have a causal role in the development of depressive-like behaviors, in a pathway that is mediated through the host's metabolism.

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INTRODUCTION

Major depressive disorder (MDD) is a debilitating mental disorder affecting up to 15% of general population and accounting for 12.3% of the global burden of disease.[1] MDD increases health-care expenditures and suicide rates.[2] In recent decades, several theories have attempted to explain the pathogenesis of MDD, including neurotransmission deficiency,[3] neurotrophic alterations,[4] endocrine-immune system dysfunction[5] and neuroanatomical abnormalities.[6] As none of these theories has been universally accepted, a definitive pathogenesis of MDD remains largely elusive, and there is a pressing need to identify novel pathophysiologic mechanisms underlying the disorder.

Previously, we found that MDD was associated with obvious disturbances in peripheral and central metabolites.[7–10] Interestingly, several altered metabolites in MDD subjects (such as hippurate, dimethylamine and dimethylglycine) are metabolic byproducts of gut microbiota.[9] Moreover, previous clinical studies have reported disturbances in the gut microbiotic compositions in limited samples of heterogeneous depressed subjects with similar findings observed in animal models of depression.[11–13] These preliminary studies highlight the potential association of gut microbiotic changes and the development of MDD. However, further investigation in larger, well-characterized MDD populations is still required, as these previous studies have not addressed whether disturbances in gut microbiota have a causative role in the onset of MDD.

In recent years, mounting evidence has demonstrated that gut microbiota can greatly influence all aspects of physiology.[14] Variations in the composition of gut microbiota have been reported to have crucial roles in the pathogenesis of several enteric and metabolic diseases, such as irritable bowel syndrome, diabetes and obesity.[15–19] Emerging evidence also suggests that gut microbiota can influence brain function and behavior through the 'microbiota-gut-brain axis'.[20–24] Germ-free (GF) mice, which are devoid of any bacterial contamination, have been widely used to investigate such phenomena.[24] Recent studies have demonstrated that GF mice display reduced non-spatial memory, social motivation and anxiety compared with their conventionally raised specific pathogen-free (SPF) counterparts.[21,23,25,26] Another recent study has described that targeted modifications in gut microbiota can correct autism spectrum disorder-related behavioral abnormalities.[27] On the basis of these findings, we hypothesized that the dysbiosis of gut microbiota may be a contributory factor to the development of depression.

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In this study, we initially assessed how gut microbiota physiologically influence the psychobehavioral characteristics of GF and SPF mice. Then, using 16S rRNA gene sequencing, the gut microbial communities of 58 MDD patients and 63 healthy controls were compared to evaluate whether alterations in the gut microbiome are associated with MDD status. Furthermore, to assess whether alterations of gut microbiota have a causal role in depression-like behavior, gut microbiome remodeling was accomplished through fecal microbiota transplantation (FMT) from either MDD patients or healthy individuals on GF mice followed by behavioral testing to assess depression-like behaviors. Finally, metagenomic and metabolomic analyses of samples from the mice harboring 'depression microbiota' were conducted to examine how the gut microbiome influences host metabolism.

MATERIALS AND METHODS

Behavioral testing

The protocols of animal experimentation were reviewed and approved by the Ethical Committee of Chongqing Medical University (ECCMU, Chongqing, China) and the Third Military Medical University (Chongqing, China). Male GF Kunming mice and SPF Kunming mice were bred in the Experimental Animal Research Center at the Third Military Medical University. GF mice were kept in flexible film gnotobiotic isolators until the beginning of experiments. All animals were fed the same autoclaved chow and water *ad libitum* under a 12-h light-dark cycle (lights on at 0730) and a constant temperature of 21–22 °C and humidity of 55 ± 5%. For each test, the mice were transferred to the experimental room for acclimation at least 1 h prior to behavioral testing. All tests described below were carried out by observers blind to the animal genotypes between 0800 and 1700. All behavioral tests were videotaped and quantified by a video-computerized tracking system (SMART, Panlab, Barcelona, Spain).[28]

Open-field test (OFT): All mice were individually tested in an open-field apparatus[29] consisting of a black square base (45 × 45 cm^2) with black walls (45 cm in height). A single mouse was gently placed in the corner of the chamber, and after 1 min of adaptation, all spontaneous activities were recorded for 5 min using the video-computerized tracking system. The total motion distance was used as an index of locomotor activity, while the proportion of distance spent in the center (inner 25% of the surface area) was construed as an index of anxiety-like behavior.

Y-maze: The Y-maze apparatus[30] consisted of three dark gray arms (45 cm in length × 10 cm in width × 29 cm in height). Each mouse was placed at the end of one arm and allowed to freely explore the maze for 8 min. The sequence and total number of arms entered was recorded. Entry into an arm was considered valid only when all four paws of the mouse were inside that arm. The percentage of alternation was the number of triads containing entries into all three arms divided by the maximum possible number of alternations (the total number of arms entered minus 2) × 100%.

Tail suspension test (TST): Mice were individually suspended by their tails[31] using adhesive tape (distance from tip of tail was 2 cm). Test sessions lasted for 6 min with the last 5 min scored for immobility. Mice that climbed on their tails were removed from further testing. Animals were considered to be immobile when they exhibited no body movement and hung passively.

Forced swimming test (FST): The mice were placed individually in a Plexiglas cylinder (30 cm in height × 15 cm in diameter) filled with 15 cm water (24 ± 1 °C).[32] Immobility was defined as the absence of all motion with the exception of movements required to keep the mouse's head above water. Test sessions lasted for 6 min with the last 5 min scored for immobility.

Subject recruitment and sample collection

The protocols of clinical experimentation were reviewed and approved by ECCMU. Written informed consent was obtained from all recruited human subjects. Recruitment of MDD and healthy subjects was performed as previously described.[7,9] Briefly, MDD diagnoses were carried out according to the Structured Psychiatric Interview using DSM-IV-TR criteria,[33] and the 17-item Hamilton Depression Rating Scale was used to quantify the severity of MDD.[34] MDD candidates were excluded on the basis of substance abuse in addition to pregnancy, nursing or current menstruation for female subjects. Healthy controls were excluded on the basis of a history of systemic medical illness or mental disorders or family history of any psychiatric disorder. A total of 58 MDD patients and 63 demographically matched healthy controls were recruited from the psychiatric center and medical examination center of the First Affiliated Hospital at Chongqing Medical University, respectively. The majority of MDD subjects ($n = 39$) were drug-naive, while the remaining MDD subjects ($n = 19$) were being treated with various anti-depressants. All MDD subjects and healthy controls who were using antibiotics or prebiotics were excluded. The detailed characteristics of these recruited subjects are shown in Supplementary Table S1.

Fecal sample collection and 16S rRNA gene sequencing

Fecal samples were collected from the recruited subjects or FMT model, frozen immediately following collection and stored at −80 °C prior to analyses. Fecal samples were pulverized with a mortar and pestle in liquid nitrogen, and bacterial genomic DNA was extracted by the standard Power Soil Kit protocol. Briefly, the fecal samples were thawed on ice. The MoBio lysis buffer was added to these fecal samples, which were further vortex mixed. Fecal suspensions were centrifuged and the supernatant placed into the MoBio Garnet bead tubes containing MoBio buffer.

Roche 454 sequencing (454 Life Sciences Roche, Branford, PA, USA): The V3-V5 regions of the 16S rRNA gene extracted from the fecal samples of recruited subjects and cecum samples from recipient mice at 2 weeks post FMT were PCR-amplified with barcoded universal primers containing linker sequences for 454-pyrose-quencing.[35]

Illumina MiSeq sequencing (San Diego, CA, USA): The V4-V5 regions of the 16S rRNA gene extracted cecum samples from recipient mice at 1 week post FMT were PCR-amplified with primers containing linker sequences for Illumina MiSeq sequencing.[36]

16S rRNA gene sequencing analysis

Raw sequences obtained from 454 sequencing were quality-filtered using Mothur (Version 1.31.2, http://www.mothur.org/) to obtain unique reads.[37] Sequences of less than 200 bp and greater than 1000 bp as well as sequences containing any primer mismatches, barcode mismatches, ambiguous bases and homopolymer runs exceeding six bases were excluded. Raw sequences obtained from MiSeq sequencing were quality-filtered using QIIME (version 1.17) with the following criteria: (i) 300-bp reads were truncated at any site receiving an average quality score of less than 20 over a 50-bp sliding window and discarding the truncated reads that were shorter than 50 bp; (ii) exact barcode matching, two-nucleotide mismatch in primer matching and reads containing ambiguous characters were removed; and (iii) only sequences that overlapped longer than 10 bp were assembled according to their overlap sequence.

All remaining sequences were assigned to operational taxonomic units (OTUs) with a 97% threshold of pairwise identity and then classified taxonomically using the RDP reference database (http://www.mothur.org/wiki/RDP_reference_files).[38] These taxonomies were used to construct summaries of the taxonomic distributions of OTUs, which can then be applied to calculate the relative abundances of microbiota at different levels. Alpha diversity was calculated by four different parameters: (i) observed species; (ii) Shannon Index; (iii) phylogenetic diversity and (iv) Simpson.[39,40] Distance matrices (Beta diversity) between samples were generated on the basis of weighted (Bray-Curtis similarity) and non-weighted (unweighted UniFrac) algorithms and reported according to principal coordinate analysis (PCoA).[41] To perform the UniFrac analysis, representative sequences for each OTU were aligned using PyNAST, and a phylogenetic tree from this alignment was constructed with Fast Tree.[42] Random Forest algorithm was carried out to identify the key discriminatory OTUs,[43] which assigns an importance score to each OTU by estimating the increase in error caused by removing that OTU from the set of predictors.

FMT

Fecal samples from randomly chosen MDD patients ($n = 5$, male, age 27–61 years) and healthy controls ($n = 5$, male, age 29–62 years) were used to colonize the guts of GF mice. The procedures of preparing the fecal samples for microbiota transplantation were as described in a previous study.[16] Briefly, fecal samples were handled under anaerobic conditions. Each fecal sample (0.1 g) was suspended with 1.5 ml of reduced sterile phosphate-buffered saline, and pools were made from equal volumes of donor suspensions. Adult (6–8-week-old) male GF Kunming mice were colonized with pooled samples derived from either MDD patients or healthy controls. The 'depression microbiota' and the 'healthy microbiota'

recipient mice were separately bred in different gnotobiotic isolators to prevent normalization of gut microbiota. Within each individual gnotobiotic isolator, either 'depression microbiota' or 'healthy microbiota' recipient mice were bred in different cages (five mice per cage). The weights of the mice were measured at the beginning of FMT experimentation and immediately prior to killing of the mice. The behavioral tests (including OFT, FST and TST) were performed on weeks 1 and 2 after microbiota transplantation. Cecal samples were collected at the time the mice were killed and immediately snap-frozen in liquid N_2 and stored at − 80 °C.

Comparisons of metabolite profiles from the FMT model

On week 2 post FMT, the depressed and control mice were killed, and cecum, serum and hippocampus samples were obtained. These samples were subsequently extracted and analyzed by gas chromatography-mass spectrometry (Agilent 7890A/5975C (Agilent Technologies, Santa Clara, CA, USA)), liquid chromatography-mass spectrometry (Agilent 6538 UHD and Accurate-Mass Q-TOF/MS) and nuclear magnetic resonance (Bruker AVANCE II 600, Bruker Biospin, Rheinstetten, Germany), based metabolomics platforms.

Gas chromatography-mass spectrometry metabolite profiles were processed according to our previously published work.[44] The resulting three-dimensional matrix—including peak indices (RT-m/z pairs), sample names (observations) and normalized peak area percentages—were introduced into SIMCA-P 12.0 (Umetrics, Umeå, Sweden). Multivariate statistical methods, such as partial least squares discriminant analysis (PLS-DA), were used to identify differential metabolites between groups.[45] The differential metabolites were identified using a statistically significant threshold of variable influence on projection values obtained from the PLS-DA model and a two-tailed Student's *t*-test. Metabolites with variable influence on projection values of greater than 1.0 and *P*-values of less than 0.05 were deemed statistically significant.[46] The Human Metabolome Database was used to comprehensively analyze the differential metabolites in terms of *in vivo* metabolic activity.

Nuclear magnetic resonance-based metabolomics analysis of cecum samples: a 70-mg fecal sample was extracted with 700 µl of phosphate buffer solution (phosphate-buffered saline, 0.1 M, pH 7.4). Then, 400 µl samples of supernatant were mixed with 200 µl of phosphate-buffered saline. After centrifugation at 12 000 r.p.m. for 10 min, 500 µl samples of supernatant were transferred into 5-mm nuclear magnetic resonance tubes. The proton spectra were collected on a 600 Spectrometer operating at 599.925 MHz ^{1}H frequency. A standard NOESYPR1D pulse sequence was used (recycle delay-90o-t1-90o-tm-90o-acquire free induction decay). PLS-DA was used to visualize discrimination between healthy controls and MDD subjects.[45] The coefficient loading plots of the model were used to identify the spectral variables responsible for sample differentiation on the scores plot. A correlation coefficient ($|r|$) of greater than 0.500 was used as the cutoff value for statistical significance based on a *P*-value of 0.05.

Liquid chromatography-mass spectrometry-based metabolomics analysis of cecum samples: an 80-µl aliquot of serum sample was mixed with 240 µl of methanol. Then, 0.01 g of hippocampal tissue was extracted with 1000 µl of buffer (chloroform/methanol = 2:1). The extracted supernatants of serum and hippocampus were subjected to Agilent 6538 UHD and Accurate-Mass Q-TOF/MS. Spectra were collected in both positive and negative ESI mode. PLS-DA were used to identify differential metabolites in GF mice relative to SPF mice.[45] The differential metabolites were identified with variable influence on projection values of greater than 1.0 and *P*-values of less than 0.05.

Shotgun metagenomic analysis of cecum samples

Samples were sequenced by Illumina HiSeq2500. Raw datasets of PE read files were run through Trimmomatic (v0.32) to remove low-quality base pairs and sequence adapters using these parameters [SLIDINGWINDOW:4:15 MINLEN:36].[47] Trimmed reads were filtered using the Fastq quality filter program (Fastx toolkit v0.0.13.2) with the parameters [-q 10 -p 10].[48] MetaPhlAn v1.7.8 and Bowtie2 v2.2.1 were used for profiling the taxonomic clades in the high-quality metagenomic datasets.[49,50] The paired-end and singleton reads were assembled using an IDBA-UD v1.1.1 assembler.[51] The open reading frames of the assembled scaffold sequence were annotated using Prodigal v2.60 gene finder with the parameter [-p meta].[52] Bowtie2 v2.2.1 aligner was used to map the reads to the assembled scaffold. The method for estimating the abundance of each predicted open reading frame from a sample has been previously described.[53] KEGG Orthology was assigned through BLAST (BLASTP v2.2.29+) search against the KEGG GENES Database v58 and eggNOG v3.0 database with an E-value cutoff of 1e-5. Bitscore (greater than or equal to 60) and minimum alignment length (greater than or equal to 15 aa) were then used to filter the blast hits.[54] Linear discriminant analysis was used to identify the differential KEGG pathway and KEGG enzyme commission number (E.C.s) representation between fecal microbiomes of the humanized depressed and healthy control mice.[55]

RESULTS

Absence of gut microbiota produces decreased immobility time in the FST

We initially assessed how the absence of gut microbiota physiologically influences psychobehavioral characteristics by comparing GF mice with SPF mice. From the OFT, representative motion tracks for a GF mouse and a SPF mouse are presented in Figures 1a and b. We found no difference in total motion distance between GF and SPF mice (Figure 1c). In contrast, the proportion of central motion distance was significantly increased in GF mice relative to SPF mice (Figure 1d), suggesting reduced anxiety-like behavior in GF mice. In the Y-maze spontaneous alternation test, the alternation percentage was significantly decreased in GF mice relative to SPF mice, indicating that GF mice displayed better memory performance compared with SPF mice (Figure 1e). In the FST, immobility time is widely used as an index of depression-like behavior. Here, we found that GF mice displayed decreased depression-like behavior as evidenced by a significantly decreased immobility time (Figure 1f). Expanding on previous studies that have observed altered anxiety and memory states from changes in gut microbiotic composition,[56–58] we have for, we believe, the first time found that the absence of gut microbiota decreases immobility time in the FST, suggesting a potential link between the 'microbiota-gut-brain axis' and depression-like behavior.

MDD patients exhibit significant alterations in their gut microbiomes

Given clinical metabonomic observations of metabolic disturbances generated from changes in gut microbiota in addition to murine studies showing that the absence of gut microbiota influences depression-like behavior, we next sought to determine whether MDD patients displayed disturbances in their gut microbiomes. The stool samples, as well as demographic and clinical data, from 58 MDD patients and 63 demographically matched healthy controls were collected. The detailed characteristics of these recruited subjects are shown in Supplementary Table S1. There were no significant differences in their key demographic characteristics (Supplementary Table S2).

A culture-independent, 16S ribosomal RNA gene-sequence-based approach was used to compare the gut microbial communities of MDD patients and healthy controls. DNA was extracted from their fecal samples. V3-V5 variable region of bacterial 16S rRNA genes was PCR-amplified. Samples were multiplexed and pyrosequenced, followed by quality filtering and chimera checking. We obtained a total of 854 639 high-quality 16S rRNA gene sequences (7063 ± 2352 reads/fecal sample, Supplementary Table S3), which were subsequently clustered into OTUs at a 97% similarity level. The majority of these OTUs belonged to only two phyla (Firmicutes and Bacteroidetes; 83.1 ± 11.9%).

Initially, the within-sample (α) phylogenetic diversity analysis showed that there were no significant difference between the two groups (Supplementary Figures S1a–d; Supplementary Table S4). In addition, the unweighted UniFrac analysis—which focuses on the degree of microbial phylogenetic similarity (β-diversity)—was used to determine the degree by which the gut microbiota within MDD subjects differed from those within healthy controls. The three-dimensional plots of unweighted UniFrac analysis showed an obvious difference in the gut microbial community

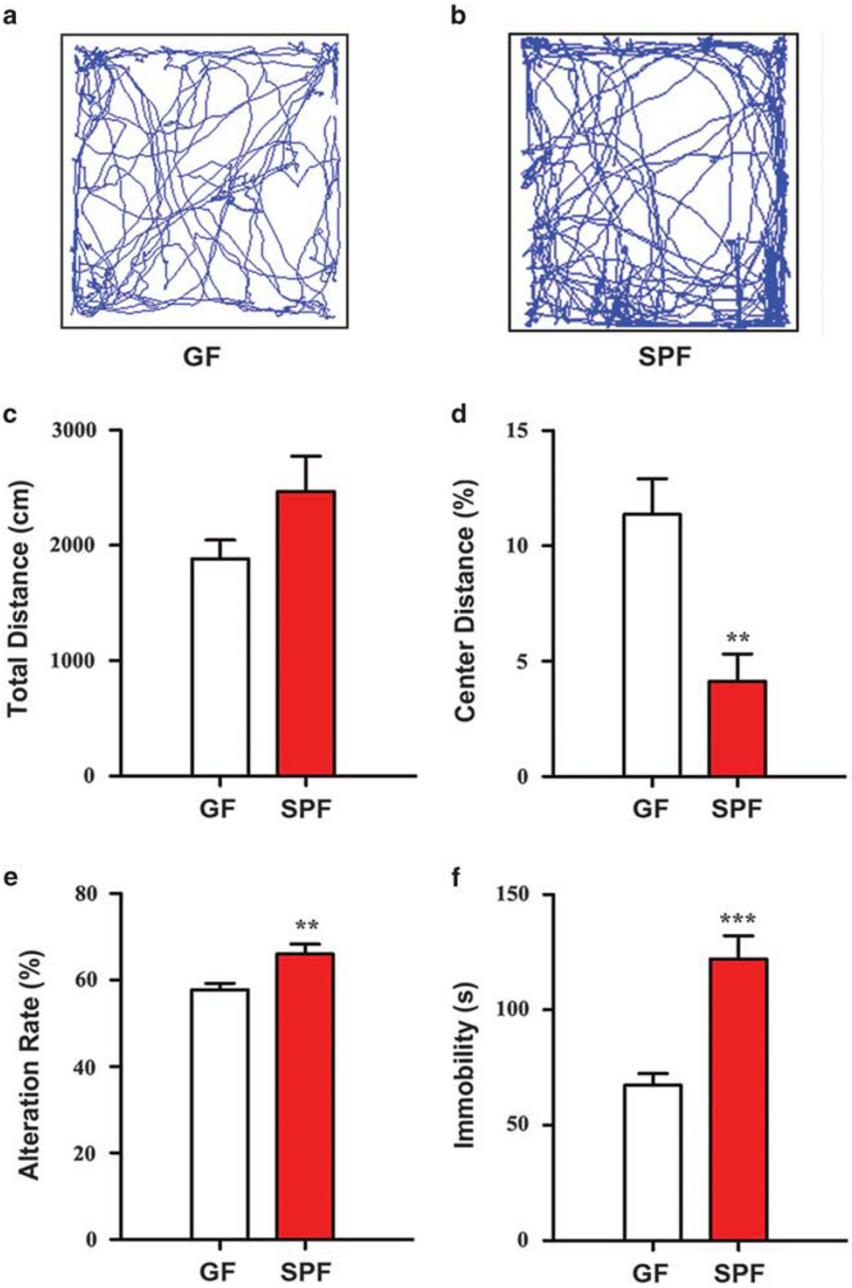

Figure 1. Effect of gut microbiota on mood-related behavior. (**a, b**) Representative motion tracks for a germ-free (GF) mouse and a specific pathogen-free (SPF) mouse. (**c**) Open-field test (OFT): the total motion distance was measured to assess locomotor activity. There was no difference in total motion distance between GF and SPF mice ($n = 22$/GF group; $n = 15$/SPF group). (**d**) OFT: the proportion of central motion distance was quantified to assess anxiety-like behavior. GF mice displayed an increased proportion of central motion distance relative to SPF mice ($n = 22$/GF group; $n = 15$/SPF group). (**e**) Y-maze test: alternation rate of GF mice was significantly decreased compared with SPF mice ($n = 17$/GF group; $n = 20$/SPF group). (**f**) Forced swimming test (FST): GF mice displayed decreased depression-like behavior as evidenced by a significant decreased immobility time ($n = 21$/GF group; $n = 15$/SPF group). Data presented as means ± standard errors of the mean. $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test.

compositions between MDD patients and healthy controls (Figure 2a). A similar discrimination between MDD and healthy control group was also observed using weighted UniFrac analysis (Supplementary Figure S2). These differences in the gut microbiomes were not significantly related to any key categorical variables (that is, sex, smoking status and antidepressant use, Supplementary Figures S3a–e) nor to any key continuous variables (that is, age and body mass index, Supplementary Figures S4a and b).

To identify the gut microbiota primarily responsible for discriminating MDD subjects from healthy controls, we applied a Random Forests classifier, which assigns an importance score to each OTU by estimating the increase in error caused by removing that OTU from the set of predictors. A total of 54 OTUs whose relative abundance reliably distinguished MDD and healthy control samples were identified (Figure 2b, Supplementary Table S5). Of these 54 differential OTUs, a total of 29 OTUs were overrepresented in MDD subjects and assigned to the families of

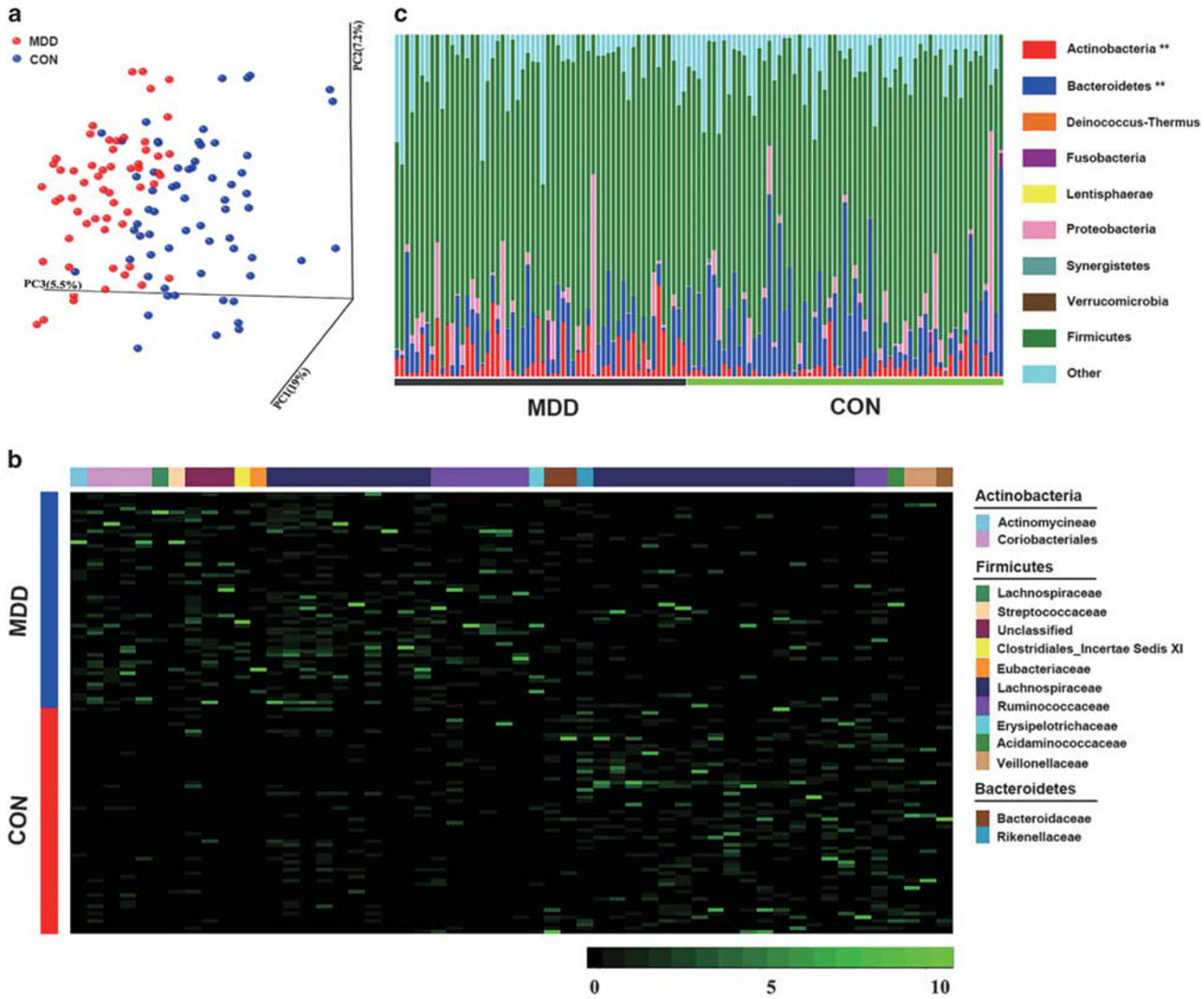

Figure 2. 16S rRNA gene sequencing reveals changes to microbial diversity in MDD. (**a**) Three-dimensional principal coordinate analysis (PCoA) of unweighted UniFrac distances showed an obvious difference in gut microbiotic composition between major depressive disorder (MDD) patients and healthy controls ($n = 58$/MDD, red plots; $n = 63$/HC, blue plots). The percentage of variation explained by principal coordinates is marked on the axes. (**b**) Heatmap of the 54 discriminative operational taxonomic units (OTUs) abundances between depressed subjects and healthy controls. The taxonomic assignment of each OTU is provided on the right. The increased OTUs in MDD subjects are arranged on the left, and the decreased OTUs are arranged on the right. As the changed directions of some discriminative OTUs sharing the same taxonomic assignment at the class level were not identical, there are some colors that are repeated across the same axis. (**c**) Relative abundances of the phyla present in samples from MDD patients (left, black bar) and healthy controls (right, green bar). The relative abundances of *Actinobacteria* and *Bacteroidetes* were significantly changed in MDD patients as compared with healthy controls. $^{**}P < 0.01$ by *t*-test.

Actinomycineae, Coriobacterineae, Lactobacillaceae, Streptococcaceae, Clostridiales incertae sedis XI (Parvimonas), Eubacteriaceae, Lachnospiraceae (Anaerostipes, Blautia, Dorea, Lachnospiracea incertae sedis), Ruminococcaceae (Clostridium IV) and Erysipelotrichaceae incertae sedis, while a total of 25 OTUs were overrepresented in healthy control subjects and assigned to the families of Bacteroidaceae, Rikenellaceae (Alistipes), Lachnospiraceae (Coprococcus, Clostridium XlVa, Lachnospiracea incertae sedis, Roseburia and Faecalibacterium), Acidaminococcaceae (Phascolarctobacterium), Veillonellaceae (Megamonas) and Sutterellaceae. Those discriminative OTUs were mainly assigned to the phyla Firmicutes (45/56, 76.7%), Actinobacteria (5/56, 10.9%) and Bacteroidetes (3/56, 5.3%). Compared with healthy controls, the relative abundances of Actinobacteria were increased in MDD subjects, while those of Bacteroidetes were decreased (Figure 2c, Supplementary Figures S5a and b). Although there were no significant differences in the overall relative abundances of Firmicutes between MDD patients and healthy controls (Figure 2c, Supplementary Figure S5c), change of Firmicutes was still one of hallmark in MDD. This is because some members of the Firmicutes OTUs (24/56) were increased in MDD patients, while others were decreased (Supplementary Table S5). In sum, we observed that MDD was associated with a disturbance in the gut microbiome characterized by alterations in specific OTUs assigned to the phyla Firmicutes, Actinobacteria and Bacteroidetes.

Transplantation of MDD patient microbiota induces depression-like behaviors in GF recipient mice

To investigate whether changes in gut microbiome contribute to the pathogenesis of MDD, FMT experiments were performed.[59] This approach has been successfully used to determine the causative role of gut microbiota in the onset of obesity, colitis and

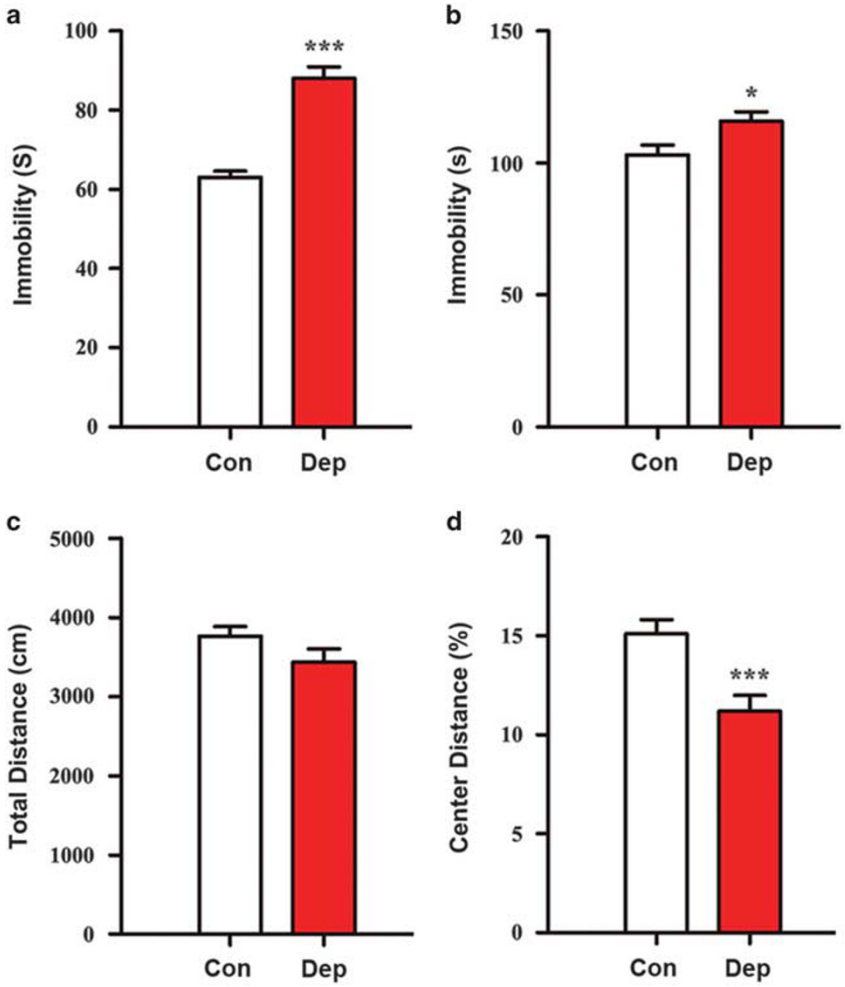

Figure 3. Comparative assessment of depression-like behavior in 'depression microbiota' and 'healthy microbiota' recipients. (**a**) Forced swimming test (FST), and (**b**) tail suspension test (TST): The 'depression microbiota' recipients displayed an increased duration of immobility in the FST and TST compared to 'healthy microbiota' recipients (FST: $n = 63$/depressive group; $n = 69$/control group; TST: $n = 55$/depressive group; $n = 63$/control group). (**c**) Open-field test (OFT): There was no difference in total motion distance between 'depression microbiota' and 'healthy microbiota' recipients ($n = 63$/depressive group; $n = 69$/control group). (**d**) OFT: Proportion of central motion distance was quantified to assess anxiety-like behavior. The 'depression microbiota' recipients displayed a decreased proportion of central motion distance relative to 'healthy microbiota' recipients. ($n = 63$/depressive group; $n = 69$/control group). Data are presented as means ± standard errors of the mean. $^{*}P < 0.05$, $^{***}P < 0.001$ by *t*-test.

type I diabetes.[15–19] Here, as previously reported, adult GF mice were colonized with pooled fecal samples randomly derived from five non-medicated MDD patients and five healthy controls without *a priori* knowledge of their gut microbial profiles. In the *post hoc* 16S rRNA gene sequence analysis of these samples, PCoA plot of unweighted and weighted UniFrac matrix showed that the gut microbial phenotypes of these randomly selected MDD and healthy control samples were similar to their corresponding groups (Supplementary Figure S6). This humanized FMT model allowed us to: (i) determine whether the depressive phenotypes of MDD patients were transmissible via their gut microbiomes and (ii) comparatively analyze the gut microbial community structure, metabolism and host-microbial co-metabolism of the 'depression microbiota' and 'healthy microbiota' recipient mice.

Initially, the weight of experimental mice was measured weekly. We found that the weight of 'depression microbiota' recipient mice was not significantly different than 'healthy control' recipient mice during FMT experimentation (Supplementary Figures S7a–c). To test whether colonization of GF mice with 'depression microbiota' results in depression-like behaviors, three well-established behavioral tests—OFT, FST and TST—were performed on weeks 1 and 2 post FMT. On week 1 post FMT, there were no significant differences in OFT, FST and TST between 'depression microbiota' and 'healthy microbiota' recipient mice (Supplementary Figures S8a–d). However, on week 2 post FMT, the 'depression microbiota' recipient mice displayed a decreased proportion of center motion distance in the OFT and an increased duration of immobility in the FST and TST as compared with those of 'healthy control' recipient mice (Figures 3a and b). However, there were no significant differences in total motion distance between the two groups in the OFT (Figure 3c). These findings show that colonization of GF mice with 'depression microbiota' resulted in increased depression-like behaviors as compared with colonization with 'healthy microbiota'. Given that anxiety is a common symptom among MDD patients,[60] anxiety-like behavior was also tested here. We found that 'depression microbiota' recipients displayed a decreased center motion distance in the OFT, which is indicative of anxiety-like behavior (Figure 3d). Therefore, in this humanized depressed model, diagnostic depressive behavior (as measured via the FST and TST) as well as non-diagnostic anxiety behavior (as measured via the OFT) associated with depression were transmissible via the gut microbiome.

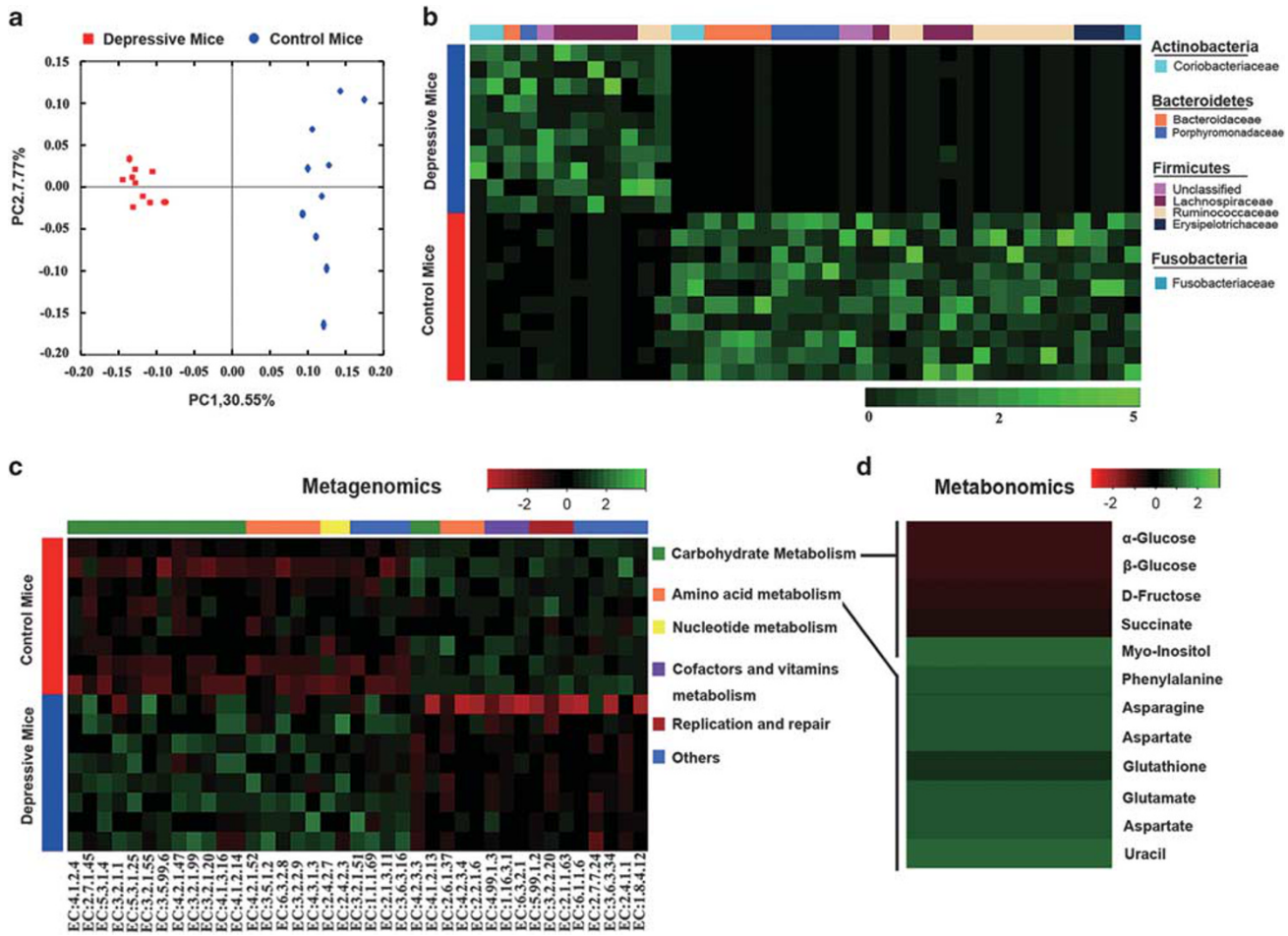

Figure 4. 16S rRNA sequencing, metagenomic and metabolomics analyses. (**a**) Two-dimensional principal coordinate analysis (PCoA) of unweighted UniFrac distances showed an obvious difference in the microbial community compositions between 'depression microbiota' recipient mice and 'healthy control' recipient mice ($n = 10$ in each group). (**b**) Relative abundance of operational taxonomic units (OTUs) responsible for discriminating depressed mice from control mice. The taxonomic assignment of each OTU is provided on the right. The increased OTUs in depressed mice are arranged on the right, and the decreased OTUs are arranged on the left. (**c**) Relative abundance of enzyme commission numbers (E.C.s) responsible for discriminating the fecal microbiomes of depressed and control recipient mice. The key metabolic pathways of these E.C.s are provided on the right. (**d**) Fecal and serum metabolites that are associated with carbohydrate metabolism and amino acid metabolism.

Characterizing gut microbiotic alterations in recipient mice

To determine whether differences in the gut microbiomes between MDD and healthy control individuals were maintained in the recipient mice, the microbial communities in the cecum stools harvested from depressed and control mice were subjected to 16S rRNA gene sequencing at 2 weeks post FMT. The PCoA plots of weighted and unweighted UniFrac analysis revealed that the key characteristic discriminative gut microbiota observed in human donors were also represented in recipient mice (Figure 4a and Supplementary Figure S9). Using a Random Forests classifier, 40 OTUs responsible for discriminating the gut microbiota in 'depression' and 'healthy control' recipient mice were identified (Figure 4b, Supplementary Table S6). These discriminative OTUs were mainly assigned to the phyla Firmicutes (22/39, 56.4%), Bacteroidetes (13/39, 33.3%) and Actinobacteria (4/39, 10.2%), which were similar to the differences observed in the gut microbiomes between MDD patients and healthy controls.

Moreover, disparities in behavioral performance between 'depression' and 'healthy control' recipient mice were observed at 1 and 2 weeks post FMT. To uncover the underlying molecular basis, the microbial communities of 'depression' and 'healthy control' recipient mice at 1 week post FMT were analyzed. Two-dimensional PCoA analysis of unweighted UniFrac distances showed that the gut microbiotic community composition of 'depression microbiota' recipient mice was different from that of 'healthy control' recipient mice (Supplementary Figure S10). In the PCoA analysis of the weighted UniFrac distances, contrasting results were obtained for the two time points, which paralleled the observed disparities in behavioral performance. The unweighted UniFrac analysis is relatively sensitive to rarer taxa, whereas the weighted UniFrac analysis is relatively sensitive to abundances of taxa. These findings suggest that the relatively high abundances of differential gut microbiota detected at 2 weeks post FMT may be responsible for the observed depression-like behaviors.

Metagenomic and metabonomic analyses reveal perturbed metabolic pathways in depressed mice

To characterize the functions encoded by the gut microbiota DNA, murine cecum stool samples were collected at the time of killing the mice (week 2 post FMT) and subjected to multiplex shotgun metagenomic analysis using the Illumina HiSeq2500 ($6.3 \times 10^7 \pm 7.6 \times 10^6$ sequences per sample; Supplementary Table

S7). These readings were annotated by comparison with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Linear discriminant analysis was used to compare the KEGG pathways and E.C.s representation in the fecal microbiomes of the humanized depressed and healthy control mice, which yielded 39 E.C.s and 7 KEGG pathways that were responsible for discriminating the fecal microbiomes of the two groups (Figure 4c and Supplementary Figure S11, Supplementary Table S8). To further validate these findings, unbiased metabolomic analysis of fecal, serum and hippocampal samples revealed that depressed mice were significantly different from control mice (Supplementary Figures S12a–h). The differential metabolites between depressed mice and healthy controls are presented in Supplementary Tables S9-11, and the non-differential metabolites are presented in Supplementary Table S12.

The 39 differential E.C.s are primarily associated with carbohydrate and amino acid metabolism. The abundance of gene copies associated with several carbohydrate metabolism E.C.s were increased in depressed mice relative to control mice (for example, pentose phosphate pathway (EC: 4.1.2.4, deoxyriboaldolase; EC:2.7.1.45, 2-dehydro-3-deoxygluconokinase), starch and sucrose metabolism (EC:3.2.1.1; alpha-amylase), fructose and mannose metabolism (EC:5.3.1.25; L-fucoseisomerase and amino sugar and nucleotide sugar metabolism (EC:3.2.1.55, alpha-N-arabinofuranosidase; EC:3.5.99.6, glucosamine-6-phosphate deaminase; EC:4.2.1.47, GDP-mannose 4,6-dehydratase)). Consistent with these findings, follow-up untargeted metabolomic analysis of fecal and serum samples showed that levels of several key carbohydrate metabolites (for example, α-glucose, β-glucose, fructose and succinate) were increased in depressed mice (Figure 4d, Supplementary Tables S9 and S10). In regard to E.C.s involved in amino acid metabolism, the changes were relatively diverse. We observed increased abundance of E.C.s gene copies involved in lysine biosynthesis (EC:4.2.1.52, dihydrodipicolinate synthase), histidine metabolism (EC: 4.3.1.3; histidinase), cysteine and methionine metabolism (EC: 3.2.2.9; S-adenosylhomocysteine nucleosidase), arginine and proline metabolism (EC:4.1.3.16, 4-hydroxy-2-oxoglutarate aldolase; EC:4.1.2.14, 2-dehydro-3-deoxy-phosphogluconate aldolase), and glutamate metabolism (EC:3.5.1.2, glutaminase) and observed downregulation of E.C.s gene copies involved in valine, leucine and isoleucine biosynthesis (EC:2.2.1.6; acetolactate synthase), phenylalanine, tyrosine and tryptophan biosynthesis (EC:4.2.3.4; 5-dehydroquinate synthase), and alanine metabolism (EC:6.3.2.1; pantothenate synthetase). Accordingly, some of these alterations were subsequently captured by untargeted metabolomic analysis of fecal and serum samples: the levels of glutamate, phenylalanine, phenylalanine biosynthesis-related metabolites (myo-inositol) and alanine-related metabolism (aspartate, asparagine, glutathione and uracil) were decreased in depressed mice relative to control mice (Figure 4d, Supplementary Tables S9 and S10). In addition, consistent with the key pathways identified by the differential E.C.s, KEGG pathway analysis showed that carbohydrate metabolism (that is, pentose and glucuronate interconversions as well as starch and sucrose metabolism) and glutamate metabolism were dysregulated in depressed mice (Supplementary Figure S10).

Given that the hippocampus is widely recognized as a key brain region involved in depression,[61] comparative hippocampal metabolic profiling of humanized depressed and control mice was also performed. In agreement with the fecal and serum findings, hippocampi from depressed mice were characterized by disturbances in carbohydrate metabolism (for example, glucose, lactose and malic acid) and amino acid metabolism (for example, phenylalanine, N-acetyl-L-aspartic acid, glycine and leucine) (Supplementary Table S11). In sum, these findings demonstrate that dysbiosis of the gut microbiome may have a causal role in the development of depression via modulating host metabolism.

DISCUSSION

In this study, we demonstrate that the absence of gut microbiota induces depression-like behavior in mice and that the composition of gut microbiota is significantly altered in MDD patients vis-a-vis healthy control individuals. More notably, this alteration was transmissible: colonization of GF mice with 'depression microbiota' resulted in increased depression-like behaviors as compared with colonization with 'healthy microbiota'. Mice harboring 'depression microbiota' mainly exhibited disturbances in microbial genes and host metabolites involved in carbohydrate and amino acid metabolism. These results highlight the role of gut microbiota as a potential causative factor in depression through their impact on host metabolism.

There have been numerous previous studies that focused on the effects of the 'microbiota-gut-brain axis'.[20,24,25] Here, we found that the absence of gut microbiota induces decreased immobility time in the FST. In line with our findings, previous GF murine studies have reported that the absence of gut microbiota physiologically influences the brain's serotonergic and neuroendocrine systems,[21,62] both of which have been linked to the pathogenesis of depression. These findings are further supported by previous studies reporting that probiotic treatment can ameliorate depression-like behaviors.[63–65]

Previously, Naseribafrouei *et al.*[11] reported that some OTUs in the Bacteroidetes phylum are positively correlated with depression, while other OTUs show a negative correlation with depression. Moreover, Jiang *et al.*[12] found that Bacteroidetes, Proteobacteria and Actinobacteria are increased, while Firmicutes is decreased, in MDD subjects vis-a-vis healthy control individuals. Here, we found that the relative abundance of Actinobacteria was increased in MDD subjects, whereas that of Bacteroidetes was decreased, as compared with healthy controls. Moreover, we found that Firmicutes was also responsible for discriminating MDD from healthy controls, although we found no significant difference in the overall relative abundance of Firmicutes between the two groups. The characteristic gut microbiota in MDD subjects identified here were significantly different than those found in previous studies. This disparity may have resulted from differences in sample sizes, the demographic and clinical characteristics of the recruited MDD subjects, and/or the statistical methods used to identify the MDD-associated gut microbiota. However, these findings consistently demonstrate that MDD is linked to marked alterations in gut microbiota composition.

Here, we found that the differences in the gut microbial communities observed between MDD subjects and healthy controls was unrelated to confounding factors such as antidepressant use, sex, age, smoking status or body mass index. Owing to the lack of detailed dietary information, we could not analyze the relationship between gut microbial composition and dietary intake. Given that the lifestyles and dietary habits of individuals within the city of Chongqing are very similar (that is, primarily dominated by rice consumption accompanied by small amounts of local meats and vegetables), this potential confounding factor likely does not significantly influence the current findings. Similar to our findings, changes in the gut microbial community from the first to the third trimester of pregnancy are also unrelated to covariates such as health status, body mass index and dietary intake.[16] However, some previous studies have also reported that dietary habits and age can influence the gut microbiomes of healthy individuals.[66,67] These discrepancies may result from differences in the recruited subjects. Taken together, by recruiting well-matching subjects, we found that MDD was associated with characteristic alterations in the gut microbiome.

To determine whether gut microbiota have a causative role in the development of depression, a bacterial colonization experiment was performed here. We demonstrated that colonization of GF mice with whole gut microbiota sampled from depressed

individuals led to increased depression-like behaviors, exhibiting that whole gut microbiota can serve as a pathogen-like entity. Significantly, the altered phylum-level bacterial taxa of the 'inputted' human 'depression microbiota' (characterized by alterations in Firmicutes, Actinobacteria and Bacteroidetes) were efficiently captured in the 'output' mouse fecal communities of 'humanized' depressed mice. These findings suggest that the alteration of gut microbiota and the resulting induction of depression-like behaviors are transmissible. On the basis of these characteristic changes in the gut microbiome, further research should identify the specific gut microbiota species that disproportionally contribute to the development of depression, as such species may serve as novel targets for depression therapy.

We also found that the gut microbiota that induced depression-like behaviors in recipient mice produced observable systemic metabolic alterations. Accordingly, a recent animal study also demonstrated that gut microbiota influence autism spectrum disorder-like behavioral symptoms through impacting host metabolism.[27] Here, humanized depressed mice were characterized by disturbances in microbial genes and host metabolites involved in carbohydrate and amino acid metabolism. The majority of carbohydrate metabolic pathways were enhanced in depressed mice relative to control mice, suggesting a higher energy demand in depressed mice. This finding may account for previous studies that have shown a reduction in glucose metabolism in brains from MDD patients.[68] In addition, we found that depressed mice were characterized by obvious disturbances in amino acid metabolism. In agreement with these findings, alterations in peripheral and central amino acid metabolism have been previously identified in MDD patients.[69,70] Here, we did not find any particular metabolite(s) that were obviously perturbed in depressed mice, suggesting that a systemic metabolic perturbation induced by gut microbiota likely contributes to the observed depression-like behavior.

There were some limitations that should be noted here. First, although we were able to identify the disturbed gut microbial communities of MDD subjects, we did not examine other neuropsychiatric disorders with similar clinical presentations to MDD. Further studies that recruit patients with other mental disorders (such as bipolar disorder and schizophrenia) are needed to assess the specificity of altered gut microbiota. Second, all depressed and healthy control subjects were recruited from the same site and are of Han Chinese ethnicity; thus, site-specific and ethnic biases in microbial phenotypes cannot be ruled out. Further studies that recruit ethnically diverse subjects from various clinical sites are required to validate the current findings. Third, the pooled fecal samples from a limited set of donors were used to perform the FMT experimentation. Additionally FMT studies using multiple independent pooled samples from different donors or individual samples from different donors would be of great value in addressing which experimental method(s) can more effectively capture the key characteristic gut microbiota in MDD patients to identify the specific gut microbiotic species that disproportionally contribute to the development of depression. Fourth, only male mice were used to perform the FMT experiment here; whether or how sex-based differences influence this FMT model requires further investigation. Finally, only hippocampal metabolite signatures were identified in depressive mice. Therefore, further studies using other brain areas that have been linked to depression (such as the prefrontal cortex and hypothalamus) are needed to further analyze the central nervous system-based metabolite changes associated with alterations in gut microbiota.

In a companion paper, we show that changes in behavior caused by (i) chronically increased stress levels, (ii) knockout of caspase 1 leading to decreased inflammasome function (iii) pharmacological treatments also result in alterations in the gut microbiome. Therefore, we suggest that the microbiota-gut-brain axis is truly bidirectional, functioning in a manner through which, as demonstrated here, modifications in microbiota affect behavior, whereas conversely, as documented in our companion paper, interventions that affect behavior through different mechanisms, result in changes in the microbiota.[71] On the basis of our results, we propose that novel strategies to target this bidirectional interface of gut microbiota and behavior may represent an approach for the treatment of MDD that is conceptually novel, when compared with existing antidepressant treatments.

CONCLUSIONS

We demonstrated in these experiments that gut microbiota can physiologically induce depression-like behavior in mice. Moreover, we document characteristic alterations in the gut microbiotic community of MDD patients and show with state-of-the-art techniques that gut microbiota can contribute to depression-like behavior through altering host metabolism. These findings provide an original perspective to uncover the pathologic mechanism(s) underlying depression as well as revealing the need for innovative gut-mediated therapies for depression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Designed the experiments: PX, HW and JY. Performed the metagenomic analysis: PZ, MLL, ZF and LZ. Performed the metabolomic analysis: CJZ, XJX, BHZ and PZ. Performed the fecal microbiotic transplantation: ZF, BHZ, MLL, XTZ, XYD and WXL. Analyzed the metagenomic and metabolomic data: JJC, SHF and XJX. Collected the clinical samples: DYY, YTY, XYD, XTZ and HQM. Drafted the manuscript: PX, HW and PZ. Revised the manuscript for intellectual content: JY, PX and NDM.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)