Rifaximin-Mediated Gut Microbiota Regulation Modulates the Polarization of Microglia During CUMS in Rat

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Abstract

Background: Chronic unpredictable mild stress (CUMS) can not only lead to depression-like behavior but also change the composition of the gut microbiome. Regulating the gut microbiome can have an antidepressant effect, but the mechanism by which it improves depressive symptoms is not clear. Short-chain fatty acids (SCFAs) are small molecular compounds produced by the fermentation of non-digestible carbohydrates. SCFAs are ubiquitous in intestinal endocrine and immune cells, making them important mediators of gut microbiome-regulated body functions. Activated M1 microglia can cause pro-inflammatory and neurotoxic effects, while M2 microglia serve anti-inflammatory and neuroprotective functions. The balance between the two phenotypes of microglia plays an important role in the occurrence and treatment of depression caused by chronic stress. We hypothesized that rifaximin exerts an antidepressant effect by changing the abundance of fecal SCFA metabolites and transforming the microglial phenotype.

Methods: We administered 150 mg/kg rifaximin intragastrically to rats exposed to CUMS for 4 weeks and investigated the composition of the fecal microbiome, the content of short-chain fatty acids in the serum and brain, microglial phenotypic profiles and hippocampal neurogenesis.

Results: Our results show that rifaximin ameliorated depressive-like behavior induced by CUMS, as reflected by sucrose preference, the open field test and the Morris water maze. Rifaximin increased the relative abundance of Ruminococcaceae, which were significantly positively correlated with high levels of butyrate in the brain. Rifaximin also increased the transformation of M1 microglia into the M2 type in the hippocampal dentate gyrus (DG) and ameliorated neurogenic abnormalities and functional deficits caused by CUMS.

Conclusions: These results suggest that rifaximin can enhance the neuroprotective effect of microglia to some extent by regulating the gut microbiome and one of its metabolites, butyrate.

1. Introduction

Depression is a pervasive neuropsychiatric disorder that is characterized by affective flattening, slow mental processing and hypobulia[1]. Globally, 300 million people suffer from depression, and the number increases every year[2]. Although it is currently recognized that the pathogenesis of depression involves many physiological, genetic and social-environmental aspects, the pathophysiological mechanisms of depression have not been fully elucidated[3]. The current clinical treatment of depression is usually based on the monoaminergic deficiency hypothesis[4], but the therapeutic effect is not optimal.

Accumulating evidence on the gut microbiome has highlighted the potential link between structural changes in the fecal microbiome and the development of depression, and researchers have proposed a microbial–gut–brain axis to explain the relationship between the microbiome and the brain[5–7]. Furthermore, the role of microglia in the neuroinflammatory response that occurs in the development of

depression has been demonstrated[8, 9]. Therefore, targeting the gut–brain axis and microglial pathology may serve as a new therapeutic strategy for depression.

The gut–brain axis involves two-way interactions between the gut microbiota and the central nervous system. Different signals from the gastrointestinal tract can regulate brain function through neural, endocrine, immune, metabolic and other pathways[10]. Importantly, changes in gut microbiota can modify their biochemical interactions with the central nervous system as a result of their altered molecular outputs, especially microbial-related metabolites[11]. Although the biological mediators that drive these effects remain largely unknown, methods that change the structure of the gut microbiome have been shown to affect the stress response, emotional and cognitive processes and behavior of animals[12, 13].

Short-chain fatty acids (SCFAs) are microbial metabolites that constitute the main bacterial fermentation products of dietary fiber in the gut. These molecules are generally considered to be key candidate factors in microbiota–gut–brain communication due to their ability to easily cross the blood–brain barrier. cumulating evidence is gradually clarifying their potential value in immune regulation[14, 15]. An SCFA mixture reduces the secretion of cytotoxins by stimulating THP-1 microglia-like cells, and the use of formate alone reduces the respiratory burst of microglial cells[16]. However, few studies have directly explored the role of SCFAs as potential mediators to affect the microbiotatargeted interventions in affective and cognitive functioning.

The hippocampus is a brain region in which active neurogenesis occurs throughout life. Adult hippocampal neurogenesis starts with the proliferation of neural progenitor cells (NPCs) located in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), which is associated with the modulation of cognitive processes, such as pattern separation and cognitive flexibility[17, 18]. The dysregulation of hippocampal neurogenesis leads to memory and learning deficits and has been associated with the onset of depression and anxiety disorders[19]. Neurons communicate with each other through synapses located on dendritic spines[20]. Moderate pressure stimulation increases the number of synapses and enhances neural function, while long-term chronic pressure inhibits the production of new neurons in the hippocampus and reduces the density of dendritic spines, leading to memory impairment[21, 22]. The number and shape of dendritic spines play important roles in synaptic plasticity, learning and memory.

Microglia are the dominant immune cells in the brain, where they regulate the dynamic balance of the neuroinflammatory environment. After stimulation by external pathogenic factors, such as lipopolysaccharide (LPS) and interferon- γ (IFN- γ), microglia transform into the pro-inflammatory M1 phenotype through classical activation and secrete a large number of inflammatory mediators and cytotoxic molecules, leading to damage in peripheral nerve cells[23, 24]. Conversely, microglia can also adopt the anti-inflammatory M2 phenotype through selective activation pathways and play a neuroprotective role by secreting neuroprotective factors, such as Interleukin-10(IL-10)[25]. However, our understanding of the effects of chronic unpredictable depression on microglia and their potential association with hippocampal neurogenesis remains very limited.

Traditional antibiotics, such as Beta-lactamics and fluoro-quinolones, in addition to their bactericidal or bacteriostatic functions, often have a profound impact on the composition of gut microbiome, mainly manifested in the reduction of probiotics, such as Bifidobacteria, and the increase of potential pathogenic bacteria, such as Enterobacteriaceae[26]. Rifaximin is a non-absorbable antibiotic that has a high safety profile due to its low systemic absorption. Rifaximin has been shown to inhibit bacterial overgrowth in the small intestine and prevent recurrent hepatic encephalopathy[27]. Rifaximin has important antibacterial and anti-inflammatory effects in the colon, including reduced bacterial virulence and translocation.

In addition, rifaximin seems to have a unique prebiotic properties in promoting the growth of beneficial bacteria such as Bifidobacteria and Lactobacilli, thereby actively regulating the composition of the gut microbiome[28, 29]. Animal models and metagenomic analysis showed that, different from the reduction of flora diversity caused by traditional antibiotics, rifaximin keeping stable the overall composition of on gut microbiome diversity[30]. After the withdrawal of rifaximin, the changes in the gut microbiome composition were stable for a long time[31]. Rifaximin also showed a potential modulate on bacterial metabolic function[32]. Therefore, study on the regulatory effect of rifaximin on gut microbiome will help to promote the understanding of gut–brain axis.

Given the evidence that the gut–brain axis and neuroinflammation are involved in depression, we hypothesized that rifaximin has a microbial-dependent antidepressant effect that involves the regulation of microglial phenotypes and neurogenesis. We investigated the effects of chronic rifaximin administration on microglial phenotypes and hippocampal neurogenesis in a chronic unpredictable mild stress (CUMS) rat model of depression. Furthermore, to explore how gut microbiome affect the activation of microglia, we assessed the relation between SCFAs and microbiome. Finally, we investigated the effect of sodium butyrate on the polarization of microglia.

2. Materials And Methods

2.1 Animals and drug administration

Male sprague dawley rats (n=56, 3 weeks old) were purchased from the experimental animal center of shandong university (Jinan, China). All rats were housed under standard environmental conditions (22±0.5 °C, 50%±5% humidity, and a 12 h light/12 h dark cycle) and maintained with free access to a standard laboratory pellet diet and water. The rats were habituated to their new environment for a week, and changes in their sucrose preference, body weight and sucrose consumption before and after drug administration were recorded. The weights and sucrose preferences in the first week were used as the baseline. After their acclimatization, the rats were divided randomly into four groups: control group (CON, n=14), rifaximin group (CON+R, n=14), CUMS model group (CUMS, n=14) and CUMS-exposed rats treated with rifaximin (CUMS+R, n=14).

2.1.1 Chronic unpredictable mild stress procedure

The CUMS procedure was performed as previously described[33] with a slight modification. Rats in the CUMS and CUMS+R groups were randomly exposed to different stressors: cage tilting for 24 h, cold swimming for 3 min (at 0 °C), water or food deprivation for 24 h, level shaking for 15 min, tail nip for 1 min (1 cm from the end of the tail), 45 °C heat stress for 5 min and inversion of the light/dark cycle for 24 h. These stressors were applied for 28 days, during which each stressor was applied 4 times. The rats were exposed to different stressors at random every day, making it impossible for the animals to predict the stimulus. The same stressor was not applied on consecutive days. The CON and CON+R group were undisturbed except for necessary procedures. Feces were collected on the last day of the CUMS procedure.

2.1.2 Pharmacological treatments

Rifaximin was purchased from Yuanye Bio-Technology Co. Ltd (Shanghai, China). Rifaximin was mixed with normal saline 0.9% in high-speed vortex state. During the 4 weeks of CUMS procedures, rats in CUMS+R and CON+R groups were treated with rifaximin (150 mg/kg) by oral gavage once daily (at 16:00) for 4 weeks. The doses of rifaximin were chosen based on a previous study[34]. The procedures were approved by the Institutional Animal Care and Committee, Shandong University.

2.2 Behavioral tests

2.2.1 Sucrose preference test (SPT)

The sucrose preference test (SPT) was administered to quantify loss of interest in rewarding stimuli. Baseline measurements were taken during adaptation, during which rats were placed in different cages and exposed continuously to two bottles for 12 hours, one containing sucrose water (1% (wt/vol)) and one containing tap water (normal water). Rats (n=14 per groups) were deprived of water and food for 12 h before the preference test. During the preference test, rats were housed in individual cages followed by free access to 2 bottles of fluids containing a sucrose solution (1% sucrose, 200 mL) and water (200 mL), respectively, for 12 h. The placement of the two bottles was changed after 6 h to prevent the possible effects of preference for a side on drinking behaviors. The consumption of the sucrose solution and tap water was calculated by weighing the bottle. The sucrose preference (SP) value was calculated as follows: $\text{sucrose intake (g)} \times 100\% / [\text{sucrose intake (g)} + \text{water intake (g)}]$.

2.2.2 Open field test (OFT)

The OFT was used to assess exploratory activities and anxiety-like behavior in an open box. The open device was a square acrylic box (150×150×50 cm) divided into 25 squares at the bottom. All of the rats were placed in the center of the open field apparatus and allowed to explore freely for 5 min. The number of squares crossed (four paws placed on a new square) and the time spent in the center area were recorded by video tracking software (SMART 2.5, Spain). After each rat was tested, the site was swabbed with a wet cloth and dried with a hot air blower.

2.2.3 Morris water maze (MWM)

Spatial learning and memory were assessed using the Morris water maze. Rats (n=14 per groups) were trained continuously for 5 days with four quadrants, and the order of the quadrants in each experiment was changed randomly. The platform was hidden 1 cm below the surface of the water and was located in the center of a quarter of the pool. The rats were directed to the platform if they could not locate it within 90 seconds. After reaching the platform, the rats remained for 10 seconds before being removed. After each experiment, the rats were dried with cotton wool, placed in cages and kept warm in a room at a constant temperature of 28 °C. Spatial memory was tested on the sixth day with the platform removed. The escape latency time (time taken to find the platform) and the percentage of time spent and entry frequency in the target quadrant (the quadrant containing the platform) were recorded. Performance was recorded by video tracking software (SMART 2.5, Spain).

2.3 16S rRNA analysis of fecal microbiota

The experiments included extracting the total DNA from samples (n=5 per groups) of the faeces. The data were analyzed on the free online Majorbio I-Sanger Cloud Platform. Total DNA was extracted according to the instructions of the E.Z.N.A.® SOIL Kit (Omega Bio-Tek, Norcross, GA, U.S.). The concentration and purity of DNA were measured using a NanoDrop 2000 spectrophotometer, and the quality of the DNA extraction was confirmed by 1% agarose gel electrophoresis. PCR amplification of the V3-V4 variable region was performed using 338F (5'-ACTCCTACGGGAGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers. The microbial composition was analyzed via 16S rRNA sequencing by Shanghai Majorbio Bio-pharm Technology (Shanghai, China) according to standard instructions.

2.4 SCFA concentration analysis

The concentrations of SCFAs (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and hexanoic acid) in the serum and brain (n=4 per groups) were determined on a Thermo TRACE 1310-ISQ system (Thermo, USA) fitted with an Agilent HP-INNOWAX column (30 mm × 0.25 mm × 0.25 µm, Agilent, United States). Standard solutions of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and hexanoic acid were diluted with ethyl ether to 0.02 µg/mL, 0.1 µg/mL, 0.5 µg/mL, 2 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL and 500 µg/mL, respectively. One hundred microliters of 15% phosphoric acid was added to each 200 µL serum sample or 50 µg of brain tissue, and then 20 µL of 75 µg/mL isohexanoic acid solution and 280 µL of diethyl ether were added. The samples were centrifuged at 4 °C and 12000 rpm for 10 min. The supernate was taken for testing. The mixture was stored at -20 °C.

2.5 Histopathological examination

The colons were soaked in 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) for 24 h, dehydrated and embedded in paraffin, cut into 5 µm slices transversely with a microtome, stained with hematoxylin and eosin (HE) and analyzed by Olympus BX53 microscope.

2.6 Immunofluorescence

Rats (n=4 per groups) were deeply anesthetized with 10% pentobarbital (10 mL /kg) and slowly perfused with 300 mL of phosphate buffer saline (PBS, pH 7.2), followed by 300 mL of 4% paraformaldehyde (PFA). The brain was removed and immobilized in 4% PFA for 72 hours and then dehydrated in 30% sucrose for 72 hours. Tissues on slides were treated with 0.2% Triton X-100 and blocked with 5% goat serum. The slide was incubated overnight at 4 °C with the following primary antibodies: Ki-67 (D3B5) Rabbit mAb (Ki-67, 9129, Cell Signaling), Anti-Doublecortin (DCX, ab254133, Abcam), Anti-Iba1 antibody (Iba-1, ab178846, Abcam), Inducible nitric oxide synthase

Polyclonal Antibody (iNOS, MA5-17139, Invitrogen) , Arginase 1 Polyclonal Antibody (Arg-1, PA5-85267, Invitrogen), Anti-PSD95 antibody (ab238135, Abcam), Anti-CD68 antibody (ab125212, Abcam), Neuronal nuclei (E4M5P) Mouse mAb (NeuN, 94403, Cell Signaling). Anti-ZO1 tight junction protein antibody (ZO-1, ab221547, Abcam) and Anti-Claudin 1 antibody (Claudin-1, ab211737, Abcam). After the primary antibodies were rinsed with PBS, the tissues were covered with Alexa Fluor® 488- and Alexa Fluor® 594-conjugated fluorescent secondary antibodies and incubated in the dark for 60 min. After rinsing the secondary antibodies with PBS, 4,6-Diamidino-2-phenylindole (DAPI) solution was added, and the slides were incubated in the dark for 3-5 min. The slides were observed with an Olympus BX53 fluorescent microscope equipped with a DP74 Microscope Digital Camera.

2.7 Golgi Staining

After the rats (n=4 per groups) were sacrificed and perfused with normal saline, the brain was removed and immediately fixed in 30% sucrose solution for 48 hours. Then, the brain was treated with a Golgi staining kit (g1069, Servicebio, China) for 5 days. After staining, tissue blocks were cut into 50-μm-thick sections, and then sections were dehydrated in absolute ethanol twice for 20 min and cleared in xylene for 30 min. The images were obtained using a VS120-S6-W system (OLYMPUS, Japan) and analyzed with Olympia ver. 2.9.

2.8 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of Interleukin-1ra(IL-1ra), IL-10, Tumor necrosis factor-α(TNF-α) and Interleukin-1ra (IL-1β) in the brain tissue(n=4 per groups) supernatant and cell cultures were measured with validated specific ELISA assays according to the manufacturer's instructions (Elabscience, Wuhan, China). Each sample (100 μL) was added to ELISA plates in triplicate. A reference standard was used to establish the working curve, and the biotinylated antibody, HRP conjugate solution, substrate reagent and terminal solution were successively added according to the instructions. The inflammatory cytokine levels were measured by a microplate reader at 450 nm absorbance.

2.9 Primary Microglia Culture and treatment

The brain tissue of newborn (P0-P5) SD rats was extracted for primary microglia culture. The brain tissue was enzymatically dissociated into single cell suspension with 0.25% trypsin and filtered with 70 μm cell

filter. Then, mixed glial cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (Gibco) at 37 °C and 5% CO₂ for 1 week. Microglia were mechanically isolated from mixed glial cultures and inoculated into 24-well plates at a density of 1×10^5 cells/cm with a humidified atmosphere of 95% air and 5% CO₂. Lipopolysaccharide (LPS, 100 ng/mL, Sigma) was used to activate microglial cells. The cells were treated with different concentrations of sodium butyrate (SB, MedChemExpress): 0.1uM, 0.3uM and 0.5uM. Then, the in vitro cultured microglia were divided into four groups: CON, LPS (treated with LPS for 24 h), SB (treated with SB for 24 h) and LPS+SB (treated with LPS and SB for 24 h). After the indicated treatments, the cells were used to assess microglial phenotypic profiles.

2.9.1 Flow cytometry analyses

Cells were incubated with fluorescently labeled Alexa Fluor® 488 anti-rat CD86 antibody (ab256270, Abcam) (1:100), APC anti-mannose receptor antibody (ab223961, Abcam) (1:100) and Anti-CD68 antibody (ab283654, Abcam) (1:100) for 30 min. FACS was performed with the BD FACS Aria II cytometer (Becton Dickinson), and the data were analyzed using Kaluza Analysis 2.1 (Beckman Coulter Life Sciences, USA).

3.0 Statistical Analysis

All experimental data are represented as the mean $\pm$ SEM. Except for the Morris water maze (MWM) test results, data were analyzed by two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. Repeated-measures two-way ANOVA followed by post hoc Bonferroni multiple comparison tests were used for the Morris water maze. The Spearman correlation test was used to analyze the correlation between the gut microbiome and short-chain fatty acids, the Spearman test and the corresponding p values were calculated by using the `cor.test()` function with two-sided alternative hypothesis, and p values were corrected for multiple comparisons using the `qvalue` package in R. 7Parameters were analyzed using GraphPad Prism 8 (GraphPad Software, USA). The analysis results were only further evaluated when a significant difference was observed. $P \leq 0.05$ or $q \leq 0.1$ was considered statistically significant.

3. Results

3.1 Rifaximin ameliorated CUMS-induced depressive-like behaviors

The experiment was designed according to the steps in Fig. 1A. The SPT was performed individually to lessen the effect of variability in sucrose preference. Low sucrose intake reflects an impaired reward response and anhedonia, one of the core symptoms of major depression. The CUMS-treated rats showed significantly decreased sucrose consumption in comparison with the CON group, and this effect was reversed in CUMS-exposed rats that received rifaximin in Fig. 1B. The OFT was used to measure

movement and exploratory behavior in new environments. The CUMS treatment resulted in a decrease in the total frequency of crossing and rearing compared with the CON group (Fig. 1C-D), and a marked decrease in the time spent in the central area was observed in the CUMS-exposed rats when compared with the CON group (Fig. 1E). These decreases in spontaneous movement (crossing, rearing and center duration) were not present in CUMS + R rats. The learning ability and spatial memory of rats were tested using the MWM. On days 3 and 4, CUMS-exposed rats had a longer escape latency than the CON and CUMS + R groups (Supplemental Fig. 1A). After removing the platform, CUMS-exposed rats spent less time in the target quadrant and crossed the platform fewer times than the CON group (Supplemental Fig. 1B-C). These results indicate that CUMS impaired learning and spatial memory. In sharp contrast, rats in the CUMS + R group spent more time in the target zone and crossed it more frequently in the search session compared with the CUMS-exposed rats, showing a circular motion centered around the platform area (Supplemental Fig. 1D). However, rifaximin treatment alone had no significant effect on learning and spatial memory compared with the CON group.

3.2 Rifaximin modulates the microbial community composition of CUMS rats

Previous studies have shown that gut microbiome alterations can affect depressive-like behaviors. Therefore, we attempted to determine whether CUMS-exposed rats exhibited alterations in the gut microbiome. Alpha diversity was analyzed by calculating the Shannon, Simpson, Chao1 and Ace indices on OTU level. The results show that the diversity of the fecal microbiome was reduced in rats in the CUMS group, as Shannon values were significantly decreased while Simpson values were significantly increased (Fig. 2A-B). Furthermore, the Ace and Chao1 indices of CUMS rats were significantly decreased compared with those of the CON group (Fig. 2C-D), indicating that CUMS reduced the richness of the fecal microbiome. Rifaximin protected against these adverse effects and did not negatively affect the richness of the gut microbiome of CON + R rats.

Beta diversity was analyzed through principal coordinate analysis (PCoA) plots using nonphylogenetic Bray-Curtis metrics to assess differences in microbial composition (OTU) between the four groups (Fig. 2E). There was a clear separation between the CUMS and CON groups along the first principal component (PC1) axis (Adonis p value = 0.001), suggesting that CUMS-induced depression can change the structure of the microbiome.

At the phylum level, we found that the richness of Firmicutes in the CUMS group was significantly lower than that in the CON group, while the richness of Bacteroides was significantly higher. Rifaximin significantly reversed the change of Firmicutes and Bacteroidetes in the CUMS + R group (Fig. 2F). Although there was no statistical difference, rifaximin showed a trend of reducing microbial richness and diversity.

To identify significant changes in the gut microbiome, taxonomic comparisons at the family level were performed for all groups (Fig. 2G). A total of six families differed in relative abundance from their levels in the CON group. Four of the six bacterial families, namely, Lactobacillaceae (Firmicutes, $P = 0.013$),

Prevotellaceae (Firmicutes, $P = 0.047$), Ruminococcaceae (Firmicutes, $P = 0.025$) and Lachnospiraceae (Firmicutes, $P = 0.018$) were decreased in the CUMS group, and two families, Muribaculaceae (Bacteroidetes, $P = 0.009$) and Bacteroidaceae (Bacteroidetes, $P = 0.007$) were increased. These results demonstrate that the relative abundances of predominant Firmicutes genera were decreased in CUMS subjects, while those of Bacteroidetes were increased, suggesting that the balance between Firmicutes and Bacteroidetes may be involved in the pathogenesis of depression. Rifaximin increased the richness of Bacteroides and Prevotellaceae in rats in the CON + R group, and no negative behavioral effects were observed.

Next we attempted to determine the effects of CUMS or rifaximin treatment on the integrity of intestinal mucosa. The results of hematoxylin-eosin staining(HE) indicated that there was no significant pathological change in colon (Supplemental Fig. 2A). However, the integrity of intestinal mucosal barrier was impaired as there was significantly decreased of the median fluorescence intensity(MFI) of zonula occludens-1(ZO-1)(Supplemental Fig. 2B-D).

3.3 The relevance between gut microbes and Short-chain fatty acid

We analyzed changes in SCFAs caused by the gut microbiome to assess their potential role as key mediators. For this purpose, we measured the SCFA contents (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and caproic acid) in the serum and brain by Gas Chromatography–Mass Spectrometer (GC–MS) (Fig. 3A-B). CUMS treatment increased the level of acetic acid and decreased those of propionic acid and butyric acid in both the serum and the brain. Rifaximin treatment significantly increased the levels of propionic acid and butyric acid in the serum of the CUMS + R group compared with CUMS rats, whereas it had no significant effect on the content of acetic acid. However, in the brain, rifaximin had a limited effect on butyric acid and no effect on acetic acid and propionic acid, the concentrations of which differed from those in the serum. Interestingly, rifaximin treatment increased the butyric acid concentrations not only in CUMS + R group but also in the CON + R group, which indicates that butyrate variation may be a specific function of rifaximin.

In order to investigate the relationship between microbes and SCFAs after CUMS treatment, we examined correlations between the six microbes enriched in CUMS rats and five SCFAs that varied between groups in the brain (Fig. 3C). Spearman's test revealed 18 significant unique microbe–SCFA correlations in the brain (q value < 0.1), with Spearman's rank correlation coefficient (Spearman r) ranging between -0.88 and 0.76 . We present these correlations between microbe abundance and SCFA levels as a matrix. There were two significant positive microbe–SCFA correlations (q value < 0.05): Prevotellaceae and propionic acid (Spearman $r = 0.76$, q value = 0.032), Ruminococcus bromii and butyric acid (Spearman $r = 0.71$, q value = 0.040). There were two significant negative correlations (q value < 0.05): Muribaculaceae and butyric acid (Spearman $r = -0.68$, q value = 0.045), Bacteroidaceae and propionic acid (Spearman $r = -0.88$, q value = 0.016). These results may reveal part of the mechanism by which rifaximin modulates depressive symptoms through gut microbiota.

3.4 Rifaximin alters the microglial phenotype in the hippocampus, accompanied by the restoration of sodium butyrate in the brain

Microglia regulate neuroinflammation in the adult brain and play an important role in homeostasis disorders caused by chronic stress. The results show that the CUMS procedure increased the number of Iba1⁺ microglia in the hippocampal DG compared with the CON group. Additionally, the number of microglia (Iba-1⁺) was decreased in the CUMS + R group compared with CUMS group (Fig. 4A,D). Furthermore, we analyzed the relative number of M1 and M2 microglia by detecting iNOS and Arg-1, which are markers of microglial cell activation. The results show that the proportion of Iba-1⁺/iNOS⁺ positive cells(M1 cells) was increased after 4 weeks of CUMS exposure, while the proportion of Iba-1⁺/Arg-1⁺ cells(M2 cells) was significantly decreased(Fig. 4B-C, E-F). In contrast, the proportion of M1 and M2 cells was substantially reversed in the DG regions of rats in the CUMS + R group.

To confirm the morphometric changes in microglial cells in response to CUMS in the hippocampal DG, a sholl analysis of microglial cells was used to characterize the complexity of the cells. CUMS treatment significantly reduced the complexity of microglia: the mean number of intersections and surface area were significantly decreased (Fig. 4H, Supplemental Fig. 3A). Our result showed that the CUMS-induced activated microglia respectively as increased soma volume and decreased ramification length(Supplemental Fig. 3B-C). These morphological alterations are the characteristics of pro-inflammatory microglia. The intersections in the CON and CUMS + R groups reached a peak at 20 micrometer, while the CUMS group had a peak at 24 micrometer (Fig. 4G), which indirectly confirms the increased microglia volume.

We further measured the levels of inflammatory cytokines in the hippocampal DG. Rifaximin treatment inhibited the CUMS-induced increase in TNF- α and IL-1 β , which are M1 microglial phenotype-related pro-inflammatory cytokines (Supplemental Fig. 3D-E), and increased IL-10 and IL-1ra, which are M2 microglial phenotype-related anti-inflammatory cytokines, in the DG (Supplemental Fig. 3F-G). These results confirm the involvement of microglia in CUMS-mediated inflammatory activation and suggest that rifaximin can regulate the phenotype and related cytokines of microglia.

In view of the evidence that SCFAs are sufficient to promote microglia-mediated neuroinflammation, we aimed to determine whether microglia are activated by abnormal changes in SCFAs. Based on the unique regulatory effect of rifaximin on cerebral butyric acid content, we hypothesized that the change in microglial phenotype might be related to this SFCA. Therefore, we used simple linear regression to analyze the interrelation of brain butyrate content with the expression of microglial phenotype-related inflammatory cytokines and anti-inflammatory cytokines. The results show that sodium butyrate content was negatively correlated with TNF- α and IL-1 β (Supplemental Fig. 3H) and positively correlated with the level of IL-10 and IL-1ra (Supplemental Fig. 3I). These results suggest that rifaximin may participate in the regulation of inflammation through sodium butyrate, triggering a change in the microglial phenotype.

3.5 Rifaximin reverses the impaired neurogenesis and integration of newborn neurons in CUMS rats

The effect of CUMS on neurogenesis in the hippocampal SGZ is related to two stages of the neurogenic process: differentiation and integration of new cells in the subgranular cell layer of the dentate gyrus. To evaluate the differentiation of newborn cells, DCX-a marker of newborn neurons-was used to determine the influence of rifaximin on neurogenesis in the hippocampus. As shown in Fig. 5A-D, significant decreases in the number and length of DCX⁺ cells were observed in CUMS rats in comparison with the CON group, suggesting the impairment of neural differentiation. However, rifaximin alleviated the negative effect of CUMS on neurogenesis. An outcome of hippocampal neurogenesis is the production of mature dentate granule neurons that migrate and are functionally integrated into the granule cell layer (GCL) of the DG. Ki67, a marker of proliferation, and NeuN, a marker of mature neurons, were used to trace the integration of immature neurons in the DG. The migration index was calculated by the ratio of the Ki67⁺/NeuN⁺ cell (immature neuron) center distance to granular cell layer width. We observed that CUMS led to a significant reduction in the number of immature neurons and significantly altered the migration patterns of immature neurons (Fig. 5E-G), as indicated by the lower migration index (Fig. 5H-I). Migration defects may alter the integration of immature neurons since their abnormal position inside the GCL affects their function, which explains the decreased learning ability and memory of CUMS rats in the Morris water maze. Rifaximin reversed these migration effects. The number of mature neurons was not affected by CUMS or rifaximin (Fig. 5J).

3.6 Rifaximin Affects Synaptic Plasticity impairment induced by CUMS

The formation and growth of dendritic spines are an important structural basis of the neural network. Next, we measured dendritic spines in the DG (Fig. 6A). We found that the density of dendritic spines and the number of mushroom spines decreased significantly in rats in the CUMS group (Fig. 6C-D). In addition, there was a higher proportion of mushroom spines in rats in the CUMS + R group compared with those in the CUMS group.

CD68 is located in the lysosomal membrane of microglia and monocytes, and is one of the most commonly used markers to describe the phagocytic function of microglia. The number of Iba-1⁺/CD68⁺ cell was significantly increased after CUMS treatment (Fig. 6B,E). We further found that the microglia of rats in the CUMS group contained more postsynaptic density protein 95 (PSD95), a marker of excitatory glutamatergic synapses on postsynaptic membrane (Supplemental Fig. 4A-B), which suggested that microglia have stronger phagocytic activity. The rifaximin treatment normalized the CUMS-induced alterations in CD68 and PSD95.

3.7 Sodium butyrate induced the phenotypic transformation microglia in vitro

Our in vivo results suggest that the ability of rifaximin to modulate microglial phenotypes may be related to sodium butyrate (SB). In order to assess whether SB regulates phenotypic changes in microglia, flow cytometry was performed on a single-cell suspension to study the polarization phenotype of activated microglia stimulated by LPS or SB (Fig. 7A). CD86 and CD206 were used as M1 and M2 phenotypic markers. First, we investigated the effects of different doses of SB on microglia (0.1uM, 0.3uM and 0.5uM) on the basis of GC–MS results. Increases in the proportion of M1 cells (CD86⁺ CD206⁻) were observed in the LPS group when compared with the CON group, indicating that LPS treatment triggered the classical activation of microglia (Fig. 7B). We also found that SB could increase the proportion of M2-like microglia (CD86⁻ CD206⁺) in a dose-dependent manner, and 0.3 uM SB significantly increased the percentage of M2 microglia (Fig. 7C). In LPS + SB0.3uM group, the proportion increased for CD206⁺ cells and decreased for CD86⁺ cells compared with LPS group, suggesting that SB can transform the LPS-induced pro-inflammatory microglia into anti-inflammatory microglia. ELISA analysis showed that LPS + SB0.3uM suppressed the LPS-induced increase in TNF- α and IL-1 β (Fig. 7D-E). LPS stimulation had no significant effect on the content of IL-10 and IL-1ra whereas SB significantly elevated anti-inflammatory cytokine levels in the medium (Fig. 7F-G). SB treatment can also effectively alleviate the strong phagocytosis of microglia induced by LPS and downregulated the expression of CD68 (Supplemental Fig. 5A-B).

Discussion

Evidence that the microbiota–gut–brain axis plays a critical role in health and disease, including neuropsychiatric disorders, is rapidly accumulating[35]. These findings suggest that changes in the gut microbiome and metabolites play an important role in emotion regulation. Metagenomic studies revealed that the microbiome changed significantly in patients with severe depression[36]. Different species of *Lactobacillus* and even different strains of *Lactobacillus* species, such as *Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* HN001, has been reported to have antidepressant effects in improving severe depression and intestinal dysfunction in humans[37, 38]. These results suggest that there is a close relationship between the gut microbiome and the pathogenesis of depression, and an understanding of the physiological mechanism underlying these relationships will help in exploring new options for clinical treatment.

In this study, using a rat model of stress-induced depression, we investigated the effect of rifaximin on depression-like behavior via alteration of the gut microbiome and analyzed the resulting microglia-mediated inflammatory response and hippocampal neurogenesis. Our evidence shows significant changes in the structure of the gut microbiome and the levels of short-chain fatty acids in the brains of depressive rats exposed to chronic unpredictable mild stress (CUMS). Furthermore, CUMS increased the number of M1 microglia in DG and enhanced the phagocytosis and the pro-inflammatory function of microglia, which was related with the dysregulation of hippocampal neurogenesis. The rats exposed to stress also exhibited depression-like behaviors, such as decreased sugar preference, reduced exploratory behavior and impaired learning and memory. The increase in butyrate content in the central nervous system induced the transformation of M1-type microglial cells to M2-type microglia and ameliorated the

inflammatory environment in the brain. The number and function of immature neurons in the hippocampal DG were significantly improved. We demonstrate that rifaximin may exert antidepressant effects by modulating the composition of the gut microbiome and limiting microglia-mediated neuroinflammation. Our findings shed light on the influence of the gut microbiome on hippocampal neurogenesis in depression and its potential mechanisms. Thus, treatment strategies targeting the gut microbiome in patients with depression may ameliorate the cognitive and behavioral changes associated with the disease.

Growing evidence supports the notion that the gut microbiome can influence the pathological process of depression through bi-directional communication between the gut and the brain[39]. Clinical studies have shown that the diversity and richness of the gut microbiome of depressed patients are significantly reduced compared with those of healthy people[40, 41]. Transplants of the gut microbiome of depressed patients into germ-free mice resulted in depression-like behavior in the recipient mice, suggesting that depressive symptoms can be induced by the alteration of gut microbiome[42], further supporting the close relationship between the gut microbiome and depression.

It is generally believed that environmental factors play an important role in the pathogenesis of depression. Chronic stress exposure is a significant inducer of depression. In the CUMS model, chronic stress not only affects the brain and stress response system but also disturbs the intestinal microbiota. Haiyin Jiang et al. found that the richness of Bacteroidetes, Proteobacteria and Actinobacteria in the gut microbiome of patients with major depressive disorder (MDD) was significantly increased, while the levels of Firmicutes were significantly decreased[43]. Zhang W et al. also stated that the proportion of Firmicutes and Bacteroides is an important factor that distinguishes patients with major depression from healthy people[44]. The results of 16S rRNA-based analysis of the gut microbiome showed that the content of Firmicutes in the gut microbiome of CUMS rats was decreased, while the content of Bacteroides was significantly increased, which is consistent with previous research evidence[45]. Our study further analyzed changes in the gut microbiome at the family level. We found that the contents of Lactobacillaceae, Prevotellaceae, Ruminococcaceae and Lachnospiraceae in Firmicutes decreased significantly, while those of Muribaculaceae and Bacteroidaceae in Bacteroides increased significantly in CUMS rats. Rifaximin, a non-absorbable antibiotic that is able to reduce bacterial virulence and translocation, effectively modulated the gut microbial composition and reversed the decrease in Firmicutes and increase in Bacteroidetes induced by CUMS. Interestingly, rifaximin significantly increased the levels of Prevotellaceae and Bacteroidaceae in the CON + R group, however, the intestinal flora composition did not change significantly. Prevotella is thought to be related to the synthesis of short-chain fatty acids (SCFAs), which can protect the intestinal mucosal barrier by improving the intestinal inflammatory environment[46]. Although many studies have reported that the relative abundance of Bacteroides was negatively correlated with depression-related brain signals[47, 48], a few studies have found that Bacteroidaceae and its metabolites may play a positive role in the occurrence of mental illness[49, 50]. As the main enteric symbiont, Bacteroidaceae plays an important role in maintaining the balance of the gut microbiome. The reason for these different conclusions may be that the function of flora is closely related to its richness, and different levels of Bacteroidaceae may play completely

opposite functions. The rifaximin-induced increase in Bacteroidaceae did not lead to depressive-like behavior. We will further explore the potential mechanism of this change in future studies. These findings may help to further explain the relationship between these fecal microbiota changes and depression.

Our results showed that there were no significant changes in colonic physiological structure and intestinal mucosal barrier integrity in CUMS rats. Therefore, the gut microbiome may affect the body through its metabolites. Metabolites are important factors in the gut microbiome-mediated regulation of the physiological function of the host. Previous studies on human and animal SCFAs have mainly analyzed bacteria from intestinal feces, and there is evidence that SCFAs in the stool are involved in the development of depression[51]. However, whether fecal SCFAs can be used as a standard to measure the overall SCFA status of the host is still in question. Although the intestinal tract is the main site of SCFA production, epithelial cells in different tissues and organs selectively take up butyrate, propionate and acetate[52]. It is possible that this biological concentration gradient confers different roles to SCFAs in different cell and tissue types.

In this study, we measured the levels of SCFAs in the serum and brain. We found that the content of acetic acid in the serum and brain tissue of CUMS rats increased significantly, while the content of butyrate and propionate decreased. After rifaximin treatment, the content of propionate and butyrate in the serum increased significantly; in the brain, rifaximin only reversed the CUMS-induced decrease in the butyrate level. The correlation between the gut microbiome and brain SCFAs was further analyzed using a Spearman correlation matrix. We found a correlation between several gut microbiota and SCFAs, for example, between *Ruminococcus bromii* and butyric acid and between Lachnospiraceae and butyric acid. With the development of metagenomics, we have gained an understanding of different SCFA sources. *Ruminococcus bromii* and Lachnospiraceae are considered to be among the main sources of intestinal butyrate[53]. *Ruminococcus bromii* can produce butyrate by fermenting dietary polysaccharides (such as starch and hemicellulose), inulin and pectin derivatives[54]. Lachnospiraceae species have genes for the synthesis of butyrate from acetyl CoA to butyryl CoA, and some genera in this family can also synthesize butyrate from acetic acid and lactic acid[55]. This may explain the regulatory effect of rifaximin on butyrate via the gut microbiome. Furthermore, we found that other correlations, such as *Lactobacillus* and Prevotellaceae, which were affected by rifaximin, were negatively correlated with acetic acid content, while Bacteroidaceae and acetic acid content were positively correlated. There is a very close relationship between bacterial species in the gut. Due to cross-feeding, a core metabolic mechanism of this microbial community, a large amount of butyric acid does not come directly from the digestion of fiber by specific species but is produced by interactions within the intestinal microbial ecosystem[56, 57]. Acetic acid, which is formed in the fermentation process, is an important contributor to butyrate production[58], so the composition of the microbial community as a whole will also affect the overall production efficiency of butyrate. Although we did not find a significant effect of rifaximin on acetic acid or propionic acid content in the brain, we believe that these correlations may indicate a potential relationship between the gut microbiome and SCFAs in rats with depression.

Microglia, the main immune cells in the central nervous system, appear to be heterogeneous with diverse functional phenotypes, ranging from pro-inflammatory M1 phenotypes to immune-suppressive M2 phenotypes[59]. The contribution of microglia to the developing brain includes controlling the number of neuronal precursor cells (NPCs) by active apoptosis induction and the removal of excess, apoptotic, or dying NPCs[60]. As the first line of defense of the central immune system, M1 microglia mainly express high levels of pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, and resist pathogenic factors. However, activated M1 microglia can induce neurotoxicity due to the excessive accumulation of pro-inflammatory factors and various neurotoxic mediators, such as superoxide and ROS[61]. Four major anti-inflammatory cytokines—IL-4, IL-1ra, IL-10 and transforming growth factor- β (TGF- β)—are employed by M2 microglia to suppress the pro-inflammatory response[62].

We observed that CUMS increased the proportion of M1 microglia in the hippocampus of rats. Morphologically, amoeba-like changes occurred in microglia, accompanied by an increase in volume and decrease in surface area, and increased levels of IL-1 β and TNF- α were detected in the DG area of the hippocampus, which suggests that CUMS promoted the transformation of microglia to the M1 type. Ben Bassetta et al. found that CUMS induced depression-like behavior in mice and increased the number of M1 microglia in the hippocampus[63], which is consistent with our study. Rifaximin increased the proportion and complexity of M2-type microglia, decreased the spatial density of total microglia and increased the levels of IL-1ra and IL-10. These results suggest that rifaximin may affect neuroinflammation in the DG by changing the number and polarization of microglia. We further found that butyric acid, which was affected by rifaximin, was negatively correlated with the content of TNF- α and IL-1ra in the DG and positively correlated with the content of IL-10 and IL-1 β , suggesting that butyric acid may be a key mediator of rifaximin in regulating the microglia phenotype.

Evidence from patients with major depression and animal models demonstrates that chronic stress can reduce the volume of the hippocampus and the number of new neurons and cause structural abnormalities and functional deficits[64, 65]. Antidepressant drugs can play a role in alleviating depression symptoms by increasing the number of new neurons and improving neurological function[66]. During neurogenesis in the hippocampus, neural progenitor cells (NPCs) in the SGZ migrate in the GCL and undergo morphological and physiological maturation to functionally integrate into the hippocampal circuitry[67]. In this study, we used juvenile rat to access how CUMS disrupt neurodevelopmental processes. Our results show that the numbers of newborn and immature neurons in the DG of CUMS rats were significantly reduced, and the levels of TNF- α and IL-1 β were increased. Neuroinflammation induced by cytoplasmic activation seems to inhibit adult neurogenesis in the DG, which is considered an indicator of neurodegenerative diseases[68]. The migration distance of immature neurons in the GCL of the DG was shortened in CUMS rats; this disturbs the normal function of the hippocampal circuitry, explaining the impairment of learning and memory functions in the MWM. Rifaximin significantly improved the low number of newborn neurons induced by CUMS and increased the migration distance of immature neurons in the GCL. These results suggest that rifaximin prevented the neurodevelopmental disorder caused by CUMS; this was behaviorally reflected in the higher crossing frequency and longer residence time in the target quadrant in the MWM.

Synapses participate in the formation of learning and memory through electrochemical signal transduction within highly specialized neural infrastructure[69]. Synapses are usually divided into three types according to their morphology: thin, mushroom and stubby. Due to the limitation of technical conditions, we only distinguished the mushroom between other types of spines. It is generally believed that mushroom spines are responsible for the formation and long-term maintenance of memory[70]. High-dose corticosterone, considered to be a critical mediator of chronic stress effects, can cause the atrophy of dendritic spines in the prefrontal lobe and inhibit the formation of new dendritic spines[71]. Antidepressants can improve depression-like behavior by reversing the synaptic loss caused by chronic stress[72]. We found that the number of mushroom spines in the DG decreased significantly and the microglia of the CUMS group contain more spine inclusions, which is consistent with previous studies. Rifaximin treatment increased the density of mushroom spines and alleviated the phagocytosis of microglia induced by CUMS, suggesting that the improvement in depression-like behavior after rifaximin treatment was associated with a reversal of microglia-mediated spine loss.

Primary microglia were used to verify whether sodium butyrate can regulate the microglial phenotype in vitro. A concentration gradient of 0.1, 0.3 and 0.5uM was used to verify the effect of sodium butyrate (SB). The results show that SB promoted the polarization of M2 microglia in a dose-dependent manner. Radhika Patnala suggested that SB could be used as an epigenetic molecular switch to modify the transition of microglia from pro-inflammatory to anti-inflammatory[73]. SB inhibits the functional extension of microglia activation through Akt activation and HDACs[74]. This further supports our results. In addition, although growing evidence suggests that SB has the ability to change the inflammatory phenotype of microglia[75], most researchers have used super-physiological doses of SB, which makes the reliability of the conclusion questionable. On the basis of the results of short-chain fatty acid determination, we explored whether a physiological dose of SB (0.3uM) under the influence of rifaximin could inhibit the LPS or CUMS induced activation of microglia in vitro. Interestingly, SB (0.3uM) effectively promoted the LPS or CUMS induced transformation of M1 microglia to M2 microglia, together with increased levels of anti-inflammatory IL-1ra and IL-10. SB(0.3uM) also reversed the increase in pro-inflammatory TNF- α and IL-1 β produced by M1 microglia, and alleviated the phagocytosis of microglia induced by LPS or CUMS and downregulated the expression of CD68. Combined with sufficient evidence that M2 microglia serve a neuroprotective effect in neurodegenerative diseases[76], these findings support the hypothesis that the induction of M2-like microglia during rifaximin treatment related to the reverse of the abnormal function of the hippocampal circuitry, which may be mediated by SB. The mechanism of sodium butyrate regulating the phenotypic transformation of microglia is not clear, which is one of our future research goals.

Conclusion

In conclusion, CUMS can lead to changes in the microglial phenotype and the secretion of inflammatory factors, resulting in the dysfunction of NPC proliferation and differentiation in the DG and leading to cognitive impairment and depressive-like behavior. The use of rifaximin, a non-absorbable antibiotic, can significantly improve depressive-like behavior and cognitive impairment, which may be related to its

ability to regulate microglia-mediated neurogenesis through sodium butyrate, a metabolite of gut microbiota. These results demonstrate that gut microbiota and their SCFA metabolites play important roles in neurogenesis in adult mental disorders. Therefore, methods that modulate the gut microbiome and its metabolites may be a promising treatment strategy for depression.

Abbreviations

Arg-1: Arginase 1

CUMS: Chronic unpredictable mild stress

DAPI: 4,6-Diamidino-2-phenylindole

DCX: Doublecortin

DG: Dentate gyrus

GCL: Granule cell layer

ELISA: Enzyme-linked immunosorbent assay

HE: Hematoxylin and eosin Staining

IFN- γ : Interferon- γ

IL-1 β : Interleukin-1 β

IL-1ra: Interleukin-1ra

IL-10: Interleukin-10

IL-4: Interleukin-10

iNOS: Inducible nitric oxide synthase

LPS: Lipopolysaccharide

MDD: Major depressive disorder

MFI: median fluorescence intensity

MWM: Morris water maze

NeuN: Neuronal nuclei

NPC: Neural progenitor cell

OFT: Open field test

OTUs: Operational taxonomic units

PC1: First principal component

PCoA : Principal coordinate analysis

PBS: Phosphate buffer saline

PFA: Paraformaldehyde

SB: Sodium butyrate

SCFA: Short-chain fatty acids

SGZ: Subgranular zone

SPT: Pucrose preference test

TGF- β : Pransforming growth factor- β

TNF- α : Tumor necrosis factor- α

Declarations

Ethics approval and consent to participate

All animal experiments in this study were approved by the Animal Experimental Committee of Shandong University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HNL designed the study, performed immunofluorescence and wrote the first draft of the paper, YJX performed flow cytometry analyses, ZMZ, WW, MYZ and ZJJ carried out the animal work and prepared samples and SYC prepared samples, FP and DXL discussed the protocols and results, DXL, RMH and CHH discussed the whole project. All authors were involved in the final version of the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

Depressive-like behavior induced by CUMS and the antidepressant effect of rifaximin. (A) Experimental flowchart. (B) Sucrose preference. (C) Frequency of crossing. (D) Frequency of rearing. (E) Duration in the center area. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the CON group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the CUMS group.

Figure 2

Effects of rifaximin on the microbial composition and integrity of intestinal mucosa of CUMS rats. (A) Shannon index. (B) Simpson index. (C) Ace index. (D) Chao1 index. (E) Principal co-ordinate analysis. (F) Relative abundance of distinguishable phyla. (G) Relative abundance of distinguishable families.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the CON group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the CUMS group. P values were verified by the Benjamini and Hochberg correction post hoc test.

Figure 3

The relevance between the short-chain fatty acid content in the brain and the gut microbiome. (A) The levels of SCFAs in the serum. (B) The levels of SCFAs in the brain. (C) A matrix is used to describe SCFA–microbe correlations. The depth of the color of the square indicates the magnitude of the correlation, where blue squares represent positive correlations and red squares represent negative correlations. White asterisks indicate the significance of the correlation (* $q < 0.1$, ** $q < 0.05$). In (A) and (B), black asterisks indicate the significance of the correlation * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the CON group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the CUMS group.

Figure 4

Effects of rifaximin on microglia in the hippocampal DG of CUMS rats. (A) Iba-1 immunoreactivity of microglia in the DG. (B) Immunofluorescence for Iba-1 (red) and iNOS(Green). (C) Immunofluorescence for Iba-1 (red) and Arg-1(Green). (D) The total number of microglia. (E)The poroportion of Iba-1+/iNOS+ (M1) cell. (F) The poroportion of Iba-1+/Arg-1+(M2) cell. (G) The number of Sholl intersections. (H)The

mean number of intersections. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. the CON group, # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. the CUMS group.

Figure 5

Effects of rifaximin on neurogenesis in the hippocampal DG of CUMS rats. (A) Immunofluorescence for DCX (red) and DAPI (blue) in the DG. (B) Length measurement of DCX+ cells. (C) The number of newborn neurons (DCX+). (D) The length of DCX+ cells. (E) The number of immature neurons (Ki67+/NeuN+). (F)

Immunofluorescence for Ki67 (red) and NeuN (green) in the DG. (G) Immunofluorescence image depicting the migration of immature neurons (Ki67 +/NeuN +). (H) The width of the GCL. (I) The migration index of immature neurons. (J) The number of mature neurons (NeuN+). Values represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the CON group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the CUMS group.

Figure 6

(A) Changes in dendritic spines in the DG were examined by Golgi staining. (B) Immunofluorescence image depicting the Iba-1+/CD68+ cells. (C) The total number of dendritic spines. (D) The number of

mushroom spines. (E) The number of Iba-1+/CD68+ cell. Values represent the mean $\pm$ SEM.
 *P<0.05,**P<0.01,***P<0.001vs. the CON group, #P<0.05, ##P<0.01, ###P<0.001 vs. the CUMS group.

Figure 7

Sodium butyrate regulates the polarization of cells in vitro. (A) Representative FACS plots showing CD86 and CD206 expression gated on microglial cells treated with LPS or SB. (B) The proportion of M1 phenotype microglia. (C) The proportion of M2 phenotype microglia. (D) The level of TNF-α. (E)The level

of IL-1 β . (F) The level of IL-10. (G) The level of IL-1ra. *P<0.05, **P<0.01, ***P<0.001 vs. the CON group, #P<0.05, ##P<0.01, ###P<0.001 vs. the LPS group.

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