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Magnoflorine alleviates colitis-induced anxiety-like behaviors through regulating gut microbiota and microglia mediated neuroinflammation

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Abstract

Background: Inflammatory bowel disease (IBD) and anxiety are often comorbid, and are interconnected through the microbiota-gut-brain axis. The therapeutic medications for anxiety are often constrained by adverse effects that limit their long-term use. The pursuit of natural, safe drug for anxiety is important, with the precise mechanisms elucidating the interplay between drugs and the gut-brain axis in modulating mood remaining elusive.

Results: We revealed a significant association between active ulcerative colitis(UC) patients and anxiety. Mendelian randomisation analysis suggested that UC has a causal relationship on anxiety, but not on depression. Next we identified *Ziziphus jujuba,* a natural plant, as a dual

therapeutic agent for both UC and anxiety through Batman database. Magnoflorine, as the predominant compound found in *Ziziphus jujuba*, exhibits promising therapeutic properties for the treatment of UC and anxiety disorders. Our experiments found that magnoflorine not only alleviated colitis, but also reduced colitis-induced anxiety behaviors through gut microbiota. Mechanistically, magnoflorine could increase the abundance of *Odoribacteraceae* and *Ruminococcus*, regulate bile acid metabolism, especially hyodeoxycholic acid (HDCA) in colitis mice. HDCA supplement could alleviate both colitis and colitis-induced anxiety. Meanwhile HDCA could inhibit the binding site of lipopolysaccharide to the TLR4/MD2 complex, thereby inhibiting microglia activation and alleviating neuroinflammation.

Conclusion: Our study unveils that magnoflorine alleviates colitis-induced anxiety-like behaviors through regulating gut microbiota and microglia mediated neuroinflammation, which has the potential therapeutic for IBD comorbid with anxiety disorders.

Keyword：Inflammatory Bowel Disease; Anxiety; Gut-brain axis; Microbiota; Microglia

Background

Inflammatory bowel disease (IBD) is a prevalent condition in northern Europe and North America, with a rapid increase reported in Asia[1]. It is characterized by chronic and incurable inflammation of the gastrointestinal

tract. Gut microbial dysbiosis has been consistently associated with intestinal inflammatory diseases, including IBD. Evidence indicates that reduced diversity and richness of gut microbiota correlate with an elevated risk of colitis[2]. The microbiota plays a crucial role in the development of IBD[3]. Metabolomic and metagenomic profiling have revealed distinct differences in microbial and metabolite compositions between IBD patients and healthy individuals[4]. Alterations in the microbiota contribute to IBD through multiple mechanisms. One key mechanism is the production of metabolites by the gut microbiota. Specific classes of metabolites, such as bile acids, short-chain fatty acids (SCFAs), and tryptophan metabolites, have been implicated in the pathogenesis of IBD. These metabolites can affect the host through various mechanisms, including modulation of immune responses and inflammation[5].

IBD has a high prevalence of comorbid anxiety and depression. Multiple studies have shown that the prevalence of anxiety is increased in patients with IBD compared to the general population. The prevalence for symptoms of anxiety in patients with IBD estimates range from 5% to 20.7%[6]. The anxiety and depression have been associated with a more aggressive presentation of IBD[7]. They can also affect disease activity and increase the risk of hospital readmissions in individuals with IBD. Anxiety and IBD are interconnected, with each influencing the other through gut-brain axis[8]. The gut-brain axis is a bidirectional communication system between the gut and the brain. It involves complex interactions between the central nervous

system (CNS), the enteric nervous system (ENS), the gut microbiota, and the immune system[9]. Changes in the gut microbiota, gut inflammation, and gut permeability can affect brain function and contribute to the development of psychiatric symptoms[6]. Individuals with IBD were 34% more likely to initiate antidepressants in the year following IBD diagnosis compared to controls without IBD[9]. The potential benefits of antidepressants in IBD are as follows: treating anxiety and depression; reducing pain and improving sleep[10]. Antidepressants can have various side effects, such as gastrointestinal effects, sexual dysfunction, weight changes, dizziness and drowsiness, changes in blood pressure and heart rate. The prevalence of antidepressant medication use among patients with IBD has been steadily increasing in recent years. However, a significant proportion of these patients, approximately two-thirds, do not adhere to the full course of treatment[9]. Finding a medication that treats both IBD and anxiety with few side effects is critical.

Magnoflorine is the main ingredient in *Ziziphus jujuba*, which is heavily used in Chinese medicine to treat insomnia and other neurological disorders. Magnoflorine has a wide range of pharmacological effects, including anti-diabetic activity; anti-inflammatory activity; cardiovascular effects; neuropsychopharmacological activity: anti-anxiety; antifungal activity; immunomodulatory activity and antioxidant activity. Based on the available studies, magnoflorine has shown no cytotoxicity to most cells and has been

considered relatively safe[11]. Hence magnoflorine has a potential function to treat IBD and anxiety with less side effects.

In this investigation, we scrutinized the impact of magnoflorine on gut microbiota, and substantiated the amelioration of colitis and anxiety-related behaviors by magnoflorine through three rescue experiments (Antibiotic Intervention Experiment, fecal microbiota transplantation and sterile fecal filtrate transplantation), and then elucidated the mode of action of magnoflorine on colitis and colitis-induced anxiety. Relying on intestinal microbiota and their metabolites, we subsequently probed the pivotal intestinal bacterial metabolite hyodeoxycholic acid (HDCA), and comprehensively probed the mechanism of action of HDCA on neuroinflammation via modulation of the TLR4/Myd88 pathway.

Methods

Clinical investigation

Patients diagnosed with UC from four hospitals participated in the completion of clinical questionnaires assessing anxiety, depression, sleep quality, and overall quality of life. Questionnaires utilized for this evaluation included Generalized Anxiety Disorder 7-item Scale (GAD-7), Patient Health Questionnaire- 9 (PHQ-9), Pittsburgh Sleep Quality Index (PSQI), and Inflammatory Bowel Disease Quality-of-Life Questionnaire (IBD-Q)

Inclusion criteria for this study encompassed individuals diagnosed with

UC and age ≥ 18. Exclusion criteria involved patients who declined participation in the clinical survey, individuals lacking comprehension of the questionnaire content, those with documented mental disorders, comorbid malignant tumors, neurological disorders, chronic disorders affecting systems beyond the gastrointestinal tract, such as heart disease and renal conditions, as well as those incapable of self-care. The clinical investigation was approved by the ethics committee of the Tianjin Medical University, Tianjin, China (Approval NO. IRB2024-YX-152-01)

Mendelian randomisation

We used two-sample MR to assess causality mediating between UC, anxiety and depression. All data were publicly available genome-wide association studies (GWAS) summary statistics. GWAS searches of summary statistics were performed to extract polymorphic genetic instrumental variables(IVs) for single nucleotide polymorphisms (SNPs) associated with UC, anxiety or depression. The genetic data for UC and depression were obtained from a recently published paper[12]. And genetic data for anxiety were obtained from PGC dataset. The IVs were chosen based on the following criteria: (1) SNPs associated with each genus at the genome-wide motif significance threshold ($P < 1.0 \times 10^{-7}$ for UC, $P < 1.0 \times 10^{-7}$ for anxiety, $P < 1.0 \times 10^{-8}$ for depression) were identified as potential IVs; (2) The clumping parameters were set to $r^2 < 0.001$, and kb = 10,000 kb in order to

ensure independence among the selected SNPs; (3) Positive stranded alleles were used to infer positive stranded alleles using allele frequency information when palindromic SNPs were present. Next, the strength of the IVs was examined using F-values (F=[beta/Se]2), weak IVs with F<10 were removed. We mainly applied inverse variance weighted (IVW) as the main analysis to combine the SNP-speciffc estimates calculated using Wald ratiosto, for detecting the effect of UC on anxiety and depression or anxiety and depression on UC. We first harmonized the exposure and outcome data to comparison of effector alleles in the positive strand, if specific or can be inferred based on allele frequencies. Echoes of the gene variants were discarded for further MR analysis. Next, Mendelian randomization analysis, heterogeneity test, multiple validity test, and graphing were performed using TwosampleMR package in R(version: 4.3.1). In addition, we applied Leave-one-out analysis to assess the impact of each SNP on the estimation.

Animal experiments

Specific pathogen free (SPF)-grade female C57BL/6J mice, aged 6-8 weeks, were obtained from Fukang (Beijing, China) and housed in the SPF-grade animal facility at the Affiliated Hospital of Chengde Medical College. The mice were housed in groups of 4-5 per cage and provided with a standard pellet diet and ad libitum access to water. The animal facility maintained a controlled environment with a 12-hour light/dark cycle and a temperature of

25 ± 2 °C. Following a 7-day acclimatization period, a colitis model was induced by administering 3% DSS (40 KDa) in the drinking water for a duration of 7 days. All animal experiments were approved by the ethics committee of the Tianjin Medical University, Tianjin, China (Approval No. IRB2022-DWFL-074)

Magnoflorine intervention

Thirty-two C57BL/6J mice were weighed after a one-week acclimatization period following their purchase. The experiment commenced when their body weight reached approximately 20 g. The mice were divided into four experimental groups, each receiving different treatments. The groups and treatments were as follows: 1. Control group: mice were provided with free access to water and received PBS solution by gavage.2. Mag group: mice were provided with free access to water and received PBS solution containing magnoflorine (10 mg/Kg/d) by gavage for 10 days. 3. DSS group: mice were provided with free access to water for the first three days, followed by ad libitum access to 3% DSS for the remaining seven days. They received PBS solution by gavage. 4. Mag + DSS group: mice were provided with free access to water for the first three days, followed by ad libitum access to 3% DSS for the remaining seven days. They received PBS solution containing magnoflorine by gavage(10 mg/Kg/d) for 10 days. The optimal administration concentration of magnoflorine was determined through our pre-experimental validation process.

Antibiotic Intervention Experiment: Eighteen 6-8-week-old C57BL/6 mice were divided into three groups: ABx-C, ABx-D, and ABx-MD. The experimental procedures for each group were the same as the Control, DSS, and Mag + DSS groups, respectively. However, before the colitis model was induced, the mice in all three groups were given an antibiotic cocktail (penicillin 200 mg/L, neomycin 200 mg/L, metronidazole 200 mg/L, and vancomycin 100 mg/L) ad libitum for 2 weeks.

During the course of the experiment, the body weight of the mice was recorded on a daily basis. The disease activity index (DAI) score was utilized to evaluate the severity of colitis based on weight loss, stool condition, and the presence of blood in the stool[13]. On the fifth day of the experiment, fecal samples were collected from each group of mice and stored at -80°C for further analysis. On the eighth day, under chloral hydrate anesthesia, Brain tissues were obtained and the hippocampal region were isolated. Some of the brain tissue samples were partially frozen at -80℃, while others were fixed in 10% formalin for subsequent investigations. The entire colon was collected, and the length was measured. A portion of the colon tissue was frozen at -80℃, while the other portion was fixed in 10% formalin for further studies.

FMT and SFF

In the present study, we conducted experiments in which we simultaneously concurrently provided nourishment to 10 mice, serving as fecal donors for subsequent fecal microbiota transplantation (FMT) and

sterile fecal filtrate (SFF). The feeding conditions were consistent with those mentioned earlier. Additionally, we administered PBS/Mag to two separate groups of mice via oral gavage, with each group consisting of 5 mice. This administration was carried out for a duration of 8 days, followed by a cessation period of 3 days. The purpose of this cessation was to collect fecal samples for subsequent FMT and SFF procedures. Then we collected the stool and dissolved that in sterile PBS at a ratio of 200 mg feces to 2 ml PBS and mixed thoroughly. The suspension was filtered through a sterile 700 mesh filter and subsequently centrifuged at 600 g for 5 minutes. This process allowed for the collection of the supernatant, which was used for FMT. The supernatant was further filtered through a sterile 0.22 um filtration membrane to ensure sterility before being used for SFF[14].

FMT: Twelve 6-8-week-old C57BL/6 mice were divided into two groups: FMT-Control and FMT-Mag. After 2 weeks of antibiotic cocktail intervention, the mice in both groups received fecal bacteria via gavage for 3 days at a volume of 200 ul/day. Following the fecal transplantation, the mice in both groups were given ad libitum access to 3% DSS for 5 days.

SFF: Twelve 6-8-week-old C57BL/6 mice were divided into two groups: SFF-Control and SFF-Mag. After 2 weeks of antibiotic cocktail intervention, the mice in both groups received a colony metabolite transplantation via gavage for 10 days at a volume of 200 ul/day. In the last 5 days, the mice in both groups were given ad libitum access to 3% DSS for 5 days.

HDCA intervention

Twenty 6-8-week-old C57BL/6 mice were divided into four groups: 1. Control group: mice were provided with free access to water and received 0.5% carboxymethylcellulose sodium(CMC) solution by gavage. 2. HDCA group: mice were provided with free access to water and received 0.5%CMC solution containing HDCA (500 mg/Kg/d) by gavage for 10 days. 3. DSS group: mice were provided with free access to water for the first three days, followed by ad libitum access to 3% DSS for the remaining seven days. They received 0.5%CMC solution by gavage. 4. HDCA + DSS group: mice were provided with free access to water for the first three days, followed by ad libitum access to 3% DSS for the remaining seven days. They received 0.5%CMC solution containing HDCA by gavage(500 mg/Kg/d) for 10 days[15].

Behavioral Experiments

The mice were given a 30-minute acclimatization period upon entering the laboratory room from the rearing room before the behavioral experiments began. During this acclimatization period, the mice were allowed to adjust to their new environment. After the acclimatization period, the behavioral experiments were initiated. The activities of each mouse were recorded for a total of 5 minutes. However, for the analysis, only the activities of the mice during the last 4 minutes of the recording were analyzed using ImageJ software. The software was used to analyze and quantify the specific

behaviors or activities exhibited by the mice during this time period.

1. Open Field Test (OFT): The Open Field Test is commonly used to assess the anxiety level and locomotor/exploratory ability of mice. In this test, mice are placed in an open arena (50 x 50 x 50 cm) and their behavior is recorded. Parameters such as total movement distance, central area movement distance, and central area exploration time are measured and analyzed to evaluate the mice's locomotor and exploratory abilities.

2. Elevated Plus Maze Test (EPT): The Elevated Plus Maze Test is another commonly used test to assess anxiety levels and exploratory behavior in mice. The maze consists of two open arms and two closed arms, elevated above the ground. Mice are placed on the maze and their behavior is recorded. Parameters such as exploration time in the open arm, stay time in the closed arm, and the number of times entering the open arm are measured to assess the mice's exploratory abilities and anxiety levels.

3. Tail Suspension Test (TST): The Tail Suspension Test is used to assess depression-related behaviors in mice. In this test, mice are suspended by their tails for a specific period of time, and their immobility time is recorded. Increased immobility time is considered an indicator of depressive-like behavior.

4. The Forced Swimming Test (FST) was employed to evaluate depression-related behaviors in mice. A transparent vessel with dimensions of 30 cm in height and 20 cm in diameter was utilized for this experiment.

The vessel was filled with tap water, and the temperature was maintained at 23-25°C. The liquid level in the vessel was standardized for each experiment. The mice were gently grasped by their tails and placed vertically into the water-filled vessel. The duration of immobility, defined as the absence of any active movement, was recorded during the test.

These behavioral tests are commonly used in preclinical research to assess the effects of various interventions or conditions on the behavior of mice, including anxiety and depression-related behaviors.

Histology analysis and PAS staining

Formaldehyde-fixed colon tissues were processed to create paraffin sections with a thickness of 5 micrometers. This was done by treating the tissues with a series of alcohol gradients, followed by xylene and paraffin embedding. Hematoxylin-eosin (H&E) staining was then applied to the sections. The stained sections were analyzed using ImageJ software to evaluate various aspects of the tissue pathology, including inflammatory infiltration, crypt structural alterations, and ulceration. The software was used to analyze the images and assign scores to quantify the severity of these pathological features[16]. In addition to hematoxylin-eosin staining, periodic acid Schiff (PAS) staining was conducted on paraffin-embedded tissue sections. The procedure involved dewaxing in xylene, rehydration in ethanol, and rinsing in distilled water. Sections were oxidized with periodic acid for

15 minutes, then stained with Schiff reagent for 10 minutes and rinsed again for 5 minutes. Counterstaining with hematoxylin was performed for 1 minute, followed by washing, dehydration, and sealing with neutral resin for microscopic examination. The number of positive cells in each crypt was subsequently counted.

Immunohistochemistry and immunofluorescence staining

Colorectal and brain tissues were processed into paraffin sections with a thickness of 5 micrometers. The sections underwent a series of steps including xylene treatment, gradient alcohol dehydration, thermal repair using citrate buffer, and antibody incubation. For the colon tissue, MUC2 antibody (1:1000, PROTEINTECHGROUP, USA) was used, while for the hippocampal tissue, IBA1 antibody (1:300, ABclonal, Wuhan, China) was employed. The incubation with primary antibodies was carried out overnight at 4°C, followed by washing with PBS three times for 3 minutes each. Subsequently, the sections were subjected to secondary antibody incubation for 20 minutes, followed by another round of PBS washing. Diaminobenzidine hydrochlo-ride (DAB) stain was performed for 1 minute, and the sections were observed after sealing. The number of MUC2-positive cells in each colonic crypt was counted, and the morphology of microglia was evaluated using ImageJ software.

For immunofluorescence staining, heat repair was performed, and the

colon and hippocampal tissue sections were blocked with 5% goat serum for 20 minutes. Anti-PV1(1:200, ABclonal, Wuhan, China) , anti-ZO-1(1:200, ABclonal, Wuhan, China) were incubated with the sections for 48 hours at 4°C. After washing with PBST (PBS with Tween-20) three times for 5 minutes each, the sections were incubated with fluorescent secondary antibodies for 1 hour. Following another round of PBST washing, the nuclei were stained with 4’,6-Diamidino-2’-phenylindole (DAPI). The sections were washed again with PBST and sealed with an antifluorescent mounting medium for fluorescence microscopy. The relative fluorescence intensity was evaluated using ImageJ software.

Cell culture and drug treatment

The BV2 microglia cell line was purchased from the Chinese Type Culture Conservation Centre. BV2 microglia cells were cultured in Dulbecco's-modified Eagle's medium F12 (DMEM F12) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) with 1% penicillin/streptomycin at 37°C in an incubation environment of 5% CO_2. BV2 microglia cells were implanted into 6-well plates and treated with HDCA (25uM, 50uM, 100uM) for 1 h, followed by Lipopolysaccharide (LPS 100ng/ml) for 24 hr. Cells were subsequently collected to extract the total RNA and protein for RT-PCR and WB.

Real-time polymerase chain reaction

Total RNA was extracted from intestinal and brain tissues using the RNeasy mini kit (Qiagen, Carlsbad, CA, USA), with RNase-free water used for elution. The concentration and purity of the obtained RNA were assessed using a nano drop spectrophotometer (Thermo Fisher Scientific, USA). For cDNA synthesis, 1000 ng of total RNA per sample was utilized and reverse transcribed using the TIANScript RT kit according to the manufacturer's instructions (TIANGEN, Inc. Beijing, China). Real-time PCR analysis was performed using the StepOnePlus Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). The primer sequences for target genes are provided in Additional file 2: Table S1.

Western blotting

Tissue samples were lysed in RIPA buffer containing protease and phosphatase inhibitors. The proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinyl dene fluoride (PVDF) membrane. Then the membrane was blocked in 5 % BSA at room temperature for 20 min. Subsequently, the primary anti-CLDN3 (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-ZO-1(1:1000, ABclonal, Wuhan, China), anti-TLR4(1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-Myd88(1:1000, Cell Signaling Technology, Danvers,

MA, USA), anti-IL-6(1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (1:5000, Cell Signaling Technology, Danvers, MA, USA) and anti-GAPDH(1:1000, ABclonal, Wuhan, China) were suppled. The chemiluminescent signal was detected using ECL following incubation with appropriate secondary antibodies. Band intensity was analyzed using ImageJ.

Molecular docking analysis

The amino acid sequence and protein domain were retrieved from PDB database (https://www.rcsb.org/). The structure of HDCA was retrieved from PubChem database. The TLR4/MD2/LPS complex was retrieved from PDB database, which was then visualized in PyMOL software (Schrodinger). The ligand-binding domain of TLR4/MD2 complex to HDCA was as the same as to LPS. The ligand was docked to TLR4/MD2 complex in Autodock software, which was then visualized in PyMOL software.

Gut microbiota analysis

16S rRNA gene sequencing was performed at the Sangon BioTech Institute (Shanghai, China) to analyze the microbial composition. Genomic DNA extraction from the total community was carried out using the E.Z.N.A™ MagBind Soil DNA Kit (Omega, M5635-02, USA). The V3-V4 region of the 16S rRNA gene was amplified using the 2x Hieff® Robust PCR Master Mix (Yeasen, 10105ES03, China) with specific PCR primers: forward primer

(CCTACGGGNGGCWGCAG) and reverse primer (GACTACHVGGGTATCTAATCC). The PCR products were then subjected to sequencing using the Illumina MiSeq system (Illumina MiSeq, USA). The resulting sequence tags were clustered into operational taxonomic units (OTUs) with a ≥ 97% similarity threshold using Usearch software (version 11.0.667). Principal coordinates analysis (PCoA) and analysis of similarities (ANOSIM) with a 999 permutations were employed to assess whether inter-group differences were significantly greater than intra-group differences. Differences between groups were compared using Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3) and Linear discriminant analysis (LDA) effect size (LEfSe) (version 1.1.0). Correlation coefficients and p-values between microbial communities/OTUs and mouse characteristics were calculated using the Weighted correlation network analysis (WGCNA) package in R software (version 4.3.1).

RNA sequencing analysis

RNA sequencing analysis was performed by Sangon Biotech, Inc. (Shanhai, China). Total RNA was extracted from colon tissues using the Total RNA Extractor (Sangon Biotech , Shanhai, China). Quality and quantity of RNA was analysed by Qubit2.0 RNA kit and Bioanalyzer FR-980A(FURI Science, Shanhai, China). Samples were submitted to Sangon Biotech for library preparation, using Hieff NGS™ MaxUp Dual-mode mRNA Library Prep Kit

and sequencing using DNBSEQ-T7 platform (Illumina). Further analyses were performed by R software, including alpha diversity, PCoA, differential expression analysis, GO analysis, KEGG analysis.

LC-MS

The main BA profiles in brain were determined by Liquid Chromatograph Mass Spectrometer (LC-MS). The brain sample weighing 30 mg was prepared by adding 100 μL of water and 500 μL of pre-cooled methanol solution. Subsequently, 10 μL of 200 ng/mL internal standard was added, followed by vortexing for 60 seconds. The sample was subjected to low-temperature sonication for 30 minutes, repeated twice, and then left at -20℃ for 1 hour. Afterward, an additional 10 μL of 200 ng/mL internal standard was added, vortexed for 60 seconds, sonicated at low temperature for 30 minutes (twice), and left at -20℃ for 1 hour. The resulting mixture was subjected to centrifugation at 14,000 rcf at 4℃ for 20 minutes. The supernatant was collected and freeze-dried. The BA concentration was quantified using LC-MS.

Statistical analysis

All data were represented as means±SEM and analyzed using GraphPad Prism 9.0 program. Data from two groups were compared using independent-samples t-test, while comparisons among more than two groups

utilized one-way ANOVA followed by Tukey’s multiple comparison tests. A p-value of less than 0.05 was considered statistically significant.

Result

UC combined with anxiety and depression affects patients' quality of life

A total of 137 patients with UC were included, including 90 patients with active stage and 47 patients with remission. Age, gender and weight were not associated with disease activity in UC patients ($P > 0.05$) (Additional file 2: Table S2).

We found the proportion of anxiety during the active stage was significantly higher than in remission ($P < 0.05$), with notable differences in anxiety severity across varying disease activity levels ($P < 0.05$). Similarly, the proportion of depression in the active stage exceeded that in remission ($P < 0.05$), and differences in depression severity were also significant among different disease activity levels ($P < 0.05$). Furthermore, the proportion of sleep disturbances was significantly higher in the active stage compared to remission ($P < 0.05$), although no differences were found across disease activity levels ($P > 0.05$). Additionally, the proportion of individuals experiencing poor quality of life was significantly greater in the active stage ($P < 0.05$) than in remission (Additional file 2: Table S3, Table S4 and Fig. 1A).

Moreover, the GAD-7, PHQ-9, and PSQI scores were higher in active UC than in remission ($P < 0.05$), and the IBD-Q scores were smaller in active UC ($P < 0.05$) than in remission (Additional file 2: Table S5). Linear regression analyses of GAD-7 and PHQ-9 with PSQI and IBD-Q scores revealed that anxiety ($R^2 = 0.379$, $P < 0.0001$), depression ($R^2 = 0.475$, $P < 0.0001$) and quality of life were linearly correlated, while anxiety ($R^2 = 0.072$, $P < 0.0001$), depression ($R^2 = 0.145$, $P < 0.0001$) and sleep disturbances were linearly correlated (Fig. 1B). These results suggested that UC combined with anxiety and depression affected the progression of disease and the quality of life.

Bidirectional two-sample Mendelian randomization revealed UC has causal relationship on anxiety

Previous investigations have unveiled a proximal interplay among anxiety, depression, and UC through observational methods. However, the precise direction of causation remains obscure. Herein, we expounded upon the causal associations between anxiety, depression, and UC employing a bidirectional two-sample Mendelian randomization approach. According to the selection criteria of IVs, the SNPs were used as IVs (Additional file 2: Table S6-S9). Our study disclosed that UC exhibited a notable linkage with anxiety (OR: 1.069, 95% CI: 1.023-1.117, $P < 0.01$), while its correlation with depression was statistically nonsignificant (OR: 1.047, 95% CI: 0.928-1.183,

P = 0.454) in the forward regression. Notably, no inheritable predisposition toward anxiety manifests a relationship with UC upon reverse analysis (OR: 1.005, 95% CI: 0.839-1.206, P = 0.949). In contrast, our bidirectional Mendelian randomization assessments fail to corroborate a significant association between UC and depression (Fig. 1C). Subsequent scrutiny through tests for heterogeneity (P > 0.05) and horizontal polytropy (P > 0.05) on IVs attests to the absence of heterogeneity or horizontal polytropy. Mendelian randomization exclusion sensitivity analysis revealed that removing any specific SNP did not affect the results (Additional file 1: Fig. S1).

Magnoflorine has potential ability to treat UC and anxiety

Utilizing the GeneCards and Batman databases, we employed a targeted approach for pharmaceutical screening to address the therapeutic management of UC and anxiety disorders (Fig. 2A). Initially, a comprehensive search operation was conducted in the GeneCards database to identify genes associated with UC and anxiety, setting a stringent threshold (Scores > 5), utilizing the keywords “Ulcerative colitis” and “Anxiety”. This exploratory phase yielded a remarkable total of 728 genes linked to UC and 190 genes linked to anxiety, with an intriguing convergence of 46 genes overlapped between the two conditions, thereby implicating these specific genetic loci in the co-occurrence of UC and anxiety.

Subsequent interrogation of the Batman database targeted TCM associated with the identified 46 overlap genes. Intriguingly, our analysis underscored *Ziziphus jujuba* as the TCM exhibiting the strongest association with these genetic targets (P = 7.41e-28). Delving into the pertinent literature pertaining to *Ziziphus jujuba*, we uncovered documented evidence regarding its potential efficacy in ameliorating symptoms of UC and anxiety[17,18]. While the precise mechanistic underpinnings remain unexplored, our investigative foray unveiled Magnoflorine as the principal bioactive constituent of *Ziziphus jujuba*, renowned for its diverse pharmacological attributes encompassing anti-inflammatory and antidepressant properties[19,20]. Motivated by these intriguing findings, we prioritize Magnoflorine as the focal point of our investigative pursuits to delineate its therapeutic impact on experimental colitis and anxiety-related behaviors through meticulously designed animal experimentation protocols.

Magnoflorine alleviated DSS-induced colitis

To evaluate the effect of magnoflorine on colitis, we induced a mouse model of colitis with 3% DSS orally for 7 days, and observed the phenotype of mice after administration of magnoflorine (10 mg/Kg/d) by gavage. The experimental procedure is shown in Fig. 2B. We determined the optimal concentration of magnoflorine in our previous pre-experiments.. In contrast to DSS, magnoflorine significantly alleviated DSS-induced colitis, and the

relevant evidence was that magnoflorine reversed weight loss, decreased DAI scores (Fig. 2C), and alleviated colon length shortening (Fig. 2D). Pathological analyses further indicated that magnoflorine reduced colonic inflammatory cell infiltration, decreased crypt destruction, and lowered pathological scores (Fig. 2F). To further investigated the effect of magnoflorine on colonic inflammation, we examined the levels of pro-inflammatory factors in colonic tissues. As shown in Fig. 2E, compared with the DSS group, the mRNA levels of TNF-α, IL-1β, and IL-6 were reduced in the Mag+DSS group.Taken together, these results demonstrated that magnoflorine can alleviate colitis symptoms and colonic damage in mice with colitis

The colonic epithelial barrier plays a pivotal role in the pathogenesis of IBD, and tight junctions are critical components of the colonic epithelial barrier. In our study, we observed reduced expressions in tight junctions including ZO-1 and Claudin 3 in colitis mice through western blotting, while magnoflorine treatment effectively reversed this decline (Fig. 3A). Additionally, we employed immunofluorescence staining to investigate the expression of ZO-1. Our data exhibited a decline in ZO-1 expression in colitis mice (Fig. 3B), which was attenuated by magnoflorine treatment. The mucins secreted by goblet cells are crucial for maintaining intestinal barrier integrity and preventing the infiltration of intestinal microbiota. In this study, we employed the PAS staining to assess the numbers of goblet cells in

colonic tissues. As shown in Fig. 3C, our findings revealed that magnoflorine effectively counteracted the reduction in goblet cell numbers observed in colitis. Furthermore, we employed immunohistochemical staining to examine the expression of MUC2, a key mucin protein, in the colonic tissues of colitis-induced mice. The results demonstrated a significant decrease in MUC2 expression, which was mitigated by the administration of magnoflorine. Collectively, these findings suggest that magnoflorine exerts its protective effects against DSS-induced colitis by preserving goblet cell function and maintaining the integrity of tight junctions.

Magnoflorine alleviated anxiety-like behaviors in mice with colitis

Patients with colitis often experience comorbid neurological disorders, such as anxiety and depression. In our study, we aimed to assess anxiety and depressive behaviors in mice subjected to colitis induction. To evaluate anxiety-like behaviors, we conducted the OFT and observed a reduction in exploration time and distance in the central area among colitis-induced mice. Additionally, in the EPT, colitis mice exhibited decreased exploration time and distance in the open arms, as well as a decrease in the number of entries into the open arms. These findings collectively indicated the presence of anxiety-like behaviors in colitis mice. However, treatment with magnoflorine reversed these results (Fig. 4A, Additional file1: Fig. S2), as evidenced by increased exploration time and distance in the center area of the OFT, as

well as increased exploration time in open arms, time in close arms, and the number of entries into the open arms in the EPT. Thus, our results demonstrated that magnoflorine ameliorated anxiety-like behaviors in colitis mice.

Furthermore, we investigated depressive-like behaviors in mice using the FST and the TST (Additional file1: Fig. S2). Surprisingly, acute colitis induced by DSS did not elicit depressive-like behaviors in the mice. Moreover, magnoflorine did not exert any impact on these behaviors.

Magnoflorine ameliorated neuroinflammation, reduced microglia activation and maintained blood-brain barrier

Anxious behavior in colitis mice has been reported to be associated with the blood-brain barrier[21]. Thus, we investigated on the blood-brain barrier, specifically focusing on ZO-1 and PV1(Fig. 4B). To demonstrate the impact, we employed immunofluorescence staining and our findings revealed a reduction in the expression of ZO-1 and PV1 within the choroid plexus of the lateral ventricle in mice with colitis. Intriguingly, the administration of magnoflorine successfully counteracted this decrease, and these results suggested that magnoflorine could maintain the blood-brain barrier.

Moreover, we assessed the activation level of microglia in the CA1 region of the hippocampus using immunohistochemical staining to label the expression of IBA1. As shown in Fig. 4C, our findings revealed that microglia

in the CA1 region of the hippocampus of colitis mice exhibited significant activation, and treatment with magnoflorine reversed this activation, as evidenced by the reduction in endpoints, and an increase of protrusion length , indicating its potential to modulate microglial activation.

Given the association between neuroinflammation and anxiety behavior, we sought to investigate the levels of pro-inflammatory factors in the hippocampal region of each experimental group. Our results indicated that colitis mice exhibited elevated expression of mRNA levels of TNF-α, IL-1β, and IL-6 (Fig. 4D). However, treatment with magnoflorine significantly reduced the expression of mRNA levels of pro-inflammatory factors. Collectively, these results suggest that magnoflorine may exert its protective effects against anxiety behavior by modulating neuroinflammation and microglial activation in the hippocampal region.

Magnoflorine regulated gut microbiota and promoted the enrichment of secondary bile acids-producing bacteria

Next, we further explored the impact of magnoflorine on the gut microbiota composition of each group via 16S rRNA gene sequencing. Magnoflorine increased the Alpha diversity of gut microbiota as shown by shannon index (Additional file1: Fig. S3A). Principal Coordinate Analysis (PCoA) revealed a distinct segregation among all experimental groups (Additional file1: Fig. S3B). Simultaneously, we observed a consistent fecal bacterial composition among all groups of mice at both the phylum and

genus levels. But in mice with colitis, the administration of magnoflorine resulted in a reduction of the Firmicutes phylum, an increase in the Verrucomicrobia phylum, and a decreased the Firmicutes/Bacteroidetes ratio (Fig. 5A). As shown in Fig. 5B and C, the application of LEfSe analysis revealed an enrichment of specific bacterial taxa within the Mag group at the family level, including *Odoribacteraceae, Clostridiales_Incertae_Sedis_XIll, Erysipelotichaceae, Streptomycetaceae, Eubadteriaceae, Pdrphyromohadaceae*, and *Sutterellaceae*, additionally, an enrichment of *Ihubacter, Odoribacter. Longibaculum, Amedibacillus, Streptomyces, Anaerofustis, Parabacteroides* and *Parasutterella* were observed at the genus level (LDA score > 2.0, $P < 0.05$). After DSS treatment, *Bifidobacteriaceae, Oxalobacteraceae* and *SpirocHaetaceae* were enriched at the family level in Mag+DSS group, and *Bifidobacterium, Ruminococcus, Butyricicoccus, Lachnospira* and *Rectinema* were enriched at the genus level in Mag+DSS group(LDA score > 2.0, $P < 0.05$). *Bifidobacterium, Ruminococcus*, *Oxalobacteraceae* and *Butyricicoccus* play a role in the regulation of bile acid metabolism[22–25]. In addition, we utilized the STAMP software to conduct an in-depth analysis of the dissimilarities between the experimental groups. Consistently, our findings revealed an enrichment of *Odoribacteraceae* in the Mag+DSS group (Additional file1: Fig. S3C), which is known to possess a secondary bile acid metabolism capability[26]. Subsequently, we employed the PICRUSt technique to predict the functional attributes of the groups,

620 followed by a differential analysis using the STAMP software. Intriguingly,
621 our results indicated a higher expression of the baiH gene in the Mag+DSS
622 group compared to the DSS group (Additional file1: Fig. S3D). Notably, this
623 gene is associated with secondary bile acid metabolism.
624 To delve deeper into the intricate association between microbial colonies
625 and their corresponding traits, we employed the WGCNA approach. This
626 method enabled us to cluster and establish a network of Operational
627 Taxonomic Units (OTUs). Furthermore, we performed power calculations
628 with a value of 7 to enhance the accuracy and reliability of our analyses
629 (Additional file1: Fig. S3E). Finally, we successfully constructed sixteen OTU
630 coexpression modules through our analysis. As shown in Fig. 5D, among
631 these modules, the blue module (eigengene value = 0.77, P = 0.001)
632 exhibited the strongest association with EPT(frequency of visits to open
633 arms), while the red module (eigengene value = 0.62, P = 0.02)
634 demonstrated the highest correlation with Mag+DSS. These two modules
635 were subsequently chosen for further investigation and analysis. At the
636 family taxonomic level, the Red module encompasses a total of 7 microbial
637 colonies, while the Blue module consists of 47 colonies. Notably, 4 colonies
638 are overlapped between these two modules (Additional file1: Fig. S3F).
639 These overlapping colonies are identified as unclassified_*Bacteroidales,*
640 *Lachnospiraceae, Ruminococcaceae*, and *Muribaculaceae*.
641

Magnoflorine alleviated colitis and anxiety-like behavior depending on microbiota

In light of the previous findings demonstrating the potential of magnoflorine to enhance the intestinal microbiota in mice, we sought to further investigate the role of the intestinal microbiota in this context. To achieve this, we employed a three-step experimental approach. Firstly, we administered an ABx to the mice as a pretreatment, followed by the administration of magnoflorine and induction of colitis using DSS(Fig. 6A). Our results showed no significant differences in body weight, DAI scores, colon length, and pathological damage between the ABx-D and ABx-MD groups (Fig. 6B, C and Additional file1: Fig. S4A and B). Furthermore, behavioral tests were conducted to assess the impact of the interventions (Fig. 6D). Notably, no significant differences were observed in the time in center area of OFT and the time in the open arms of EPT between the ABx-D and ABx-MD groups, interestingly, there were significant difference between the ABx-C and ABx-D groups. These findings provide valuable insights into the potential influence of the intestinal microbiota on the observed effects of magnoflorine in the context of colitis.

To elucidate the potential involvement of the microbiota in the observed effects, we conducted FMT experiments (Fig. 6E). Remarkably, our findings demonstrated that FMT-Mag effectively ameliorated the weight loss, DAI elevation, colon shortening, and histological damage observed in the colitis

mice (Fig. 6F, G and Additional file1: Fig. S4C and D). Moreover, behavioral assessments revealed notable improvements (Fig. 6H), as evidenced by an increased the time in the center area of OFT and the time in the open arms of EPT.

The previous findings indicated that FMT-Mag exhibited potential in ameliorating anxiety-like behaviors in colitis mice. However, the underlying mechanism by which the microbiota exerted its effects remained unclear, as the bacteria were unlikely to directly cross the blood-brain barrier. Therefore, we hypothesized that the beneficial effects might be mediated by the metabolites produced by the microbial colony. To investigate this, we conducted the SFF experiment (Fig. 6I). Remarkably, our results demonstrated that SFF-Mag exhibited the same effects as FMT-Mag, SFF-Mag effectively ameliorated the weight loss, DAI elevation, colon shortening, and histological damage observed in the colitis mice (Fig. 6J, K and Additional file1: Fig. S4E and F), and improved colitis-induced anxiety behiviours (Fig. 6L). That suggested that the metabolites derived from the magnoflorine-treated microbiota were responsible for the observed improvements in anxiety behavior.

These findings provide valuable insights into the potential mechanisms underlying the beneficial effects of microbiota regulated by magnoflorine and highlight the importance of microbial metabolites in mediating gut-brain axis communication.

The key bacteria altered by magnoflorine is associated with genes

We conducted RNA-seq analysis on colon tissue samples obtained from Mag+DSS group and DSS group. The results illustrated significant differences in the transcriptomes between two groups (Fig. 7A). Subsequently, we conducted a comprehensive analysis of the differentially expressed genes to identify enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Additional file1: Fig. S5A and B). As shown in Fig. 7B, we identified specific KEGG pathways that were significantly associated with the differentially expressed genes($P < 0.05$), including neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway and MAPK signaling pathway. These results provide valuable insights into the molecular mechanisms underlying these biological processes and pathways, which may have implications for next study.

Furthermore, we employed the Spearman correlation analysis to examine the associations between the differentially abundant microbiota and the differentially expressed genes (Fig. 7C). Specifically, we observed a positive correlation between Gabrg2 and all the differentially abundant microbiota, except for *Akkermansia*. Conversely, Twist1 displayed a negative correlation with the remaining components of the differentially abundant microbiota. These results shed light on the complex dynamics between host genes and the intestinal microbiota, emphasizing the potential significance of these

interactions in shaping host physiology and health.

Magnoflorine altered the bile acid metabolism in brain

Utilizing ADMETlab 2.0[27], we computed a LogP value of 0.395 and a BBB Penetration of 0.147 for magnoflorine, suggesting its limited ability to traverse the blood-brain barrier. Additionally, a comprehensive literature review revealed consistently low levels of magnoflorine and its metabolites within brain tissue[28,29], further corroborating its restricted brain accessibility. Given the previous findings highlighting the potential involvement of the intestinal microbiota and its predicted functions, particularly in bile acid metabolism, we hypothesized that magnoflorine may exert its effects on anxiety behavior in colitis mice by modulating bile acid metabolism. To test this hypothesis, we employed targeted metabolomics to assess the levels of bile acids in the brain tissues of colitis mice. Our results revealed the detection of a total of 27 bile acids, we identified a significant increase in HDCA and 7-ketolithocholic acid (fold change >2, $P < 0.05$) in the Mag+DSS group (Fig. 8A). However, HDCA showed the most significant difference in abundance between groups, so we chose HDCA for the follow-up study. These findings provide novel insights into the potential mechanisms underlying the effects of magnoflorine on anxiety behavior in colitis mice, highlighting the potential role of bile acid metabolism in mediating gut-brain axis communication.

HDCA alleviated anxiety-like behavior and neuroinflammation via TLR4/Myd88 pathway

To evaluate the effect of HDCA on colitis-induced anxiety, we induced a mouse model of colitis with 3% DSS orally for 7 days, and observed the phenotype of mice after administration of HDCA (500 mg/Kg/d) by gavage (Fig. 8B). HDCA could alleviate colitis (Additional file1: Fig. S6A and B) and colitis-induced anxiety (Fig. 8C), as evidence by increasing exploration time in the center area of the OFT, as well as increasing exploration time in open arms of EPT. HDCA significantly reduced the expression of mRNA levels of pro-inflammatory factors as shown in Fig. 8D. These suggested that HDCA had a role in alleviating colitis-induced anxiety

Previous studies and the literature have reported the role of microglia-mediated neuroinflammation involved in the development of anxiety[30], we conducted an experiment using BV2 cells. We exposed these cells to LPS and simultaneously treated them with HDCA. Our results revealed that LPS administration led to a significant upregulation of TNF-α, IL-1β, and IL-6 mRNA levels and active BV2 cells (Additional file1: Fig. S6C and D). However, the presence of HDCA inhibited this increase in a dose-dependent manner. This suggested that HDCA could alleviate microglia-mediated neuroinflammation.

To elucidate the underlying mechanism by which HDCA ameliorated

752 microglia mediated neuroinflammation, we conducted a thorough
753 investigation of relevant genes in microglia using the Genecard database and
754 performed KEGG pathway analysis (Fig. 8E). Our analysis revealed a
755 significant enrichment of relevant genes in the Toll-like receptor signaling
756 pathway ($P < 0.05$). Subsequently, we conducted molecular docking
757 experiments and observed that the ligand-binding domain (LBD) of
758 TLR4/MD2 complex to HDCA was consistent with that to LPS (Fig. 8F).
759 Based on these findings, we hypothesized that HDCA may competitively
760 inhibit the binding of LPS to the TLR4/MD2 complex, thereby suppressing
761 the downstream transmission of TLR4 signaling. To validate this hypothesis,
762 as shown in Fig. 8G, we induced an inflammatory response in BV2 cells using
763 LPS and observed a significant increase in the protein levels of Myd88 and
764 the downstream effector molecule IL-6. The same phenomenon has been
765 observed in animal experiment (Fig. 8H), indicating HDCA's potential role in
766 alleviating neuroinflammation. So, treatment with HDCA inhibited the
767 increase in these proteins, providing further evidence for our hypothesis.
768
769 **Discussion**
770 The intricate communication relationship between the gut and the brain,
771 known as the "gut-brain axis", is a crucial factor in the pathogenesis of
772 neurological disorders. Anxiety is a major global challenge, and its
773 prevalence in IBD has reached epidemic proportions, ranging from

774 5%-20.7%[6]. In this study, we aimed to investigate the specific role of the
775 gut-brain axis in the behavioral mechanism of colitis-induced anxiety. Our
776 findings demonstrated that magnoflorine regulated the intestinal microbiota
777 and increased the HDCA level in brain, alleviated colitis, colitis-induced
778 anxiety behavior and microglia mediated neuroinflammation.

779 Patients with UC are at a high risk of comorbid anxiety, which often
780 predicts a poor prognosis[31,32]. Guidelines recommend screening for
781 depression and anxiety for patients with IBD, and drugs for anti-neurological
782 disorders had effective results in IBD patients[33], but these medications often
783 come with side effects. Recent study has demonstrated that magnoflorine
784 possesses both anti-inflammatory and moderating effects on neurological
785 disorders[34], thus showing potential for development as a dual therapeutic
786 agent for IBD and anxiety-depression. A study investigating the gut
787 microbiota composition of UC patients with and without anxiety and
788 depression revealed distinct alterations in microbial diversity and abundance.
789 Notably, patients with UC combined with anxiety and depression exhibited a
790 reduced abundance and diversity of gut flora compared to those without
791 these psychological comorbidities[35]. Furthermore, specific bacterial taxa
792 were found to be associated with depression in IBD patients. *Ruminococcus*
793 and *Lachnospiraceae* were negatively correlated with depression severity[36].
794 These findings suggest a potential link between the depletion of certain
795 beneficial gut bacteria and the development of depressive symptoms in IBD

patients and that improving the gut microbiota can alleviate IBD-related anxiety and depression. In our study, magnoflorine administration alleviated colitis-induced anxiety behavior by suppressing microglia mediated neuroinflammation. Our study provides evidence for the potential therapeutic role of magnoflorine in treating colitis-induced anxiety through modulation of the gut microbiota and its metabolites..

The gut barrier is a multifaceted structure comprised of a mucus layer and microbiota, an epithelial layer, and a closely interconnected intrinsic layer of immune cells. This barrier acts as a defense mechanism, preventing the entry of harmful substances into the body through the gut, thereby safeguarding the body from potential harm. Disruption of its functionality has been linked to IBD[37]. The integrity of the intestinal barrier function is crucial in preventing the entry of pathogen-associated molecular patterns (PAMPs) such as LPS into the body, which can result in the disruption of the blood-brain barrier and ultimately lead to the development of anxiety[21,38]. The negative correlation between barrier function and anxiety development is evident in both the intestinal blood barrier and the blood-brain barrier[38,39]. Our investigation demonstrated that magnoflorine confered protection to the intestinal barrier function. This protection included the up-regulation of tight junction proteins such as ZO-1 and CLDN3, as well as an increase in the expression of goblet cells and MUC2. Furthermore, magnoflorine has been shown to enhance the integrity of the blood-brain barrier by upregulating the

818 expression of ZO-1 and PV1.

819 The regulation of gut barrier function is intricately associated with the
820 intestinal microbiota[40]. Certain probiotics, such as *Bifidobacterium*[41] and
821 *Ruminococcus*[42], have the potential to enhance intestinal barrier function.
822 Our investigation has provided evidence for the ability of magnoflorine to
823 modulate bacterial microbiota in both healthy and colitis mice. Specifically,
824 we observed an increase in the Verrucomicrobia phylum and a decrease in
825 the Firmicutes/Bacteroidetes ratio. Additionally, magnoflorine
826 supplementation led to an increased abundance of beneficial bacteria,
827 specially *Odoribacteraceae,* and a decreased abundance of harmful bacteria.
828 Subsequent FMT experiments demonstrated that FMT from
829 magnoflorine-treated mice effectively protected colitis. This suggests that
830 *Odoribacteraceae* may play a crucial role in preserving intestinal barrier
831 function. However, there is a notable absence of relevant studies
832 investigating the specific protective mechanisms of *Odoribacteraceae* in
833 colitis.

834 In addition, we also conducted a comprehensive transcriptome analysis of
835 colitis tissues. Our analysis revealed that differentially expressed genes were
836 significantly enriched in various pathways, including neuroactive
837 ligand-receptor interaction, MAPK signaling pathway, and PI3K Akt signaling
838 pathway. Correlation analysis further unveiled intriguing associations,
839 indicating that *Odoribacteraceae* and *Ruminococcus* exhibited a positive

correlation with Gabrg2, while displayed a negative correlation with Twist1. Notably, Twist1 is known to regulate immune cell function and upregulated in patients with corticosteroid-resistant UC[43,44]. This suggests that *Odoribacteraceae* may confer protection against colitis by inhibiting the expression of Twist1. However, it is imperative to conduct further experimental investigations to validate this hypothesis.

Inflammation plays a pivotal role in the pathogenesis of both IBD and neurological disorders. The association between neuroinflammation and anxiety has been extensively documented in numerous studies[36] . Neuroinflammation occurring in various brain regions has been linked to anxiety[30]. The hippocampus, situated between the thalamus and the medial temporal lobe, is a crucial component of the limbic system involved in cognitive function regulation. It exhibits an intricate neural network of connections with brain regions associated with emotions. Activation of microglia (identified by IBA1 labeling) in the hippocampus leads to the production of pro-inflammatory factors such as TNF-α, IL-1β, and IL-6, consequently promoting anxiety-like behaviors[30]. LPS serves as a potent stimulant in this regard[21]. Conversely, administration of an oral microglia inhibitor to mice alleviates anxiety-like behavior[46]. Notably, the morphology of microglia reflects their activation status. Our experimental findings revealed heightened microglia activation in the hippocampus and elevated levels of the aforementioned pro-inflammatory factors in mice with

colitis-induced anxiety. However, the administration of magnoflorine effectively inhibits microglia activation.

A study reported that proteins associated with UC combined with anxiety and depression clustered in the acute inflammatory response pathway[35], In the context of IBD, the intestinal tract serves as a conduit for transmitting inflammatory signals to the brain via three distinct pathways: the systemic-humoral pathway, cellular immune pathway, and neuronal pathway[47]. Disruption of the intestinal barrier function allows the entry of intestinal toxins and inflammatory response factors into the body through the gut-blood barrier. Consequently, these inflammatory factors and toxins, including LPS, can breach the blood-brain barrier and gain access to the brain[48]. This breach triggers the activation of immune cells within the brain, such as microglia, thereby provoking an inflammatory response that ultimately leads to neurological dysfunction, such as anxiety[21,48]. Similarly, enterobacterial metabolites can enter the body, some of which exhibit protective effects on the blood-brain barrier and can inhibit microglia activation. For instance, SCFAs[49] and bile acid[50] fall into this category. Our study utilized SFF experiments to demonstrate the ability of enterobacterial metabolites to mitigate anxiety behaviors induced by colitis. Our findings indicated that the distinct microbiota was linked to bile acid metabolism, as revealed by correlation and differential microbiota analyses. Consequently, we proceeded to investigate the levels of bile acids in brain tissues and

observed a significant increase in HDCA levels in the Mag+DSS group. However, there were still shortcomings in this experiment, as we only examined the concentration of bile acids in brain tissue, but did not performed faecal and blood metabolite assays.

HDCA, a natural secondary bile acid, has been shown to alleviate non-alcoholic fatty liver disease by inhibiting intestinal FXR and up-regulating hepatic CYP7B1[51]. Intriguingly, both FXR activation and inhibition in microglia did not modulate the production of pro-inflammatory mediators[52].Additionally, HDCA can alleviate sepsis by competitively inhibiting the binding of LPS to the TLR4/MD2 complex, and This inhibitory effect was confirmed by surface plasmon resonance (SPR) and cellular experiments[53]. Furthermore, HDCA has been demonstrated to alleviate LPS-induced inflammation by regulating the TGR5/AKT/NF-κB signaling pathway in microglia[54]. Tao Y et al[55]. report that TGR5 knockout mice display anxiety-like behavior. LPS can penetrate the brain by disrupting the blood-brain barriers[21], leading to microglia activation via the TLR4/Myd88 signaling pathway and ultimately resulting in neuroinflammation. Our experiments further confirmed that.

Conclusions

In conclusion, our investigation demonstrates that the oral administration of magnolforine modulates the gut-brain axis and mitigates colitis and colitis-induced anxiety behaviors by influencing intestinal *Odoribacteraceae*

and *Ruminococcus*, as well as the enterobacterial metabolite HDCA. Furthermore, HDCA competitively inhibits LPS binding to the TLR4/MD2 complex, leading to the inhibition of the TLR4/Myd88 signaling pathway and subsequent attenuation of neuroinflammation (Fig.9). Our investigation presents a novel perspective on the therapeutic approach to anxiety associated with IBD.

Authors' contributions

HLC and LW conceived and designed the study. LW, MFL, YD, JYW and SQQ performed the experiments. LYL and SQQ performed the analysis of the data. LW and MFL wrote the manuscript. BMW, BQL, and HLC revised the manuscript. All authors read and approved the final manuscript

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Data availability

The sequencing data are available on the NCBI site with the project's code

PRJNA1067339 and PRJNA1067185.

Declaration of Interest

Ethics approval and consent to participate

The clinical investigation was approved by the ethics committee of the Tianjin Medical University, Tianjin, China (Approval NO. IRB2024-YX-152-01). All animal experiments were approved by the ethics committee of the Tianjin Medical University, Tianjin, China (Approval No. IRB2022-DWFL-074)

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Fig.1 Relationship between anxiety, depression and UC. (**A**) Proportions of anxiety, depression, sleep disturbance and poor quality of life in UC patients with different disease activity. (**B**) Linear relationship between GAD-7, PHQ-9 and PSQI, IBDQ. (**C**) Mendelian randomization: Scatterplot of genetic association between anxiety, depression and UC. Statistical significance was determined using Tukey test. ****$P < 0.0001$, ns, not significant.

Fig.2 Magnoflorine alleviated DSS-induced colitis. (**A**) Schematic drug

screening. (**B**) Experimental scheme of the mouse trial (n = 8). Oral PBS and magnoflorine treatments were indicated. (**C**) Daily body weight and daily DAI scores changes throughout the DSS treatment duration of the study. (**D**) Images of the colon and the colon length in each group (n = 8). (**E**) Concentrations of three representative pro-infammatory cytokines at mRNA level in colon. (**F**) H&E staining of colon sections and pathological scores of colons (n = 8). Statistical significance was determined using one-way ANOVA, followed by Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig.3 Magnoflorine protected intestinal barrier function. (**A**) Magnoflorine increased ZO-1, CLDN3 expression in colon tissue. Representative Western blot images and densitometric quantification of ZO-1 and CLDN3. Protein levels were normalized to β-actin or GAPDH. (**B**) Representative immunofluorescence images and average fluorescence intensity of ZO-1. Magnification: 400 ×, Scale bars: 50μm. (**C**) Representative pictures of PAS staining and MUC2 immunohistochemical staining in colon tissue. Magnification: 400 ×, Scale bars: 50μm. PAS-positive cells and MUC2 production in colonic goblet cells was significantly enhanced in Mag+DSS group compared to DSS group. Statistical significance was determined using one-way ANOVA, followed by Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig.4 Magnoflorine alleviated colitis-induced anxiety behavior, relieved neuroinflammation and protected the blood-brain barrier in

mice. (**A**) Representative images of mouse trajectories in OFT and EPT experiments (n = 7). Magnoflorine increased the time and distance in center area of OFT experiments, increased the time in open arms and decreased the time in closed arms of EPT experiments. (**B**) Representative immunofluorescence images of ZO-1 and PV-1. Increased expression of ZO1 and PV1 in the choroid plexus of the lateral ventricle in the Mag+DSS group compared with the DSS group. (**C**) Representative immunohistochemistry images of Iba-1 in hippocampus CA1 region. Increased endpoints and decreased process length of microglia in the Mag+DSS group compared to the DSS group (n = 3). (**D**) Concentrations of three representative pro-infammatory cytokines at mRNA level in hippocampus. Statistical significance was determined using one-way ANOVA, followed by Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig.5 Magnoflorine regulated the composition and function of intestinal microbiota. (**A**) The relative abundance of fecal bacterial in phyla level. (**B**) LEfSe analysis of differences in the microbial taxa between the Control group and Mag group, and between the DSS group and Mag+DSS group (LDA > 2, $P < 0.05$). (**C**) The relative abundance of *Odoribacteraceae* and *Ruminococcus* between different groups. (**D**) WGCNA detected module-traits associations. In the heatmap, each row corresponded to a module eigengene (ME) and each column to a trait. Each cell contained the corresponding correlation and p-value. The traits of OFT refers to the time in

central area of OFT. The traits of EPT refers to the times enter in open arms.

Fig.6 Magnoflorine alleviated colitis and anxiety-like behavior depending on microbiota. (A) Study design for the ABx experiment (n = 5-6). (**B**) The colon length in each group of ABx. (**C**) DAI scores change throughout the DSS treatment duration of the ABx experiment. (**D**) Representative images of mouse trajectories in OFT and EPT experiments of the ABx experiment. (**E**) Study design for the FMT experiment (n = 5-6). (**F**) The colon length in each group of FMT. (**G**) DAI scores change throughout the DSS treatment duration of the FMT experiment. (**H**) Representative images of mouse trajectories in OFT and EPT experiments of the FMT experiment. FMT-Mag increased the time in center area of OFT experiments, increased the time in open arms of EPT experiments compared to FMT-Control. (**I**) Study design for the SFF experiment (n = 5-6). (**J**) The colon length in each group of SFF. (**K**) DAI scores change throughout the DSS treatment duration of the SFF experiment. (**L**) Representative images of mouse trajectories in OFT and EPT experiments of the SFF experiment. SFF-Mag increased the time in center area of OFT experiments, increased the time in open arms of EPT experiments compared to SFF-Control. Data were presented as means±SEM. Statistical significance was determined using one-way ANOVA, followed by Tukey test, or using Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig.7 Magnoflorine regulated transcriptome expression in colitis

tissues. (A) Volcano plots showed differentially up-regulated and down-regulated genes with magnoflorine treatment based on single-dimensional statistical analysis. Red dots indicated the up-regulated genes and the green dots indicated the down-regulated genes (log2FC > 1, $P < 0.05$). (**B**) The KEGG enrichment showed the top 30 representative differential pathways for DEG after magnoflorine treatment ($P < 0.05$). (C) The heatmap showed sperman correlation between gut bacteria and hub genes. Each cell contained the p-value (*$P < 0.05$, **$P < 0.01$).

Fig.8 HDCA inhibited neuroinflammation through TLR4/Myd88 pathway. (**A**) Targeted bile acid metabolism in brain tissue were measured between the DSS and Mag+DSS mice by LC-MS analysis. (**B**) Study design for the HDCA experiment (n = 5-6). (**C**) Representative images of mouse trajectories in OFT and EPT experiments (n= 5-6). HDCA increased the time in center area of OFT experiments, increased the time in open arms of EPT experiments. (**D**) Concentrations of three representative pro-infammatory cytokines at mRNA level in hippocampus. (**E**) The top 30 KEGG pathway based on microglia-related genes of Genecard database. (**F**) Molecular docking analysis of HDCA on LBD of TLR4/MD2 complex. (**G**) Western blotting analysis of TLR4, Myd88 and IL-6 in BV2 cell stimulated by LPS and HDCA. Protein levels were normalized to β-actin. (**H**) Western blotting analysis of TLR4, Myd88 and IL-6 in brain hippocampus tissue in HDCA treatment colitis mice. Protein levels were normalized to β-actin. Statistical

significance was determined using one-way ANOVA, followed by Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig.9 Schematic summary for the role of magnoflorine in the pathogenesis of colitis and colitis-induced anxiety. Mice with colitis exhibited gut microbiota dysbiosis, impaired gut barrier, disrupted blood-brain barrier, activated microglia, and neuroinflammation, leading to anxiety. Magnoflorine can remodel gut microbiota, increase the abundance of *Odoribacteraceae* and *Ruminococcus,* enhance the concentration of HDCA in the brain. HDCA can alleviate neuroinflammation by inhibiting the TLR4/Myd88 signaling pathway, thereby alleviating colitis-induced anxiety.

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Additional file1

Fig. S1 Mendelian randomization Leave-one-out analysis. (**A**) Leave-one-out plots for UC on anxiety. (**B**) Leave-one-out plots for anxiety on UC. (**C**) Leave-one-out plots for UC on depression. (**D**) Leave-one-out plots for depression on UC.

Fig. S2 Magnoflorine affected mice behavior. Magnoflorine increased the times enter in open arms of EPT experiments, but not affected immobility time in TST and FST experiments. Statistical significance was determined using one-way ANOVA, followed by Tukey test. *$P < 0.05$, ns, not significant.

Fig. S3 Magnoflorine regulated colitis microbiota and WGCNA analysis. (**A**) α-diversity upon magnoflorine therapy represented by the

Shannon index, *$P < 0.05$. (**B**) The PCoA plots upon magnoflorine therapy. (**C**) STAMP analysis of microbiota at family level between DSS group and Mag+DSS group. (**D**) By STAMP analysis, 16sRNA sequencing data analyzed by PICRUSt prediction yielded that K15873 was enriched in the Mag+DSS group. K15873: baiH, NAD+-dependent 7beta-hydroxy-3-oxo bile acid-CoA-ester 4-dehydrogenase. (**E**) WGCNA analysis. The left diagram showed clustering sampletree and cutHeight = 42, the right diagram showed network topology analysis for different soft-threshold power. The green arrow indicates the selected power value. (**F**) Venn diagram showing the common part of red and blue ME.

Fig. S4 Magnoflorine alleviated colitis depending on microbiota. (**A**) Daily body weight change throughout the DSS treatment duration of the ABx experiment. (**B**) H&E stained colon sections and Pathological scores of colons of the ABx experiment. (**C**) Daily body weight change throughout the DSS treatment duration of the FMT experiment. (**D**) H&E stained colon sections and Pathological scores of colons of the FMT experiment.(**E**) Daily body weight change throughout the DSS treatment duration of the SFF experiment. (**F**) H&E stained colon sections and Pathological scores of colons of the SFF experiment.. Data were presented as means±SEM. Statistical significance was determined using Tukey test. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig. S5 GO and KEGG analysis based on DEG between DSS group and

Mag+DSS group. (**A**) GO functional enrichment analysis, including BP, CC, and MF. (**B**) KEGG functional enrichment analysis.

Fig. S6 HDCA alleviated colitis and neuroinfalmmation. (**A**) Daily body weight and DAI scores change throughout the DSS treatment duration of the HDCA treatment experiment. (**B**) Images of the colon and the colon length in HDCA treatment experiment. (**C**) Concentrations of three representative pro-infammatory cytokines at mRNA level in BV2 cells. (**D**) Representative immunofluorescence images of IBA-1 in BV2 cells. Magnification: 200×, Scale bars: 50μm. Data were presented as means±SEM. Statistical significance was determined using Tukey test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Additional file2

Table S1 The primer sequences of target genes. **Table S2** General Clinical Features with Remission and Activity in Patients of UC. **Table S3** Anxiety, Depression, Sleep Quality, and Quality of Life in UC Patients in Activity Compared with in Remission. **Table S4** Anxiety, Depression, Sleep Quality, and Quality of Life in UC Patients in Mild, Moderate, Severe Activity. **Table S5** Scores of GAD-7, PHQ-9, PSQI, and IBD-Q in UC Patients in Activity Compared with in Remission. **Table S6** IVs used in MR analysis of the association between UC and anxiety. **Table S7** IVs used in MR analysis of the association between anxiety and UC. **Table S8** IVs used in MR analysis of the association between UC and depression. **Table S9** IVs used in MR

analysis of the association between depression and UC.

Figures

Figure 1

() Proportions of anxiety, depression, sleep disturbance and poor quality of life in UC patients with different disease activity. () Linear relationship between GAD-7, PHQ-9 and PSQI, IBDQ. () Mendelian randomization: Scatterplot of genetic association between anxiety, depression and UC. Statistical significance was determined using Tukey test. **** < 0.0001, ns, not significant.

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. () Schematic drug screening. () Experimental scheme of the mouse trial (n = 8). Oral PBS and magnoflorine treatments were indicated. () Daily body weight and daily DAI scores changes throughout the DSS treatment duration of the study. () Images of the colon and the colon length in each group (n = 8). () Concentrations of three representative pro-infammatory cytokines at mRNA level in colon. () H&E staining of colon sections and pathological scores of colons (n = 8). Statistical significance was determined using one-way ANOVA, followed by Tukey test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

Figure 3

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Figure 5

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Figure 9

() Targeted bile acid metabolism in brain tissue were measured between the DSS and Mag + DSS mice by LC-MS analysis. () Study design for the HDCA experiment (n = 5–6). () Representative images of mouse trajectories in OFT and EPT experiments (n = 5–6). HDCA increased the time in center area of OFT experiments, increased the time in open arms of EPT experiments. () Concentrations of three representative pro-infammatory cytokines at mRNA level in hippocampus. () The top 30 KEGG pathway based on microglia-related genes of Genecard database. () Molecular docking analysis of HDCA on LBD of TLR4/MD2 complex. () Western blotting analysis of TLR4, Myd88 and IL-6 in BV2 cell stimulated by LPS and HDCA. Protein levels were normalized to β-actin. () Western blotting analysis of TLR4, Myd88 and IL-6 in brain hippocampus tissue in HDCA treatment colitis mice. Protein levels were normalized to β-actin. Statistical significance was determined using one-way ANOVA, followed by Tukey test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

Figure 10

Schematic summary for the role of magnoflorine in the pathogenesis of colitis and colitis-induced anxiety. Mice with colitis exhibited gut microbiota dysbiosis, impaired gut barrier, disrupted blood-brain barrier, activated microglia, and neuroinflammation, leading to anxiety. Magnoflorine can remodel gut microbiota, increase the abundance of and , enhance the concentration of HDCA in the brain. HDCA can alleviate neuroinflammation by inhibiting the TLR4/Myd88 signaling pathway, thereby alleviating colitis-induced anxiety.

Figure 11

Figure 12

Figure 13

Figure 14

Figure 15

Figure 16

Figure 17

Figure 18

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