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Gegen Qinlian decoction alleviates depression-like behavior by modulating the gut microenvironment in CUMS rats

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Abstract

Background Gegen Qinlian Decoction (GQD) is a classical traditional Chinese medicine (TCM) formula primarily utilized for treating gut disorders. GQD showed therapeutic effects on several diseases in clinical and animal studies by targeting gut microbes. Our recent studies also found that GQD efficiently alleviated anxiety in methamphetamine-withdrawn mice via regulating gut microbiome and metabolism. Given that various studies have indicated the link between the gut microbiome and the development of depression, here we endeavor to explore whether GQD can manage depression disorders by targeting the gut microbiome.

Methods and materials The depression-like model was induced in rats through chronic unpredictable mild stress (CUMS) and the depression levels were determined using the sucrose preference test (SPT). To address the depression-like behavior in rats, oral administration of GQD was employed. The colon microbiome and metabolite patterns were determined by 16s rRNA sequencing and untargeted metabolomics, respectively.

Results We found 6 weeks of CUMS can induce depression-like behavior in rats and 4 weeks of GQD treatment can significantly alleviate the depression-like behavior. GQD treatment can also ameliorate the histological lesions in the colon of CUMS rats. Then, CUMS increased the abundance of gut microbes, while GQD treatment can restore it to a lower level. We further discovered that the abundances of 19 bacteria at the genus level were changed with CUMS treatment, among which the abundances of *Ruminococcus*, *Lachnoclostridium*, *Pygmaiobacter*, *Bacteroides*, *Pseudomonas*, and *Pseudomonas Family_XIII_AD3011_group* were stored by GQD treatment. Besides, we identified the levels of 36 colon metabolites were changed with CUMS treatment, among which the levels of Fasciculic acid B, Spermine, Fludrocortisone acetate, alpha-Ketoglutaric acid, 2-Oxoglutaric acid, N'-(benzoyloxy)-2-(2,2-dichlorocyclopropyl) ethanimidamide, N6-Succinyl Adenosine Oleanolic acid, KQH, Ergosta-5,7,9(11),22-Tetraen-3-beta-Ol, Gentisic acid, 4-Hydroxyretinoic Acid, FAHFA (3:0/16:0), Leucine-enkephalin and N-lactoyl-phenylalanine can be restored by GQD treatment.

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Conclusion Our findings provide evidence supporting the therapeutic efficacy of GQD in alleviating depression-like behavior in CUMS rats, potentially being targeted on colon bacteria (especially the abundance of *Ruminococcus* and *Bacteroides*) and metabolites (especially the level of Oleanolic acid).

Keywords Depression, Gegen Qinlian decoction, Gut bacteria, Colon metabolites

Introduction

Depressive Disorders have been regarded as one serious mental health condition [1]. Despite the rapid development of antidepressants such as selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), therapeutic strategies for depression remain limited. Currently, the major challenge for the applications of antidepressants that target the central neuro system is the significant side effects such as insomnia, nausea, resistance, and even suicidal ideation [2, 3]. Accordingly, there is an urgent need to develop alternative targets to treat depression.

In the last decade, increased emphasis has been given to the role of gut microbiota in regulating depressive disorder [4, 5]. Recent findings provide strong evidence that depression disrupts the balance of gut microbiota homeostasis [6, 7]. Conversely, gut microbiota imbalance exacerbates depressive symptoms through disturbing gut metabolite microenvironments. Further, major depressive disorder (MDD) patients had distinct gut microbe patterns from healthy people [8]. Additionally, fecal microbiota transplantation (FMT) assays demonstrated that the transfer of microbes from depressed animals or patients to healthy animals resulted in the induction of depression-like behaviors [9–11]. Notably, probiotics have already been used in treating patients with refractory depression [12]. This compelling evidence has proven the gut microbiota as a novel avenue for developing antidepressants or complementary adjuncts to conventional treatments.

Traditional Chinese Medicine (TCM) for the treatment of psychiatric disorders is widely recognized in clinical practice due to its efficacy and minimal side effects and has emerged as a promising alternative strategy for depression [13–17]. Gegen Qinlian Decoction (GQD) is a classical TCM formula composed of four herbs: *Pueraria lobata* (Willd.) Ohwi (Gegen), *Scutellaria baicalensis* Georgi (Huangqin), *Coptis chinensis French* (Huanglian), *and Glycyrrhiza uralensis* Fisch (Gancao). GQD is traditionally used in treating enteritis, diarrhea, and ulcerative colitis [18]. Besides, GQD primarily exerts its therapeutic effects on several non-gastrointestinal disorders by targeting the intestinal microenvironment, such as diabetes, steatohepatitis, colorectal cancer, and influenza, in clinical and animal studies [19–25]. Additionally, our previous study found that GQD treatment efficiently alleviated anxiety-like behavior in methamphetamine (METH)-exposed mice by regulating colon bacteria and metabolites [11]. Further, previous studies have revealed the anti-depression effects of active components from GQD [26–31]. As such, we hypothesize that GQD can also alleviate depressive symptoms by regulating the homeostasis of gut microbiota and gut metabolism.

In the present study, a depression-like model was established in rats through the application of CUMS. Subsequently, intragastric gavage of GQD was employed as an intervention to treat depression-like behaviors in the CUMS rats. The study aimed to assess the effectiveness of GQD in alleviating depression-like behaviors and to target potential gut microbiota and metabolites associated with GQD treatment.

Materials and methods

Rats breeding

The experimental procedures involving rats, including animal breeding and behavioral tests, were conducted under the approval and supervision of the Institutional Animal Care and Use Committee (IACUC) at Nanjing University of Chinese Medicine (The animal experimental ethics number is 202103A097). Sprague DawLey (SD) rats, aged 8 weeks, were obtained from Cavens Biotechnology Co., ltd. (Nanjing, China) and utilized in our model. Rats were allowed to acclimate for at least 3 days before the start of the experiment procedure. Throughout the study, the rats were housed under controlled environmental conditions, maintaining a constant temperature of 23±2℃ and humidity of 50±5%. They were subjected to a 12-hour light/dark cycle with lights on at 8:00 AM and had continuous access to water and food. Where necessary, rats were euthanized under anesthesia by exposure to 2% Isoflurane gas for 5 min to collect tissue samples while they were unconscious.

GQD preparation

GQD was prepared as the identical batch and the quality control was done with high-performance liquid chromatography (HPLC) as we previously illustrated [32]. Briefly, 24 g *Pueraria lobata* (Willd.) Ohwi, 9 g *Coptis chinensis* French, 9 g *Scutellaria baicalensis* Georgi, and 6 g *Glycyrrhiza uralensis* Fisch were provided from The First Affiliated Hospital of Nanjing University of Chinese Medicine. These herbs were soaked in 500 ml of water at 10 ℃ for 30 min and subsequently heated until boiling, then followed by gentle heat for an additional 30 min. The resulting solution was filtered to obtain the first decoction. The remaining residue was mixed with 500 ml

of water at 10 ℃, boiled, and gently heated for 30 min before being filtered to obtain the second decoction. The first and second decoctions were combined, thoroughly filtered to remove herb residues, and then subjected to rotary evaporation. The concentrated solution was subsequently lyophilized and stored at 4 ℃ for future use. Prior to administration, the lyophilized GQD was re-dissolved in 20 ml of water and administered intragastrically to rats at a dosage of 18 g/kg, where the 18 g is calculated based on the total crude weight of the four herbs. The chosen dosage of 18 g/kg is approximately 1.5 times to the clinical dosage in human beings [18]. The reagents and quality assessment of GQD are conducted and supervised by the Jiangsu Key Laboratory for TCM Formulae Research, Nanjing University of Chinese Medicine, China.

Sucrose preference test

The test was performed as previously reported with minor modifications [33]. Briefly, before the test, rats were individually housed in new cages, each containing two visually identical water bottles. The experiment was divided into three stages: the training stage, the fasting and water deprivation stage, and the testing stage. The training stage lasted for two days. On the first day, both bottles contained a 1% sucrose solution, and after 12 h, the positions of the two bottles were switched. On the second day, one bottle contained a 1% sucrose solution, and the other bottle contained sterile water. Again, after 12 h, the positions of the two bottles were switched. The fasting and water deprivation stage lasted for a total of 24 h. During this period, the rats had no access to food or water. The testing stage also lasted for 24 h. In each cage, one bottle contained a 1% sucrose solution, and the other bottle contained sterile water. After 12 h, the positions of the two bottles were switched. Before the testing phase, all water bottles were carefully labeled and weighed. During the testing period, a quiet environment was maintained. After the testing concluded, all water bottles were weighed again, and the sucrose preference index was recorded. The sucrose preference rate (SPR) was calculated as follows: (weight of sucrose water) / (weight of sucrose water + weight of sterile water) × 100%.

Chronic unpredictable mild stress modeling and GQD treatment

The CUMS (Chronic Unpredictable Mild Stress) was conducted based on the previous literature with slight modifications [34]. The experimental cohort of SD rats were first subjected to 7 days of adaptation to the housing environment, then underwent baseline testing for sucrose preference using the SPT for 4 days and randomly separated into the control group (CON, $n=16$) and CUMS group (CUMS, $n=25$) based on the baseline SPT score. During the 6 weeks of CUMS modeling, The CUMS group was subjected to a daily administration of diverse mild stressors. These stressors were chosen randomly, ensuring that consecutive exposures did not entail repetition. Following each stressor exposure, a subsequent set of stressors was administered. The stressors applied in this study encompassed the following protocols: a 24-hour fasting period, a 24-hour deprivation of water access, continuous illumination for a duration of 24 h, a 24-hour provision of wet bedding with the addition of 250 mL of water per cage, a 45° tilt of the cage sustained for a 24-hour interval, a 90-second tail clamp, a 6-minute session of forced swimming in cold water maintained at 4 °C, and a 6-minute session of forced swimming in hot water at 42 °C. The weight of each rat was measured at 6:00 PM on the first day of every week, and the SPT score was measured on the first day of every week. After 6 weeks, the CON rats were randomly intragastric gavaged with either vehicle (V, saline) or GQD for the next 5 weeks, dividing into CON+V group ($n=8$) and CON+GQD group ($n=8$). The CUMS rats were also randomly intragastric gavaged with either V, GQD, or FLX for the next 5 weeks, dividing into CUMS+V ($n=8$), CUMS+GQD ($n=8$), and CUMS+FLX group ($n=9$). FLX (fluoxetine) here was used as a positive control drug for treating depression-like behaviors in CUMS rats (Fig. 1A). Likewise, the SPT score was measured on the first day of every week.

Histology

The colon tissues were obtained from 4% PFA-prefixed rats and further fixed with 4% PFA for 4 h. After serious dehydration, the tissues were cleared in xylene and embedded in paraffin. Sections of 5 μm thickness were prepared for H&E staining following the manufacturer's instructions (SOLARBIO, G1120). Briefly, the paraffin-embedded tissue sections were dewaxed in xylene and rehydrated through graded alcohol solutions. They were then immersed in hematoxylin solution for 5 min to stain cell nuclei. Following this, sections were briefly dipped in acid alcohol solution (1% hydrochloric acid in 70% ethanol) until the desired blue staining intensity was achieved and then rinsed in tap water. Subsequently, the sections were immersed in eosin solution for 2 min to stain cytoplasm, followed by rinsing in tap water. Finally, sections were dehydrated through graded alcohol solutions and cleared in xylene.

16s rRNA sequencing and analysis

Upon sacrificing the rats, fecal samples were collected from the colon and immediately snap-frozen in liquid nitrogen. These samples were stored at -80℃ for subsequent analysis.

For the absolute quantification of gut microbial 16s RNA, Shanghai Genesky Biotechnologies (China)

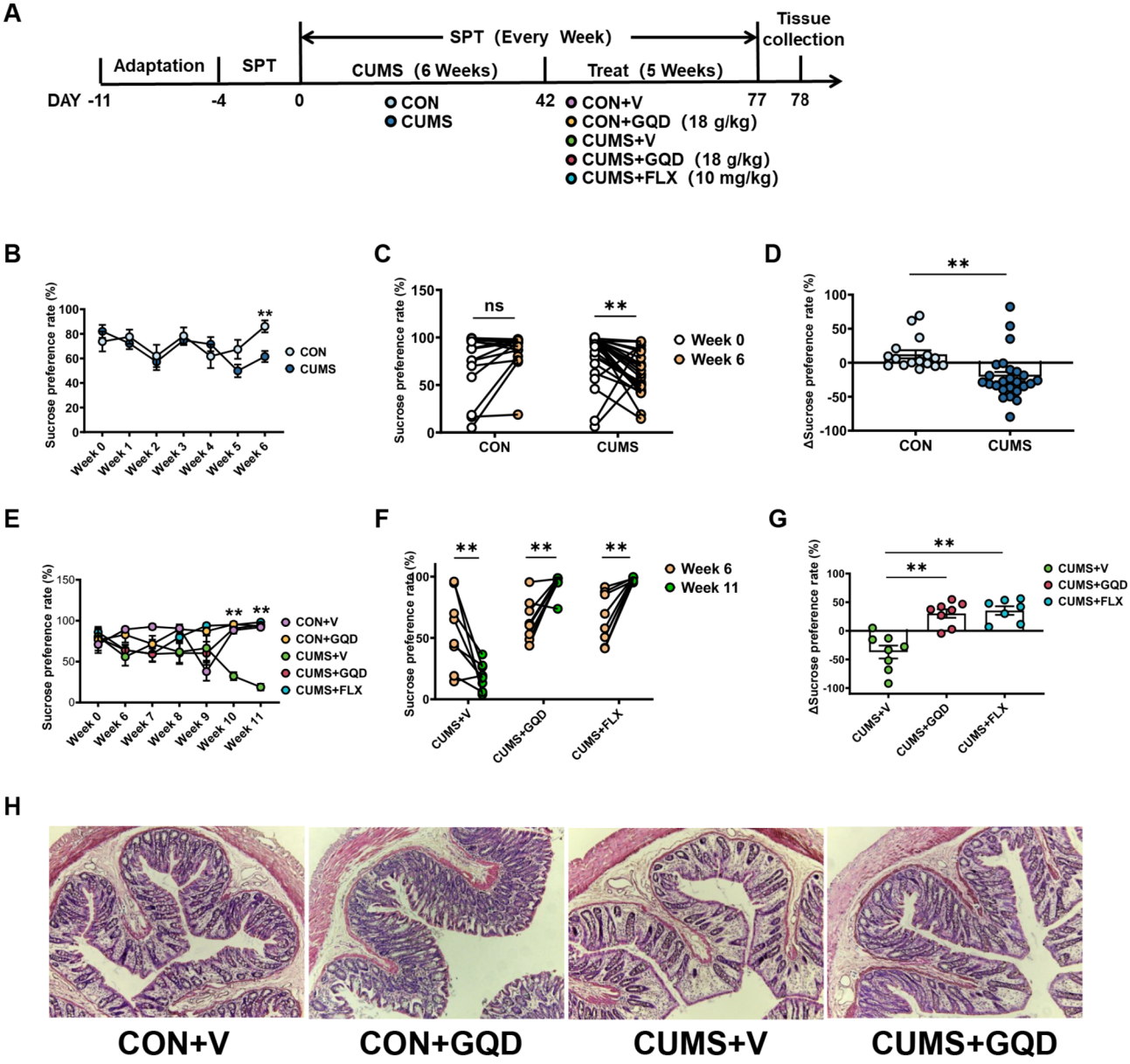

Fig. 1 GQD alleviates the depression-like behavior in CUMS rat. **(A)** The experimental timeline settings with male rats. **(B)** The sucrose preference rates (SPR) were measured every week for 6 weeks with rats exposed to chronic unpredictable mild stress (CUMS) and controls (CON). (*t*-test, day 0: $P=0.3761$, $n=41$; Week 5: $P=0.0565$, $n=41$; Week 6: $P=0.0010$, $n=41$). **(C)** The values of SPR for each corresponding CUMS or CON rat at day 0 (baseline) and day 42 (6 weeks) were compared. (Two-way ANOVA, Week 6: CON, $P=0.2214$; CUMS, $P=0.0037$) **(D)** The delta values of SPR for each corresponding CUMS or CON rat at day 42 (6 weeks) and day 0 were calculated and compared. (*t*-test, $P=0.0019$, $n=41$). **(E)** After 6 weeks, CUMS rats were randomly divided into three groups, including vehicle treatment (CUMS + V), GQD treatment (CUMS + GQD), and Fluoxetine treatment (CUMS + FLX) groups, while CON rats were randomly divided into two groups, including vehicle treatment (CON + V), GQD treatment (CON + GQD) groups. SPR of rats in each group was measured every week for the next 5 weeks. (One-way ANOVA, Week 0: $P=0.8858$; Week 10: $P<0.0001$; Week 11: $P<0.0001$). **(F)** The values of SPR for each corresponding rat in the three groups at week 6 and week 11 were compared. (Two-way ANOVA, CUMS + GQD: $P=0.0065$; CUMS + FLX: $P=0.0012$; CUMS + V: $P=0.0009$). **(G)** The delta values of SPR for each corresponding rat in the three groups at week 11 and week 6 were calculated and compared. (One-way ANOVA, CUMS + GQD: $P<0.0001$; CUMS + FLX: $P<0.0001$). **(H)** The typical H&E staining photos of the colons of rats in the indicated group. CON + V, control rats with vehicle treatment. CON + GQD, control rats with GQD administration. CUMS + V, rats with CUMS followed by vehicle treatment. CUMS + GQD, rats with CUMS followed by GQD treatment. N.S., $P>0.05$; *, $P<0.05$; **, $P<0.01$ versus the indicated controls

conducted the analysis based on the reported relevant literature [35, 36]. Genomic DNA from bacteria was extracted using the FastDNA® SPIN Kit (MP Biomedicals, Santa Ana, CA, United States). The quality of genomic DNA was assessed using agarose gel electrophoresis, while its concentration and purity were determined using Nanodrop 2000 and Qubit3.0 spectrophotometer. To enable quantification, polymorphic spike-ins containing

conserved regions identical to 16s rRNA and variable regions with randomized sequences (GC%~40%) were utilized as labels. These spike-ins, mixed with genomic DNA in varying copy numbers, were used for amplifying the V3-V4 regions of the 16 S rRNA and spike-ins using primers 341 F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). The resulting amplified products were sequenced using the Illumina NovaSeq 6000 sequencer following the manufacturer's guidelines. For the relative 16 S rRNA sequencing of fecal bacterial genomic DNA, the same process was employed, excluding the spike-ins in the amplification mixtures.

Data analysis was carried out using QIIME2, DADA2, and Greengenes (v.13.8). The raw data underwent Operational Taxonomic Units (OTU) cluster analysis, followed by community composition analysis, diversity analysis, taxonomy analysis, and nonparametric multidimensional scaling using Mothur, UPARSE, and R, respectively.

Untargeted metabolomics analysis

The feces samples were freshly collected from the rats' colon, 40 min after the behavioral tests. The samples were denounced with liquid nitrogen and resuspended in prechilled 80% methanol. After incubating the mixture on ice for 5 min, the clear supernatant was obtained by centrifugation twice at 15,000 g at 4 ℃ for 20 min. Subsequently, the supernatant was injected into the LC-MS/MS system for analysis, as described in reference [37].

UHPLC-MS/MS analyses were conducted using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q ExactiveTMHF-X mass spectrometer (Thermo Fisher, Germany) at Novogene (Beijing, China), following the manufacturer's instructions. The raw data files generated by UHPLC-MS/MS were processed using Compound Discoverer 3.1 (CD3.1, ThermoFisher) based on the optimized protocols by Novogene. The resulting accurate qualitative and relative quantitative results were obtained by normalizing peak intensities to the total spectral intensities using mzCloud (https://www.mzcloud.org/). For the processed data, statistical analyses were performed using Software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). Metabolite classification annotation utilized the KEGG database (https://www.genome.jp/kegg/pathway.html), HMDB database (https://hmdb.ca/metabolites), and LIPIDMaps database (http://www.lipidmaps.org/). Principal components analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) were conducted at metaX. Volcano plots were generated using ggplot2 in R language to filter metabolites of interest. Additionally, clustering heat maps and differential metabolites correlation analysis were performed using the pheatmap package in R language. The correlation analysis used Pearson analysis to get the correlation coefficient r and *P* value, and the heatmap was drawn by the 'corrplot' package in the R language.

Data and statistical analyses

Statistical analyses were performed by GraphPad Prism 8. All data were presented as the mean ± SEM. The SPT behavioral data were analyzed by ANOVA with Sidak's multiple comparisons, and other data was analyzed by *t-test*. The data of omics were analyzed as indicated. Statistical significance was set as $P<0.05$.

Results

GQD alleviated the depression-like behavior and ameliorated the colon pathological changes in CUMS rat

As shown in Fig. 1A, the CUMS group was subjected to unpredictable mild stress described in Materials and Methods for 42 days (6 weeks), during which SPT was performed for each rat every week. As a result, the body weights of CUMS rats were significantly decreased from week 3 compared to control (CON) rats, while no difference observed between the two groups at baseline on day 0 (Fig. S1A). In the SPT assay, compared to CON, CUMS group exhibited a decreasing trend at Week 5 and a significant decrease at Week 6 in sucrose preference rates (SPR) while no difference between the two groups was observed at day 0 (Fig. 1B). Compared to the corresponding baseline (week 0), CUMS rats but not CON rats exhibited a significant decrease in SPR at Week 6 (Fig. 1C), and the delta-SPR showed less in CUMS than that in CON rats (Fig. 1D). These results indicated that the CUMS-treated rats were developing depression-like behaviors during 6 weeks.

After 6 weeks, the rats in CON and CUMS groups were then divided into two and three groups respectively, as indicated in Methods and Material (Fig. 1A). As shown in Fig. S1B, there was no difference in the body weights among these groups, and no difference in SPR was observed at baseline (week 0) among three CUMS groups. Both CUMS+GQD and CUMS+FLX rats exhibited higher SPR than CUMS+V rats at week 10 and week 11 (Fig. 1E). Further, Either GQD treatment or FLX treatment increased SPRs in CUMS rats at week 11, and CUMS+V rats even had a further drop in SPR when compared to their corresponding SPRs at week 6 (Fig. 1F), while no difference was observed in CON+V group and CON+GQD group (Fig. S1C). The delta-SPR of week 6 and week 11 was significantly lower in CUMS+V rats than CUMS+GQD and CUMS+FLX rats (Fig. 1G), while no difference between CON+V group and CON+GQD group (Fig. S1D). Collectively, these results indicated that GQD could alleviate

depression-like behaviors in CUMS rats without affecting these behaviors in normal rats.

To evaluate the effect of GQD on the histological alternations in colons of CUMS rats, the H&E staining was conducted in CON+V, CON+GQD, CUMS+V, and CUMS+GQD groups. As illustrated in Fig. 1H, compared to CON+V rats, the colon of CUMS+V was characterized by a decline in the density of the mucosal lamina propria, an uneven distribution of intestinal glands with increased inter-glandular spacing, and a concomitant decrease in the abundance of goblet cells, while CUMS+GQD rescued these pathological changes in colon histology. Additionally, we observed that GQD treatment did not affect the histological patterns of the colons in control rats (CON+GQD group). These colons displayed an intact mucosal layer structure, evenly distributed villi, a thick muscular layer, a dense mucosa's lamina propria containing long and dense crypts, and a reasonable number of goblet cells (Fig. 1H). These results indicated a potentially protective effect of GQD on CUMS-induced pathological changes in the colon.

GQD improved the composition of gut bacteria in CUMS rat

To investigate colon bacteria, the feces of CON+V, CON+GQD, CUMS+V, and CUMS+GQD rats were collected at the end of week 11 (Fig. 1A) and subjected to 16s rRNA sequencing. As shown in Fig. 2A, Principal Component Analysis (PCA) showed that the divergence of gut microbes in CUMS+V group was significantly greater than in other groups. Further, the composition of gut microbes in CUMS+GQD rats was separated from CUMS+V rats. In parallel, the chao1 index showed that CUMS increased the abundance of gut microbes, while GQD treatment significantly restore it to a lower level (P=0.0335, Fig. 2B). Figure 2C shows the top 10 gut microbes at the genus level that had significant changes in their relative abundances among the four groups.

As to absolute abundances in colon bacteria at the genus level, 11 floras were upregulated in CUMS+V group compared to CON+V group, including *Acetatifactor* (P=0.0103), *Eubacterium_ventriosum_group* (P=0.0022), *Eubacterium_xylanophilum_group* (P=0.0450), *Lachnoclostridium* (P=0.0283), *Lachnospiraceae_FCS020_group* (P=0.0063), *Roseburia* (P=0.0410), *Colidextribacter* (P=0.0160), *UCG-007* (P=0.0315), *Pygmaiobacter* (P=0.0259), *Ruminococcus* (P=0.0396) and *Family_XIII_AD3011_group* (P=0.0199, Fig. S2A), while 8 floras were downregulated including *Bifidobacterium* (P=0.0071), *Bacteroides* (P=0.0373), *Prevotellaceae_NK3B31_group* (P=0.0136), *Parabacteroides* (P=0.0214), *Allobaculum* (P=0.0467), *Christensenellaceae_R-7_group* (P=0.0458), *Parasutterella* (P=0.0013) and *Pseudomonas* (P=0.0291, Fig. S2B) with Metastats analysis. Besides, compared to CUMS+V rats, 5 floras were upregulated in CUMS+GQD rats, including *Bacteroides* (P=0.0375), *Prevotella* (P=0.0061), *Helicobacter* (P=0.0439), *Phascolarctobacterium* (P=0.0179), and *Pseudomonas* (P=0.0158), while 11 flora were downregulated including *Eubacterium_ruminantium_group* (P=0.0016), *Lachnoclostridium* (P=0.0206), *Marvinbryantia* (P=0.0424), *UCG-009* (P=0.0151), *NK4A214_group* (P=0.0305), *Fournierella* (P=0.0368), *Pygmaiobacter* (P=0.0271), *Ruminococcus* (P=0.0009), *Family_XIII_AD3011_group* (P=0.0213), *Christensenellaceae* (P=0.0172) and *Clostridia_vadinBB60_group* (P=0.0122, Fig. S2C). Among these changed bacteria, we found that the *Bacteroides* and *Pseudomonas* were decreased by CUMS but restored by GQD treatment (Fig. 2D), and the *Ruminococcus*, *Lachnoclostridium*, *Pygmaiobacter*, *Family_XIII_AD3011_group* were increased with CUMS but restored by GQD treatment (Fig. 2E), indicating that these colon bacteria might be GQD targets in CUMS rats.

The linear discriminant analysis effect size (LEfSe) algorithm was used to identify significant changes in bacterial communities with potential biological relevance to CUMS and GQD. As shown in Fig. 2F between CON+V and CUMS+V rats, at the genus level, Dubosiella、Akkermansia、Allobaculum were enriched in CON+V, while Ruminococcus was enriched in CUMS+V group. Between CUMS+V and CUMS+GQD rats, Desulfovibrio and Ruminococcus were enriched in CUMS+V, while Bacteroides were enriched in CUMS+GQD group (Fig. 2G). At the genus level, Ruminococcus and Bacteroides were identified as significantly related to CUMS and GQD by both the Metastats abundance and the LEfSe algorithm analysis. Taken together, these results indicated that GQD might alleviate depression-like behaviors through modulating gut bacteria in CUMS rats.

GQD treatment restored changes in colon metabolism of CUMS rat

Here, untargeted metabolomics was employed to identify potential colon metabolites that may be involved in the therapeutic effects of GQD on depression-like behaviors in CUMS rats. The Pearson correlation analysis indicated the desired reliability and robustness of samples and the obtained data (Fig. S3A). Overall, we identified 14 (6 positives+8 negatives) upregulated and 22 (14 positives+8 negatives) downregulated colon metabolites in CUMS+V rats when compared to the CON+V rats (Fig. 3A, Fig. S3B), as well as 71 (41 positives and 30 negatives) upregulated and 39 (25 positives and 14 negatives) downregulated colon metabolites in CUMS+GQD group when compared to CUMS+V group (Fig. 3B, Fig. S3C). Comparing the analysis of Fig. 3A with Fig. 3B, the levels of Fasciculic acid B, Spermine, Fludrocortisone acetate, alpha-Ketoglutaric acid, 2-Oxoglutaric

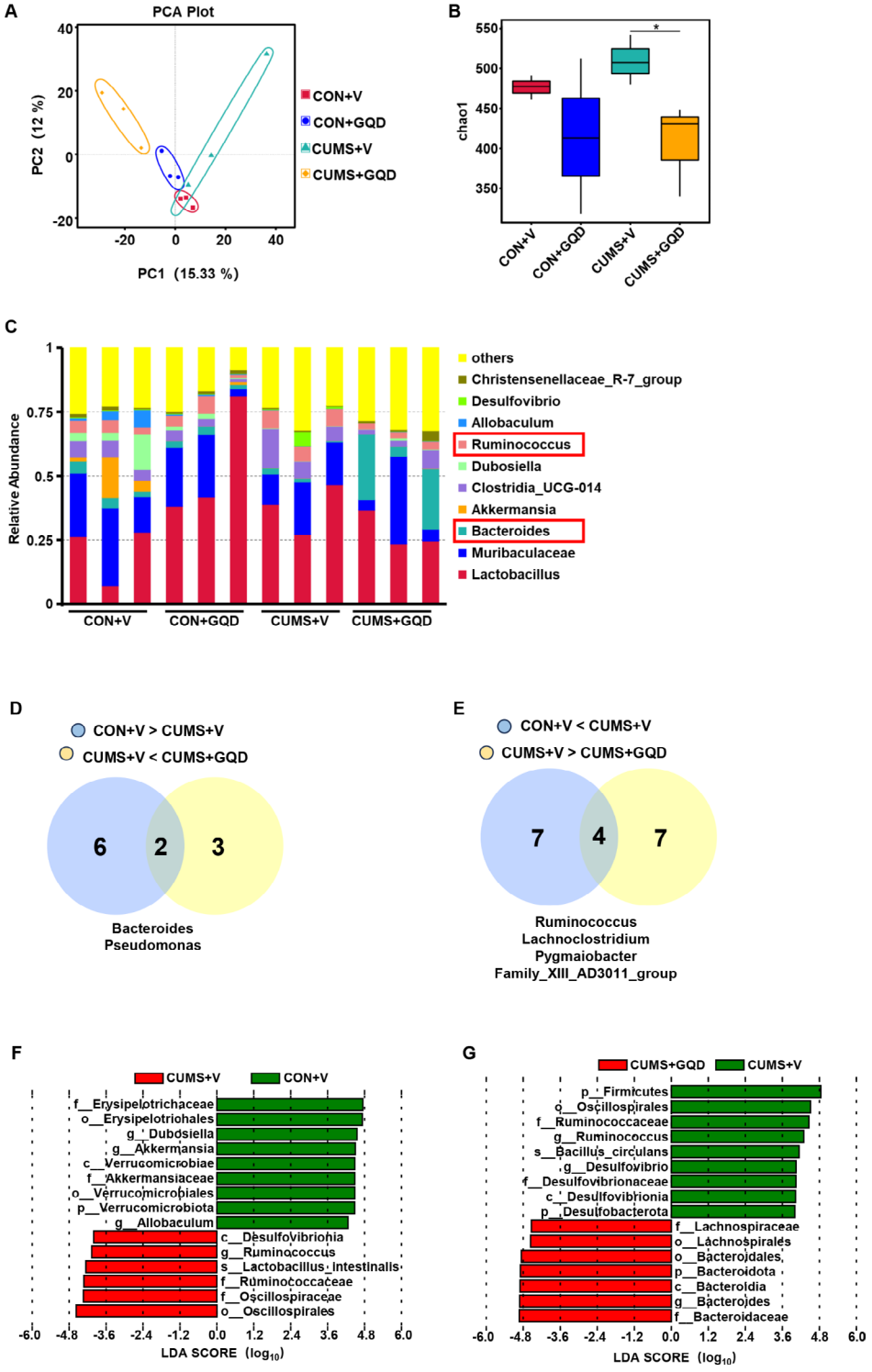

Fig. 2 GQD improves the composition of gut bacteria in CUMS rat. **(A)** The Principal Component Analysis (PCA) of the 16s rRNA data of gut microbes of rats in the indicated group. **(B)** α diversity analysis of the gut microbes of rats in the indicated group illustrated by the chao1 index. **(C)** The column graph illustrates the relative abundances of the top 10 gut bacteria at the genus level. A red box indicates the significantly changed bacteria between CON + V, CUMS + V, and CUMS + GQD groups. **(D, E)** The Venn diagrams display the bacteria that were restored by GQD treatment in CUMS rats. **(F, G)** The LDA scores of gut bacteria changed significantly between CON + V and CUMS + V as well as between CUMS + V and CUMS + GQD groups by LDA Effect Size(LEfSe)analysis. CON + V, control rats with vehicle treatment. CON + GQD, control rats with GQD administration. CUMS + V, rats with CUMS followed by vehicle treatment. CUMS + GQD, rats with CUMS followed by GQD treatment. *, $P < 0.05$ versus the indicated group

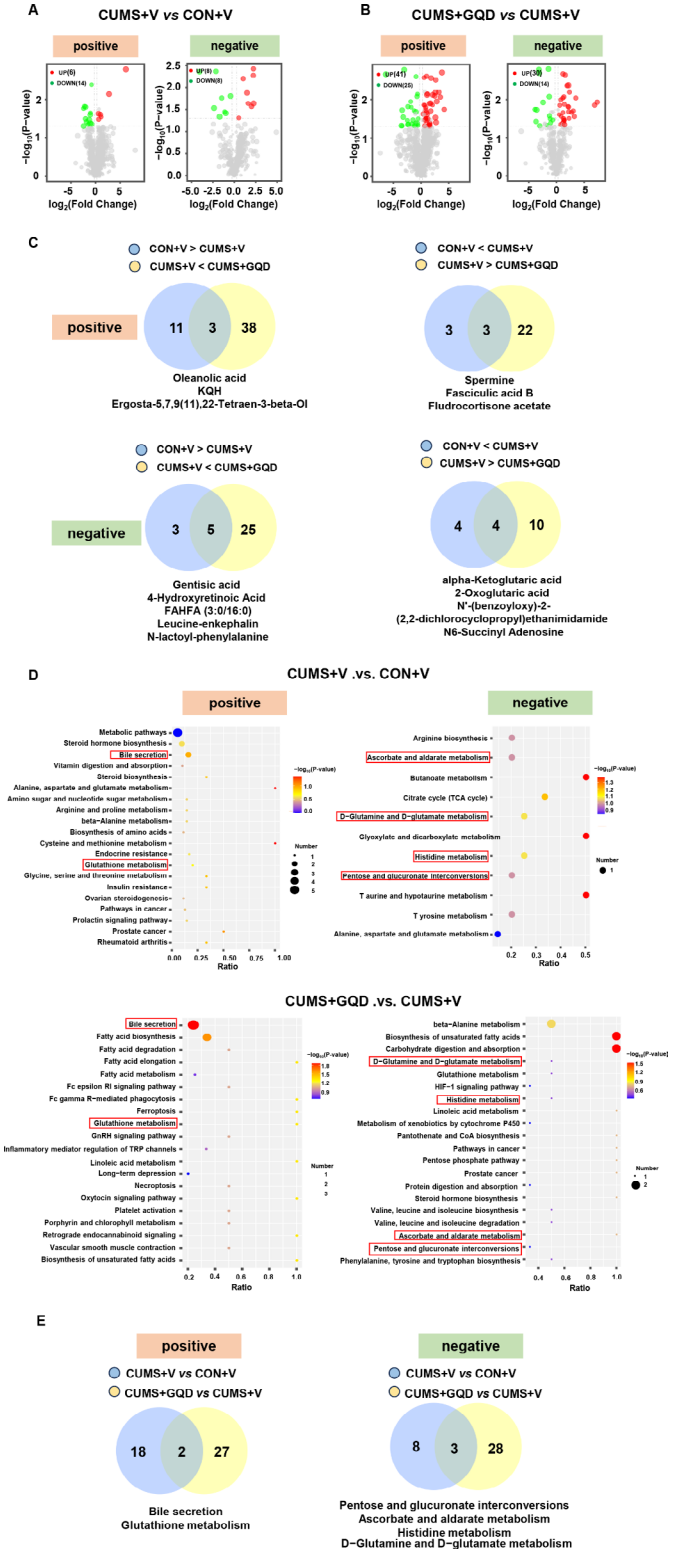

Fig. 3 GQD treatment ameliorates the colon metabolism in CUMS rat. **(A, B)** Volcano plot graphs exhibit the differential metabolites between indicated groups with Variable Importance in the Projection (VIP) > 1.0, Fold change (FC) > 1.2 or < 0.833, and *P*-value < 0.05. **(C)** Venn diagrams illustrate the metabolites that restored by GQD administration in the colons of CUMS rats. **(D)** KEGG bubble charts illustrate the top 20 changed metabolic pathways between indicated groups. **(E)** Venn diagrams show the number of changed pathways and the names of the overlapping changed pathways between indicated groups. CON + V, control rats with vehicle treatment. CON + GQD, control rats with GQD administration. CUMS + V, rats with CUMS followed by vehicle treatment. CUMS + GQD, rats with CUMS followed by GQD treatment

acid, N'-(benzoyloxy)-2-(2,2-dichlorocyclopropyl) ethanimidamide, N6-Succinyl Adenosine was elevated but restored by GQD treatment in CUMS rats. In parallel, the levels of Oleanolic acid, KQH, Ergosta-5,7,9(11),22-Tetraen-3-beta-Ol, Gentisic acid, 4-Hydroxyretinoic Acid, FAHFA (3:0/16:0), Leucine-enkephalin, N-lactoylphenylalanine were reduced but restored by GQD treatment in CUMS rats (Fig. 3C). As shown in Fig. 3D and E, with KEGG analysis, we found 2 positive and 4 negative common changed metabolites pathways in CUMS+V vs. CON+V group and CUMS+GQD vs. CUMS+V group. These changed metabolites and related pathways may be the potential metabolic targets for the treatment of GQD on depression-like behaviors in CUMS rats.

Discussion

Both clinical and animal studies are increasingly focusing on treating depression by influencing the gut system through the "gut-brain axis" [38–40]. Here, we found that GQD efficiently alleviated the depression-like behavior and rescued the colon histological lesions in CUMS rats. Evidence have shown the active components of GQD have therapeutic effects on neurobiological diseases. For example, baicalin has potential therapeutic effects on lipopolysaccharide-induced, chronic unpredictable stress-induced, and repeated restraint stress-induced depressive-like behavior [28, 29, 41]. Berberine improves depressive-like behavior in mice by activating tryptophan hydroxylase 1, inhibiting NLRP3 inflammasome, and suppressing synaptic plasticity [30, 31]. Puerarin, an isoflavone compound isolated from *Pueraria lobata*, was reported to have multiple roles against several neurological disorders, such as cognitive disorders, Parkinson's disease, Alzheimer's disease, and depression [42, 43]. Network pharmacology analysis also revealed the phytoconstituents of *Licorice* can protect against long-term depression, aging-associated diseases, and addiction related disorders [44]. Early in 2015, Xu et al. [20] reported that GQD performed its anti-diabetic effects by enriching the amounts of gut-beneficial bacteria. From then on, more and more evidence demonstrated that GQD played a therapeutic role in diabetes [19, 22], ulcerative colitis [45], and even tumors [21] largely through modulating gut microbiome. Here, our data showed that GQD decreased the diversity of gut microbes in control rats, consistent with the findings of our previous study [11], and restored the aberrantly elevated diversity in CUMS rats. Additionally, GQD treatment ameliorated the histological lesions in the colon, restored colon bacteria and metabolites in CUMS rats. Given the lack of conclusive evidence indicating a direct influence of GQD on the neurophysiology of the brain, we posit that the antidepressant effects of GQD are likely mediated through the modulation of the gut microenvironment.

Traditional Chinese Medicine (TCM) formulas are meticulously crafted to harness the synergistic interactions among their constituent components, with the overarching goal of achieving a comprehensive and holistic therapeutic effect [46–48]. The synergy among these components is believed to enhance their therapeutic efficacy, providing a nuanced and well-rounded approach to healing. This holistic perspective aligns with the TCM principle that views the body as an integrated whole, where different organs and systems are interconnected and influence one another. It's essential to note that this holistic approach may also contribute to the minimization of potential side effects. The multifaceted nature of TCM formulas allows for a nuanced modulation of physiological processes, often mitigating the risk of adverse reactions associated with more targeted interventions [49–52]. As discussed above, many active components of GQD have already been proven to have anti-depression effects, here we sought to explore the synergetic effects of GQD in depression disorder. As a result, we found GQD alleviated depression-like behavior in CUMS rats in a four-week treatment period, comparable to the effect of fluoxetine, a classical selective serotonin reuptake inhibitor (SSRI) for treating depression. So far, no significant side effects have been observed with the use of GQD in the treatment of conditions such as colitis, diabetes, cancer, and other diseases in animal or clinical studies. Therefore, we anticipate that GQD could serve as an effective alternative therapy for treating depression with minimum side effects.

In the current research, we have identified a multitude of altered bacteria in rats subjected to CUMS or GQD. Among the 19 bacteria that changed by CUMS treatment in this study, 10 of these taxa, at the genus level, have been previously documented as exhibiting alterations in depressive states in humans or animals, including *Eubacterium_ventriosum*, *Lachnoclostridium*, *Lachnospiraceae*, *Colidextribacter*, *Ruminococcus*, *Bifidobacterium*, *Bacteroides*, *Parabacteroides*, *Parasutterella*, and *Allobaculum* [6, 9, 53–58]. Besides, in our assay, GQD treatment altered the abundance of 16 gut bacteria at the genus level of CUMS rat, 6 of which have been previously reported changed by GQD administration, including *Bacteroides*, *Prevotella*, *Phascolarctobacterium*, *Clostridia*, *Ruminococcus*, and *Christensenellaceae* [18, 19, 24, 45, 59]. Among these bacteria, the abundances of 6 genus bacteria were subsequently reversed with the addition of GQD in CUMS rats. With further analysis with the LEfSe algorithm, *Ruminococcus* was shown as the most likely candidate for the key regulator for GQD therapy. *Ruminococcus* or *Ruminococcus gnavus* (R. gnavus), was initially isolated in 1974 as an anaerobic bacterium residing in the human gastrointestinal tract [60]. It is considered a normal bacterium present in the healthy

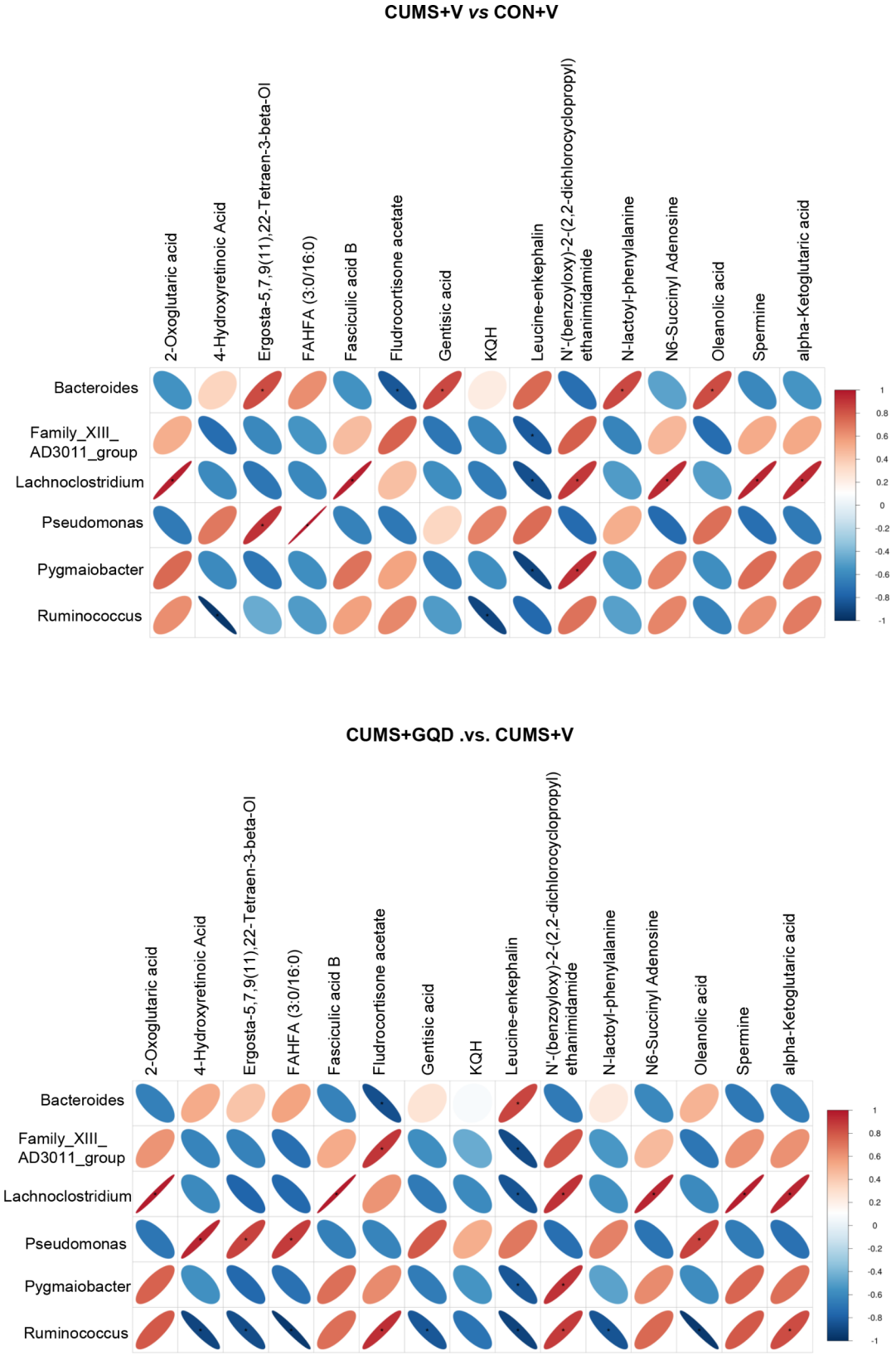

Fig. 4 Correlation analysis between gut bacteria and metabolites. The correlation analysis was performed between the gut bacteria and metabolites that were altered by CUMS but then restored by GQD treatment. The legend on the right indicates the correlation coefficient, where the redder the color, the stronger the positive correlation, and the bluer the color, the stronger the negative correlation. The flatter of the ellipsis indicates the higher the absolute value of the correlation. The sections marked with asterisks (*) in the results indicate $P \leq 0.05$

gut microbiota from infancy and plays a vital role in food digestion and the production of essential nutrients. Over the years, mounting evidence has associated changes in the abundance of *Ruminococcus* with various gastrointestinal disorders, including Crohn's disease, inflammatory bowel disease, and colorectal cancer, as well as non-gastrointestinal conditions such as obesity, coronary heart/artery disease, and even Covid-19 [54]. Recent studies have shown a significantly altered level of *Ruminococcus* in several psychological disorders, including anxiety, epilepsy, and depression [54]. Chahwan et al. demonstrate a positive correlation between *Ruminococcus* level and the Depression, Anxiety, and Stress Scale (DASS) depression score in patients [61]. Additionally, two other studies reported that the administration of TCM or antidepressants resulted in decreased abundances of *Ruminococcus*, which aligns well with our findings of increased *Ruminococcus* levels in rats with CUMS-induced depression, followed by downregulation upon GQD treatment accompanied by alleviation of depression-like behaviors in rats.

To explore the potential targets of GQD on colon metabolism, the untargeted metabolism analysis was performed in CUMS rat. The 16 gut metabolites were identified with altered levels by CUMS treatment but then restored by GQD addition. Among these metabolites, Fasciculic acid B and Spermine have anti-inflammation activities in the gut microenvironment, and gut inflammation is believed closely related to depression disorders [62]. Alpha-ketoglutaric acid and 2-oxoglutaric acid, both key components in the TCA cycle, play pivotal roles in regulating energy metabolism, and it was recognized that abnormal energy metabolism was one of the important mechanisms for the development of depression [63]. Gentisic acid, known for its potent antioxidant and anticarcinogenic effects, may hold promise as an adjunct therapeutic agent in the treatment of neurodegenerative diseases, such as Parkinson's disease [64]. Among them, Oleanolic acid and its derivatives have significant direct antidepressant effects, and the mechanism of action may be related to the 5-HT1A receptor and BDNF-ERK-CREB signaling pathway [65–67]. Furthermore, accumulating evidence underscores the robust anti-inflammatory properties of Oleanolic acid and its derivatives [68–71], further emphasizing their pivotal role in regulating the gut microenvironment. Combining our observation that CUMS treatment can significantly decrease Oleanolic acid level that was then stored by GQD administration, it is strongly postulated that Oleanolic acid serves as the key gut metabolite responsible for the antidepressant effects of GQD in the process of depression-like behavior in CUMS rat.

Next, the correlation analysis was performed between the gut bacteria and metabolites that changed with CUMS but then restored by GQD administration. The results showed that *Lachnoclostridium* exhibits a strong correlation with 2-Oxoglutaric acid, Fasciculicacid B, Leucine-enkephalin, N'-(benzoyloxy)-2-(2,2-dichlorocyclopropyl)ethanimidamide, N6-Succinyl Adenosine, Spermine, alpha-Ketoglutaric acid; *Pseudomonas* exhibits a strong correlation with Ergosta-5,7,9(11) 22-Tetraen-3-beta-Ol, FAHFA (3:0/16:0); *Pygmaiobacter* exhibits a strong correlation with N'-(benzoyloxy)-2-(2,2-dichlorocyclopropyl)ethanimidamide, Leucine-enkephalin; *Ruminococcus* has strong correlation with 4-Hydroxyretinoic Acid, not only in CUMS+V vs. CON+V group but also in CUMS+GQD vs. CUMS+V group with the same trends (Fig. 4). The lack of in-depth exploration of the correlation between gut microbiota and gut metabolism in the treatment of depression with GQD is a limitation of the current study, which will be further addressed in the future.

Conclusion

The present study presents evidence supporting the therapeutic effects of GQD on depression-like behavior in CUMS rats. With GQD administration, a notable enhancement in the composition of gut microbiota and rectification of the metabolism, which had been perturbed by CUMS, were observed. Among these, *Ruminococcus*, *Lachnoclostridium*, and *Bacteroides*, along with various metabolites, including Oleanolic acid, emerge as potential mediators responsible for modulating the antidepressant effects of GQD in the context of CUMS rat models. Our discoveries provide valuable insights into the mechanisms by which GQD may exert its therapeutic influence on depressive manifestations. This opens potential avenues for further research and clinical applications.

Abbreviations

GQD	Gegen Qinlian decoction
TCM	Traditional Chinese Medicine
CUMS	Chronic Unpredictable Mild Stress
SPT	Sucrose Preference Test
SPR	Sucrose Preference Rate
CON	Control group
FLX	Fluoxetine
V	Vehicle-saline

Supplementary Information

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Supplementary Material 1

Author contributions

Peng Y and Liu D: Investigation, Conceptualization; Hu T, Mai Y, Wang Z and Du Y: Assisted Investigation; Peng Y, Song H, Pan W, and Cai Q: Formal analysis. Fan Y: Visualization; Liu D and Guan X: Writing – original draft; Du Y, Zhang Y, Ge F, and Kim HY: Manuscript Revision. Zhang Y, Ge F and Kim HY: Data re-analysis

and Verification. Guan X: Conceptualization, Writing - review & editing, Funding acquisition, Supervision.

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Data availability
The datasets generated during the current study are available in the NCBI sequence read archive, under the accession ID: PRJNA1100179 (https://www.ncbi.nlm.nih.gov/bioproject).

Declarations

Ethics approval
All animal breeding, care, and experimental protocols had been approved by Nanjing University of Chinese Medicine Institutional Animal Care and Use Committee (the animal experimental ethics number is 202103A097). All experiments were performed in accordance with Nanjing University of Chinese Medicine Guide for the Care and Use of Laboratory Animals, China. The study is reported in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Consent to participate
Not applicable.

Consent for publication
The authors all agree with the submission of the manuscript.

Competing interests
The authors declare no competing interests.

Clinical trial number
Not applicable.

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