Chinese Herbal Medicines 16 (2024) 612–621

Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

Deciphering relationship between depression and microbial molecules based on multi-omics: A case study of Chaigui Granules

Qi Wang [a,b], Yingxia Zhao [a,b], Xuemei Qin [a,b,*], Junsheng Tian [a,b,*]

[a] Modern Research Center for Traditional Chinese Medicine, Shanxi University, Taiyuan 030006, China
[b] Key Laboratory of Effective Substances Research and Utilization in TCM of Shanxi Province, Shanxi University, Taiyuan 030006, China

ARTICLE INFO

Article history:
Received 9 August 2023
Revised 4 October 2023
Accepted 16 December 2023
Available online 28 February 2024

Keywords:
arginine metabolism
Chaigui Granules
depression
gut microbiota
microbial molecules

ABSTRACT

Objective: To decipher the antidepression effect of Chaigui Granules (CGKL) from the relationship between depression and microbial molecules based on multi-omics.
Methods: Male SD rats were subjected to chronic unpredictable mild stress (CUMS) for seven weeks. The antidepressants CGKL extract and CGKL were administered for the following four weeks. The behavior test and the content of monoamine neurotransmitters were used to evaluate the efficacy of CGKL. The 16S rRNA sequencing, LC-MS technology and molecular biological techniques were used to explore the pharmacological mechanism of CGKL.
Results: CGKL treatment obviously alleviated the depressive behavioral indicators and regulated the content of monoamine neurotransmitters, and presented dose-dependent manner. CGKL could also improve the arginine metabolism disorder of gut microbiota in the jejunum. Meanwhile, the contents of arginine and its metabolites in the serum and hippocampus were regulated to normal levels. Further investigation indicated that the expression of related rate-limiting enzyme genes and proteins in the hippocampus was validated by qRT-PCR and Western blotting. The results showed that the gut microbiota, metabolites, and genes or proteins of rate-limiting enzymes involved in the arginine pathway were significantly regulated by CGKL.
Conclusion: The present study demonstrates that CGKL might exert antidepressant effects through regulating arginine metabolism, and its mechanism may be related to modulating the gut microbiota and related metabolic enzyme.

1. Introduction

Depression, as one of the most common mental disorders, causes significant problems, and the number of affected patients continues to increase. Affected individuals indicate having depressed moods, feeling inferior, and experiencing sleep disorders and apathy in most things (Gu et al., 2020; Jiao et al., 2018). With the increasing number of affected patients, it is becoming urgent to find antidepressant drugs that are effective and safe (Yao et al., 2015).

Xiaoyao San has been one of the representative prescriptions for treating depression, with records of use since the Song Dynasty. In modern pharmacological research, it has been found that Xiaoyao San is able to improve the symptoms of depression patients and depression-like behaviors of rats caused by chronic unpredictable mild stress (CUMS) (Feng et al., 2014; Liu et al., 2019; Liu et al., 2020; Ji et al., 2022). Chaigui Granules (CGKL), a novel type of antidepressant traditional Chinese medicine with clinical approval, was obtained by the research group after the prescription of Xiaoyao San was disassembled and reconstituted. The chemical compositions were analyzed by UPLC-MS, and 95 compounds were discovered, including paeoniflorin, atractylenolide III, and saikosaponin A (Gao et al., 2020; Zhao et al., 2021). Most of these components are the material basis for the efficacy in treating depression (Zhou et al., 2021; Tian et al., 2021; Wang et al., 2019). We found that CGKL at 8.3 g/kg could improve gastric electrical function and small intestinal peristalsis to improve gastrointestinal function and regulate the level of trimethylamine oxide (TMAO) produced by gut microbiota metabolism (Liu et al., 2019). It was suggested that CGKL played an antidepressant role by improving gastrointestinal function and regulating the metabolites of gut microbiota, but its precise mechanism was not clear.

Accumulating evidence suggests that the gut microbiota influences both the physiology and behavior of the gastrointestinal function and the central nervous system by regulating the

* Corresponding authors.
E-mail addresses: qinxm@sxu.edu.cn (X. Qin), jstian@sxu.edu.cn (J. Tian).

https://doi.org/10.1016/j.chmed.2023.12.003
1674-6384/

microbiota-gut-brain axis (Evrensel & Ceylan, 2015; Luna & Foster, 2015). Recent investigations suggested that the gut microbiota composition of depressive patients differed from healthy individuals through a 16S rRNA gene sequencing approach (Dash et al., 2015). The appearance of depressive behaviors also accompanies reduced richness and diversity of the gut microbiota, suggesting that the gut microbiota and depression may interact (Winter et al., 2018). Thus, changes in the gut microbiota may be prospective indicators of the pathogenesis of depression.

A growing body of literature has focused on the underlying biomarkers and associated metabolic pathways to explain the pathogenesis of depression and the treatment mechanisms of antidepressants (Pan et al., 2018; Nasca et al., 2018). Metabolomics studies revealed changes in the levels of the arginine precursor substance glutamate (Glu) and its metabolites citrulline and spermidine (Spd) in different tissues of depressed patients and depressed rats and indicated that the arginine pathway was speculated to be involved in the development of depression (Zhou et al., 2011). Arginine catabolism could influence the condition of individuals with depression through the dysregulation of oxidative and nitrosative stress functions (Ali-Sisto et al., 2018). Furthermore, the effects of the gut microbiota on metabolite and metabolic functions can be explored through relative quantification. Research reports have proven that there are alterations in arginine and related metabolites in depression and that these changes are involved in depression, anxiety, and stress severity. Agmatine and putrescine (Put) are two other important metabolites of arginine that have been implicated in counteracting some of these neurodegenerative effects of depression (Liu et al., 2009). Administration of agmatine to rats may exert antidepressant and anxiolytic activity, cognitive enhancement, and neuroprotective effects in rat models.

Arginine was also reported to influence some neurotransmitters in the prefrontal cortex of the brain, such as dopamine (DA), γ-aminobutyric acid (GABA), and Glu, which are primarily considered to play important roles in cellular bioenergetics and oxidative stress. Stevens et al. reported that single-nucleotide exact amplicon sequence differences of the human gut microbiota were able to identify depression phenotypes (Stevens et al., 2021). Depressed patients had slightly higher proportions of taxonomic groups that produced GABA. GABA production and degradation occur mainly through the following pathways: "arginine-putrescine and GABA degradation superpathway", "arginine and ornithine degradation superpathway", and "arginine degradation AST superpathway". These findings show that the gut microbiota is involved in the arginine metabolism pathway in depressed patients (Strandwitz et al., 2019). Stress leads to damage to the intestinal barrier function and increased permeability, which causes the activation of the intestinal immune system, thereby affecting the central nervous system, which is considered to be a potential pathophysiological mechanism leading to major depressive disorder (Farzi et al., 2018). It has been clarified that the gut microbiota is able to maintain intestinal barrier function (Sanders et al., 2021). Therefore, it was significant to assess the alterations of arginine metabolism related to the gut microbiota. The pathophysiological mechanism of depression and the role of gut microbiota in the development of depression could also be further understood.

In this study, behavior tests and monoamine neurotransmitter determinations were used to evaluate the efficacy of CGKL extract and CGKL. Then, we chose the clinical preparation of CGKL to further study the relationship between its antidepressant effect and the gut microbiota. 16S rRNA sequencing and LC-MS technology were used to analyze the improvement effects of CGKL, including the structural composition of the gut microbiota in CUMS-induced rats, as well as the regulation of metabolite levels in arginine metabolic pathways involved in the microbiota. From the perspective of gut microbiota, molecular biological techniques, including Western blotting and qRT-PCR, were employed to provide an in-depth study of the mechanism by which CGKL exerts antidepressant effects through the pathway of arginine metabolism mediated by the gut microbiota.

2. Materials and methods

2.1. Reagents and materials

Mass spectrometry-grade acetonitrile was obtained from Fisher Scientific (Fisher Scientific, Pittsburgh, PA). Purified water (18.2 MΩ) was prepared using a Milli-Q water purification system (Millipore, USA). Formic acid, *L*-arginine (Arg, No.403C021), *L*-ornithine (Orn, No.903H021) and *L*-citrulline (Cit, No.116A024) were purchased from Solarbio (Beijing, China); Glu (No.Y5V5I-NH) and GABA (No.635QO-KP) were purchased from TCI Development Co., Ltd. (Shanghai, China); and Put (No. Q10M9D61065) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China), spermine (Spm, No. T3007/71-44-3) and Spd (No. T4893/124-20-9) were purchased from Topscience Co., Ltd. (Shanghai, China). Tryptophan (Trp, 089K00082V), 5-hydroxytryptamine (5-HT, BCBR6328V), 5-hydroxyindoleacetic acid (5-HIAA, BCBM0853V), tyrosine (Tyr, BCBG4813V), norepinephrine (NE, 091M1746V), DA(BCBD7743V) and 3,4-dihydroxybenzylamine hydrobromide (DHBA, No. MKBS7646V) were purchased from Sigma-Aldrich (Louis, USA). All other reagents and chemicals were of analytical grade.

Venlafaxine hydrochloride capsules from Chengdu Kanghong Pharmaceutical Group Co., Ltd. (Chengdu, China) (Lot No. 181203) and Shugan Jieyu Capsule from Sichuan Jishengtang Pharmaceutical Co., Ltd. (Chengdu, China) (Lot No. 180101) were the positive control drugs. The Chaigui Granules (CGKL) extract was supplied by the Shanxi Institute of Traditional Chinese Medicine Co., Ltd. (Shanxi, China). It was composed of the following six medicinal herbs: *Bupleuri Radix* (Chaihu in Chinese) (Lot No. 1805215131), *Paeoniae Radix Alba* (Baishao in Chinese) (Lot No. 1806436111), *Angelicae Sinensis Radix* (Danggui in Chinese) (Lot No. 1803583111), *Atractylodis Macrocephalae Rhizoma* (Baizhu in Chinese) (Lot No. 1809657151), *Glycyrrhizae Radix* et *Rhizoma* (Gancao in Chinese) (Lot No. 1810013171), and *Menthae Haplocalycis Herba* (Bohe in Chinese) (Lot No. 1802017131) in a specific proportion of 6: 6: 6: 6: 3: 2. These medicinal herbs were purchased from Hebei Anguo Qiao Traditional Chinese Medicine Tablets Co., Ltd. (Baoding, China) authenticated by Prof. Xuemei Qin in Pharmacognosy Department of Shanxi University. The preparation and quality control of CGKL were described in Figs. S1–S2.

2.2. Rats and treatments

Specific pathogen-free (SPF) Sprague Dawley male rats [8 weeks, (200 ± 20) g] were obtained from Beijing Vital River Laboratory Co., (Beijing, China) with license No. SCXK 2016–0006. The rats were housed in cages with a temperature of (24 ± 2) °C and a relative humidity of (50 ± 5)% under a 12 h/12 h light–dark cycle, with standard chow and water available ad libitum. After adaptability time, the rats were randomly categorized into eight groups: CON group (control), CUMS group (model), VEN group (CUMS + Venlafaxine, 35 mg/kg), SHU group (CUMS + Shugan Jieyu Capsule, 0.15 g/kg), EXT-L group (CUMS + low dose of CGKL extract, 4.2 g/kg), EXT-M group (CUMS + medium dose of CGKL extract, 8.3 g/kg), EXT-H group (CUMS + high dose of CGKL extract, 16.6 g/kg), and CGKL group (CUMS + CGKL, 8.3 g/kg), with 12 rats in each group. In the previous research, we have determined that the clinical dosage of CGKL is 8.3 g/kg, which is equivalent to the medium

dosage of CGKL extract mixed with a certain proportion of excipients (Tian et al., 2022). In order to further evaluate the impact of preparation process on drug efficacy, we simultaneously set up three doses of CGKL extract groups and CGKL group. All animal experiments were carried out strictly following the NIH Guidelines for Care and Use of Laboratory Animals (USA) and the Prevention of Cruelty to Animals Act (1986) of China. The research project was approved by the Committee of Scientific Research at Shanxi University (CSRSX) with approval No. SXULL20180006 that was approved on June 2, 2018.

The CUMS procedure was carried out as described in previous reports (Fu et al., 2018; Tian et al., 2022). The specific stress methods and arrangements were described in the supplementary materials. All procedures were shown in Fig. 1.

2.3. Tissue sample collection and preparation

Blood was collected under anesthesia by intraperitoneal injection 53 d after CUMS model establishment. Jejunum contents and hippocampal tissue were collected and snap-frozen into liquid nitrogen for further analysis. All samples were separated and stored at −80 °C.

2.4. 16S rRNA microbial community analysis

Based on the results of behavioral indicators, six rats from each group were selected. The total DNA of the microbiota in intestinal contents was extracted for 16S V3-V4 high-throughput sequencing. The 16S rRNA gene sequences were selected and compared to determine the relative abundance of microbiota taxa. Then, the filtered sequences were clustered into operational taxonomic units (OTUs) according to representative sequences using Usearch and classified against the Greengenes Database with a threshold of 97% sequence similarity (DeSantis et al., 2006; Zhang et al., 2018). Microbiotal analysis of Bray-Curtis dissimilarities was calculated according to the levels of altered gut microbiota using R software.

2.5. LC-MS instrumentation and method for determining monoamine neurotransmitters and arginine-related metabolites

LC-MS analysis was performed using a 1290 Infinity binary pumps Liquid Chromatography System (Agilent Technologies) equipped with a Waters ACQUITY UPLC BEH C_{18} Column (100 mm × 2.1 mm, 1.7 μm) and a Waters ACQUITY UPLC T3 Column (100 mm × 2.1 mm, 1.7 μm) with a 3 200 Q Trap (AB Sciex, Boston, USA) mass spectrometer. The separation mobile phases were a water/formic acid (0.1%) mixture (solvent A) and acetonitrile (solvent B). The method for the determination of monoamine neurotransmitters referred to the previous quantitative method established by the research group (Zhao et al., 2018; Zhou et al., 2018). The method for the determination of arginine-related metabolites was described in the supplementary material. The elution gradient program for the samples was shown in Table S1. Optimal multiple-reaction monitoring (MRM) transitions were further identified for the analyses of individual AAs (Table S2). The most abundant MRM transition for each analyte was considered a quantifier ion. The methodological validation was described in the supplementary material. Data acquisition and analysis were all performed with Analyst 1.6.3 software (AB SCIEX).

2.6. Western blot analysis

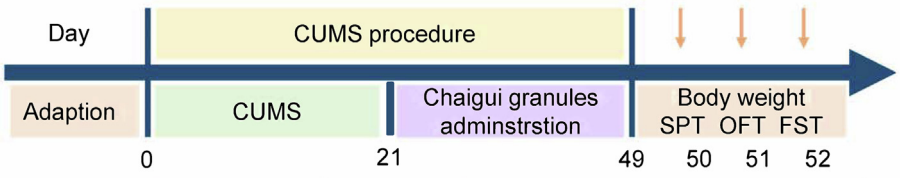
The experimental processes of protein extraction and Western blotting were based on the manufacturer's instructions, and the specific details were described in the supplemental material (Langille et al., 2013). The primary antibodies included spermidine/spermine N1-acetyltransferase 1 (SAT1) (1:1 000, Bioss, 17244R), ornithine decarboxylase (ODC) (1:1 000, Bioss, 1294R), recombinant agmatine ureohydrolase (AGMAT) (1:1 000, absin, 134271) and β-actin (1:1 000, Abcam 4970S).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

To determine the expression of mRNA in the rat hippocampus, the experimental procedures followed the manufacturer's recommendation (Liu et al., 2019). The primer sequences were shown in Table S8.

2.8. Statistical analysis

All data were statistically analyzed using SPSS 20.0 software (IBM, Chicago, IL, USA). Data with normal distribution and



Experiment Design

Groups	0–21 d	22–49 d
CON	No-CUMS	
CUMS	CUMS	CUMS
VEN		+ Venlafaxine, 35 mg/kg
SHU		+ Shuganjieyu Capsule, 0.15 g/kg
EXT-L		+ CGKL-extract, 4.2 g/kg
EXT-M		+ CGKL-extract, 8.3 g/kg
EXT-H		+ CGKL-extract, 16.6 g/kg
CGKL		+ CGKL, 8.3 g/kg

Fig. 1. Antidepressant effects of CGKL in rats after CUMS. Schedule of CUMS model, treatment, behavioral tests, and time of sample collection. CUMS was performed from day 1 to day 21, and therapeutic administration was performed on day 22. Saline and CGKL were administered i.g. into CUMS rats. Behavioral tests and biological samples were collected at end of schedule.

homogeneity of variance were statistically processed using a One-way analysis of variance (ANOVA). The *t*-test was used for comparisons between every two groups. Quantitative data with a normal distribution are expressed as the means ± standard deviations of the mean (SD). The statistical analyses were conducted with GraphPad Prism 8.0.1 software (La Jolla, CA, USA). The *n* value indicates the number of rats per group, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. CGKL improved depression-like state of CUMS rats

3.1.1. CGKL alleviated depressive-like behavior induced by CUMS

The results of behavioral tests showed that CUMS-induced rats exhibited depressive-like behavior ($P < 0.05, 0.01$) compared with the control group after three weeks of stress (Fig. S3). However, this depressive behavior could be significantly improved with the administration of different drugs after a 4-week treatment period ($P < 0.05, 0.01$). The low and medium doses of CGKL extract and CGKL could ameliorate body weight, reward response, motor function and desperate response. We found that the effect of the EXT-M group was the best of the three doses, and CGKL also showed a considerable effect on the results of the behavior tests. Compared with the Venlafaxine hydrochloride capsules and Shugan Jieyu Capsule, the effects of CGKL showed equivalent to those of above positive control drugs.

3.1.2. CGKL alleviated monoamine neurotransmitter disorder induced by CUMS

The monoamine neurotransmitters in the 5-HT and NE pathways in the serum of CUMS rats were significantly affected. As shown in Fig. S4, the levels of Trp and 5-HIAA in the model group were increased significantly ($P < 0.01$), and the levels of 5-HT, Tyr, DA and NE were decreased significantly ($P < 0.05, 0.01$) compared with the CON group. All substances were significantly adjusted by CGKL treatment. However, 5-HT, Try and NE were only increased by CGKL extract, and there was no significant difference. Similar to the above behavioral results, CGKL had the same effect as EXT-M and positive drugs.

All of these observations suggested that rats show depressive-like behavior and abnormalities in contents of monoamine neurotransmitters after seven weeks of CUMS stress. These effects were mitigated by drug treatment, and Chaigui Granule showed the same effect as the extract of CGKL according to the results between the group of CGKL and the EXT-M. Meanwhile, the effect of CGKL was also comparable with the two positive drugs. Since we focused on the study of CGKL on the gut microbiota of CUMS and its related metabolites, we would focus on the CON group, CUMS group and CGKL group in the following experiment.

3.2. Gut microbiota changed following CUMS intervention and CGKL treatment

The gut microbiota diversity index changed in CUMS-induced depression. It has been shown that CUMS-induced depressive behaviors may be ameliorated by modulating the gut microbiota. The specific composition of the microbiota community in each sample at each classification level and the number of OTUs from phylum to species fluctuated greatly among samples due to the differences among individuals. As shown in Fig. S5A, there was a large difference in the number of OTUs in the jejunum. The number of taxa contained in different samples was calculated at each classification level. As seen from Fig. S5B, the gut microbiota was rich in the jejunum.

In this study, the diversity of the gut microbiota structural composition in rats with CUMS was disrupted. In the jejunum, the Chao 1 index, Observed_species, Faith_pd and Shannon index of diversity in the CUMS group were less than those in the control group, indicating that the microbial diversity was reduced after CUMS intervention and that CGKL could effectively increase the species diversity and richness (Fig. 2, Table S9).

The principal component analysis (PCA) plot indicated an obvious separation between the two groups along the PC1 axis, and the treatment group was near the control group, indicating that CGKL had a good regulatory effect on the gut microbiota (Fig. 3A). Furthermore, the non-metric multidimensional scaling (NMDS) analysis plots of unweighted UniFrac showed that the control group was more similar to the CGKL-treated group than CUMS group (Fig. 3B). Thus, it was likely that CGKL was of a potency that could improve the altered CUMS-induced rat gut microbiota composition.

Linear discriminant analysis effect size (LEfSe) emphasizes the search for robust differential species among groups (Fig. S6). To identify microbiota taxa that differed in relative abundance in the antidepressant-treated rats in comparison to the control, we analyzed the sequencing results using the LEfSe algorithm. The results showed that the proportion of varieties of species in the jejunum was approximately 76.4%.

If the gut microbiota in the body becomes imbalanced, it can harm intestinal function (Du et al., 2016). Picrust2 analysis revealed significant alterations in the abundances of functional pathways in the gut microbiota. From these results, except for unclassified microbiota, it was evident that the differential microbiota in the jejunum was more involved in the arginine pathway, which was one of the altered pathways in the potential pathogenesis of depression. These microbiotas included members of the genera *Acinetobacter*, *Thermus*, and *Ochrobactrum*, which are involved in the arginine biosynthetic pathway. *Pseudomonas*, *Leptothrix* and *Acinetobacter* are also involved in an arginine catabolic pathway (Fig. 4). From the abovementioned results, it was concluded that there was a strong relationship between depression and the arginine pathway, confirming the results shown above.

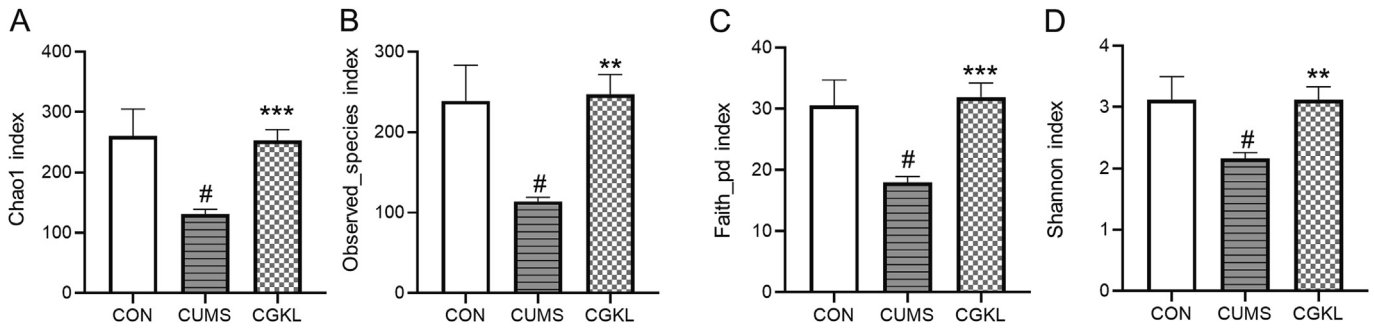


Fig. 2. Regulation of α diversity index of jejunum microbiota by CGKL. (mean ± SD, $n = 4$) $^{\#}P < 0.05$ *vs* CON; $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* CUMS.

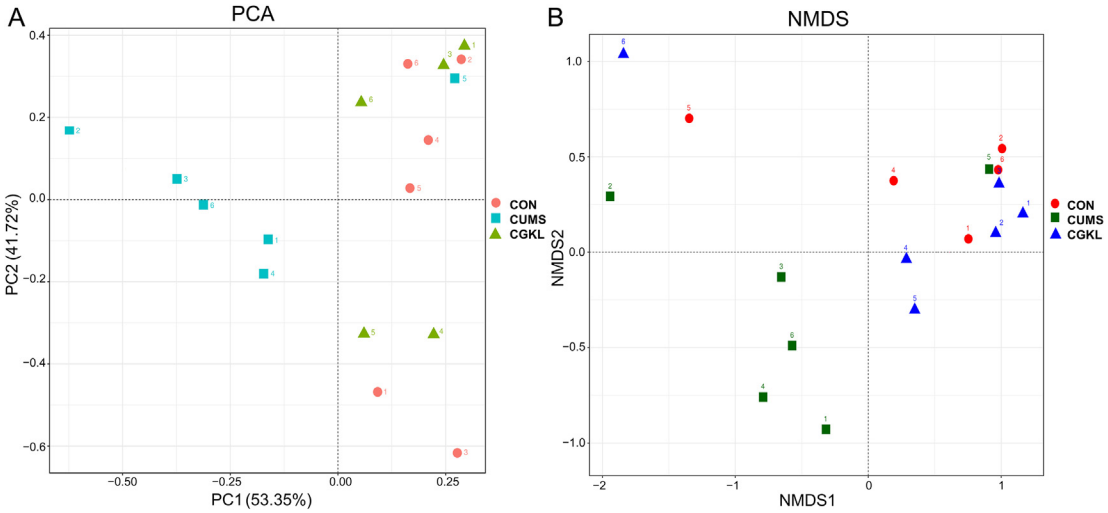


Fig. 3. PCA of gut microbiota data. PCA plots among three groups (A); NMDS analysis plots of unweighted UniFrac among three groups (B).

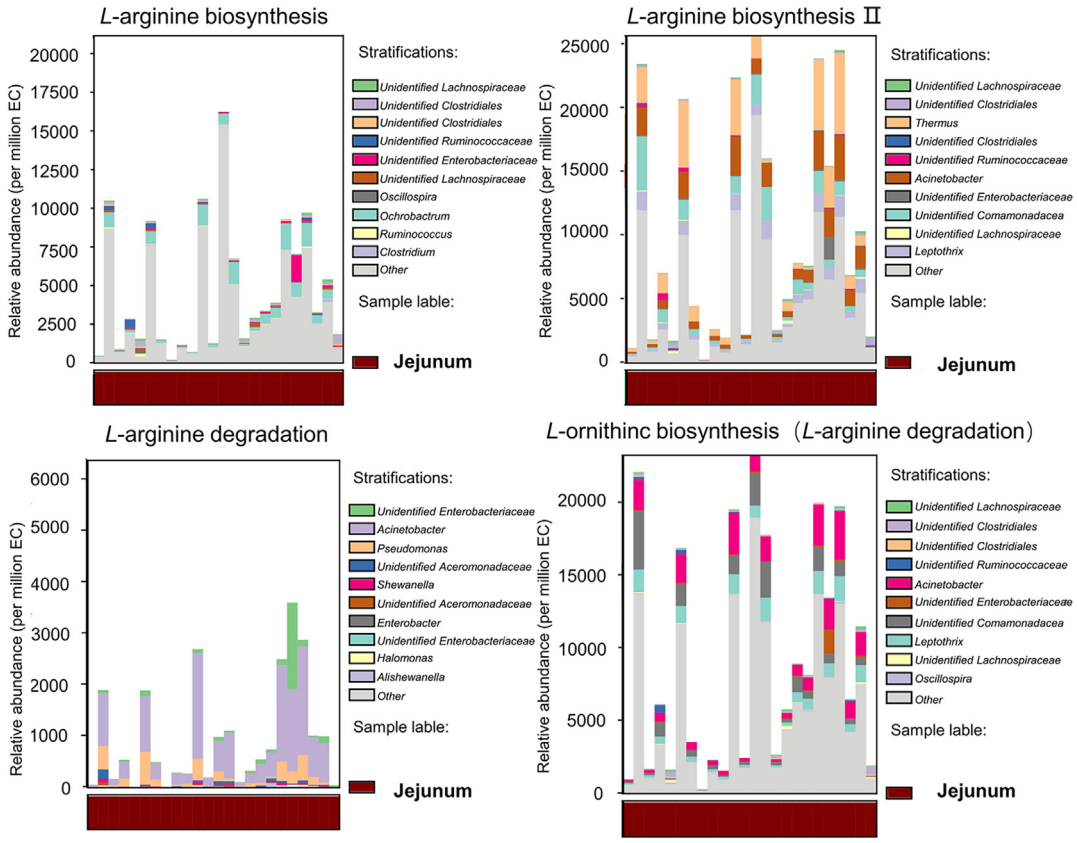


Fig. 4. Differential composition of gut microbiota species identified through MetaCyc metabolic pathways. The abscissa shows samples of different parts, which are grouped by different colors, and samples in group are sorted according to similarity of data; ordinate shows relative abundance of metabolic pathway; contribution values of different taxa to metabolic pathway are displayed by different colors at same taxonomic level.

Increased levels of arginine have been reported in the hippocampal tissues of CUMS rats, and the arginine levels in the sera of depression patients were increased (Zhou et al., 2019). It was proposed that arginine metabolites were related to the pathology and physiology of depression, and putrescine may become a new biomarker to forecast the severity of depression (Ozden et al., 2020; Limon et al., 2016). Arginine and its metabolites play a key role in host and microbiota (Nüse et al., 2023). Taken together, our results suggested that depression was predicted by gut microbiota composition and function. Therefore, it was necessary to further study the relationship between the arginine metabolism pathway and depression.

3.3. Changes in arginine metabolic profiling of CUMS-induced depression

Changes in the metabolite content of the arginine metabolic pathway in CUMS-induced depressed rats were detected through the LC-MS technique. In the LC-MS base-peak chromatograms of various metabolites, the other peaks detected are indicated with the respective numbers of the identified metabolites stated in Fig. S7. The optimized MS parameters for the detection of the selected arginine metabolites were summarized in Table S2. The methodological validation results were shown in Tables S3–S7. Compared with the control group, the contents of arginine and ornithine in the CUMS group were significantly increased in the serum, while the contents of the metabolized polyamines Spd and Spm and their metabolite GABA were significantly decreased. The levels of each metabolite were decreased and trended significantly after CGKL treatment, and ornithine, the downstream substance derived from arginine metabolism, was regulated more significantly. In the hippocampus (Fig. 5A), the contents of glutamate, arginine, citrulline and ornithine were significantly higher in the CUMS group. Conversely, the contents of polyamines, Spd, Spm, Put and its corresponding metabolite GABA, produced from arginine metabolism, were significantly decreased, which indicated that the pathway of arginine metabolism was disordered in CUMS rats, consistent with the above serum results. Each metabolite level was significantly improved after CGKL treatment (Fig. 5B). Combined with the serum results, the CGKL group showed some degree of regulation of the arginine metabolic pathway. In sum-

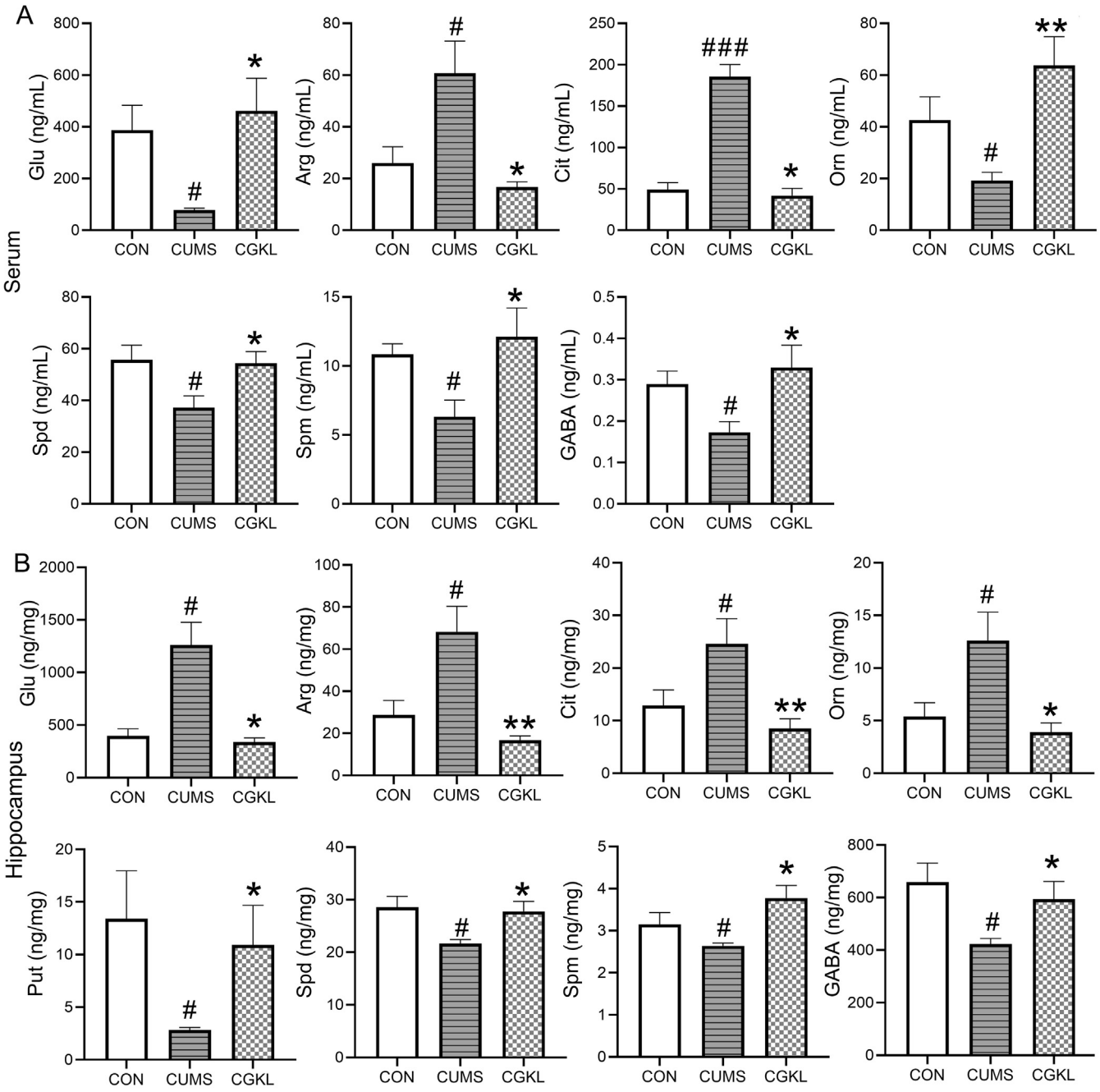


Fig. 5. Changes in metabolites of arginine metabolic pathway in CUMS-induced depression rats (mean ± SD, n = 6). The regulatory trends of CGKL on various substances in serum (A) and hippocampus (B), respectively. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs* CON; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* CUMS, ns not significant.

mary, CUMS-induced depression changed the structure/composition of the gut microbiota and disturbed the arginine metabolic pathway, and CGKL showed the expected regulating effect of substantially altered metabolic levels.

3.4. Gene- and protein-level differential expression analysis

To evaluate the impact of CGKL on the regulation of the arginine pathway, we further analyzed genes and proteins related to arginine metabolism pathways. In our results, the *Odc*, *Agmat*, spermine synthase (*Spms*), and spermidine synthase (*Spds*) gene expression levels in the CUMS-induced model rats were significantly higher than those in the other groups, indicating an arginine metabolism disorder in the CUMS-induced rats (Fig. 6A–D). In addition, the *Sat1* gene expression decreased in CUMS rats (Fig. 6E). While the administration of CGKL could regulate the expression of these gene in the arginine metabolic pathway.

The study demonstrated that the downregulation of SAT1 (Fig. 7D), the key rate-limiting enzyme involved in polyamine catabolism, AGMAT and ODC increased significantly in the model group (Fig. 7B and C). Both the key rate-limiting enzymes ODC and SAT1 regulate polyamine levels. These alterations may be related to the occurrence of depression. From the data above, we may conclude that a primary consequence of changing the expression levels of the ODC, SAT1, and AGMAT proteins was the alteration of depression (Fig. 7). These findings may be particularly relevant to arginine catabolism-associated proteins, the presentation of which changed in response to the antidepressant treatment in the study. Among them, hyperactivation of the ODC pathway can increase neuronal excitation. Neurons were excessively excitatory, resulting in neurotoxic excitation, which eventually caused depression-like behavior.

Consequently, the above results suggested that CUMS-mediated regulation of the gut microbiota was a critical pathway mediating depression-like determination in the arginine metabolism pathway. In general, combined with the improvement of CGKL in the composition and structure of the gut microbiota, the findings ultimately indicated that CGKL might play an antidepressant role by regulating the structure of the gut microbiota involved in the arginine metabolic pathway.

4. Discussion

In behavioral tests of this study, the results assessed depression-like behaviors in rats. This study reported that CGKL effectively improved depression-like behaviors induced by CUMS.

Based on the significant efficacy demonstrated by the group's previous experiments, the distribution of gut microbiota in different parts of CUMS rats was investigated, and the distribution and diversity of flora in different parts of the intestine were found to differ in the structural composition of flora at each taxonomic level. The results of diversity analysis showed that CGKL regulated the diversity of jejunum microbiota more significantly in both the α- and β-diversity levels, as determined by 16S rRNA gene sequencing. Therefore, this study revealed a special regulatory effect of CGKL on the arginine pathway involved in the jejunum microbiota. Therefore, the analysis of arginine and its metabolites was performed by LC-MS to observe the regulatory effect of CGKL. Subsequently, the gene and protein expression levels of key rate-limiting enzymes in the pathway were validated by molecular biology methods to detect the antidepressant-like effects of CGKL. Meanwhile, the metabolism of the host was also disturbed after CUMS induction. Furthermore, the jejunum microbiota was involved in the arginine metabolism pathway, which indicated that depression both altered the gut microbiota and influenced the host metabolic phenotype, ultimately leading to metabolite disorders in the host. These outcomes could provide new views into revealing novel antidepressant effects of CGKL and understanding the function of the gut microbiota in central nervous system disorders, indicating that modulating the gut microbiota could be a new therapeutic tool.

Growing evidence suggests that disturbed gut microbiota may contribute to depression, but the exact triggering mechanism remains unclear. In this study, it was observed that CUMS depres-

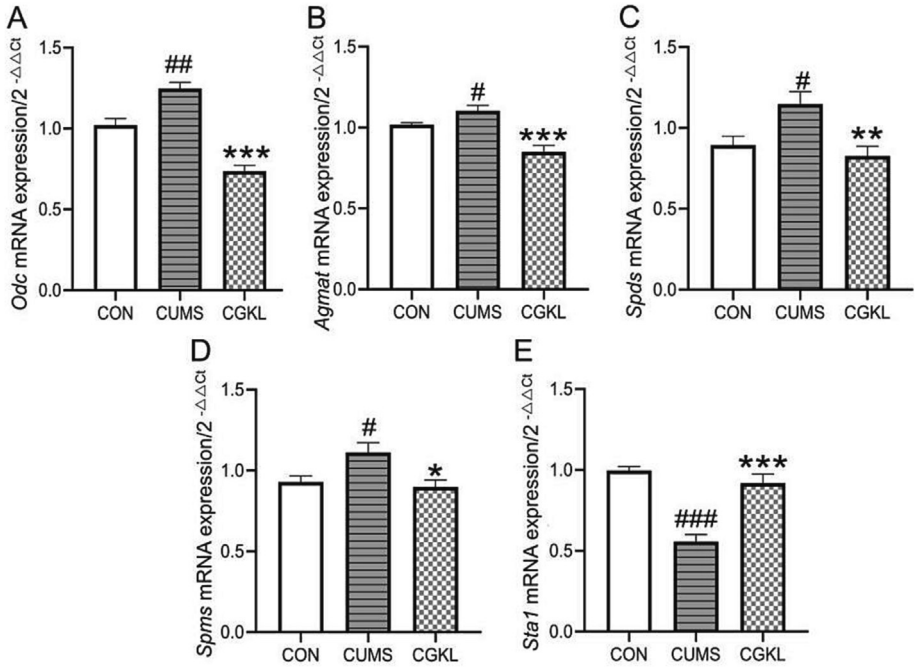


Fig. 6. Expression of arginine-metabolizing enzymes in hippocampus of CUMS rats (mean ± SD, $n = 6$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* CON; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* CUMS, ns not significant, the colors in figure represent different groups.

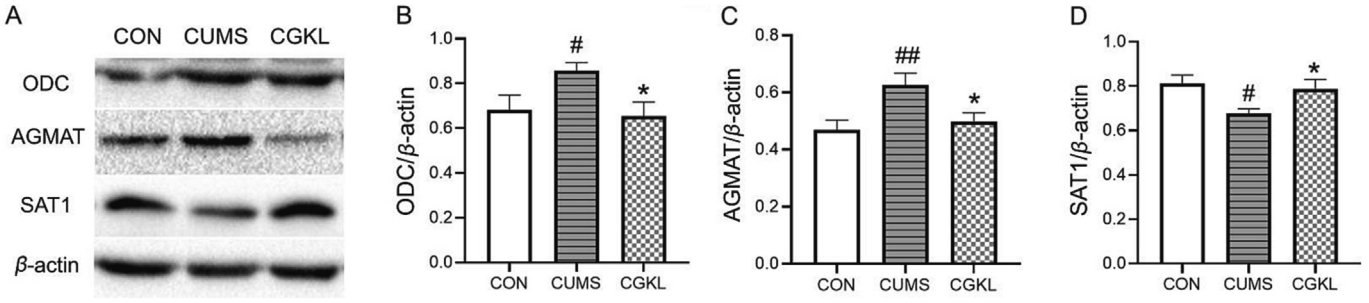


Fig. 7. Regulatory effect of CGKL on arginine metabolism-related proteins in hippocampi of CUMS rats (mean ± SD, n = 6). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* CON; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* CUMS, ns not significant, the colors in the figure represent different groups.

sion rats were characterized by alterations in microbial composition, function, and arginine metabolic pathways. In terms of gut microbiota functional prediction, it has gradually been recognized in the literature that the value of gut microbiota studies lies in the importance of the interactive ecology of microbial metabolism pathways over differences in taxonomy (Visconti et al., 2019). The gut microbiota appeared to share a core set of metabolic activities including fermentation of amino acids (Van Treuren & Dodd, 2020). The rate of arginine synthesis in the intestine is higher than in other metabolically active tissues (Nüse et al., 2023). Chronic stress is one of the most critical factors in the onset of depressive disorders, and the hippocampus is a critical site during CUMS-induced alterations in depressive subjects; however, the underlying neural mechanisms remain unclear. This study used CUMS to simulate depression and found that CGKL can significantly improve the expression levels of arginine metabolism-related genes and proteins in the hippocampus. Moreover, the pharmacological mechanism of CGKL on depression was uncovered by molecular biology analysis (Fig. 8). These findings demonstrate a critical role for gut microbiota and arginine metabolism modulation in CUMS-induced depressive-like behaviors.

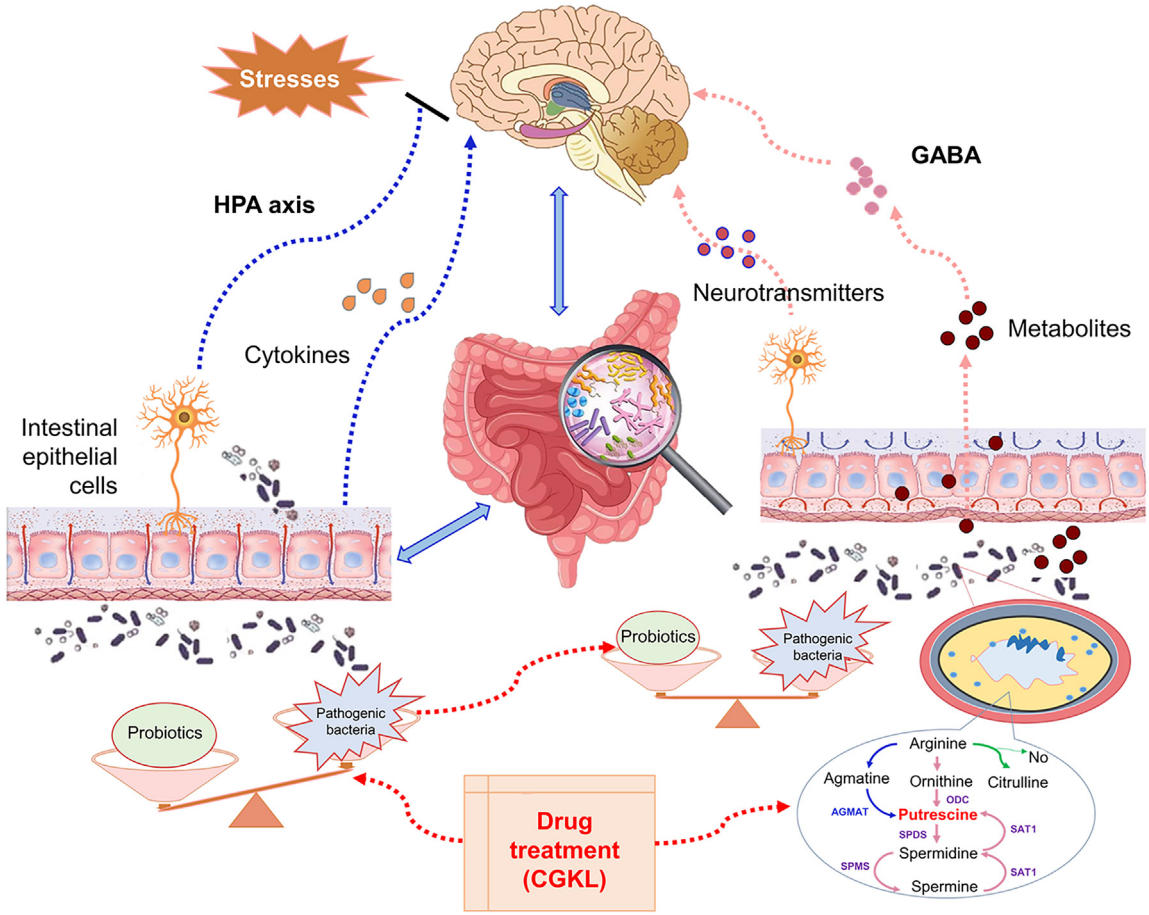


Fig. 8. Schematic diagram of antidepressant mechanism of Chaigui Granule (CGKL) regulating intestinal flora-mediated arginine metabolism. Long-term stress leads to depressed brain–gut axis dysfunction, which affects enteric nervous system through brain-gut axis, causing intestinal inflammation, which in turn leads to imbalance of intestinal flora, impaired intestinal barrier, increased intestinal permeability, and cytokine release. The other parts of body eventually become damaged. After drug CGKL was administered, intestinal flora stabilized. The flora participated in arginine metabolism pathway, and the levels of its metabolites could also be adjusted. Arginine and its metabolites can enter body through intestinal epithelial cells and produce GABA to stimulate brain, thereby improving depression.

The present amplicon sequence variants (ASV) approach revealed the analysis of the gut microbiota MetaCyc pathway in depression, in contrast to the trend to control pathways in control reference subjects (Caspi et al., 2020). In the present results, amino acid pathways were prominent in the gut microbiome metabolism of depression, especially the arginine degradation pathway, and the microbiota involved in the arginine pathway happens to be regulated by CGKL. It has been reported that arginine can be degraded into polyamines. Under stress stimulation, the metabolism of polyamine was accelerated, especially the degradation of arginine to putrescine, in a process also called the polyamine stress reaction (PSR) (Gilad & Gilad, 2003). Polyamines can interact with the gut microbiota, which is an important metabolite of the gut microbiota that can promote the balance of the body by inhibiting inflammation (Ramos-Molina et al., 2019). The polyamine level was regulated by the rate-limiting enzyme SAT1, and its expression was found to decrease in the brains of depressed patients, indicating that the arginine pathway played an important role in the development of depression (Pantazatos et al., 2015).

Previous studies have reported that oral administration of arginine to rats did not increase putrescine levels in the gut after treatment with antibiotics, indicating that putrescine may be mainly produced by gut microbiota (Kibe et al., 2014). Combined with the results of the present study, CGKL was found to have a significant modulatory effect on the gut microbiota involved in the arginine pathway in the jejunum of CUMS-induced depression rats. It was indicated that the antidepressant effect of CGKL was related to the arginine pathway mediated by the gut microbiota. Agmatine from the diet was found to be minimal, and its main source, arginine metabolism by gut commensal microbiota, was detected in the body (Laube & Bernstein, 2017). These data suggested that the gut microbiota played a dominant role in changes of the arginine metabolism pathway.

When stressed, the intestinal villi epithelium could be injured, the intestinal mucosa barrier could become damaged, and the composition and structure of the gut microbiota would also be changed (Chen et al., 2007). CGKL improved the structure of the gut microbiota, which indicates that the improvement of the gut microbiota may play a role in improving the intestinal barrier. Among them, polyamines can repair the intestinal barrier through endogenous supply and exogenous supplementation and play a role in protecting the intestine (Gao et al., 2013). Polyamines may be related to the imbalance of polyamine metabolism in depression in maintaining intestinal epithelial mucosal integrity and microbiota stability, which play an important role in maintaining the balance between excitatory and inhibitory neurons (Cao et al., 2020).

There was growing interest in the research of arginine catabolism in the development and progression of depression. Our research results clarified the associations between several key rate-limiting enzyme protein expression levels (ODC, SAT1, and AGMAT) and further illustrated the correlations among the proteins in the arginine pathway of depression (Cao et al., 2020). Interestingly, the CUMS group exhibited increases in the mRNA expression levels of multiple genes, while the rats in the CGKL group demonstrated a small decline in mRNA. Combined with the literature, we further illustrate that CGKL exerted antidepressant effects by mediating arginine metabolism through regulating the gut microbiota.

To the best of our knowledge, this was a study on microbiota sequencing of depressed rats with gut microbiota as the entry point, and it was the first time that CGKL was explored to improve the relationship between depression and the arginine metabolism pathway and related genes and proteins. The results of this study provide further evidence that the spermidine metabolite putrescine predicts depression.

5. Conclusion

Collectively, the multi-omics included microbiomics and target metabolomics revealed the effects of CGKL on the improvement of gut microbiota composition and the regulation of metabolites in the arginine metabolism pathway in CUMS rats. The relationship of gut microbiota with its molecules and depression should also be paid more attention.

CRediT authorship contribution statement

Qi Wang: Data curation, Formal analysis, Visualization, Writing – original draft. **Yingxia Zhao:** Writing – review & editing. **Xuemei Qin:** Project administration, Validation, Writing – review & editing. **Junsheng Tian:** Conceptualization, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 82074147, 82374153); the Science and Technology of Shanxi Province (No. 202103021224026); and the Key Laboratory of Effective Substances Research and Utilization in TCM of Shanxi Province (No. 202105D121009).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2023.12.003.

References

Ali-Sisto, T., Tolmunen, T., Viinamäki, H., Mäntyselkä, P., Valkonen-Korhonen, M., Koivumaa-Honkanen, H., Honkalampi, K., Ruusunen, A., Nandania, J., Velagapudi, V., & Lehto, S. M. (2018). Global arginine bioavailability ratio is decreased in patients with major depressive disorder. *Journal of Affective Disorders, 229*, 145–151.

Cao, B., Deng, R. Z., Wang, D. F., Li, L., Ren, Z. Y., Xu, L. X., & Gao, X. (2020). Association between arginine catabolism and major depressive disorder: A protocol for the systematic review and meta-analysis of metabolic pathway. *Medicine, 99*(27), e21068.

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., Ong, W. K., Paley, S., Subhraveti, P., & Karp, P. D. (2020). The MetaCyc database of metabolic pathways and enzymes - a 2019 update. *Nucleic Acids Research, 48* (D1), D445–D453.

Chen, J., Rao, J. N., Zou, T., Liu, L., Marasa, B. S., Xiao, L., Zeng, X., Turner, D. J., & Wang, J. Y. (2007). Polyamines are required for expression of Toll-like receptor 2 modulating intestinal epithelial barrier integrity. *American Journal of Physiology. Gastrointestinal and Liver Physiology, 293*(3), G568–G576.

Dash, S., Clarke, G., Berk, M., & Jacka, F. N. (2015). The gut microbiome and diet in psychiatry: Focus on depression. *Current Opinion in Psychiatry, 28*(1), 1–6.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., & Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology, 72*(7), 5069–5072.

Du, H. L., Wang, K. Q., Su, L., Zhao, H. X., Gao, S. Y., Lin, Q. S., Ma, X. F., Zhu, B. K., Dong, X., & Lou, Z. Y. (2016). Metabonomic identification of the effects of the Zhimu-Baihe saponins on a chronic unpredictable mild stress-induced rat model of depression. *Journal of Pharmaceutical and Biomedical Analysis, 128*, 469–479.

Evrensel, A., & Ceylan, M. E. (2015). The gut-brain axis: The missing link in depression. *Clinical Psychopharmacology and Neuroscience: The Official Scientific Journal of the Korean College of Neuropsychopharmacology, 13*(3), 239–244.

Farzi, A., Fröhlich, E. E., & Holzer, P. (2018). Gut microbiota and the neuroendocrine system. *Neurotherapeutics: The Journal of the American Society for Experimental Neurotherapeutics, 15*(1), 5–22.

Feng, G. M., Tian, J. S., Wu, Y. F., Zhao, S. J., Zhang, L. Z., & Qin, X. M. (2014). Clinical study of Xiaoyao Powder in treating depression. *Liaoning Journal of Traditional Chinese Medicine, 41*, 512–516.

Fu, X. Y., Chen, H. H., Zhang, N., Ding, M. X., Qiu, Y. E., Pan, X. M., Fang, Y. S., Lin, Y. P., Zheng, Q., & Wang, W. Q. (2018). Effects of chronic unpredictable mild stress on ovarian reserve in female rats: Feasibility analysis of a rat model of premature ovarian failure. *Molecular Medicine Reports, 18*(1), 532–540.

Gao, J. H., Guo, L. J., Huang, Z. Y., Rao, J. N., & Tang, C. W. (2013). Roles of cellular polyamines in mucosal healing in the gastrointestinal tract. *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society, 64*(6), 681–693.

Gao, X. X., Li, Y., Meng, M. D., Wang, P., Feng, Y., Jia, J. P., & Qin, X. M. (2020). Exploration of chemical composition and absorption characteristics of Chaigui granules based on UHPLC-Q-orbitrap-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis, 187*, 113293.

Gilad, G. M., & Gilad, V. H. (2003). Overview of the brain polyamine-stress-response: Regulation, development, and modulation by lithium and role in cell survival. *Cellular and Molecular Neurobiology, 23*(4–5), 637–649.

Gu, F., Wu, Y., Liu, Y., Dou, M., Jiang, Y., & Liang, H. (2020). *Lactobacillus casei* improves depression-like behavior in chronic unpredictable mild stress-induced rats by the BDNF-TrkB signal pathway and the intestinal microbiota. *Food & Function, 11*(7), 6148–6157.

Ji, C., Han, Y. M., Zhao, W. D., Liu, X. Y., Linghu, T., Tian, J. S., & Qin, X. M. (2022). Effects of Xiaoyaosan on behavior and skeletal muscle mitochondrial structure and function in depressed rats. *Drug Evaluation Research, 45*(9), 1763–1769.

Jiao, H., Yan, Z., Ma, Q., Li, X., Jiang, Y., Liu, Y., & Chen, J. (2018). Influence of Xiaoyaosan on depressive-like behaviors in chronic stress-depressed rats through regulating tryptophan metabolism in hippocampus. *Neuropsychiatric Disease and Treatment, 15*, 21–31.

Kibe, R., Kurihara, S., Sakai, Y., Suzuki, H., Ooga, T., Sawaki, E., Muramatsu, K., Nakamura, A., Yamashita, A., Kitada, Y., Kakeyama, M., Benno, Y., & Matsumoto, M. (2014). Upregulation of colonic luminal polyamines produced by intestinal microbiota delays senescence in mice. *Scientific Reports, 4*, 4548.

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., & Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology, 31*(9), 814–821.

Laube, G., & Bernstein, H. G. (2017). Agmatine: Multifunctional arginine metabolite and magic bullet in clinical neuroscience? *The Biochemical Journal, 474*(15), 2619–2640.

Limon, A., Mamdani, F., Hjelm, B. E., Vawter, M. P., & Sequeira, A. (2016). Targets of polyamine dysregulation in major depression and suicide: Activity-dependent feedback, excitability, and neurotransmission. *Neuroscience and Biobehavioral Reviews, 66*, 80–91.

Liu, P., Jing, Y., & Zhang, H. (2009). Age-related changes in arginine and its metabolites in memory-associated brain structures. *Neuroscience, 164*(2), 611–628.

Liu, X. J., Liu, C. C., Tian, J. S., Gao, X. X., Li, K., Du, G. H., & Qin, X. M. (2020). Plasma metabolomics of depressed patients and treatment with Xiaoyaosan based on mass spectrometry technique. *Journal of Ethnopharmacology, 246*, 112219.

Liu, Y., Ding, X. F., Wang, X. X., Zou, X. J., Li, X. J., Liu, Y. Y., Li, J., Qian, X. Y., & Chen, J. X. (2019). Xiaoyaosan exerts antidepressant-like effects by regulating the functions of astrocytes and EAATs in the prefrontal cortex of mice. *BMC Complementary and Alternative Medicine, 19*(1), 215.

Luna, R. A., & Foster, J. A. (2015). Gut brain axis: Diet microbiota interactions and implications for modulation of anxiety and depression. *Current Opinion in Biotechnology, 32*, 35–41.

Nasca, C., Bigio, B., Lee, F. S., Young, S. P., Kautz, M. M., Albright, A., Beasley, J., Millington, D. S., Mathé, A. A., Kocsis, J. H., Murrough, J. W., McEwen, B. S., & Rasgon, N. (2018). Acetyl-l-carnitine deficiency in patients with major depressive disorder. *Proceedings of the National Academy of Sciences of the United States of America, 115*(34), 8627–8632.

Nüse, B., Holland, T., Rauh, M., Gerlach, R. G., & Mattner, J. (2023). *L*-arginine metabolism as pivotal interface of mutual host-microbe interactions in the gut. *Gut Microbes, 15*(1), 2222961.

Ozden, A., Angelos, H., Feyza, A., Elizabeth, W., & John, P. (2020). Altered plasma levels of arginine metabolites in depression. *Journal of Psychiatric Research, 120*, 21–28.

Pan, J. X., Xia, J. J., Deng, F. L., Liang, W. W., Wu, J., Yin, B. M., Dong, M. X., Chen, J. J., Ye, F., Wang, H. Y., Zheng, P., & Xie, P. (2018). Diagnosis of major depressive disorder based on changes in multiple plasma neurotransmitters: A targeted metabolomics study. *Translational Psychiatry, 8*(1), 130.

Pantazatos, S. P., Andrews, S. J., Dunning-Broadbent, J., Pang, J., Huang, Y. Y., Arango, V., Nagy, P. L., & John Mann, J. (2015). Isoform-level brain expression profiling of the spermidine/spermine N1-Acetyltransferase1 (SAT1) gene in major depression and suicide. *Neurobiology of Disease, 79*, 123–134.

Ramos-Molina, B., Queipo-Ortuño, M. I., Lambertos, A., Tinahones, F. J., & Peñafiel, R. (2019). Dietary and gut microbiota polyamines in obesity- and age-related diseases. *Frontiers in Nutrition, 6*, 24.

Sanders, D. J., Inniss, S., Sebepos-Rogers, G., Rahman, F. Z., & Smith, A. M. (2021). The role of the microbiome in gastrointestinal inflammation. *Bioscience Reports, 41*(6), BSR20203850.

Stevens, B. R., Roesch, L., Thiago, P., Russell, J. T., Pepine, C. J., Holbert, R. C., Raizada, M. K., & Triplett, E. W. (2021). Depression phenotype identified by using single nucleotide exact amplicon sequence variants of the human gut microbiome. *Molecular Psychiatry, 26*(8), 4277–4287.

Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T. R., Lekbua, A., Mroue, N., Liston, C., Stewart, E. J., Dubin, M. J., Zengler, K., Knight, R., Gilbert, J. A., Clardy, J., & Lewis, K. (2019). GABA-modulating bacteria of the human gut microbiota. *Nature Microbiology, 4*(3), 396–403.

Tian, D. D., Wang, M., Liu, A., Gao, M. R., Qiu, C., Yu, W., Wang, W. J., Zhang, K., Yang, L., Jia, Y. Y., Yang, C. B., & Wu, Y. M. (2021). Antidepressant effect of paeoniflorin is through inhibiting pyroptosis CASP-11/GSDMD pathway. *Molecular Neurobiology, 58*(2), 761–776.

Tian, J. S., Qin, P. F., Xu, T., Gao, Y., Zhou, Y. Z., Gao, X. X., Qin, X. M., & Ren, Y. (2022). Chaigui granule exerts anti-depressant effects by regulating the synthesis of estradiol and the downstream of CYP19A1-E2-ERKs signaling pathway in CUMS-induced depressed rats. *Frontiers in Pharmacology, 13*, 1005438.

Wang, Y. S., Shen, C. Y., & Jiang, J. G. (2019). Antidepressant active ingredients from herbs and nutraceuticals used in TCM: Pharmacological mechanisms and prospects for drug discovery. *Pharmacological Research, 150*, 104520.

Winter, G., Hart, R. A., Charlesworth, R. P. G., & Sharpley, C. F. (2018). Gut microbiome and depression: What we know and what we need to know. *Reviews in the Neurosciences, 29*(6), 629–643.

Van Treuren, W., & Dodd, D. (2020). Microbial contribution to the human metabolome: Implications for health and disease. *Annual Review of Pathology, 15*, 345–369.

Visconti, A., Le Roy, C. I., Rosa, F., Rossi, N., Martin, T. C., Mohney, R. P., Li, W., de Rinaldis, E., Bell, J. T., Venter, J. C., Nelson, K. E., Spector, T. D., & Falchi, M. (2019). Interplay between the human gut microbiome and host metabolism. *Nature Communications, 10*(1), 4505.

Yao, Y., Huang, H. Y., Yang, Y. X., & Guo, J. Y. (2015). Cinnamic aldehyde treatment alleviates chronic unexpected stress-induced depressive-like behaviors via targeting cyclooxygenase-2 in mid-aged rats. *Journal of Ethnopharmacology, 162*, 97–103.

Zhang, T., Zhao, F., Zhang, X., Gao, X. X., Zhou, Y. Z., Tian, J. S., & Qin, X. M. (2018). Antidepressant effect of Compound Chaigui prescription and its mechanism of regulating 5-HT metabolic pathway. *Chinese Traditional and Herbal Drugs, 49*, 1338–1344.

Zhao, F., Li, Q., Liang, M. L., Yan, Y., Xing, J., Qin, X. M., & Gao, X. X. (2018). Simultaneous determination of eight kinds of neurotransmitters in rat blood serum by ultra-high performance liquid chromatograph-tandem mass spectrometry. *Chinese Journal of Analytical Chemistry, 46*(1), 121–128.

Zhao, Y. X., Xu, T., Tian, J. S., & Qin, X. M. (2021). Effect of Chaigui Granule on gut microbiota in chronic unpredicted mild stress induced depression rats model. *Chinese Traditional and Herbal Drugs, 52*(3), 736–743.

Zhou, X., Liu, L., Lan, X., Cohen, D., Zhang, Y., Ravindran, A. V., Yuan, S., Zheng, P., Coghill, D., Yang, L., Hetrick, S. E., Jiang, X., Benoliel, J. J., Cipriani, A., & Xie, P. (2019). Polyunsaturated fatty acids metabolism, purine metabolism and inosine as potential independent diagnostic biomarkers for major depressive disorder in children and adolescents. *Molecular Psychiatry, 24*(10), 1478–1488.

Zhou, Y., Huang, S. H., Wu, F. L., Zheng, Q. Y., Zhang, F. S., Luo, Y. X., & Jian, X. H. (2021). Atractylenolide III reduces depressive- and anxiogenic-like behaviors in rat depression models. *Neuroscience Letters, 759*, 136050.

Zhou, P., Xie, W. J., Luo, Y., Lu, S., Dai, Z. R., Wang, R. Y., Sun, G. B., & Sun, X. B. (2018). Protective effects of total saponins of Aralia elata (Miq.) on endothelial cell injury induced by TNF-α via modulation of the PI3K/Akt and NF-κB signalling pathways. *International Journal of Molecular Sciences, 20*(1), 36.

Zhou, Y. Z., Lu, L. L., Li, Z. F., Gao, X. X., Tian, J. S., Zhang, L. Z., Wu, B. W., & Qin, X. M. (2011). Antidepressant-like effects of the fractions of Xiaoyaosan on rat model of chronic unpredictable mild stress. *Journal of Ethnopharmacology, 137*(1), 236–244.