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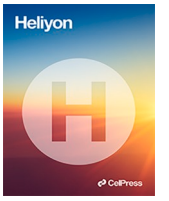

Cinnamon oil solid self-microemulsion mediates chronic mild stress-induced depression in mice by modulating monoamine neurotransmitters, corticosterone, inflammation cytokines, and intestinal flora

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ABSTRACT

Cinnamon oil (CO) is a classic Chinese medicine with excellent soothing effects on exhaustion, weakness and depression. Cinnamaldehyde is the main active ingredient of cinnamic oil. Although CO have antidepression-like effects, limited information is available. Furthermore, the disadvantages of CO, such as low oral availability and difficult portability, limit its development. In this study, a Cinnamon Oil Solid Self-Microemulsifying Drug Delivery System (CO-S-SME) was designed, prepared. In addition, we explored the effects and mechanisms of CO-S-SME on chronic unpredictable mild stress (CUMS)-induced depression-like behavior, monoamine neurotransmitters, inflammatory factors, intestinal flora in mice. Mice were subjected CUMS to establish the depression model. The antidepressant effect of CO-S-SME was evaluated by behavioral tests. In addition, the expression levels of neurotransmitters, corticosterone (CORT) and inflammatory factors in CUMS mice were analyzed by enzyme-linked immunosorbent assay. In addition, we explored the effects of CO-S-SME on the diversity and richness of intestinal flora of mice in each group. Behavioral tests showed that CO-S-SME could effectively improve depression-like behaviors in CUMS mice. Specifically, CO-S-SME treatment effectively increased neurotransmitter levels and reduced the expressions of corticosterone and inflammatory factors in CUMS mice. CO-S-SME also changed the intestinal flora composition, decreased the ratio of Firmicutes to Bacteroidetes, reduced relative abundances of Lactobacillus, modulated Alpha diversity and beta diversity. These results suggest that CO-S-SME an act as a good antidepressant, exhibiting effects via monoamine neurotransmitters, CORT, inflammation cytokines, and intestinal flora.

Abbreviations: CO, cinnamon oil; CUMS, chronic unpredictable mild stress; CORT, corticosterone; CO-S-SME, Cinnamon Oil Solid Self-Microemulsifying Drug Delivery System; HPA, Hypothalamic-pituitary-adrena; SME, Self-Microemulsifying Drug Delivery System.

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1. Introduction

Depression is a serious and common disease characterized by persistent mood depression, slow response, lack of interest, low self-esteem and sleep disorders, has become a serious social problem that affects people's mental health and quality of life [1,2]. Clinical treatment of depression mainly involves drug therapy combined with psychotherapy. At present, the main drugs for treatment of depression include serotonin (5-HT) reuptake inhibitors and norepinephrine uptake inhibitors. Although, these drugs have few adverse reactions, there are still some common problems, such as incomplete drug response and emotional blunting of patients [3,4], that lead patients with depression to seek new, safer, and more effective drugs. The pathogenesis of depression is extremely complex and involves multiple stages [5]. The monoamine hypothesis suggests that dysfunction of serotonin, norepinephrine (NE), and dopamine (DA) is the main cause of depression [6]. The inflammatory cytokine hypothesis suggests that the pathogenesis of depression is related to elevated levels of inflammatory cytokines [7]. Hypothalamic-pituitary-adrenal (HPA) axis hyperactivity is also generally accepted to be involve in the pathogenesis of depression. The brain-gut axis is a two-way information conversion pathway between the brain and gut. Abnormal microbiota impairs one or more pathways of the brain-gut axis, which may lead to brain-gut axis dysfunction and depression [8,9]. Therefore, how to explore more effective drugs for depression based on all of these assumptions is obviously important.

Cinnamomum cassia Presl (*C. cassia*), an aromatic tree species belonging to the *Lauraceae* family, is mainly produced in tropical countries and regions such as India and Malaysia, as well the Guangdong and Guangxi regions of China. Its dried bark and twigs are commonly used in traditional Chinese medicine, which has the effects of supplementing fire to support Yang and warming the interior to disperse cold. Because *C. cassia* can be used as both medicine and food, it has high economic value. At present, more than 160 components have been isolated and identified from the *C. cassia* [10]. Among them, CO is the main bioactive component of cinnamon, which has antibacterial [11–13], anti-inflammatory [14–17], anti-tumor [18,19], hypoglycemic [20,21], and other pharmacological activities. Currently, several studies have shown the efficacy of CO and its components in the prevention and treatment of certain neurological diseases [22]. However, in the previous report, CO of antidepressant mechanism has not been widely studied. At the same time, CO is a hydrophobic functional component with low bioavailability and difficult portability, which seriously hinders its clinical application of CO.

Self-microemulsifying drug delivery systems (SME) are excellent carriers of hydrophobic or difficult-to-absorb drugs. Due to the formation of nano-sized droplets with large absorptive surface areas, SME can improve digestion rate and, release rates, as well as mucus layer diffusion and epithelial cell permeability [23]. In addition, SME promotes secretion of endogenous bile salts and phospholipids, transmembrane absorption of drugs after gastrointestinal digestion, production of lipoprotein/chylomicron by reducing first-pass metabolism and lymphatic transport through the transcellular pathway; and inhibits P-glycoprotein and other efflux pumps to promote drug absorption [24,25]. At the same time, drug clearance and elimination can be reduced by changing the distribution in the tissue [26]. Solid SME (S-SME) can effectively improve the stability of drugs and is convenient for patients to carry and use, which improves medication compliance [27], and allows realization of individual drug administration through easy dose adjustment [28].

Therefore, in this study, CO–S-SME was prepared using mannitol as a lyoprotectant during freeze-drying after the successfully establishing the Liquid SME formulation. Moreover, we explore the antidepressant effects of CO–S-SME on neurotransmitters, CORT, inflammatory factors, and intestinal flora in a CUMS mouse model.

2. Materials and methods

2.1. Chemicals and reagents

Cinnamon oil was purchased from Jiangxi Hengcheng natural flavor oil Co., Ltd (85% cinnamaldehyde, Jiangxi, China). Polyoxyethylene hydrogenated castor oil (RH-40) was purchased from BASF SE (Ludwigshafen, Germany). Caprylic/capric triglycerride (GTCC) was purchased from Shandong Yousuo Chemical Technology Co., Ltd (Linyi, China). Anhydrous ethanol and mannitol were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China). Fluoxetine (FLU) hydrochloride dispersible tablets (20 mg/tablet) were purchased from Shandong Linuo Pharmaceutical Co., Ltd (Shandong, China). ELISA kit was purchased from Shanghai Enzyme Link Biotechnology Co., Ltd (Shanghai, China). Fast DNA SPIN extraction kit was purchased from MP Biomedicals (California, USA). PicoGreen dsDNA analysis kit was purchased from Invitrogen (Carlsbad, USA). NovaSeq 6000 SP Reagent Kit was purchased from Wuhan Frasergen Bioinformatics Co., Ltd (Wuhan, China).

2.2. Preparation of CO–S-SME

In brief, the total amount of fixed materials is 2 g, CO and GTCC are used as oil phase and the ratio is 8:2. The optimal formulation of CO-L-SME optimized by simplex lattice design is: oil phase (28.83%), surfactant (30%), co-surfactant (41.17%). According to optimal formula, 0.46128 g CO was added to 0.1148 g GTCC in a separate beaker. The calculated 0.6 g emulsifier (RH-40) and 0.8234 g co-emulsifier (anhydrous ethanol) were mixed in a vial on a magnetic stirrer. The CO-loaded GTCC was mixed in RH-40 and anhydrous ethanol mixture under continuous stirring until the self-emulsification was complete. Then, a weighed amount of mannitol was added, continue to stir in a constant temperature water bath for 10 min. After freeze-drying, CO–S-SME is obtained. According to the calculation of cinnamaldehyde content, the CO loadings of CO–S-SME is 150.50 mg g^{-1}.

2.3. Stability test

A certain amount of CO–S-SME was placed in a clean glass Petri dish and placed in a drug stability test instrument. The physical morphology and particle size distribution after self-emulsification were investigated at 25 °C ± 2 °C and relative humidity 60% ± 5% for 1, 3 and 6 months, and the stability was evaluated.

2.4. Animals

All experimental procedures were approved by the Biological and Medical Ethics Committee of Jiamusi University (JMSU-244, Jiamusi, China). 70 SPF grade male healthy Kunming mice (body weight 18–22 g, license number: SCXK(Ji)-2020-0002.) were purchased from Changchun Yisi Laboratory Animal Technology Co., Ltd (Changchun, China). The animals were housed and acclimatized under a normal 12 light-dark cycle of temperature (20–26 °C) and relative humidity (40–70%) before the experiments. Mice were acclimated for 14 days. Litter is changed every two days. After adaptive feeding, all mice were subjected to sucrose preference test (SPT), forced swimming test (FST) and open field test (OFT). The mice with abnormal behavior were excluded, and the experimental results were used as the behavioral baseline of each mouse. The qualified mice were randomly divided into 6 groups (10 mice·group^{-1}), which were the blank control group (KB group), depression model group (M group), and FLU positive control group (2.6 mg kg^{-1}, Y group), CO–S-SME high-dose group (200 mg kg^{-1}, G group), CO–S-SME medium-dose group (100 mg kg^{-1}, Z group), CO–S-SME low-dose group (40 mg kg^{-1}, D group). There is no effective dose of cinnamon oil in the clinical treatment of depression at present, it was found through pharmacokinetic tests that an oral dose that is too low may not achieve effective plasma drug concentration. Preliminary results showed that 500 mg kg^{-1} dose of SMEDDS resulted in death of mice. According to the results of the gradient dose experiment, 200 mg kg^{-1} was used as the dose of obvious and non-toxic effect. So, 200 mg kg^{-1} was taken as the high-dose group, and 100 mg kg^{-1}, half of the high-dose group, was taken as the medium-dose group and 40 mg kg^{-1} as the low-dose group.

2.5. CUMS procedure

In this experiment, the mice in each group (except for KB group) followed the CUMS modeling method with some modifications [29]. 1–2 kinds of stress stimuli were randomly given every day, and each kind of stimuli should not appear continuously. The stressors included swimming in cold water, deprivation of fasting, tail suspension, day and night reversal, restraint, tail clipping, wet litter, LED light stroboscopic stimulation, *etc*. Regarding the behavioral tests of depression, SPT, FST, OFT are widely used in research, and the test results are reliable [30], all of which can show memory impairment, mental retardation, and abnormal behavior similar to those of patients with depression, *etc*. phenomenon. After the CUMS procedure started, the mice were tested for SPT and FST every two weeks, and OFT test was performed every four weeks to evaluate the model establishment. From the fifth week, at the same time of modeling, each treatment group was given corresponding drugs (10 mL kg^{-1}) by gavage 30 min before daily stimulation, and the KB group and M group were given an equal volume of purified water daily until the completion of 8 weeks.

2.6. Behavioral tests

2.6.1. SPT

The test was divided into two phases without water or food deprivation. The first 3 days were sucrose preference adaptive training. On the first day, 2 bottles of 1% sucrose solution were placed, and on the second day, 1 bottle of 1% sucrose solution and 1 bottle of pure water were placed for 24 h (the position of the water bottle was changed in the middle). On the third day, fasting for 6 h and no water for 24 h. The fourth day is the test experiment, 2 bottles of 1% sucrose solution and purified water are placed respectively, the position of the water bottle is changed after 12 h, and the consumption of the 2 bottles of solution within 24 h is recorded and calculated.

2.6.2. FST

Briefly, mice were placed one by one into a transparent glass cylinder filled with water (22 ± 2 °C) and forced to swim in the vessel for 6 min. In the last 5 min, the camera system was used to record the immobility time of the animals (the mice floated on the water, did not struggle, just kept breathing). After the test, the mice were blown dry and returned to the cage.

2.6.3. OFT

The OFT was carried out in a black opaque plastic box (50 cm × 50 cm × 50 cm). The bottom of the box was divided into 25 equal squares (10 cm × 10 cm). The mice were placed gently in the same corner. Each mouse to explore freely for 6 min. In the last 5 min, using a computerized video tracking system to analyze the movement of each mouse. After each mouse was tested, the excrement was cleaned and wiped with 75% alcohol to prevent test bias caused by odor. The behavioral test content of mice includes time in center, total distance, upright times, number of enteies into the center.

2.7. Assay of neurotransmitters, CORT and inflammatory factors in tissue

After the behavioral test, the mice were sacrificed by cervical dislocation on the second day. Blood was collected from the eyeballs of the mice in each group, and serum and plasma were obtained by different treatment methods. The colonic feces and brain (left) were

taken out one by one. After the feces were taken out, they were quickly frozen in liquid nitrogen. The left brain was weighed and minced, added 9 times the volume of pH 7.4 PBS solution, homogenized at low temperature, centrifuged (4 °C, 3500 rpm, 15 min) to take the supernatant. ELISA kits were used to measure neurotransmitters, CORT and inflammatory factors levels in tissues.

2.8. Analysis of gut microbiota by 16S rRNA sequencing

Fecal samples of mice in each group were collected, and total DNA was extracted from the fecal samples according to the instructions of the DNA kit. The required target gene fragment was amplified by PCR. The amplified products were purified and recovered for fluorescence quantification. Illumina NovaSeq platform was used for high-throughput sequencing of community DNA fragments after constructing sequencing library, and the results were annotated with Green Genes database.

2.9. Statistical analyses

All results were expressed as mean ± SD. Statistical significance was determined using analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test (GraphPad Prism 8 software), and $P < 0.05$ indicated statistically significant difference.

3. Results

3.1. Stability test results

As shown in Table 1, CO–S-SME did not change significantly within 6 months (temperature 25 °C ± 2 °C, relative humidity 60% ± 5%), and formed blue opalescent translucent emulsions after microemulsification without phase separation and drug precipitation, indicating good stability of CO–S-SMEDDS.

3.2. Study on the antidepressant effect of CO–S-SME

3.2.1. SPT

As shown in Fig. 1, compared with the KB group, the sucrose consumption rate of mice in each group decreased significantly after stress, indicating that the CUMS model well simulated the symptom of lack of interest in patients with depression. After 8 weeks of gavage, mice in Y, Z and D group displayed improvements in sucrose preference compared with the M group. Although the G group improved compared with the fourth week, this change was not obvious compared with the other groups.

3.2.2. FST

As shown in Table 2, a significant decrease in the active swimming period during the forced swimming of the CUMS mice. After administration, the results of each treatment group (except for Z group) showed that the immobility time of mice was significantly reduced ($P < 0.01$).

3.2.3. OFT

The open field behavior of mice, including time in center, total distance, number of upright, and number of entries into the center, was used to evaluate anxiety. Compared with the KB group, CUMS significantly reduced the emotional and autonomous activities of mice ($P < 0.01$, Fig. 2A, B, C and D). Moreover, compared with the M group, the autonomous activity of each treatment group was enhanced (Fig. 2A, B, C and D).

3.3. Determination of monoamine neurotransmitter levels

As shown in Table 3, FLU, a selective inhibitor of 5-HT reuptake, could significantly increase 5-HT levels in the brain of depressed mice ($P < 0.01$). Compared with M group, neurotransmitter levels were increased in all treatment groups. A moderate dose of CO–S-SME (100 mg kg^{-1}) had a positive effect on the nervous system of mice. Its effect on monoamine neurotransmitters was more obvious, and its regulatory effects on DA and NE levels in the brains of depressed mice was better than that of the positive control drug FLU.

Table 1
The stability of CO–S-SMEDDS.

Time (months)	Character	Particle size (nm)
0	Free flowing white powder	25.34 ± 1.62
1	No sign of instability	25.92 ± 1.06
3	No sign of instability	27.74 ± 1.28
6	Slight aggregation of powder without colour change	28.49 ± 1.66

Data are presented as mean ± SD (n = 3).

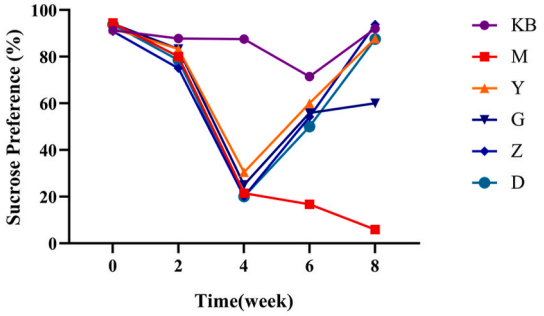

Fig. 1. Results of SPT

Table 2
The immobility time of mice in each group.

Group	immobility time (s)				
	0	second week	fourth week	sixth week	eighth week
KB	62.24 ± 15.71	61.38 ± 19.32	63.71 ± 30.76	68.13 ± 9.40	69.38 ± 23.63
M	68.75 ± 41.74	115.60 ± 44.16**	155.35 ± 57.04**	194.68 ± 64.08	192.00 ± 39.57**
Y	75.91 ± 41.01	112.75 ± 35.17*	178.13 ± 52.68**	61.81 ± 19.48	42.50 ± 18.86$^{\#\#}$
G	62.00 ± 11.98	110.38 ± 20.42**	171.88 ± 47.17**	55.00 ± 15.38$^{\#\#}$	35.49 ± 42.51$^{\#\#}$
Z	65.48 ± 28.56	119.22 ± 48.74	157.11 ± 39.87**	64.00 ± 25.33$^{\#}$	44.47 ± 17.48$^{\#\#}$
D	73.11 ± 27.35	124.22 ± 22.99*	176.44 ± 48.73**	96.61 ± 40.80	73.89 ± 22.76$^{\#\#}$

Data are presented as mean ± SD (n = 10), compared with KB, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with M, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

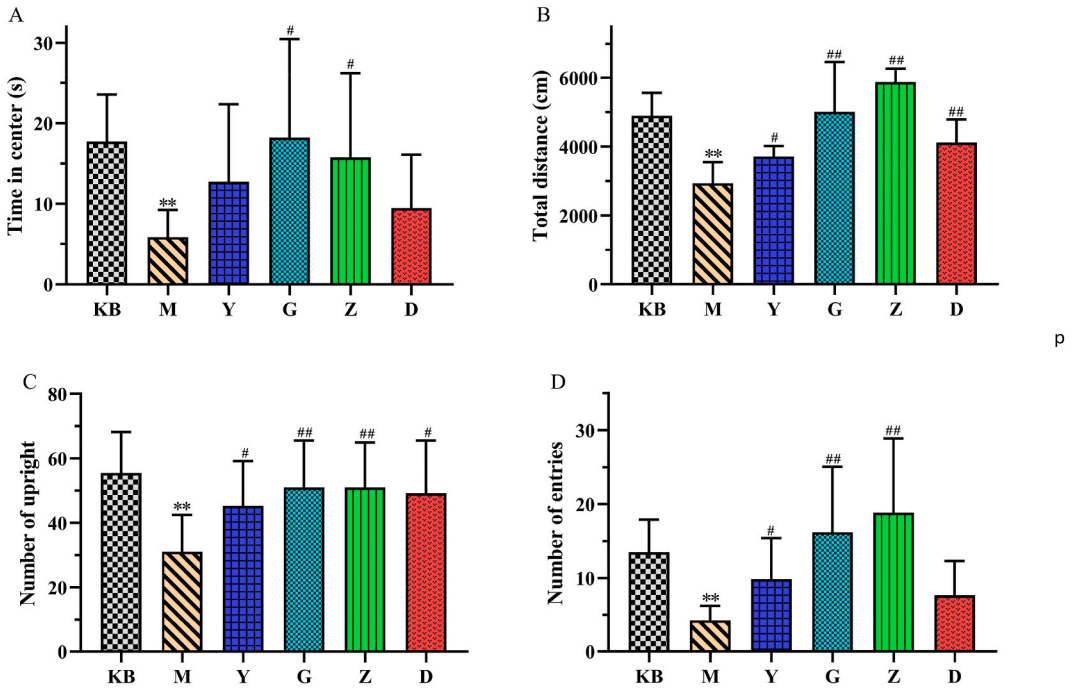

Fig. 2. Results of OFT in week 8, (A) Time in center (B) Total distance (C) Number of upright (D) Number of entries into the center, data are presented as mean ± SD (n = 10) compared with KB, $^{**}P < 0.01$, compared with M, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

3.4. Determination of CORT levels

As shown in Table 4, compared with the KB group, plasma CORT levels of mice in the M group were significantly increased ($P < 0.01$). After administration, CORT levels were decreased in all treatment groups. G group had a positive effect on the CORT of mice.

Table 3
Expression of neurotransmitters in each group.

Group	Expression of neurotransmitter		
	5-HT	DA	NE
KB	186.75 ± 14.32	104.63 ± 7.18	4.92 ± 0.57
M	139.28 ± 10.6**	93.20 ± 8.22*	3.81 ± 0.23**
Y	178.22 ± 8.58##	102.02 ± 8.75	3.86 ± 0.22
G	134.67 ± 13.48	115.76 ± 2.54##	4.23 ± 0.42
Z	176.72 ± 18.26##	103.00 ± 4.60#	4.36 ± 0.14##
D	131.85 ± 15.92	97.51 ± 8.59	4.17 ± 0.58

Data are presented as mean ± SD (n = 10), compared with KB, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with M, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

Table 4
Expression of CORT in each group.

Group	Expression of CORT
KB	158.48 ± 10.77
M	182.99 ± 11.12**
Y	164.44 ± 12.22
G	154.40 ± 9.45##
Z	164.49 ± 3.20#
D	166.99 ± 9.00

Data are presented as mean ± SD (n = 10), compared with KB, $^{**}P < 0.01$, compared with M, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

3.5. Determination of inflammatory factors levels

As shown in Table 5, compared with the KB group, serum levels of inflammatory cytokines IL-6, IL-1β, and TNF-α in the M group were significantly increased ($P < 0.05$). The G group had no significant regulatory effect on IL-6 or TNF-α compared with the M group ($P > 0.05$). Moreover, compared with other treatment groups, levels of pro-inflammatory factors were higher in the G group. Although the underlying mechanism needs further investigation, our results show significantly down-regulated levels of three pro-inflammatory factors in the Z group ($P < 0.05$).

3.6. Effects of CO–S-SME on relative abundance of intestinal flora in CUMS mice

AS shown in Fig. 3. *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are the dominant phyla. Compared with the KB group, *Firmicutes/Bacteroidetes* (F/B) values of the intestinal flora of mice in the M group were significantly increased, and the abundance of *Proteobacteria* was significantly increased. Compared with the M group, the Z group displayed reduced abundances of *Proteobacteria* and F/B values.

As shown in Fig. 4, relative abundance of *Lactobacillus* in the intestinal flora of the mice in the M group were significantly increased compared with the KB group, while the relative abundance of *Sutterella* was significantly decreased. Compared with the M group, each treatment group displayed significantly reduced relative abundance of *Lactobacillus*, while the relative abundance of *Lactobacillus* was significantly increased.

3.7. Effects of CO–S-SME on bacterial OTU number and alpha diversity in CUMS mice

The number of OTUs shared by each group is 1179 (Fig. 5). The number of OTUs in KB group, M group, Y group, G group, Z group

Table 5
Expression of inflammatory factor in each group.

Group	Expression of inflammatory factor		
	IL-6	IL-1β	TNF-α
KB	89.56 ± 2.35	86.18 ± 9.10	327.04 ± 21.83
M	100.06 ± 6.57*	119.65 ± 19.75*	415.68 ± 15.36**
Y	94.00 ± 6.97	80.89 ± 5.31##	362.37 ± 34.00#
G	98.99 ± 15.03	91.61 ± 4.74#	398.95 ± 40.62
Z	88.39 ± 8.31#	87.74 ± 10.64#	368.20 ± 29.76#
D	89.38 ± 2.55#	90.38 ± 4.37#	406.31 ± 18.20

Data are presented as mean ± SD (n = 10), compared with KB, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with M, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

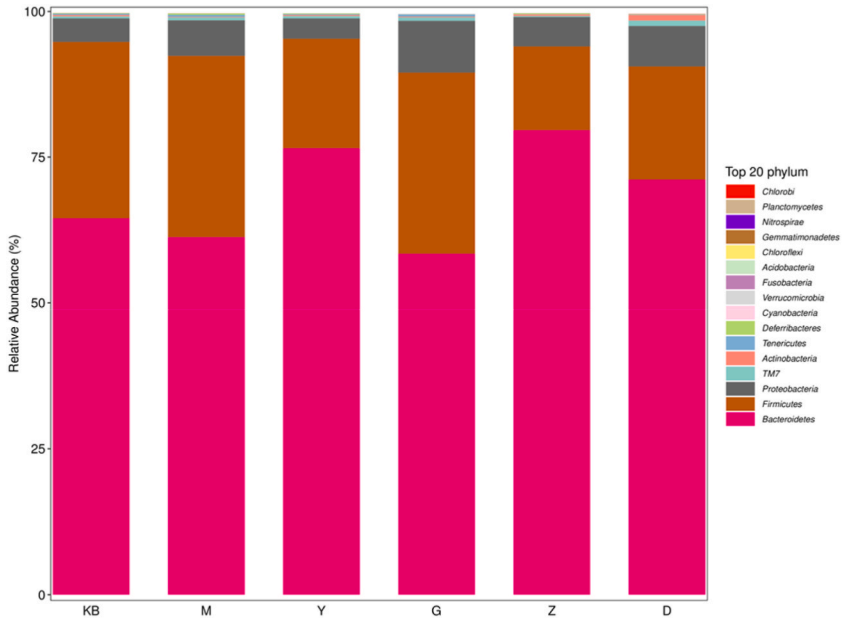

Fig. 3. Histogram of horizontal species composition of phylum.

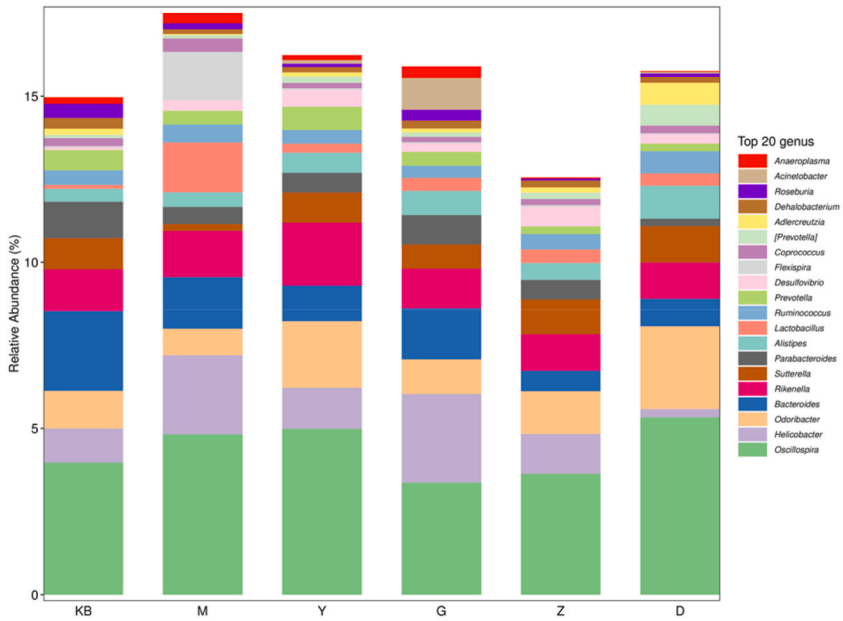

Fig. 4. Histogram of horizontal species composition of genus.

and D group were 5890, 5146, 5944, 6153, 8590 and 4096, respectively. The number of OTUs in the M group was significantly decreased compared with the KB group, indicating that CUMS caused intestinal flora disorder in mice. After the administration, the number of OTUs of mice in each group (except for G group) increased.

Chao1 and Observed Species represent species richness, while Shannon and Faith's Phylogenetic Diversity (Faith's PD) represent species and evolutionary diversity, respectively. Compared with the KB group, the richness and diversity of the M group was reduced, but there was no significant difference ($P > 0.05$, Fig. 6A, B, C and D). Compared with the M group, all treatment groups showed varying degrees of improvement in the Alpha diversity index. The Y group displaying significantly improve species diversity ($P < 0.05$, Fig. 6C). The Z group exhibiting significantly improve species richness and diversity ($P < 0.05$, Fig. 6A, B and C).

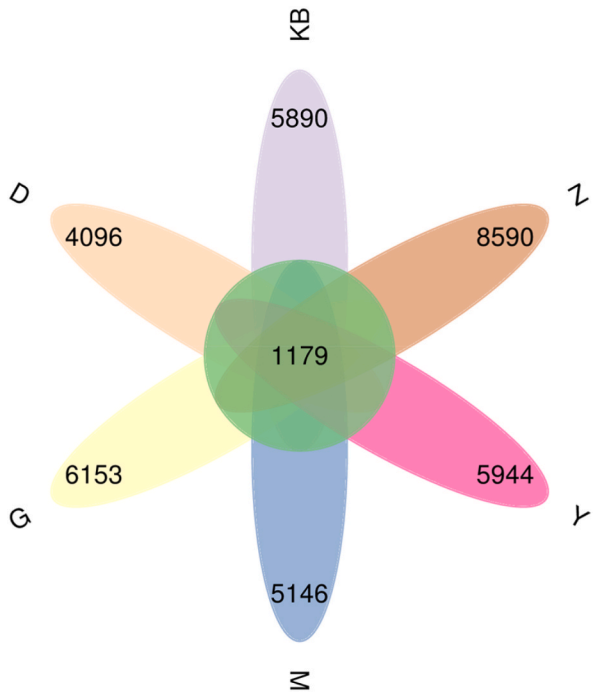

Fig. 5. The number of OTUs.

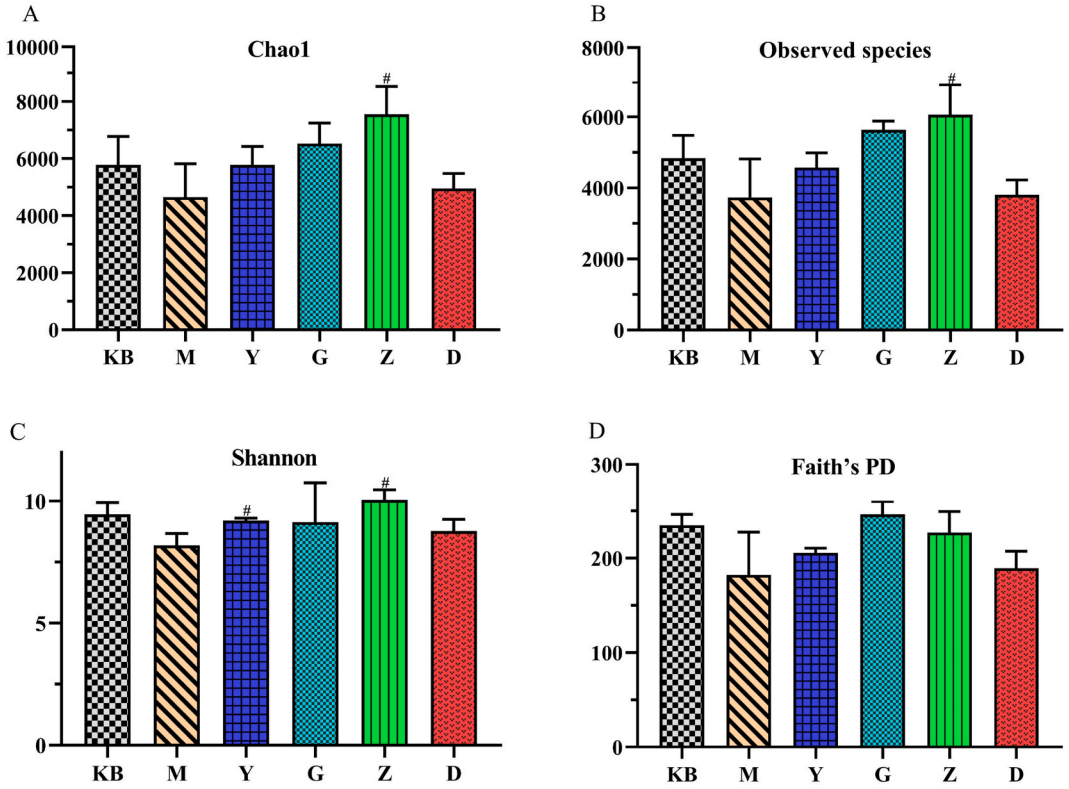

Fig. 6. Alpha diversity index(A) Chao1 (B) Observed specie (C) Shannon (D) Faith's PD, data are presented as mean ± SD (n = 3), compared with M, $^{\#}P < 0.05$.

3.8. Effects of CO–S-SME on beta intestinal diversity in CUMS mice

As shown in Fig. 7A and B, both the Bray-Curtis distance and weighted UniFrac distance principal coordinate analysis (PCoA) confirmed that KB and M group had significant differences in intestinal flora composition. Most of treatment group deviates from the M group and approached the KB group, suggesting that CUMS-induced depression can alter the microbiota structure. As shown in Fig. 7C, the stress value of the non-metric multidimensional scaling (NMDS) results is 0.14 (<0.2), indicating that the results of this analysis are reliable. NMDS was ranked, and the results show a large difference between M and KB groups. Moreover, the results of each treatment group were close to those of the KB group, consistent with the PCoA analysis.

4. Discussion

We selected three behavioral tests to assess the establishment of depression model and evaluated the improvement of depressive behavior by CO–S-SME. The depression model showed behavioral changes in behavioral experiments, indicating the establishment of this model. SPT is used to reflect the anhedonia of rodents [30], ultimately indicating their depressive state. CUMS causes anhedonia, as is reflected by reduction in sucrose preference. FST is used to evaluate the degree of despair in rodents [31]. We found that treatment with CO–S-SME was comparable with treatment with FLU in this behavioral test. Selective norepinephrine-targeting antidepressants and 5-HT neurotransport-related drugs have been shown to increase receptor activity [32]. Subsequent experiments also demonstrated the regulatory effect of CO–S-SME on neurotransmitter expression. OFT is used to assess the emotions of rodents and their autonomy, exploratory ability and nervousness in unfamiliar environments [30]. Regarding the behavioral tests of depression, SPT, FST, TST, OFT, and EPM tests are widely used in research, and the test results are reliable, all of which can show memory impairment, mental retardation, and abnormal behavior similar to those of patients with depression, *etc*. Our result showed that CO–S-SME can decrease the level of depression in CUMS mice.

The monoamine neurotransmitter hypothesis posits that the occurrence of depression is mainly related to abnormally low levels of monoamine neurotransmitters, such as 5-HT, NE, and DA in the patient's brain [33]. Recent studies have shown that dysfunction of the DA and 5-HT systems contributes to depression [34]. NE is involved in regulation of emotions and cognition, and increases social interactions. NE can cause loss of interest and social dysfunction when its secretion is disordered [35]. Measurements of neurotransmitters indicate that expression levels of neurotransmitters were negatively correlated with the severity of depressive behaviors in mice. CO–S-SME could increase expression levels of neurotransmitters in CUMS mice, which may be one of the antidepressant mechanisms of CO.

Because the HPA axis plays important roles in both short- and long-term stress responses, dysfunction of this system has a strong link to depression [36]. Both synthesis and release of glucocorticoids are induced when the HPA axis is hyperactive, CORT, a glucocorticoid that controls gene expression during stress, has long-term effects on neural circuits. Hyperactivity of the HPA axis is the most prevalent neurobiological change in patients with depression, and excessive CORT indicates reduced resistance to negative motivation [37]. Determination of CORT levels indicate that CUMS can stimulate the HPA axis to release CORT. The results of CORT measurement combined with behavioral test results show that CORT expression was positively correlated with the severity of depressive behavior in mice, indicating that the antidepressant effect of CO may be due to its inhibition of HPA axis hyperactivity to a certain extent.

Studies implicated that the HPA axis in control of stress responses. Stress can lead to an increase in inflammatory factors, thereby changing levels of neurotransmitters [38], suggesting that the treatment of depression should be jointly regulated from multiple perspectives. Because the excessive release of pro-inflammatory cytokines can affect the sexual and exploratory behaviors of rodents, along with other behavioral indicators, it is believed that CUMS stress can increase levels of inflammatory factors in animals and aggravate the occurrence of depression [39]. In the brain, cytokines produced by cells of the central nervous system are key positive regulators of many central nervous system functions and help to maintain neuroplasticity. When inflammatory cytokines are over-expressed, they interfere with neurotransmitter synthesis, reuptake, and release, which in turn affects the function of emotional and

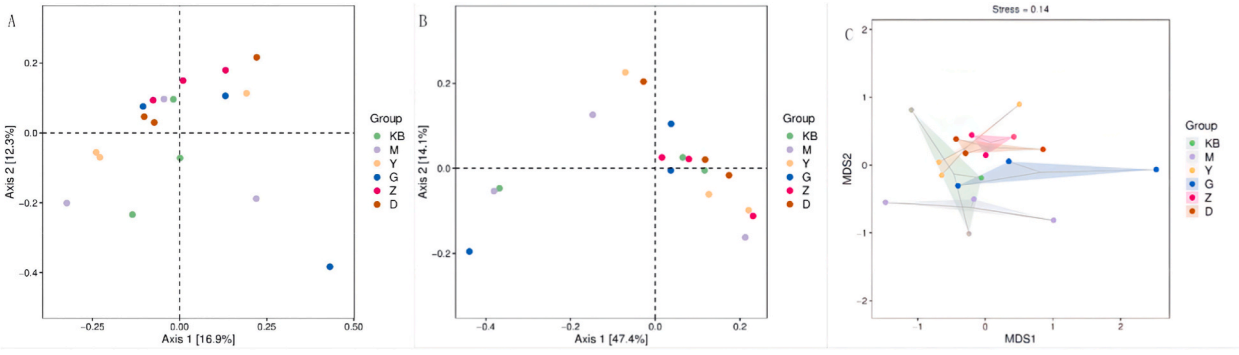

Fig. 7. Effect of CO–S-SME on the beta diversity of mice intestinal mucosal bacteria, (A) Bray-Curtis distance (B) weighted UniFrac distance (C) NMDS.

cognitive related neural circuits [40]. In psychoneuroimmunology, the most studied inflammatory factors are IL-6, TNF-α, and IL-1β. Our result suggest that CO–S-SME could reduce expression levels of inflammatory factors in CUMS mice, which may be one of the antidepressant mechanisms of CO.

The dynamic homeostasis of the gut environment is maintained by the interrelationships and interactions between beneficial and pathogenic microorganisms in the gut. This disorder can cause long-term harm to health. Recent research suggests that some intestinal flora can affect the function of neurotransmitters [41,42]. In our study, we found that each index of the alpha diversity of intestinal flora was decreased in the model group, indicating that CUMS affected the richness and diversity of intestinal flora in mice. After CO–S-SME intervention, the alpha diversity index of mice intestinal flora increased (except for Faith's PD), indicating that CO can improve the intestinal microbial disturbance induced by CUMS. The results of beta diversity experiment also showed that CO was helpful to restore intestinal flora diversity.

To further analyze the effect of CO on the intestinal microbiota of CUMS mice, we compared the relative abundance changes at the phylum level in different groups of mice. An abundance of *Firmicutes* and *Bacteroidetes* were relatively high, and their ratio (F/B) can reflect the health status of the body to a certain extent [43], as 90% of the intestinal flora are in *Firmicutes* and *Bacteroidetes* phyla. *Firmicutes* can metabolize carbohydrates and ferment them into short-chain fatty acids, which induce T cell differentiation and promote inflammation. With intestinal barrier disruption, proinflammatory cytokines are more likely to enter the blood and cross the blood-brain barrier, leading to central neuron inflammation and depression [39]. CUMS can reportedly induce a significant increase in the abundance of *Proteobacteria* [37]. F/B values of the intestinal flora of mice in the M group indicates that CUMS affected the intestinal microflora of mice. After CO–S-SME intervention, F/B decreased significantly. It was speculated that CO was beneficial to the recovery of *Firmicutes* and *Bacteroidetes* in CUMS mice. It has been shown that that *Sutterella* correlated with proinflammatory cytokines and has anti-inflammatory effects [44]. It has been shown that some members of Lactobacillus genus are probiotics, but some members can promote inflammasome activation [45–47], which can to promote anxiety-like behaviors, which is consistent with our study [48].

5. Conclusions

The occurrence and development of depression, an affective disorder, involves a complex multi-factor process. Current drug development for depression is no longer limited to a certain hypothesis. In this study, CO was used as a model material to develop a novel self-microemulsion, which was systematically evaluated for pharmacodynamic characteristics, and effects on intestinal flora. In view of the multiple pharmacological effects of CO and available experimental evidence, CO–S-SME can elicit play a strong antidepressant effect by regulating monoamine neurotransmitters, resisting HPA axis hyperfunction, and regulating inflammatory status. CO–S-SME can also effectively improve the composition of intestinal flora, which may be one of the mechanisms by which CO exerts antidepressant effects. This antidepressant approach, which is in line with the concept of traditional Chinese medicine treats diseases through multiple pathways and multiple targets, provides a promising new strategy for clinical treatment of depression with the potential for extensive medical applications.

Author contributions

Tianyu Ma performed experiments and wrote the manuscript. Bingjie Tang, Yan Wang and Mengting Shen contributed to animal experiments. Tianyu Ma and Lihong Wang analyzed and organised the data. Yang Ping and Jin Su designed the ideas. All the authors read and approved the final version of the manuscript before submission.

Declaration of competing interest

All authors disclosed no relevant relationships.

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