Biochemical and Biophysical Research Communications 566 (2021) 170–176

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The outer membrane protein Amuc_1100 of *Akkermansia muciniphila* alleviates the depression-like behavior of depressed mice induced by chronic stress

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ARTICLE INFO

Article history:
Received 20 May 2021
Accepted 4 June 2021

Keywords:
Amuc_1100
Serotonin
Microbiota-gut-brain axis
Depression

ABSTRACT

Akkermansia muciniphila is a symbiotic intestinal bacterium with a high medicinal value. Amuc_1100 is the outer membrane protein of *A. muciniphila* and plays an important role in the interaction between *A. muciniphila* and its host. The objective of this study was to evaluate the antidepressant activity of Amuc_1100 in a chronic unpredictable mild stress (CUMS) model. Amuc_1100 intervention ameliorated CUMS-induced depression-like behavior and CUMS-induced down-regulation of serotonin (5-hydroxytryptamine, or simply, 5-HT) in the serum and colon of mice. Microbial analysis of mouse feces showed that Amuc_1100 could improve the gut microbiota dysregulation induced by CUMS. In addition, Amuc_1100 intervention could also improve the down-regulation of brain-derived neurotrophic factor (BDNF) and inflammation in the hippocampus induced by CUMS. These results suggest that Amuc_1100 has a good antidepressant effect, and the mechanism may be related to the improvement of gut microbiota, the up-regulation of the BDNF level, and the inhibition of the neuroinflammatory response.

1. Introduction

Depression is a prevalent mental disease with a high recurrence rate [1]. Some studies have shown that the gut microbiota plays an important role in the pathogenesis of depression [2,3]. In the process of evolution, the gut microbiota has established a two-way communication relationship with the host, and the gut microbiota is regarded as a virtual endocrine organ that communicates with the central nervous system (CNS) through the microbiota–gut–brain axis [4,5]. A growing body of evidence suggests that altered gut microbiota can affect brain function. There is also a bidirectional interaction between gut microbes and the brain, which not only affects neurogenesis and neurodegenerative diseases but also is related to cognition, mood, and behavior [6–8].

The mechanism of the effect of the microbiota–gut–brain axis on depression is unclear, but there is evidence that the gut microbiota signals to the brain through a variety of pathways, including immune activation, production of microbial metabolites, activation of the vagus nerve, and production of various neurotransmitters and neuromodulators in the gut [9]. Brain-derived neurotrophic factor (BDNF) is a downstream signal molecule of cAMP response element-binding protein (CREB). Deficiency of BDNF is a risk factor for the development of depressive symptoms [10]. Decreased expression of BDNF has been detected in the serum samples and brain tissues of experimental animals and patients with depression [11–13]. In addition, it has been reported that there are increased levels of pro-inflammatory cytokines in the CNS of patients with depression, including IL-6, IL-1β, and TNF-α, which are the most widely studied factors with antidepressant effects, and they can affect the severity of depression [14,15].

Stress is one of the major risk factors for depression, and chronic unpredictable mild stress (CUMS) is a reliable model of depression in rodents [16]. Based on the CUMS model, Tian et al. [17–19] demonstrated that *Bifidobacterium breve* M2CF22M7 and

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https://doi.org/10.1016/j.bbrc.2021.06.018
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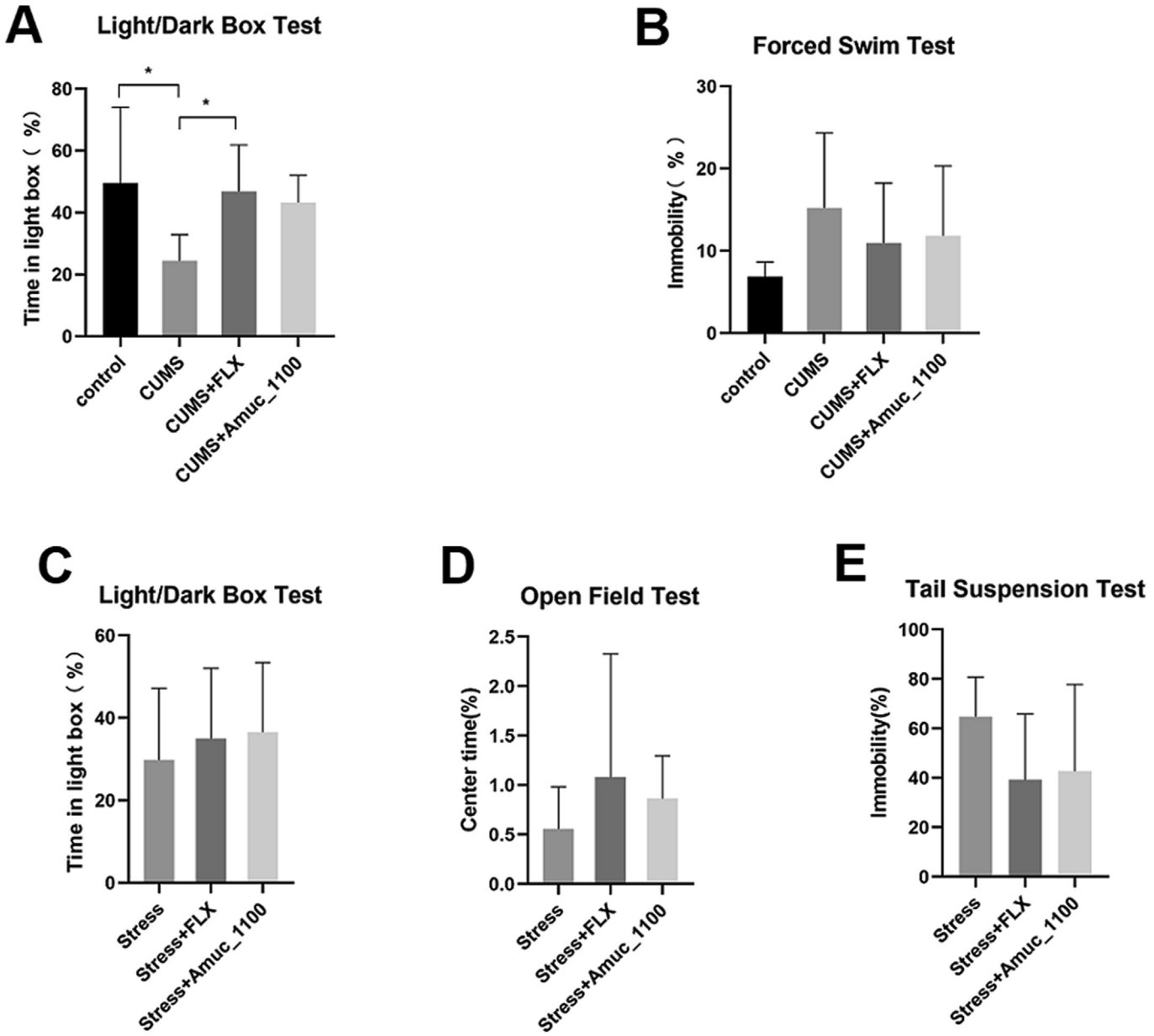

Fig. 1. Amuc_1100 can improve CUMS-induced depression-like behavior in mice. (A) The time (%) spent in the light chamber in the LDB test of intervention program. (B) Percentage immobility time in the FST of intervention program. (C) The time (%) spent in the light chamber in the LDB test of treatment program. (D) The time (%) spent in the central area in the OFT of treatment program. (E) Percentage immobility time in the TST of treatment program. Data are mean ± 95% CI, n = 4–8 per test. *P < 0.05, post hoc test.

CCFM1025 as well as *Bifidobacterium longum* subspecies *infantis* strains E41 and CCFM687 showed antidepressant effects in mice, partly due to the regulation of gut microbiota and BDNF. Yang et al.'s [20] chronic treatment of CUMS mice with minocycline demonstrated its antidepressant effect by inhibiting neuroinflammation and regulating gut microbiota. Cao et al. [21] demonstrated that the Chinese medicine formula Kai-Xin-San improved the depression-like behavior of CUMS mice by regulating the gut microbiota–inflammation–stress system. These existing studies all indicate that the gut microbiota plays an important role in the pathogenesis of depression.

Akkermansia muciniphila is part of the new generation of probiotics. The abundance of *A. muciniphila* is negatively correlated with host enteritis, obesity, diabetes, and other metabolic diseases [22–25]. The outer membrane protein Amuc_1100 of *A. muciniphila* plays a direct and important role in the interaction between *A. muciniphila* and its host. Our previous results showed that Amuc_1100 up-regulated the rate-limiting enzyme tryptophan hydroxylase 1 (Tph1) of serotonin (5-hydroxytryptamine, or simply, 5-HT) synthesis through the TLR2 signal pathway and also down-regulated the expression of serotonin transporter (SERT), resulting in an increase in the expression of 5-HT in the intestine [26].

Besides being a traditional neurotransmitter, 5-HT also plays an important role in various physiological functions as a hormone that can regulate peripheral metabolism by increasing short-term energy supply and promoting long-term energy saving; changes in 5-HT levels alter the composition of the gut microbiota, thereby affecting intestinal inflammation [27]. In addition, the intestinal 5-HT system plays a key bridge and hub role in the interaction

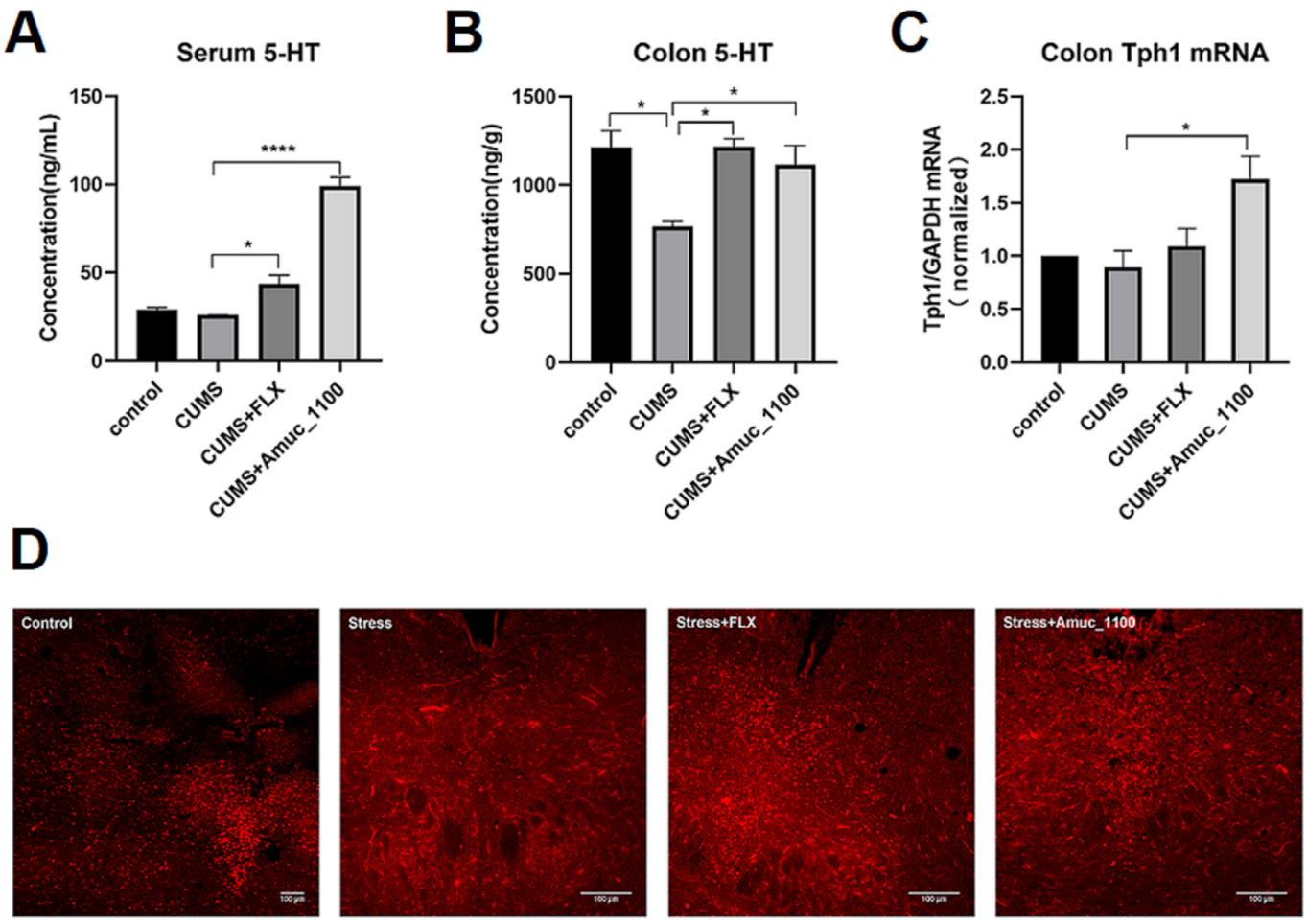

Fig. 2. The level of 5-HT in each group of mice. (A) 5-HT in serum. (B) 5-HT in the colon. (C) Tph1/GAPDH mRNA in the colon. (D) 5-HT staining of DRN. The scale bar represents 100 μm. Data are mean ± 95% CI, n = 3 per test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. CUMS. One-way ANOVA followed by Fisher's LSD post hoc test.

between gut microbiota and the host, and it is an effective way for microbes to affect the physiological function of the host [27]. In view of the existing achievements of our group and the bidirectional interaction between 5-HT and gut microbiota, we carried out this study to explore the antidepressant activity of Amuc_1100.

2. Materials and methods

2.1. Preparation of Amuc_1100

Amuc_1100 was prepared according to a previously reported method [28]. Briefly, the recombinant protein was expressed in *E. coli* BL21 (DE3), purified by Ni-NTA, and eluted with a buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl). His-tag was removed by TEV enzyme, and excess TEV and His-tag proteins were separated by Ni-NTA. The Amuc_1100 without a tag was purified by gel filtration in a buffer solution containing phosphate buffer (pH 7.2) using a HiLoad 16/60 Superdex 200 column (GE Healthcare).

2.2. Animal experiments

Male C57BL/6 mice aged 5–six weeks were housed in an environment with controllable temperature and humidity and a 12/12-h light/dark cycle in a specific-pathogen-free room, and they were able to eat and drink freely. All the procedures were approved by the Ethics Committee of Experimental Animals at Anhui University.

The experiments were mainly divided into two programs: simultaneous stress intervention and post-stress treatment, which are hereafter referred to as the intervention program and treatment program. The antidepressant fluoxetine (FLX) was used as a positive control in both programs. After one week of adaptation, the intervention program mice were divided into four groups (n = 7–8 in each group): control, CUMS, CUMS + FLX, and CUMS + Amuc_1100. Except for the control group, the other three groups were subjected to chronic stress for six weeks and were given gavage at the same time of stress. The control group and the CUMS group were gavaged daily with 200 μl of PBS. CUMS + FLX mice were gavaged daily with FLX in sterile PBS at a dose of 20 mg/kg body weight. CUMS + Amuc_1100 mice were gavaged daily with 80 μg of Amuc_1100 in sterile PBS with a volume of 200 μl. Treatment program is mainly a supplement to the behavioral experiment of intervention program; specifically, after one week of adaptation, the mice were subjected to chronic stress for six weeks, and then the mice were divided into three groups (n = 4–5 in each group), Stress, Stress + FLX, and Stress + Amuc_1100, and were treated with FLX and Amuc_1100 after stress. The Stress mice were gavaged daily with PBS, the Stress + FLX mice were gavaged daily with FLX, and the Stress + Amuc_1100 mice were gavaged daily with Amuc_1100. At the end of the two programs, behavioral tests were performed on the mice in each group, including the light/dark box test (LDB), open field test (OFT), tail suspension test (TST), and forced swim test (FST). Supplemental materials include detailed

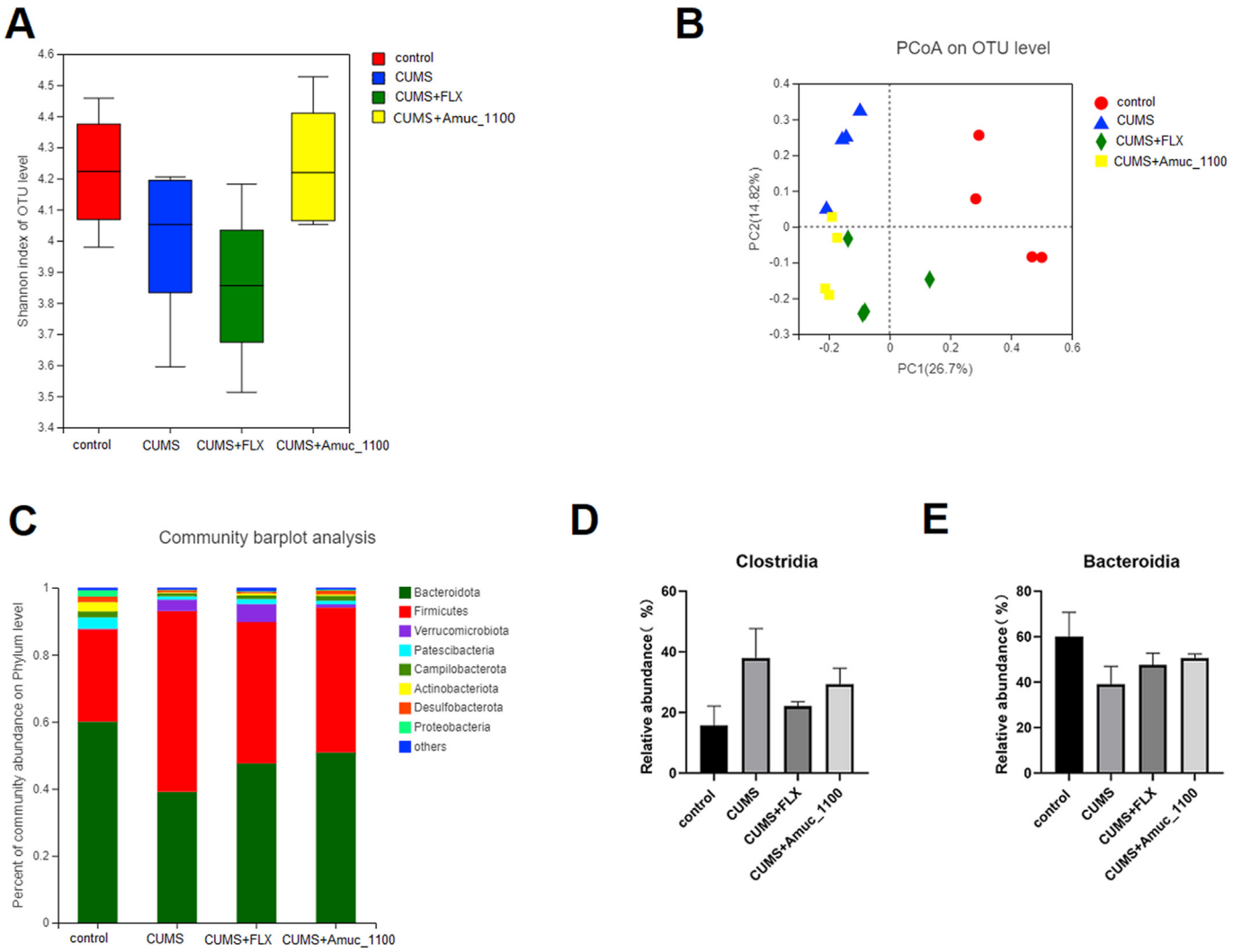

Fig. 3. Amuc_1100 alters the composition of the gut microbiota in CUMS mice. (A) Shannon index. (B) PCoA of the four groups. (C) Microbial distribution at the phylum level. (D–E) The relative abundance at the class level. (D) Clostridia. (E) Bacteroidia. Data are mean ± 95% CI, n = 4 per test. One-way ANOVA followed by Fisher's LSD post hoc test.

methods of behavioral testing.

2.3. Chronic unpredictable mild stress (CUMS)

The stress procedure was performed daily according to a previously described method with some modifications [18]. The procedure included 24 h of food/water deprivation, 24 h of 45° cage tilting, 24 h of wet bedding, 24 h of no bedding, 10 min of forced swimming, 9 min of tail clipping, 3 h of restraint, 15 min of cage shaking, and 24 h of space crowding. The mice received 1–2 mild stresses per day, and the same stress was not applied for two consecutive days.

2.4. 5-HT measurements by ELISA

To measure 5-HT in serum, blood samples were solidified at room temperature for 2 h. Then, each blood sample was centrifuged at 5000 g for 15 min. The collected supernatant was the serum. 5-HT levels in the serum were detected by a mouse 5-HT ELISA Kit according to the manufacturer's instructions (Jianglaibio, Shanghai, China).

To measure 5-HT in the colon, colon tissue 5-HT was detected as previously described [26]. Colon tissue samples were thawed, weighed, and homogenized with PBS (w/v, 1:9) on ice. The homogenizer was centrifuged at 5000 g for 15 min at 4 °C. 5-HT levels in the colon tissue were detected by a mouse 5-HT ELISA Kit according to the manufacturer's instructions (Ruixinbio, Quanzhou, China).

2.5. Immunohistochemistry

The brain tissues were fixed in 4% paraformaldehyde solution at 4 °C overnight. The fixed tissues were transferred to a 15% sucrose solution for overnight dehydration and then transferred to a 30% sucrose solution to be dehydrated for 5–7 days. After dehydration, the brain tissues were cut into frozen slices with a thickness of 40 μm and were placed on slides for immunohistochemistry staining. Nonspecific staining was blocked with 0.5% Triton X-100 and 5% BSA. Then, the tissues were stained using a primary antibody, sheep anti-mouse 5-HT (1:1000; Abcam), and a secondary antibody, donkey anti-sheep 5-HT (1:2000; Abcam).

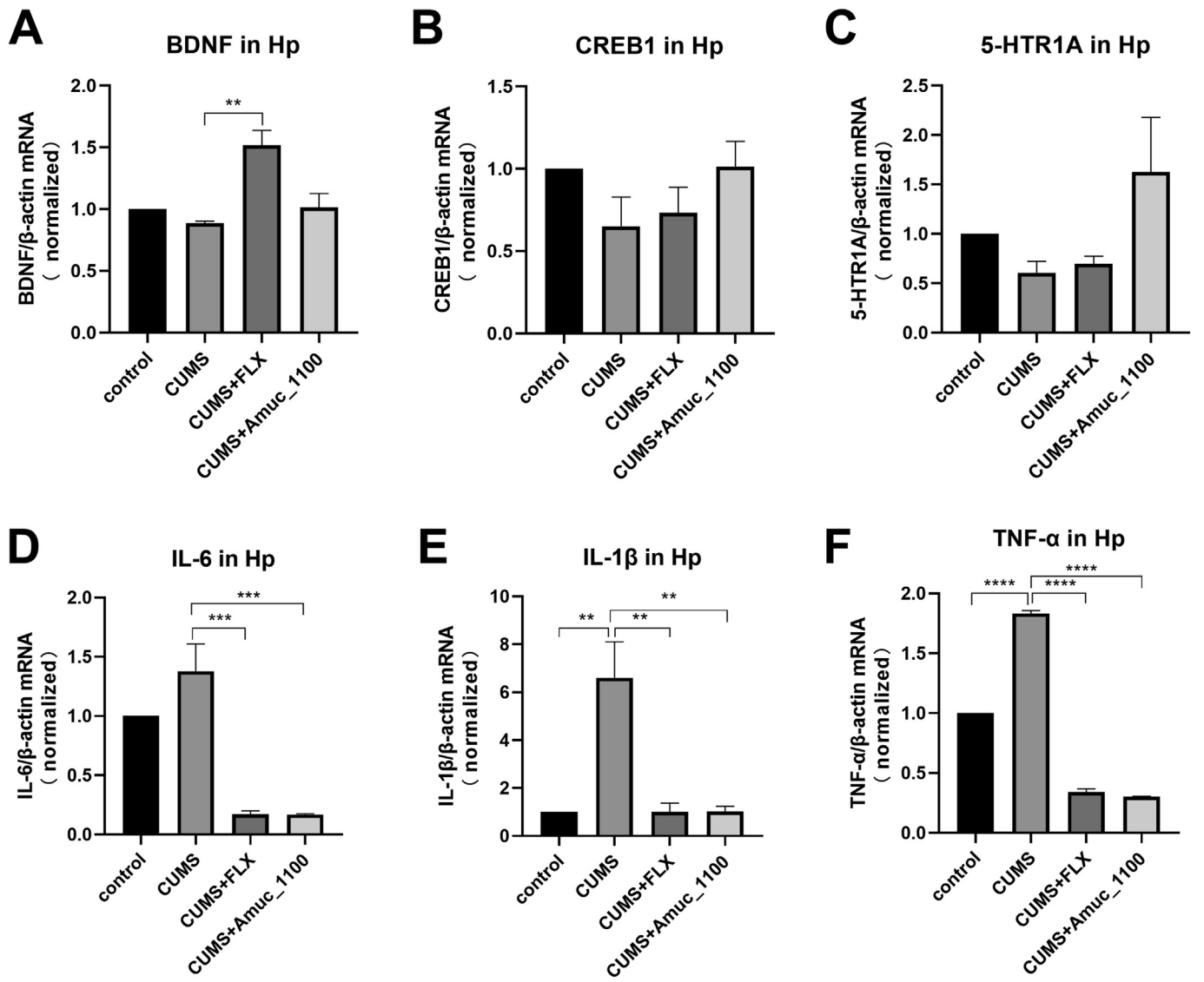

Fig. 4. Levels of related factors in the Hp of mice. (A) BDNF/β-actin mRNA in Hp. (B) CREB1/β-actin mRNA in Hp. (C) 5-HTR1A/β-actin mRNA in Hp. (D) IL-6/β-actin mRNA in Hp. (E) IL-1β/β-actin mRNA in Hp. (F) TNF-α/β-actin mRNA in Hp. Data are mean ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ****P < 0.0001 vs. CUMS. One-way ANOVA followed by Fisher's LSD post hoc test.

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the colon and hippocampus (Hp) tissue samples with TRIzol reagent (GenStar, Beijing, China). Total RNA was quantified using a OneDrop spectrophotometer (OneDrop, China). The RNA purity was evaluated by the absorbance ratio at 260/280 nm. According to the manufacturer's instructions, the RNA was reverse-transcribed into cDNA using the HiScript III cDNA Synthesis Kit (Vazyme, Nanjing, China). Relative quantitative RT-PCR amplification was performed using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The primer sequences for GAPDH, β-actin, Tph1, CREB1, BDNF, 5-HTR1A, IL-1β, IL-6, and TNF-α are listed in Table S1. GAPDH and β-actin were used as the reference genes. Quantitative real-time RT-PCR (qRT-PCR) was performed with a SYBR green fluorescent dye using a Bio-Rad CFX96 real-time PCR Detection System (Bio-Rad, Hercules, CA). The data were analyzed according to the $2^{-\Delta\Delta CT}$ method and are expressed as relative abundances (mean ± standard error of the mean (SEM)) [26].

2.7. Gut microbiota analysis

According to the manufacturer's instructions, total bacterial DNA was extracted from fecal samples using the TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China). The V3–V4 region of bacterial 16S rRNA was amplified with primers (338F: 5′-ACTCC-TACGGGAGGCAGCAG-3′ and 806R: 5′-GGACTACHVGGGTWTC-TAAT-3′). Bioinformatics analysis of 16S rRNA sequence data was performed after sequencing.

2.8. Statistics

The data were analyzed using one-way ANOVA and Fisher's least significant difference (LSD) tests for multiple comparisons. The values were represented by the average ± SEM, and P < 0.05 was considered significant.

3. Results

3.1. Amuc_1100 improves depression-like behavior caused by CUMS

A CUMS-induced depression mouse model was adopted in this study. After six weeks of chronic stress, the CUMS mice showed depression-like behaviors, mainly manifested as decreased time in the light box and increased immobility time in the FST. Treatment with Amuc_1100 at the same time (Fig. 1A and B) or after stress (Fig. 1C-E) improved the behavioral disorder induced by CUMS. The intervention of Amuc_1100 at the same time of chronic stress increased the time of mice in the light box and reduced the immobility time of FST. When treatment with Amuc_1100 after chronic stress induced disordered behavior in mice, the time in the light box and the central area in the OFT increased, and the immobility time in TST decreased. Although these changes were not significant, it could be seen that Amuc_1100 had a tendency to improve depression-like behavior in the mice.

3.2. Amuc_1100 can increase the level of 5-HT in the peripheral and central nervous system of CUMS mice

Amuc_1100 can increase the expression of 5-HT synthesis rate-limiting enzyme Tph1 in the intestines and can inhibit the cell re-uptake of 5-HT. FLX can inhibit SERT, so we detected the levels of 5-HT in the colon and serum of different experimental mice. The results showed that the levels of 5-HT in the serum and colon of CUMS mice were lower than those of control mice, and the use of FLX and Amuc_1100 interventions during chronic stress significantly increased the levels of 5-HT in the colon and serum (Fig. 2A and B). Amuc_1100 also significantly improved the expression of Tph1 in the colon of mice (Fig. 2C). The study conducted by Deng et al. [29] in 2020 showed that the intervention of the antidepressant citalopram increased the levels of 5-HT in the serum and colon and of Tph1 in the colon of mice induced by CUMS, and we observed a similar effect with FLX and Amuc_1100 (Fig. 2A-C). The dorsal raphe nucleus (DRN) of CNS is also the main part of serotonin neurons, so we performed immunohistochemical staining for 5-HT in this area of CUMS mice. The results showed that the level of 5-HT in the DRN decreased in CUMS mice. Intervention with FLX and Amuc_1100 significantly increased the level of 5-HT in DRN (Fig. 2D).

3.3. Amuc_1100 regulates the composition of the gut microbiota in CUMS mice

Gut microbiota can influence the host's response to external stress, thus affecting behaviors such as depression or anxiety. Gut microbiota can also affect the level of 5-HT, and 5-HT in turn affects the composition and abundance of gut microbiota. Based on 16S rRNA sequencing of the gut microbiota in experimental mice, the Shannon index showed that alpha diversity in the CUMS group was lower than that in control mice, although the difference was not statistically significant (Fig. 3A). Using UniFrac PCoA to visualize the similarity of the gut microbiota composition, we found that the control group and the CUMS group had unique gut microbiota structures (Fig. 3B). The histogram shows the microbial species and their relative abundance at the phylum level (Fig. 3C). The abundance of Bacteroidota decreased and the abundance of Firmicutes increased in the CUMS group, and both FLX and Amuc_1100 intervention could reverse these changes. At the class level (Fig. 3D and E), the CUMS group had higher levels of Clostridia and lower levels of Bacteroidia. Intervention of FLX and Amuc_1100 at the same time during CUMS could partially reverse these changes, although the reversals were not statistically significant. According to the results, we speculate that FLX and Amuc_1100 can alleviate or restore the changes of gut microbiota caused by chronic stress by increasing the level of 5-HT in the intestine, improving the gut microbiota of CUMS mice, and then improving the response of the mice to stress.

3.4. Amuc_1100 affects the expression of related factors in the brains of CUMS mice

Gut microbes can affect the brain through the microbiota–gut–brain axis, and the changes in microbes will affect the level of related factors in the brain. We detected the levels of BDNF, CREB1, and 5-HTR1A in the Hp. The results showed that the mRNA levels of BDNF, CREB1, and 5-HTR1A decreased in the CUMS group, but gavage of Amuc_1100 or FLX at the same time of chronic stress restored the levels of BDNF, CREB1, and 5-HTR1A (Fig. 4A–C). Therefore, we believe that Amuc_1100 may resist the cognitive impairment caused by chronic stress through the 5-HTR1A–CREB–BDNF pathway.

The effect of gut microbiota on the immune system and its two-way connection with the CNS have drawn attention to the interaction between inflammation, microbes, and depression [30], so we detected the inflammatory factors in the Hp of mice. The results showed that the levels of IL-6, IL-1β, and TNF-α in the Hp of CUMS mice were increased but could be significantly decreased by intervention with FLX and Amuc_1100 (Fig. 4D–F). This suggests that Amuc_1100 may also play an antidepressant role by inhibiting the neuroinflammatory response.

4. Discussion

A. muciniphila is a beneficial microorganism in the host's intestines, and its outer membrane protein Amuc_1100 can directly affect the host's 5-HT system and increase the level of 5-HT in the host's intestines. In this study, depression-like mice induced by CUMS were used as an animal model, and the effects of Amuc_1100 on the behavior and gut microbiota of the mice were determined, with FLX as the effect control. A change in the 5-HT level in the intestines can directly affect the gut microbiota and cause changes in the composition and abundance of microbes, and the changes or disorders in the composition of gut microbiota can regulate neuroinflammation by affecting the activation of peripheral immune cells, can regulate the release of monoamine neurotransmitters, can change the activity and function of the hypothalamic–pituitary–adrenal (HPA) axis, and can change the abundance of BDNF. Thus, Amuc_1100 can change the host's response to stress, causing changes in host behavior, or neurological diseases, such as depression [30,31]. Existing clinical studies have also shown that there is a correlation between CREB/BDNF levels and depression [32,33], and higher levels of inflammation also increase the risk of de novo depression [34]. Therefore, we speculate that Amuc_1100 can improve the gut microbiota of CUMS mice by regulating the intestinal 5-HT level and then improving the decrease of hippocampal BDNF, CREB1, and 5-HTR1A induced by CUMS. The inflammatory response in the hippocampus of CUMS mice was activated, resulting in increased levels of IL-6, IL-1β, and TNF-α, but Amuc_1100 may have attenuated the inflammatory response through changes in gut microbiota affected by intestinal 5-HT. More and more studies have shown the effects of 5-HT–gut microbiota–brain on host behavior, cognition, and emotion. Therefore, the study of Amuc_1100's antidepressant activity is helpful for further understanding the microbiota–gut–brain axis, and it lays a foundation for the development of new drugs.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

The study was financially supported by the Key Research Program of the Education Department of Anhui Province (grant no. KJ2019ZD02 to M. Z.). We thank Pro. Yongzhong Wang and Dr Kangliang Sheng for their good advice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.06.018.

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