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The modified outer membrane protein Amuc_1100 of *Akkermansia muciniphila* improves chronic stress-induced anxiety and depression-like behavior in mice†

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Akkermansia muciniphila is a next-generation probiotic. The interaction between outer membrane protein Amuc_1100 of *A. muciniphila* and toll-like receptor 2 (TLR2) in intestinal epithelial cells influences the level of intestinal 5-hydroxytryptamine (5-HT). Amuc_1100$^{\Delta 80}$ is a truncated form of Amuc_1100 lacking the first 80 N-terminal amino acids and has a higher affinity for TLR2 than the wild-type protein. Here, we report that Amuc_1100$^{\Delta 80}$ could significantly reduce anxiety and depression-like behavior of mice when they were exposed to chronic unpredictable mild stress (CUMS). The experimental results of the rat insulinoma cell line RIN-14B showed that Amuc_1100$^{\Delta 80}$ also induced a significantly higher upregulation of tryptophan hydroxylase 1 (Tph1), a rate-limiting enzyme of intestinal 5-HT synthesis. The imbalance of the gut microflora could be diminished when CUMS mice were fed with Amuc_1100$^{\Delta 80}$. These results reveal that Amuc_1100$^{\Delta 80}$ could affect the 5-HT level and the downstream 5-HTR1A-CREB-BDNF signal pathway *via* interacting with TLR2 and by altering the gut microbial composition. In parallel, the downregulation exerted by Amuc_1100$^{\Delta 80}$ on the inflammation and hyperactivated HPA axis was closely related to the improvement of depression-like symptoms in CUMS mice. This study not only provides new insights into the antidepressant effect of *A. muciniphila* and its outer membrane protein Amuc_1100 but also identifies new potential targets and pathways in the gut for future research and the development of antidepressant drugs.

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1. Introduction

Depression is a common mental disorder and represents a major contributor to the overall global burden of disease. The main characteristics of depression include loss of interest, low mood, and inattention. Patients with severe depression have an increased risk of suicide.[1–5] A link between depression and the gut microbiota has been established, indicating that the gut microbiota plays an important role in the pathogenesis of depression.[6–9] For example, ablation of the gut microbiota by antibiotic treatment reduces sucrose preference in mice.[10,11] The imbalance of the microbiota–gut–brain axis has been proposed to explain depression pathogenesis. The gut microbes can communicate with the central nervous system in many ways.[11] Notably, gut microbes can cause emotional and depressive disorders by affecting neural, endocrine, and immune pathways. They can influence the synthesis and release of neurotransmitters, including 5-hydroxytryptamine (5-HT) and gamma-aminobutyric acid (GABA). For example, they can regulate the activity of the hypothalamic–pituitary–adrenal (HPA) axis, and the level of pro-inflammatory cytokines. Gut microbes can reduce the upregulation of pro-inflammatory cytokines in patients with depression, which can increase the severity of this disease, and they can reduce the level of pro-inflammatory cytokines.[12–16] In addition, gut microbes can upregulate the level of the brain-derived neurotrophic factor (BDNF) and thus affect the development of depressive behaviors.[11]

Akkermansia muciniphila is a beneficial gut bacterium with great value for improving host metabolic functions and immune responses. It is also valuable in cancer immunotherapy.[17–22] *A. muciniphila* has become a marker of a healthy intestine and can improve the integrity of the intestinal barrier in humans and mice, conferring it with a high

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degree of significance for human health.[23–25] Amuc_1100, the outer membrane protein of *A. muciniphila*, can activate the TLR2 signaling pathway, which is likely to play a critical role in maintaining host intestinal immune and mucosa homeostasis and improving the gut barrier function. In addition, previous research has revealed that TLR2 may act in intestinal pathophysiology by regulating the gut 5-HT levels.[26] Further studies revealed that Amuc_1100 could affect the host 5-HT system through the TLR2 signaling pathway, reshape the gut microbes of mice, and improve chronic stress-induced depression-like behavior in mice.[26,27]

The Amuc_1100$^{\Delta 80}$ used in this study is a truncated protein with 80 amino acids truncated from the N-terminus of Amuc_1100. The α1 helix, which is predicted to be a coiled-coil domain (at positions 65–80), influences the oligomeric state of Amuc_1100. Amuc_1100$^{\Delta 80}$ exists as a dimer in solution due to the deletion of the α1 helix. Biofilm interference experiments showed that Amuc_1100$^{\Delta 80}$ has a higher affinity for TLR2 than wild-type Amuc_1100,[28] which demonstrated that the deletion of the α1 helix does not affect protein function. Given that Amuc_1100 can exert beneficial effects on the host through TLR2 signaling, its truncated protein is safe for hosts. Animal studies have shown that Amuc_1100 could regulate the intestinal barrier, alter the gut microbiota composition, and alleviate colonic inflammation,[26,27] indicating that Amuc_1100 has high bioavailability in the mouse intestine. Since Amuc_1100 has a major impact on the gut, the gut microbiota plays an important role in the development of depression. In the current study, we aimed at elucidating the antidepressant effect of Amuc_1100$^{\Delta 80}$ and exploring the relationship between the gut microbiota, inflammation and depression. The results may reveal the underlying mechanism of the antidepressant effect of Amuc_1100$^{\Delta 80}$ and provide the foundation for new treatment approaches towards depression by targeting the microbiota–gut–brain axis.

2. Materials and methods

2.1. Preparation of Amuc_1100 and Amuc_1100$^{\Delta 80}$

Amuc_1100 and Amuc_1100$^{\Delta 80}$ were prepared according to the previously reported method.[28] Briefly, recombinant proteins were produced with a His-tag in the *Escherichia coli* strain BL21 (DE3), and purified on a nickel–nitrilotriacetic acid (Ni-NTA) column. Amuc_1100 and Amuc_1100$^{\Delta 80}$ were eluted in a buffer (containing 100 mM NaCl and 50 mM Tris–HCl at pH 7.5, and pH 8.0, respectively). The His-Tag was removed by overnight digestion with the Tobacco Etch Virus (TEV) enzyme, after which the residual TEV and His-tag proteins were separated using Ni-NTA. Untagged Amuc_1100 and Amuc_1100$^{\Delta 80}$ were purified by gel filtration in phosphate buffer (pH 7.2) using a HiLoad 16/60 Superdex 200 column (GE Healthcare).

2.2. Cell culture and treatments

The rat insulinoma cell line RIN-14B is a useful model for studying the functions of EC cells.[29] RIN-14B cells were cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. RIN-14B cells were inoculated at a density of 7 × 10^5 cm^{-2} in 12-well plates and cultured at 37 °C for 48 h. The cultured RIN-14B cells were treated with Amuc_1100 or Amuc_1100$^{\Delta 80}$ at a concentration of 5 μg mL^{-1} for 24 h. To investigate the effect of Amuc_1100$^{\Delta 80}$ on 5-HT pathways through TLR2 signaling, the cells were treated with the TLR2 inhibitor CU-CPT22 at 1 μM for 1 h prior to the addition of Amuc_1100 or Amuc_1100$^{\Delta 80}$ at 5 μg mL^{-1}. RIN-14B cells treated with Hank's balanced salt solution (HBSS) were used as negative control. After treatment, RNA was extracted from the cells treated under different conditions and stored at −80 °C for further analysis.

2.3. Animal experiments

Male C57BL/6 mice aged 5–6 weeks were maintained in a specific pathogen-free environment with a controlled temperature and humidity, a 12/12 h light/dark cycle, and food and drinking water *ad libitum*.

The antidepressant fluoxetine hydrochloride (FLX) was used as a positive control. After one week of adaptation, mice were randomly divided into five groups (n = 6, each group): control, chronic unpredictable mild stress (CUMS), FLX, Amuc_1100 and Amuc_1100$^{\Delta 80}$. Except for the control group, the mice in the four other experimental groups were subjected to chronic stress for three weeks. At the same time as being exposed to unpredictable mild stress, the mice received oral treatments by gavage. The experimental procedure is shown in Fig. 1. The mice in the control and CUMS groups were treated with 200 μl of PBS per day; the mice in the FLX group received FLX at 20 mg per kg body weight; the mice in the Amuc_1100 group received the Amuc_1100 protein at 80 μg per day, and the mice in the Amuc_1100$^{\Delta 80}$ group received the Amuc_1100$^{\Delta 80}$ protein at 80 μg per day. FLX, Amuc_1100, and Amuc_1100$^{\Delta 80}$ were all dissolved in sterile PBS. At the end of three weeks of chronic stress, behavioral tests, including the light/dark box (LDB), open field test (OFT), elevated plus maze (EPM), forced swimming test (FST), and tail suspension test (TST), were performed on the different groups of mice. The supplemental materials include the detailed methods of behavioral testing. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Anhui University and approved by the Animal Ethics Committee of Anhui University, China.

2.4. Chronic unpredictable mild stress (CUMS)

The chronic stress procedure was carried out according to the previously described methods,[27,30] including food deprivation (24 h), water deprivation (24 h), 45° cage tilt (24 h), wet bedding (24 h), no bedding (24 h), forced swimming (10 min), tail clipping (9 min), restraint (3 h), cage shaking (15 min), and space crowding (24 h). Mice were exposed to 1–2 mild stresses per day. Different stressors were used between two consecutive days to minimize mouse adaptability.

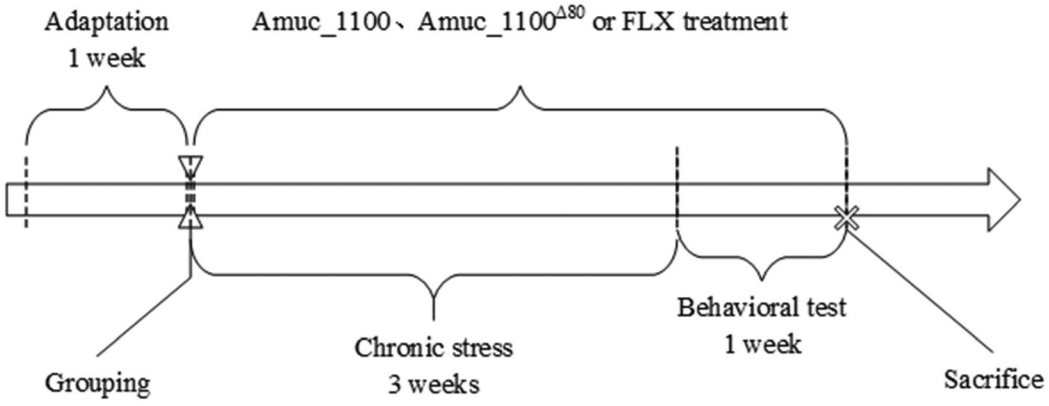

Fig. 1 Protocol of animal experiments. The whole experimental cycle was of five weeks' duration, including a one week adaptation period and three weeks of simultaneous chronic stress and treatment with Amuc_1100, Amuc_1100$^{\Delta 80}$, and FLX. All mice underwent behavioral tests in the fifth week, during which they were also treated with proteins or FLX.

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

The total RNA from the cells, colon, ileum, hippocampus (Hp), and frontal cortex (FC) was extracted using TRIzol reagent (Vazyme, Nanjing, China). The extracted total RNAs were quantified with a OneDrop spectrophotometer (OneDrop, China), and the purity was evaluated by calculating the absorbance ratio at 260/280 nm. The RNA was reverse transcribed into cDNA using a HiScript III cDNA synthesis kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) was used for relative quantification by RT-PCR amplification on a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). The *Ct* value was normalized to GAPDH or β-actin using the $2^{-\Delta\Delta Ct}$ method. All the primer sequences are shown in Table S1.†

2.6. Enzyme-linked immunosorbent assay (ELISA)

For the detection of inflammatory factors, corticosterone and 5-HT in serum, the blood samples were left to coagulate at room temperature for 2 h and centrifuged at 5000*g* for 15 min. The serum was collected and assessed with an enzyme-linked immunosorbent assay (ELISA) kit (Jianglaibio, Shanghai, China).

For the detection of 5-HT in the colon and Hp, the tissues were homogenized in PBS and centrifuged at 12 000*g* for 20 min. An ELISA kit (Jianglaibio, Shanghai, China) was used for detection.

2.7. Gut microbiota analysis

Total bacterial DNA was extracted from fecal samples using the TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China). The sequence of the 16S rRNA V3–V4 region was amplified using the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Sequencing was performed according to the standard procedure of Majorbio (Shanghai, China) using an Illumina MiSeq platform (San Diego, CA, USA). PE reads obtained by Miseq sequencing were first spliced according to the overlap relation, and sequence quality was controlled and filtered using QIIME61 (version 1.17). Operational taxonomic units (OTUs) were clustered using Uparse (version 7.0.1090) with 97% similarity, and chimeric sequences were identified and removed using UCHIME. The taxonomy of each sequence was analyzed using the RDP Classifier against the Silva (SSU132) 16S rRNA database with a 70% confidence threshold. Multiple diversity index analysis could be carried out based on OTUs. The OTUs can be used for multiple diversity index analysis and sequencing depth detection. Based on taxonomic information, the community structure can be statistically analyzed at various taxonomic levels.[25] All these data were analyzed on the online platform of Majorbio Cloud Platform.

2.8. Statistical analysis

The data were compared using the one-way ANOVA and Fisher minimum significant difference (LSD) test. The data are expressed as means ± SEM, which were analyzed using GraphPad Prism 8 software. As for the analysis of the gut microbiota, the Student *t*-test was used to compare the diversity of intestinal microbiota in each group.

3. Results and discussion

3.1. Amuc_1100$^{\Delta 80}$ promotes tryptophan hydroxylase 1 (Tph1) expression in RIN-14B cells through the TLR2 signaling pathway

It was previously reported that the interaction between Amuc_1100 and TLR2 could promote the synthesis and release of 5-HT.[26] Moreover, bio-layer interferometry showed that the affinity of Amuc_1100$^{\Delta 80}$ for TLR2 was higher than that of wild-type Amuc_1100.[26–28] 5-HT deficiency can be considered a main feature of depression.[31,32] Because the antidepressant effect of Amuc_1100 has been previously demonstrated, we speculated that Amuc_1100$^{\Delta 80}$ could also upregulate the 5-HT level and have efficient antidepressant effects. Tryptophan

hydroxylase 1 (Tph1) is a rate-limiting enzyme of intestinal 5-HT synthesis.[33–35] Thus, we first studied the effect of Amuc_1100$^{\Delta 80}$ on Tph1 expression in the RIN-14B cell line. RIN-14B cells were pre-treated with the TLR2 inhibitor CU-CPT22 or HBSS for 1 h and then exposed to Amuc_1100 or Amuc_1100$^{\Delta 80}$. Twenty-four hours later, the Tph1 mRNA level in these cells was assessed. Both Amuc_1100 and Amuc_1100$^{\Delta 80}$ promoted Tph1 expression, while CU-CPT22 pre-treatment prevented this effect (Fig. 2). This result suggests that both Amuc_1100 and Amuc_1100$^{\Delta 80}$ increase Tph1 expression through interactions with TLR2. The effect of Amuc_1100$^{\Delta 80}$ is more prominent than that of Amuc_1100.

3.2. Amuc_1100$^{\Delta 80}$ reduces anxiety and depression-like behavior in chronically stressed mice

The results obtained from the cell culture experiments supported our conjecture regarding a potential antidepressant effect of Amuc_1100$^{\Delta 80}$. Therefore, we established an animal model to further explore this hypothesis. Behavioral tests showed that the mice exposed to chronic stress for three weeks developed anxiety and depression-like behavior. For mice of the CUMS group, the number of times they entered and stayed in a light chamber decreased (Fig. 3A and B). Furthermore, the total distance travelled during exercise in the OFT (Fig. 3C) and the number of times they entered the open arm in the EPM test decreased significantly (Fig. 3D). These results indicated that the CUMS mice exhibited anxious behavior. Depression-like behavior was reflected by the increased immobility time in the FST and TST (Fig. 3E and F). The frequency of mice entering the light chamber and the time they stayed there increased with both Amuc_1100 and Amuc_1100$^{\Delta 80}$ treatments (Fig. 3A and B). In addition, these treatments increased the number of times they entered the open arm in the EPM (Fig. 3D), while they significantly decreased their immobility time in the FST and TST (Fig. 3E and F). However, only Amuc_1100$^{\Delta 80}$ treatment could increase the total distance travelled during exercise in OFT, whereas there was no significant difference between Amuc_1100-treated mice and CUMS mice in this test (Fig. 3C). Overall, these behavioral tests showed that Amuc_1100$^{\Delta 80}$ reduces anxiety and depressive behavior in mice.

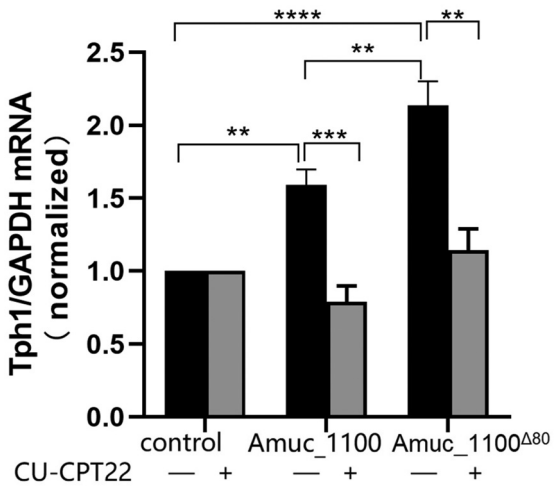

Fig. 2 Amuc_1100$^{\Delta 80}$ promotes the expression of Tph1 in RIN-14B cells through the TLR2 signaling pathway. Data are means ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

3.3. Amuc_1100$^{\Delta 80}$ restores the gut microbiota of CUMS mice

The hypothesis of the imbalance of the microbiota–gut–brain axis is often used to explore antidepressant mechanisms. The gut microbes can affect the central nervous system through a variety of mechanisms, including immune activation, the production of microbial metabolites, activation of the vagus nerve, and production of various neurotransmitters and neuroregulators in the intestinal tract.[11,36] Based on this theory, we further explored the potential mechanisms underlying Amuc_1100$^{\Delta 80}$ antidepressant activity.

Comparison of the 16S rRNA sequences from the gut microbes of different experimental groups by calculating the Sobs and Chao indexes showed that the Alpha diversity in the CUMS group was significantly lower than that in the control group, and could be increased by FLX, Amuc_1100 or Amuc_1100$^{\Delta 80}$ treatment (Fig. 4A and B). Analysis of the community composition at the phylum level revealed that the relative abundance of *Firmicutes* was increased in CUMS mice, while that of the *Bacteroidota* was decreased, which was consistent with previous reports.[27] All of FLX, Amuc_1100, and Amuc_1100$^{\Delta 80}$ limited these changes (Fig. 4C). In addition, in CUMS mice, the relative abundance of *Actinobacteriota* was increased (Fig. 4D), and that of *Campilobacteria* was decreased (Fig. 4E). Such a change in *Actinobacteriota* abundance had been previously reported, whereas the change in *Campilobacteria* has rarely been documented. The Circos circle map representation allows the visualization of the distribution of dominant species within each experimental group, as well as the distribution of dominant species across different groups. This representation highlighted changes in *Lachnospiraceae* and *Prevotellaceae* in CUMS mice compared with the control group (Fig. 4F). Therefore, we analyzed *Lachnospiraceae* and *Prevotellaceae* separately. This analysis showed that the abundance of *Lachnospiraceae* increased in CUMS mice, which was consistent with previous research on the gut microbes of patients with depression.[37] The abundance of *Lachnospiraceae* could be reduced using FLX, Amuc_1100, or Amuc_1100$^{\Delta 80}$ treatment (Fig. 4G). Some studies have shown that the abundance of *Prevotellaceae* increases in patients with schizophrenia and decreases significantly in mice with colitis induced by a high-sugar diet,[38] indicating that *Prevotellaceae* may play an important regulatory role in human health. Our results showed that the abundance of *Prevotellaceae* decreased in CUMS mice (Fig. 4H), whereas FLX, Amuc_1100 and Amuc_1100$^{\Delta 80}$ upregulated the relative abundance of this phylum. Thus, Amuc_1100$^{\Delta 80}$ corrects the imbalanced gut microbiota in mice exposed to chronic stress.

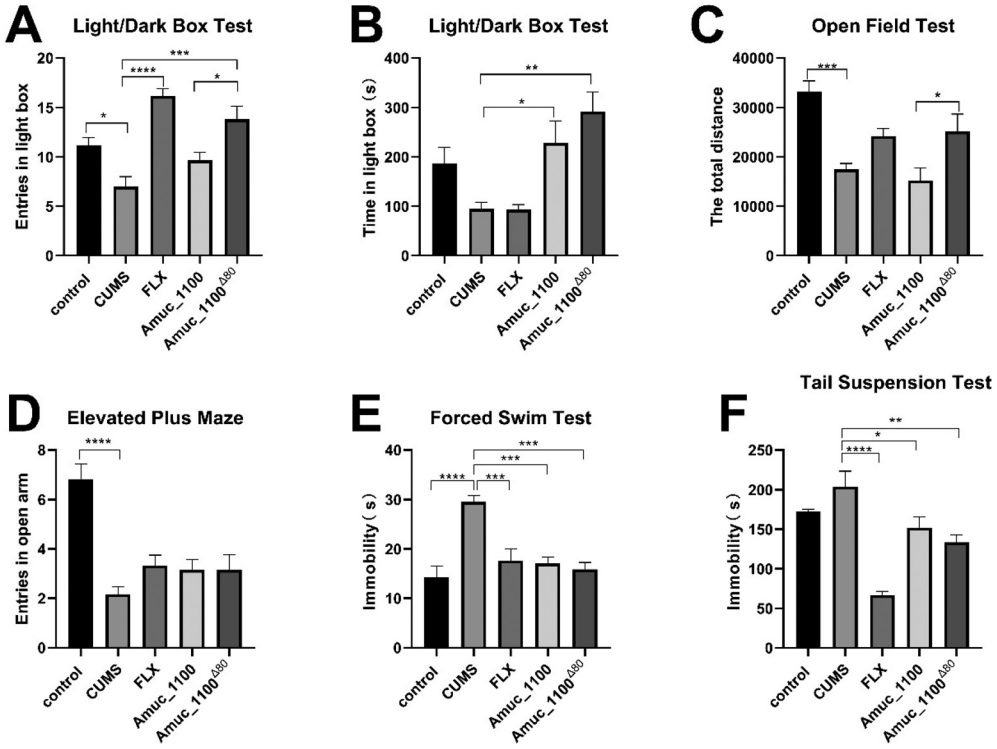

Fig. 3 Amuc_1100$^{\Delta 80}$ improves anxiety and depression-like behavior of mice exposed to chronic stress. (A) The number of times mice enter the light chamber in the LDB. (B) The time spent in the light chamber in the LDB. (C) The total distance in the OFT. (D) The number of times mice enter the open arm in the EPM. (E) The immobility time in the FST. (F) The immobility time in the TST. Data are means ± 95% CI, n = 6 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

A recent study found that bacterial metabolite 4-ethylphenyl sulfate can enter the brain and alter oligodendrocyte function and myelin sheath patterns to promote anxious behavior.[39] Furthermore, short-chain fatty acids (SCFAs) are perhaps one of the most deeply studied metabolites derived from the gut microbiota. SCFAs may interact with the enteroendocrine cells and promote indirect signaling to the brain by systemic circulation or vagal pathways, inducing the production of several hormones and neurotransmitters like GABA and serotonin in the gut.[40] SCFAs can mediate the inhibition of histone deacetylases, which may lead to the hyperacetylation of histones H3/H4 and hence increase BDNF expression, showing antidepressant effects in mice.[41] Based on the results of the previous study and the regulation effect of Amuc_1100$^{\Delta 80}$ on the gut microbiota, the conclusion can be drawn that Amuc_1100$^{\Delta 80}$ may exert antidepressant effects by regulating the gut microbiota and affecting bacterial products.

3.4. Amuc_1100$^{\Delta 80}$ influences the 5-HT system in CUMS mice

The gut microbes can interact with the 5-HT system and influence depression. Therefore, we investigate 5-HT regulation in the different experimental groups. Serum and Hp 5-HT levels were lower in the CUMS mice than in the control mice. However, FLX, Amuc_1100, and Amuc_1100$^{\Delta 80}$ increased serum and Hp 5-HT levels in the stressed mice (Fig. 5). This result suggests that Amuc_1100$^{\Delta 80}$ can influence the 5-HT system and thus affect depression.

3.5. Amuc_1100$^{\Delta 80}$ improves the 5-HTR1A-BDNF-CREB signaling pathway in CUMS mice

Under chronic stress, the expression of 5HTR1A was reduced in the central nervous system,[30] and the expression of the cAMP response element binding protein 1 (CREB1), mediated by 5-HT or norepinephrine (NE) and c-AMP/PKA signal transduction, was significantly decreased.[42] BDNF is the downstream signal molecule of CREB and can be combined with this protein to enhance transcription. The Hp 5-HTR1A level in CUMS mice was decreased, but could be restored by treatment with Amuc_1100 or Amuc_1100$^{\Delta 80}$ (Fig. 6A). Changes in the 5-HTR1A level can affect the CREB1 level, and consequently, the level of downstream BDNF. CREB1 and BDNF mRNA levels in the Hp of CUMS mice were decreased but could be increased by treating the mice with Amuc_1100 or Amuc_1100$^{\Delta 80}$ (Fig. 6B and C).

3.6. Amuc_1100$^{\Delta 80}$ downregulates the level of pro-inflammatory cytokines in CUMS mice

The pathogenesis of depression is closely related to inflammatory reactions. The upregulation of pro-inflammatory cytokines increases the risk of neonatal depression, in which IL-1β, IL-6, and TNF-α are the most studied factors related to antidepress-

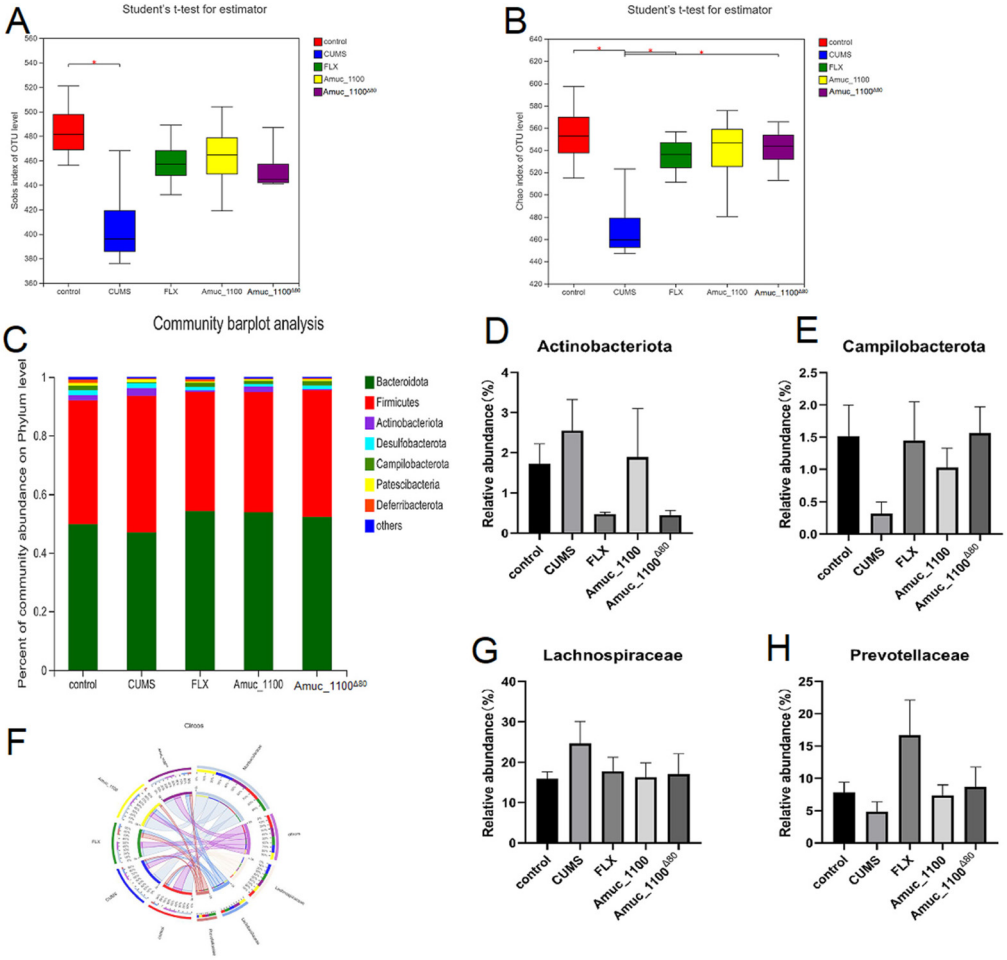

Fig. 4 Amuc_1100$^{\Delta 80}$ reduces the gut microflora imbalance in mice exposed to chronic stress. (A) Sobs index. (B) Chao index. (C) The community composition at the phylum level. (D) *Actinobacteriota*. (E) *Campilobacterota*. (F) Analysis of the distribution proportion of dominant species at the family level. (G) *Lachnospiraceae*. (H) *Prevotellaceae*. Data are means ± 95% CI, n = 4 per test. One-way ANOVA followed by Fisher's LSD *post hoc* test.

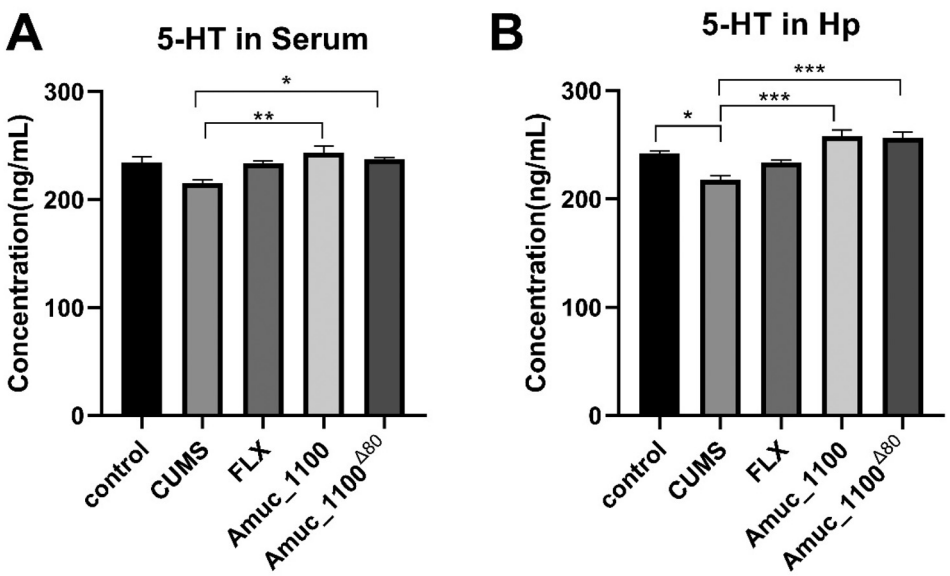

Fig. 5 Levels of 5-HT in serum and Hp in mice from the different groups. (A) 5-HT in serum. (B) 5-HT in Hp. Data are means ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

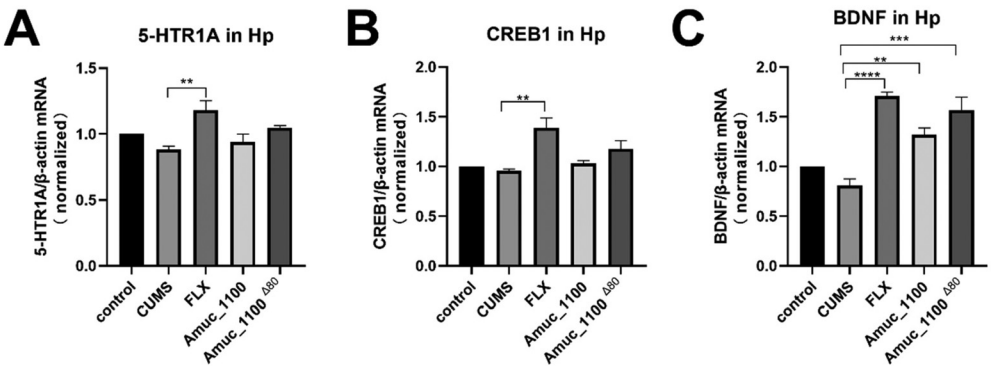

Fig. 6 Amuc_1100$^{\Delta 80}$ affects the BDNF signal pathway in depressed mice. (A) 5-HTR1A/β-actin mRNA in Hp. (B) CREB1/β-actin mRNA in Hp. (C) BDNF/β-actin mRNA in Hp. Data are means ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

ant effects.[43–45] Furthermore, a study on this issue found intestinal swelling in depressed mice during dissection. Therefore, we became more convinced that the antidepressant effects of Amuc_1100 and Amuc_1100$^{\Delta 80}$ could be related to inflammation. To test this hypothesis, we measured inflammatory factors in the central and peripheral nervous systems of mice from different groups. The analysis of intestinal inflammatory factors revealed that IL-1β, IL-6, and TNF-α mRNA levels were significantly increased in the colon and ileum of chronically stressed mice. Amuc_1100 and Amuc_1100$^{\Delta 80}$ could reduce the mRNA levels of these three inflammatory cytokines in the intestine, whereas FLX only reduced the inflammation of the ileum, without an obvious therapeutic effect on colonic inflammation (Fig. 7). Similarly, serum and FC IL-1β, IL-6, and TNF-α mRNA levels were significantly upregulated in CUMS mice. Treatment with FLX, Amuc_1100, and Amuc_1100$^{\Delta 80}$ could dampen these inflammatory markers (Fig. 8).

Several antidepressants can reduce the endogenous production of pro-inflammatory cytokines.[46] Tricyclic antidepressants inhibit the release of pro-inflammatory cytokines IL-6,

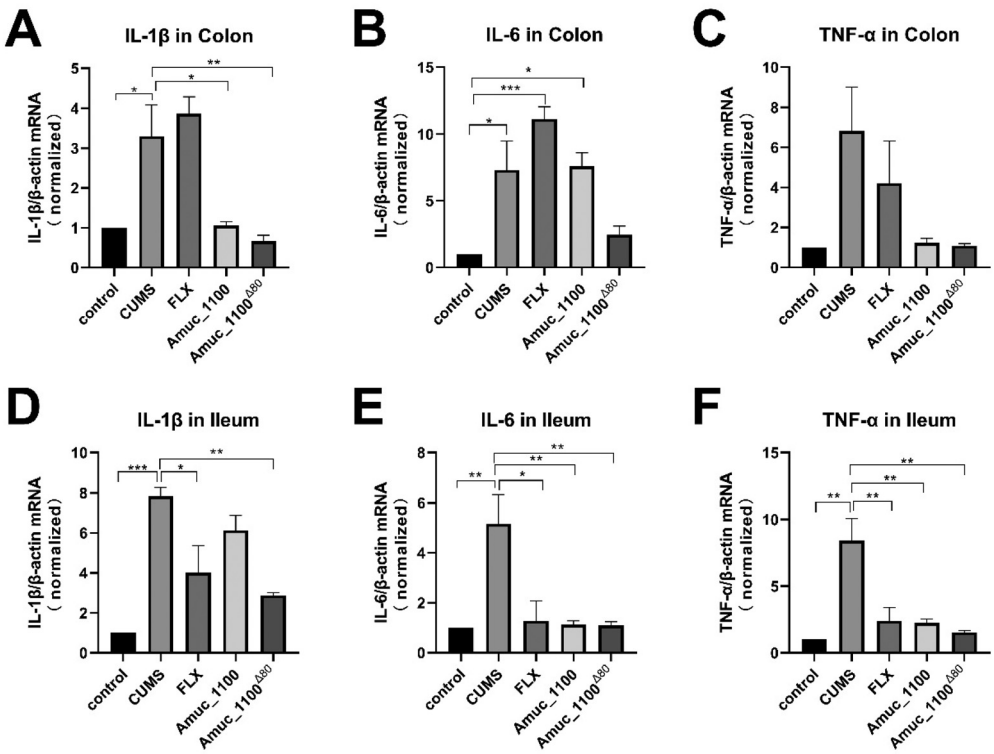

Fig. 7 mRNA levels of inflammatory cytokines in the colon and ileum of mice from different groups. (A–C) The levels of IL-1β, IL-6 and TNF-α mRNA in the colon. (D–F) The levels of IL-1β, IL-6 and TNF-α mRNA in the ileum. Data are means ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

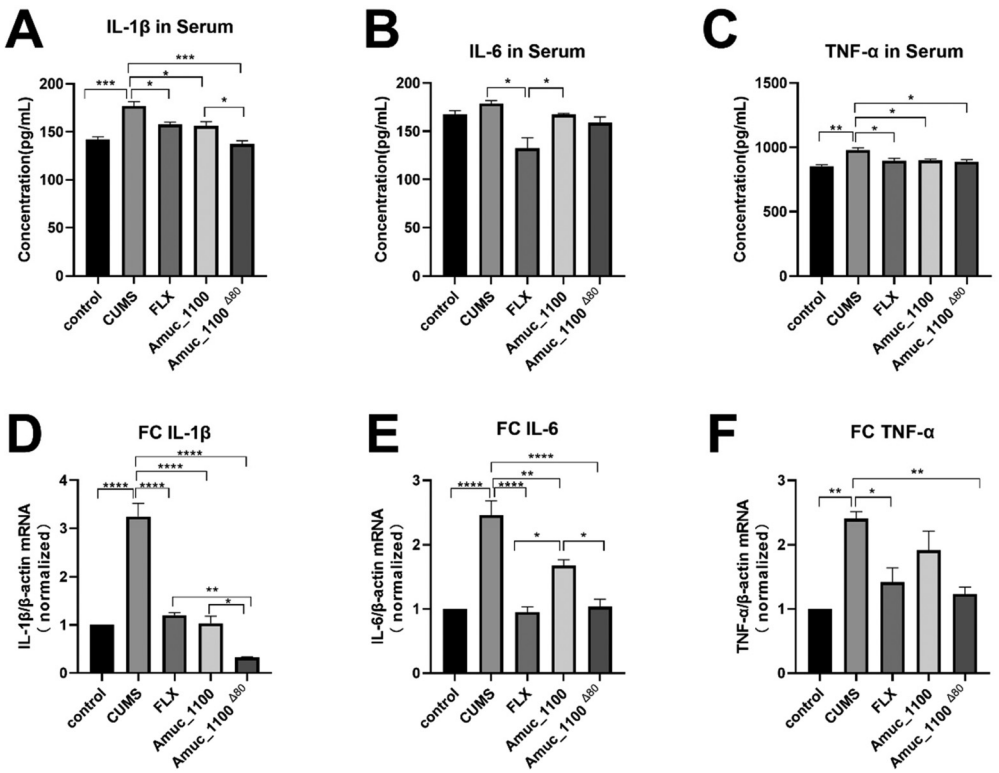

Fig. 8 mRNA levels of inflammatory cytokines in serum and FC of mice from different groups. (A–C) The mRNA levels of IL-1β, IL-6 and TNF-α in serum. (D–F) The levels of IL-1β, IL-6 and TNF-α mRNA in FC. Data are means ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

IL-1β, and TNF-α. Additionally, the next-generation antidepressant ketamine has been shown to decrease depressive symptoms *via* a decrease in circulating IL-1β levels.[47] The gut microbiota has a fundamental effect on the gut inflammatory and immune responses. It is reported that several genera including *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium*

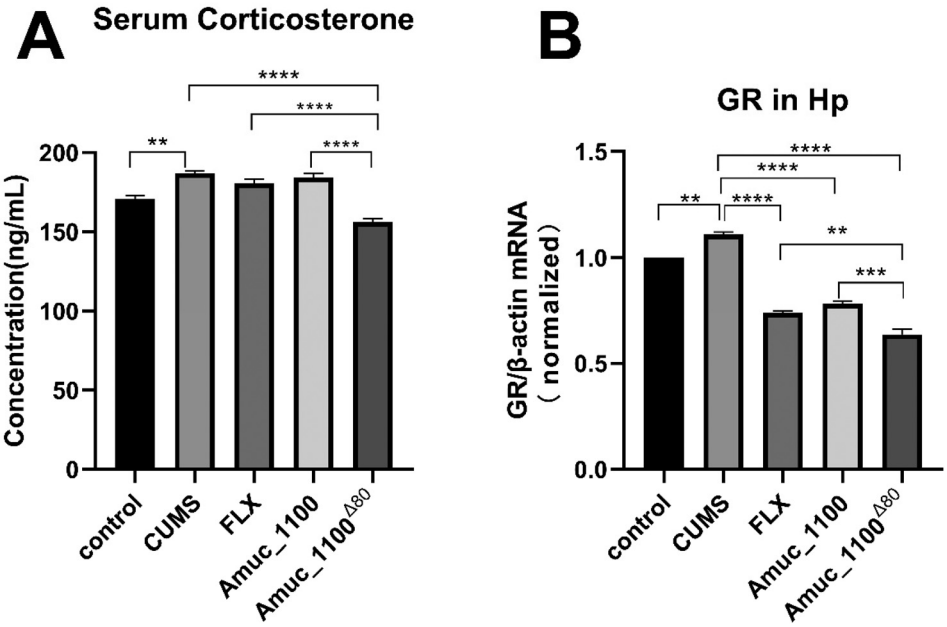

Fig. 9 Amuc_1100$^{\Delta 80}$ affects the activity of the HPA axis. (A) Corticosterone in serum. (B) GR/β-actin in Hp. Data are means ± 95% CI, n = 3 per test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One-way ANOVA followed by Fisher's LSD *post hoc* test.

have been shown to stimulate anti-inflammatory cytokines including IL-10 and downregulate inflammatory cytokines.[48,49] These findings suggest that the gut microbiota dysbiosis may play a role in the pathophysiology of depression induced by immune and inflammation responses. Some rodent experiments confirmed the link between the gut microbiota and depression *via* inflammatory markers. For example, Zhang *et al.* reported that socially stressed mice showed an increased fecal *Oscillospira* and a decreased fecal *Firmicutes*/*Bacteroidetes* ratio while developing depression-like symptoms. IL-6 receptor antibody (MR16-1) treatment rescued these symptoms, significantly decreased the *Oscillospira* count, and attenuated the decrease in the fecal *Firmicutes*/*Bacteroidetes* ratio at the same time.[50] Therefore, we speculate that Amuc_1100$^{\Delta 80}$ may perform its anti-depressive action where the gut microbiota, inflammation, and depression are linked.

3.7. Amuc_1100$^{\Delta 80}$ diminishes the hyperactivated HPA axis in CUMS mice

When the body faces stress, the HPA axis becomes hyperactive. Stress signals can cause the hypothalamus to secrete the corticotropin-releasing hormone (CRH), which induces corticotropin (ACTH) production by the anterior pituitary. ACTH stimulates the adrenal gland to produce more glucocorticoids (GCs). A high level of corticosterone, the main endogenous GC, plays a negative role in CRH and ACTH secretion, which terminates the stress response through a negative feedback loop.[51] The HPA axis mainly causes GC release into the peripheral blood. GC can mediate a negative feedback mechanism by binding to a glucocorticoid receptor (GR) in the central nervous system through the blood–brain barrier. The GR is the key intermediate of the negative feedback operating on the HPA axis.[52] Excessive expression of the GR in the Hp makes this organ vulnerable to the toxic effects of excessive GC.

Based on the understanding of the HPA axis, we investigated the effect of three weeks of chronic stress on the function of the HPA axis. We found that corticosterone as the main GC in rodents was significantly increased in the serum (Fig. 9A). Moreover, the expression of GR was significantly increased in Hp (Fig. 9B). These results indicated that the HPA axis was highly active in the CUMS mice. FLX, Amuc_1100, or Amuc_1100$^{\Delta 80}$ alleviated HPA axis hyperactivity, as evidenced by the fact that they reduced the levels of corticosterone and GR (Fig. 9).

4. Conclusion

To summarize, this study demonstrates that Amuc_1100$^{\Delta 80}$ has a better antidepressant effect than Amuc_1100 on modifying chronic stress–induced depression-like behavior in mice. Furthermore, the potential mechanism could be related to the gut microbiota modulation. Changes in the gut microbes affect the function of the central nervous system through neurotransmitters and the immune system, and thereby influence the intestinal mucosal barrier and the blood–brain barrier. Our research lays the foundation for the development of new antidepressants based on these molecular pathways.

Author contributions

Conceptualization: M. Z.; methodology: R. R. C., H. Y. Z., Y. S., T. R. H. and M. Z.; formal analysis: R. R. C., H. Y. Z. and Y. S.; investigation: R. R. C.; data acquisition: R. R. C. and H. Y. Z.; data curation: R. R. C. and M. Z.; writing—original draft preparation: R. R. C.; writing—review and editing: R. R. C., H. Y. Z. and M. Z.; funding acquisition: M. Z. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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