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ORIGINAL RESEARCH

Probiotic Bifico Ameliorates Depression- and Anxiety-Like Behaviors Induced by Estrogen Deficiency via NLRP3 Inflammasome Inhibition

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Purpose: Dysregulation of the microbiota-gut-brain (MGB) axis and activation of the NOD-like receptor protein 3 (NLRP3) inflammasome are implicated in estrogen deficiency induced depression and anxiety disorders. This study aims to investigate the effects of the probiotic Bifico on neuroinflammation and behaviors in ovariectomized (OVX) rats.

Methods: After OVX rats were treated with Bifico for 6 weeks, depression- and anxiety-like behaviors were evaluated using the sucrose preference test, forced swimming test, open field test and elevated plus maze. Furthermore, 16S rRNA sequencing was used to analyze changes in gut microbiota. Hematoxylin-eosin (HE) staining was used to observe the changes in tissue structure (intestinal tissue and hippocampus). Enzyme-linked immunosorbent assay (ELISA) was used to detect inflammatory factors. Western blot was used to detect tissue protein levels.

Results: The treatment of Bifico for 6 weeks can ameliorate depression- and anxiety-like behaviors induced by estrogen deficiency. In addition, Bifico increased the abundance of gut microbiota, especially *Lactobacillus sp*. and *Desulfovibrio*, and significantly ameliorated histological injuries in the ileum, colon and hippocampus. Bifico up-regulated the expression of tight-junction proteins zona occludens 1 (ZO-1) and Occludin in the colon and hippocampus, and down-regulated the expression of NLRP3 inflammasome signaling pathway-related proteins, including NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), Caspase-1, interleukin (IL)-1β, IL-18, toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), phosphorylated (P)-P65 and P65. Meanwhile, Bifico also reduced the levels of IL-6 and tumor necrosis factor (TNF)-α in serum and hippocampus.

Conclusion: Our findings suggest that probiotics ameliorate the depression- and anxiety-like behaviors in OVX rats by alleviating gut microbiota dysbiosis and reduce gut inflammation, thereby dampening neuroinflammation via inhibition of the NLRP3 inflammasome signaling pathway in the hippocampus. Therefore, NLRP3 inflammasome activation mediated by the MGB axis may be a potential therapeutic target for estrogen deficiency-induced affective disorders.

Keywords: microbiota-gut-brain axis, NLRP3 inflammasome, depression, estrogen, hippocampus

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Introduction

Depression is a chronic neuropsychiatric disease characterized by low mood and anhedonia. Epidemiological survey data shows that approximately 350 million people worldwide are affected by depression annually,[1] and the number of women affected is twice that of men.[2–4] Among women, the perimenopausal period, when estrogen decreases sharply, is the window period of vulnerability for the onset of depression.[1,5] At present, the main treatment methods for perimenopausal depression (PMD) are estrogen replacement therapy and conventional drug therapy. However, both estrogen replacement therapy and conventional drug therapy have certain limitations, which may lead to an increased risk of endometrial cancer, breast cancer and other diseases.[6] Therefore, it is urgent to find the best alternative treatment drugs with high safety and few side effects.

In recent years, the role of gut microbiota in the microbiota-gut-brain (MGB) axis has been increasingly recognized. Gut microbes communicate bidirectionally with the brain through the neuroendocrine, immune and autonomic nervous systems, and have been confirmed to be closely related to depression.[6–10] In addition, fecal microorganisms from patients with major depressive disorder (MDD) transplanted into the gut of rodents can induce depression- and anxiety-like behaviors in animals, indicating that gut microbiota dysbiosis may precede MDD onset.[7] Further studies have found that there is an interaction between gut microbiota and estrogen.[5,11] For example, clinical studies have found that decreased serum estrogen levels in postmenopausal women significantly alter the composition and abundance of gut microbiota.[11] An interesting recent animal study found that 3β-hydroxysteroid dehydrogenase, which is expressed by *Klebsiella aerogenes* in the gut of ovariectomized (OVX) mice, efficiently degrades estradiol (E_2), thereby leading to depression-like behaviors in OVX mice.[5] Thus, the MGB axis may be an important target for the treatment of affective disorders related to estrogen deficiency, but the specific mechanism of action remains unclear.

More and more studies have shown that neuroinflammation plays an important role in nervous system diseases.[10,12–15] Recently, some scholars have proposed the new concept of "microbiota-gut-inflammasome-brain axis", which proposes that the interaction between gut microbiota and inflammasomes will affect the intestinal microecological balance and the physiological function of the brain.[16] The most intensively studied inflammasome is the NOD-like receptor protein 3 (NLRP3) inflammasome. In addition, toll-like receptor 4 (TLR4), as an important pattern recognition receptor in innate immunity, has been shown to play an important role in neuroinflammation.[17] In intestinal epithelial cells and immune cells, lipopolysaccharide (LPS) activates TLR4, and activated TLR4 participates in the activation of NLRP3 inflammasome through the TLR4/nuclear factor (NF)-κB signaling pathway.[17] The activation of NLRP3 inflammasome promotes the maturation and release of interleukin (IL)-1β and IL-18, two proinflammatory cytokines involved in neuroimmune regulation, neuroinflammation and neurodegeneration.[17] A large number of studies have shown that hippocampal changes are an important mechanism for the pathogenesis of depression. Postmenopausal women may experience abnormalities in hippocampus-related functions, such as memory, attention, cognition and autonomic control.[18] The activation of the NLRP3 inflammasome in hippocampus has been confirmed to be related to the depression- and anxiety-like behaviors induced by estrogen deficiency in animals.[19–21] These above results suggest that MGB axis-mediated NLRP3 inflammasome in the hippocampus may be an important target for the treatment of PMD. However, the interplay between gut microbiota and NLRP3 inflammasome is poorly understood.

Recent literature has shown that probiotics have antidepressant effects. Among them, Bifico, or *Bifidobacterium* triple viable, is a probiotic mixture containing *Bifidobacterium, Lactobacillus acidophilus* and *Enterococcus faecalis*. The regulatory function of Bifico on intestinal microbes and its anti-inflammatory effect on gastrointestinal diseases have been confirmed by a large number of studies.[22–27] However, whether Bifico exerts antidepressant effects by modulating NLRP3 inflammasome activation via the MGB axis remains uninvestigated. To address this, we used bilateral ovariectomy to establish a rat model of PMD, mimicking the low estrogen state in perimenopausal women, and aimed to validate whether Bifico alleviates depression by regulating MGB-axis-mediated NLRP3 inflammasome activation. This study seeks to clarify the molecular mechanisms underlying estrogen deficiency induced depression and provide a theoretical basis for PMD prevention, treatment, and targeted drug development.

Materials and Methods

Animals

Specific pathogen-free female Sprague-Dawley (SD) rats, approximately 8 weeks old and weighing 200 ± 10 g, were used in the study. All SD rats were housed at the Laboratory Animal Center of the 900th Hospital of PLA Joint Logistics

Support Force (Fuzhou, China). Animal housing temperature was controlled at 22 ± 2°C with a relative humidity of 60 ± 5% and a 12 hours light/dark cycle. Before the start of the experiment, all SD rats were adaptively housed for 1 week, with free access to food and water. All procedures were conducted in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and approved by the Ethics Committee of the 900th Hospital of PLA Joint Logistics Support Force (Ethical Approval No. 2021–22).

Experimental Design

Female SD rats were randomly divided into the Sham group (n = 8) and ovariectomized (OVX) group (n = 24). The rats were anesthetized intraoperatively by intraperitoneal injection of 3% sodium pentobarbital (45 mg/kg). In the OVX group, bilateral ovaries were removed, while in the Sham group, bilateral ovaries were preserved and only adipose tissue of similar volume adjacent to the ovarian tissue was removed. After 1 week of postoperative recovery, the OVX rats were randomly divided into three experimental groups: OVX group (n = 8), OVX-E_2 group (n = 8) and OVX-probiotics (OVX-PB) group (n = 8). The rats in the OVX-E_2 group were given 17β-E_2 (30 μg/kg/day) by abdominal subcutaneous injection, while rats in the OVX-PB group were given Bifico solution (176.4 mg/kg/day) by gavage for 6 weeks. After the behavioral test, rats were euthanized, and the feces, serum and tissues were collected for subsequent experimental studies (Figure 1).

Behavioral Tests

Body Weight Measurements

The day of OVX surgery was recorded as beginning week 0, and the body weights of the rats were measured then and once a week thereafter. The changes in body weights of rats in the Sham, OVX, OVX-E_2 and OVX-PB groups were observed and recorded.

Sucrose Preference Test

Adaptive training was performed before the sucrose preference test (SPT). Rats were exposed to 1% sucrose solution for 1 week. After adaptation, the SPT was carried out for 24 h, after which sucrose and water consumption (mL) were recorded. The sucrose preference rate was calculated using the formula: sucrose consumption / (sucrose consumption + water consumption) × 100%.

Forced Swimming Test

The forced swimming test (FST) was used to determine the depression-like behavior of the animals. The test used a clean transparent cylinder (diameter 20 cm, height 50 cm) containing water 35 cm high, with water temperature maintained at 25°C. During the experiment, the rats were placed in the cylinder and forced to swim for 6 min, and the immobility or floating time was recorded for the last 4 min.

Open-Field Test

The open field test (OFT) was performed in a black test box (100 cm × 100 cm × 48 cm), and each rat was placed in the center of the test box for 5 min. The Smart 3.0 Animal Behavior Analysis System (Shenzhen RWD Life Technology Co., Ltd., China) was used to record the movement trajectory of the rats. The distance traveled and time spent in the central area were used to evaluate the anxiety-like behavior of the animals.

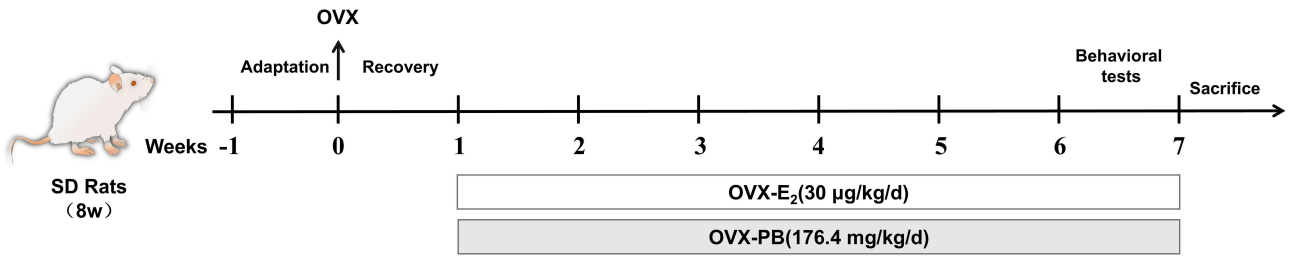

Figure 1 Schematic diagram of the experimental procedures.

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Elevated Plus Maze

The elevated plus maze (EPM) apparatus consisted of the EPM, a high-speed camera and the Smart 3.0 Animal Behavior Analysis System. The rats were placed in turn in the central area of the EPM, and the movement trajectory of the rats for 5 min was recorded by the Smart 3.0 Animal Behavior Analysis System. The time spent in the open arms and number of entries into the open arms were recorded.

Gut Microbiota Analysis

Total bacterial DNA was isolated from fecal samples using the QIAamp stool DNA kit (Qiagen, Duesseldorf, Germany). Polymerase chain reaction (PCR) was used to amplify the bacterial 16S rRNA gene in different regions of the transcript (V3-V4), using upstream primer sequence 5′-ACTCCTACGGGAGGCAGCA-3′ and downstream primer sequence 5′-GGACTACHVGGGTWTCTAAT-3′. PCR amplification reaction conditions were set as follows: pre-denaturation at 98°C for 2 min, denaturation at 98°C for 15s, annealing at 55°C for 30s, extension at 72°C for 30s, for a total of 25–30 cycles, and finally maintenance at 72°C for 5 min. PCR products were quantified and purified using a Nanodrop NC 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) after electrophoresis in an agarose gel at 1.2% concentration. Libraries were constructed and evaluated using the Tru Seq Nano DNA LT Library Prep Kit (Illumina, USA), followed by sequencing analysis on the Illumina high-throughput sequencing platform. Library construction and sequencing analysis were performed by Shanghai PaisenNuo Biotechnology Co., Ltd. (Shanghai, China).

Hematoxylin-Eosin Staining

Hippocampus, colon, and ileum tissues were fixed in 4% paraformaldehyde solution. The fixed tissue was embedded in paraffin and sectioned into 5 μm tissue sections using a sectioning machine. After deparaffinization, hematoxylin-eosin (HE) staining, dehydration and sealing were performed, and histopathological changes were observed under a light microscope.

ELISA Analysis for Inflammatory Cytokines and Hormones

After adding RIPA lysis buffer (Solarbio, Beijing, China), hippocampal tissues were placed in a high-throughput tissue grinder to fully homogenize, and the homogenates were centrifuged at 15,000 g for 20 min. The concentrations of IL-6 and tumor necrosis factor alpha (TNF-α) in hippocampal homogenate supernatant and serum were detected using enzyme-linked immunosorbent assay (ELISA) kits (ABclonal, Wuhan, China). The concentration of serum E_2 was also detected using an ELISA kit (Sino-UK Institute of Biological Technology, Beijing, China). For the ELISA procedure, the enzyme standard reagent and sample diluent were mixed in a 37°C incubator for 1 h, after which chromogenic agent was added in the dark for 15–20 min, followed by reaction termination and detection of absorbance at 450 nm.

Western Blot Analysis

Hippocampal supernatants were prepared as described in Method 2.6. Protein concentrations were determined using the BCA Protein Assay kit (BOSTER, Wuhan, China). The extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Epizyme, Shanghai, China) and transferred to polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). After blocking with 5% nonfat cow milk (BOSTER), the membranes were incubated with primary antibodies against β-actin (Abcam, ab8226, 1:2000), TLR4 (Abcam, ab217274, 1:1000), P65 (CST, #8242, 1:1000), P-P65 (Abcam, ab76302, 1:1000), MyD88 (CST, #4283, 1:1000), NLRP3 (Abcam, ab263899, 1:1000), IL-1β (Abcam, ab283818, 1:1000), IL-18 (Abcam, ab191860, 1:1000), Caspase-1 (CST, #83383, 1:1000), Caspase-3 (Abcam, ab184787, 1:1000), ASC (Abcam, ab180799, 1:1000), Occludin (Abcam, ab216327, 1:1000) or ZO-1 (Bioss, bs-1329R, 1:600) overnight at 4°C in the refrigerator. Specific bands were visualized using an enhanced chemiluminescence (ECL) kit (Santa Cruz, Dallas, TX, USA) and chemiluminescence imaging system (ChemiScope6100, Clinx Science Instruments Co., Ltd., Shanghai, China). The relative expression of the above proteins was calculated using Image J (NIH; Bethesda, MD, USA), with β-actin as a reference, and assays were repeated three times.

Statistical Analysis

The experimental data were statistically analyzed by GraphPad Prism 9.0 software (GraphPad Software, Boston, MA, USA), and the data were expressed as means ± SEM. GraphPad Prism 9.0 software was used to make statistical charts. The normal distribution and homogeneity of variance were verified before comparison of data between multiple groups. One-way or multivariate analysis of variance was used for analysis of variance if the variance was homogeneous, and Tukey's multiple comparisons post-hoc test was used for comparisons between groups. Non-parametric data were analyzed using the Kruskal–Wallis test with Dunnett's multiple comparisons test. $p<0.05$ was considered statistically significant.

Results

Probiotics Can Improve Depression- and Anxiety-Like Behaviors in OVX Rats

To explore the effects of probiotics on behavior and intestinal microecology in OVX rats, an OVX rat model was established and probiotics were administered for comparison to untreated Sham animals. The results showed that compared with the Sham group, the body weight of the rats in the OVX group increased significantly ($p<0.01$, Figure 2A), while the uterine weight and serum E_2 concentration decreased significantly ($p<0.01$, Figure 2B and C, respectively). Compared with the OVX group, the body weight of the rats in the OVX-E_2 group was lower ($p<0.01$, Figure 2A), and the E_2 concentration and uterine weight were higher ($p<0.01$, Figure 2B and C, respectively), indicating that estrogen replacement therapy for OVX rats for 6 weeks could effectively inhibit the OVX effects. There were no significant changes in body weight, uterine weight or serum E_2 level in the OVX-PB group ($p>0.05$, Figure 2B and C, respectively).

The SPT and FST were used to detect the depression-like behavior of rats. The results showed that compared with the Sham group, the percentage of sucrose consumption in the OVX group was significantly decreased ($p<0.01$, Figure 2D), while the immobility time was prolonged ($p<0.01$, Figure 2E). Compared with the OVX group, the percentage of sucrose consumption in the OVX-E_2 group ($p<0.01$, Figure 2D) and the OVX-PB group ($p<0.05$, Figure 2D) was significantly increased, and the immobility time was significantly shortened ($p<0.01$, Figure 2E). These results suggested that bilateral ovariectomy increased depression-like behaviors in rats, which could be inhibited by estrogen or probiotics.

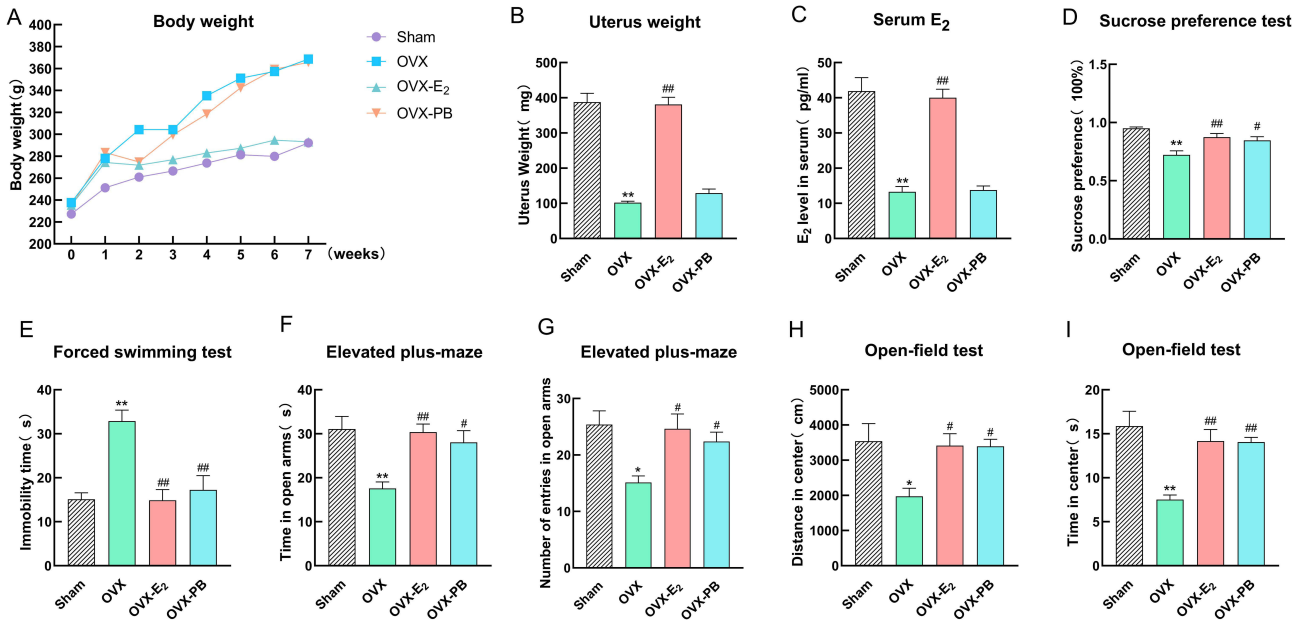

Figure 2 Assessment of depression- and anxiety-like behaviors in OVX rats. (**A**) Body weight changes of rats; (**B**) uterine weight changes in rats; (**C**) serum E_2 levels; (**D**) SPT; (**E**) immobility time in the FST; (**F**) time spent in the open arms of the EPM; (**G**) the number of entries into the open arms of the EPM; (**H**) distance traveled in the central area in the OFT; (**I**) time spent in the central area in the OFT. *$p<0.05$, **$p<0.01$ vs Sham, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs OVX.

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EPM and OFT are commonly used to evaluate anxiety-like behaviors in animals. When animals enter the open arm, a decreasing number of times in the EPM, and the distance traveled and duration of central activities are reduced in the OFT, this is taken to indicate that animals have increased anxiety-like behaviors. The results showed that in the EPM, compared with the Sham group, the OVX group had a significant reduction in the time to enter the open arm ($p<0.01$, Figure 2F) and a reduction in the number of times the rats entered the open arm ($p<0.05$, Figure 2G). Compared with the OVX group, the OVX-E_2 group and the OVX-PB group had a significant increase in the time and frequency of entering the open arm ($p<0.01$, $p<0.05$, Figure 2F and G, respectively). In the OFT, the central activity distance ($p<0.05$, Figure 2H) and central activity duration ($p<0.01$, Figure 2I) of OVX-group rats were significantly reduced. Compared with the OVX group, the OVX-E_2 group and the OVX-PB group displayed a significant reversal of the above phenomena ($p<0.01$, $p<0.05$, Figure 2H and I, respectively). These above results indicated that estrogen or probiotics were able to significantly improve anxiety-like behaviors in OVX rats.

Effects of Probiotics on Gut Microbiota and the Intestinal Barrier in OVX Rats

16S rRNA sequencing technology was used to evaluate the changes in gut microbiota at the genus level and the sequencing results are shown in Figure 3A. Compared with Sham group, the abundance of *Lactobacillus sp., Barnesiella*

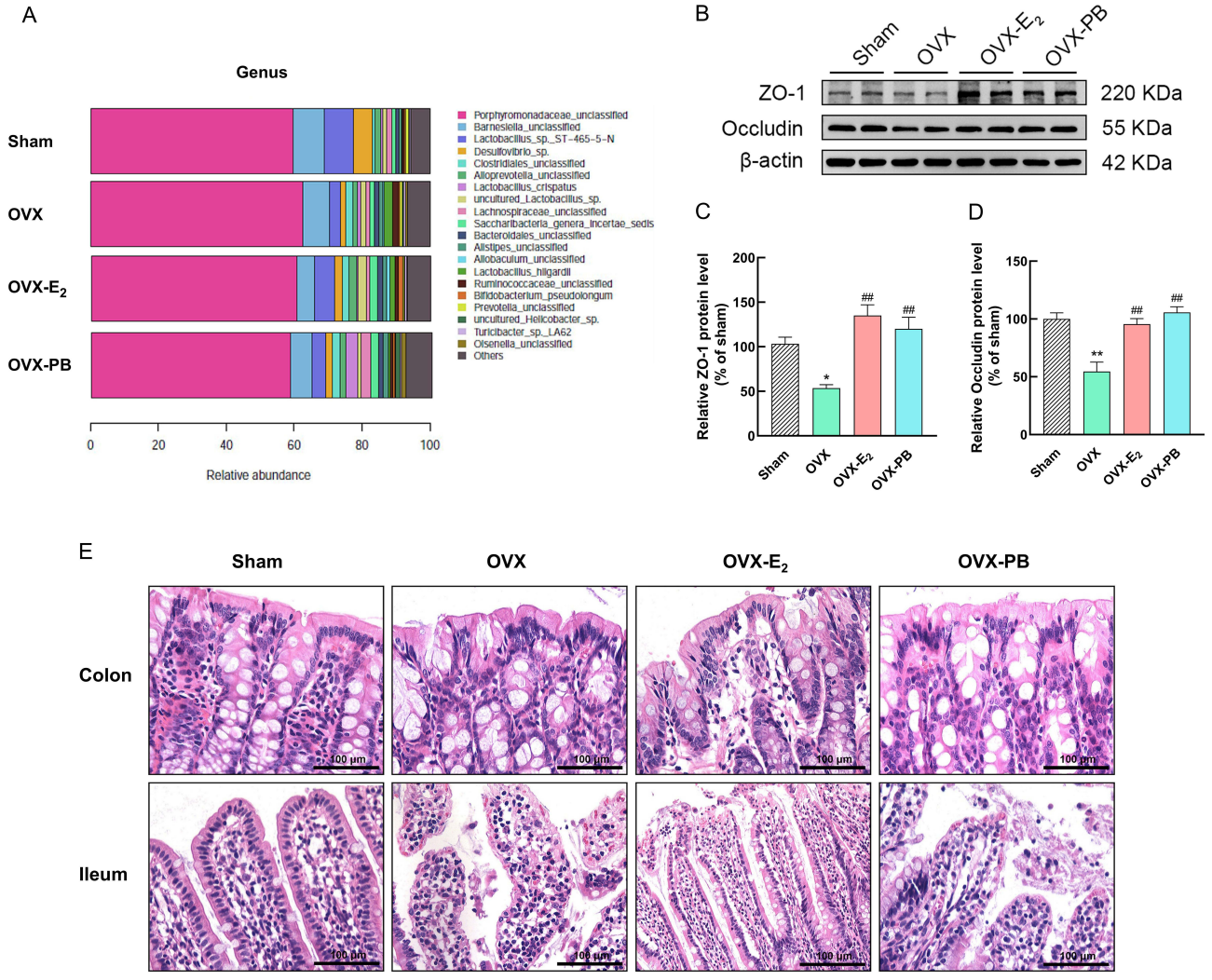

Figure 3 Assessment of gut microbiota and intestinal barrier in OVX rats. (**A**) Effects of probiotics on genus level of gut microbiota in OVX rats; (**B**) Western blot protein bands; (**C**) ZO-1 protein level; (**D**) Occludin protein level. (**E**) HE staining. Magnification 200×. Scale bar = 100 μm. Data are presented as means ± SEM (n = 3 in each group). *$p<0.05$, **$p<0.01$ vs Sham, $^{\#\#}p<0.01$ vs OVX.

and *Desulfovibrio* decreased, while the abundance of *Porphyromonadaceae* increased in the intestine of OVX rats. After estrogen or probiotics treatment, compared with the OVX group, the OVX-E_2 and OVX-PB groups had a significant reduction in the abundance of *Porphyromonadaceae* and *Barnesiella*, and an increase in the abundance of *Lactobacillus sp*. and *Desulfovibrio*. These results suggested that estrogen or probiotics can improve disorders of gut microbiota, and that gut microbiota may be an important therapeutic target for PMD.

Disruption of gut microbiota disrupts the intestinal mucosal barrier and triggers intestinal and peripheral inflammatory responses. In this study, HE staining and Western blot were used to observe whether estrogen and probiotics could improve the damage to the intestinal mucosal barrier (colon and ileum) in OVX rats. The results of HE staining are shown in Figure 3E. The intestinal epithelial cells in the Sham group were neatly arranged, the structure was clear, and the goblet cells were closely distributed. In the OVX group, the intestinal tissue structure was disordered; some villi were shortened or had disappeared, goblet cells were reduced, and intestinal mucosa was destroyed, indicating that the intestinal mucosal barrier was damaged in OVX rats. After 6 weeks of continuous administration, compared with the OVX group, the damage to intestinal tissue structure in the OVX-E_2 group and the OVX-PB group was effectively improved. Western blot results showed that compared with the Sham group, the expression levels of ZO-1 ($p<0.05$, Figure 3B and C, respectively) and Occludin ($p<0.01$, Figure 3B and D, respectively) in the colon of OVX rats were significantly decreased. After 6 weeks of estrogen or probiotic treatment, compared with the OVX group, the expression levels of tight-junction proteins ZO-1 and Occludin in the OVX-E_2 and OVX-PB groups were significantly increased ($p<0.01$, Figure 3B–D, respectively). These results suggested that estrogen or probiotics can effectively improve the damage to intestinal mucosal barrier in OVX rats by up-regulating the expression of tight-junction proteins.

Probiotics Can Inhibit the Activation of NLRP3 Inflammasome in the Colon and Serum Inflammatory Factors in OVX Rats

Studies have shown that the interaction between gut microbiota and the NLRP3 inflammasome changes dynamically in intestinal and peripheral inflammatory diseases. Western blot and ELISA were used to detect respectively the expression of NLRP3 inflammasome-related proteins (NLRP3, ASC, Caspase-1, IL-1β and IL-18) in the colon, and serum levels of inflammatory factors (IL-6 and TNF-α). Western blot results showed that compared to the Sham group, the expression of NLRP3 ($p<0.01$, Figure 4A and B, respectively), ASC ($p<0.01$, Figure 4A and C, respectively), Caspase-1 ($p<0.05$, Figure 4A and D, respectively), IL-1β ($p<0.01$, Figure 4A and E, respectively) and IL-18 ($p<0.01$, Figure 4A and F, respectively) proteins in the colons of the OVX group was significantly increased. These results indicated that estrogen deficiency can lead to activation of the NLRP3 inflammasome and up-regulation of the downstream inflammatory factors IL-1β and IL-18. Compared with the OVX group, the expression levels of NLRP3 ($p<0.05$, Figure 4A and B, respectively), Caspase-1 ($p<0.01$, Figure 4A and D, respectively), IL-1β ($p<0.01$, Figure 4A and E, respectively) and IL-18 ($p<0.01$, Figure 4A and F, respectively) proteins in the colons of the OVX-E_2 group were significantly decreased, while ASC protein also showed a downward trend. However, the trend was not statistically significant ($p>0.05$, Figure 4A and C, respectively). In addition, compared with the OVX group, the expression levels of NLRP3 ($p<0.05$, Figure 4A and B, respectively), ASC ($p<0.05$, Figure 4A and C, respectively), Caspase-1 ($p<0.01$, Figure 4A and D, respectively), IL-1β ($p<0.01$, Figure 4A and E, respectively) and IL-18 ($p<0.01$, Figure 4A and F, respectively) proteins in the colons of the OVX-PB group were significantly decreased.

ELISA results are shown in Figure 4. Compared with the Sham group, the expression levels of TNF-α and IL-6 in the serum of the OVX group were significantly increased ($p<0.01$, Figure 4G and H, respectively). After 6 weeks of continuous administration, compared with the OVX group, the expression levels of IL-6 and TNF-α in the serum of the OVX-E_2 group and the OVX-PB group were significantly decreased ($p<0.01$, Figure 4G and H, respectively).

These above results suggested that estrogen or probiotics can inhibit the expression of NLRP3 inflammasome-related proteins (NLRP3, ASC, Caspase-1, IL-1β and IL-18) in the colon of OVX rats and down-regulate the expression levels of serum inflammatory factors (IL-6 and TNF-α), thus reducing intestinal inflammation and peripheral inflammation.

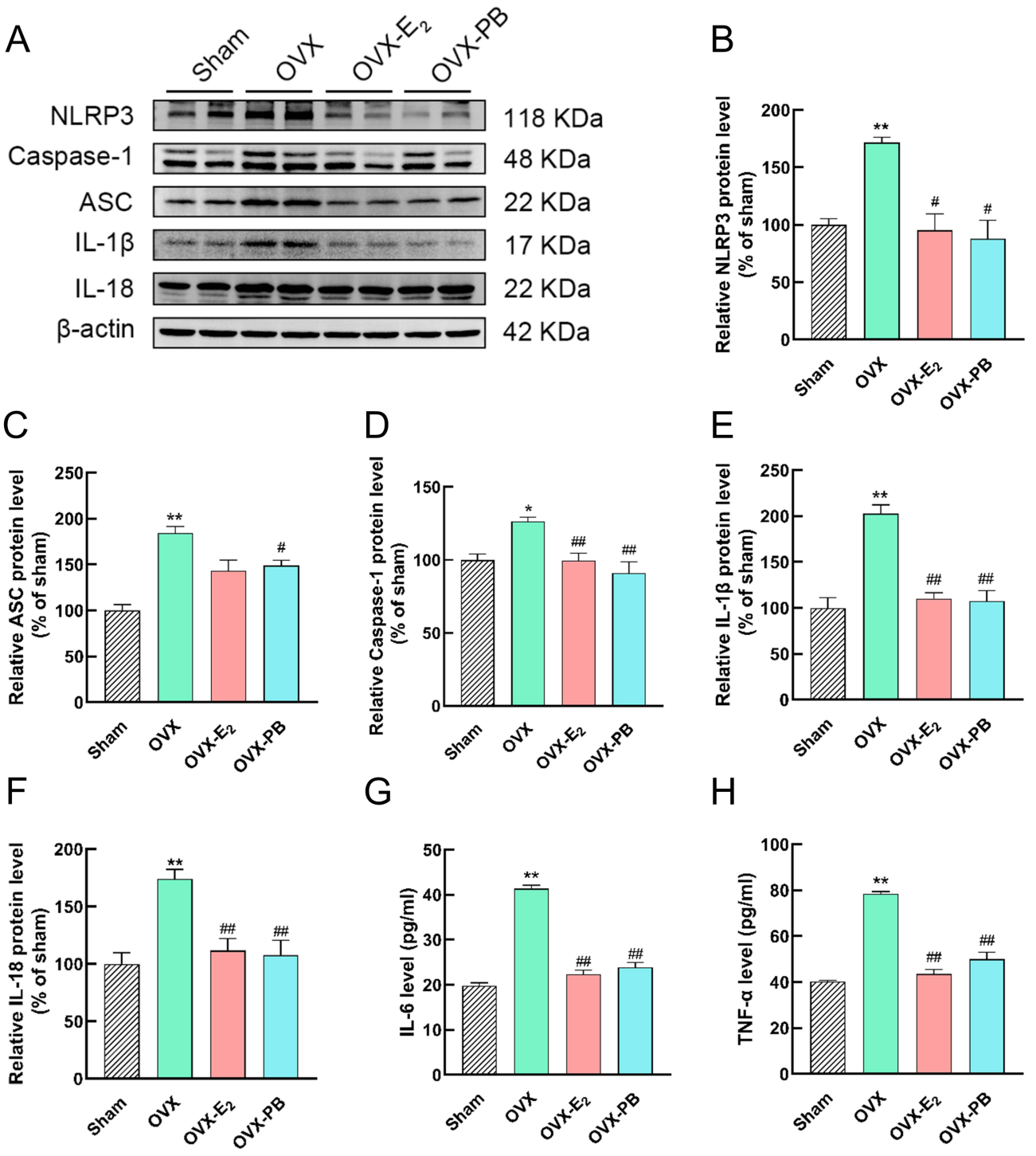

Figure 4 Assessment of NLRP3 inflammasome-related proteins and serum inflammatory factors in the colons of OVX rats. (**A**) Western blot protein bands; (**B**) NLRP3 protein level; (**C**) ASC protein level; (**D**) Caspase-1 protein level; (**E**) IL-1β protein level; (**F**) IL-18 protein level; (**G**) serum IL-6 level; (**H**) serum TNF-α level. Data are presented as means ± SEM (n = 3 in each group). $^{*}p<0.05$, $^{**}p<0.01$ vs Sham, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs OVX.

Probiotics Can Inhibit Activation of the TLR4/NF-κB Signaling Pathway in the Colon of OVX Rats

TLR4 is an important pattern recognition receptor in innate immunity and a major risk factor for inflammatory bowel disease (IBD). TLR4 activates the nuclear transcription factor-κB (NF-κB) signaling pathway, which leads to the secretion

of inflammatory factors such as IL-6 and TNF-α in the intestine and systemic circulation, and participates in the activation of NLRP3 inflammasome. Therefore, in this study, Western blot was used to further detect the expression levels of TLR4/NF-κB signaling pathway-related proteins (TLR4, MyD88, P-P65 and P65) in the colon, and the ratio of P-P65/P65 protein expression was calculated. The results are shown in Figure 5. Compared with the Sham group, the expression of TLR4 ($p<0.01$, Figure 5A and B, respectively), MyD88 ($p<0.01$, Figure 5A and C, respectively), P-P65 ($p<0.01$, Figure 5A and D, respectively) and P65 ($p<0.01$, Figure 5A and E, respectively) protein in the colon of the OVX group was significantly increased, and the ratio of P-P65/P65 protein expression was increased ($p<0.05$, Figure 5A and F, respectively). These

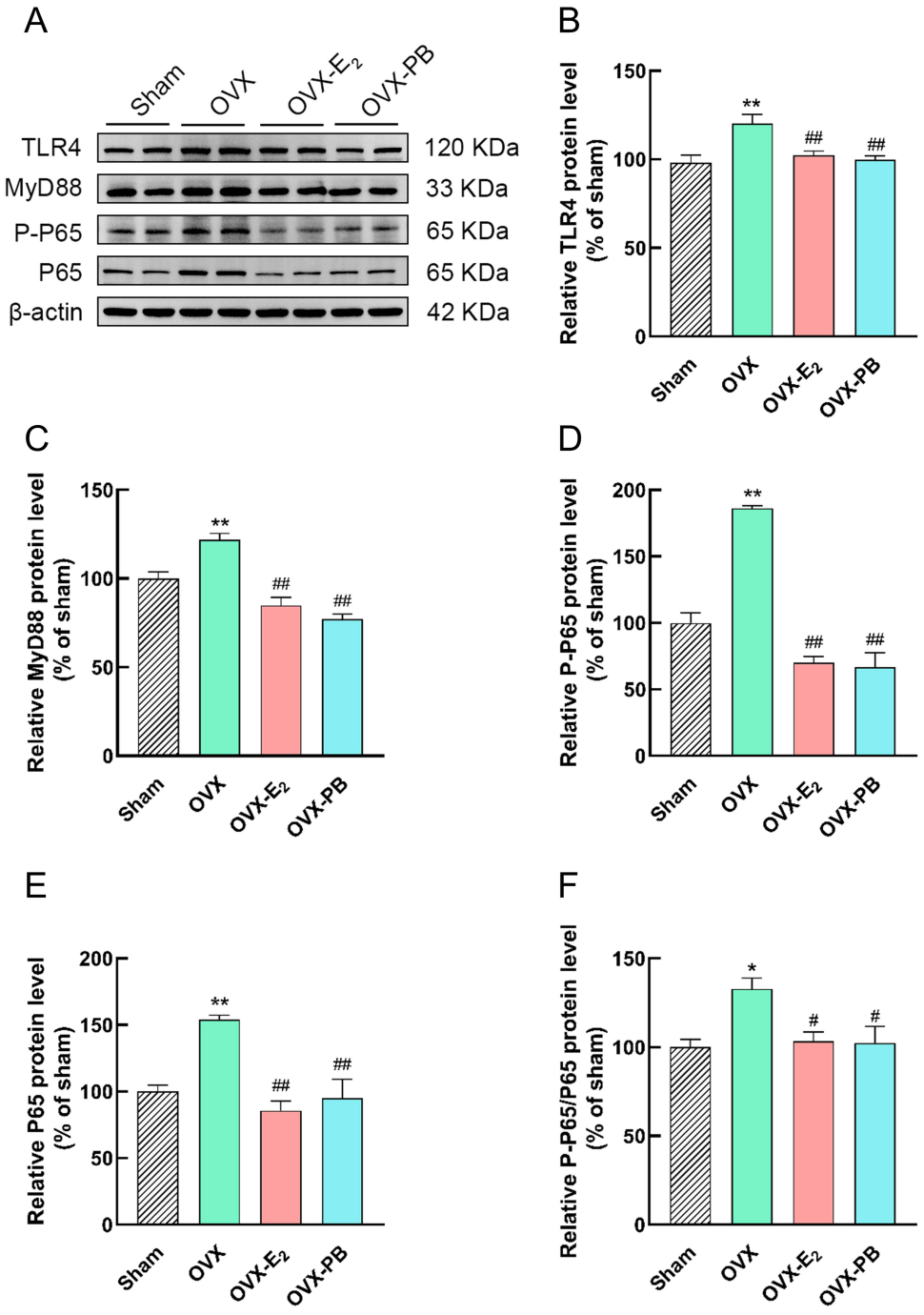

Figure 5 Assessment of the TLR4/NF-κB signaling pathway in the colon of OVX rats. (**A**) Western blot protein bands; (**B**) TLR4 protein level; (**C**) MyD88 protein level; (**D**) P-P65 protein level; (**E**) P65 protein level; (**F**) P-P65/P65 protein expression ratios. Data are presented as means ± SEM (n = 3 in each group). *$p<0.05$, **$p<0.01$ vs Sham, #$p<0.05$, ##$p<0.01$ vs OVX.

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results indicate that estrogen deficiency can lead to activation of the TLR4/NF-κB signaling pathway in rat colon. After 6 weeks of continuous treatment with estrogen or probiotics in OVX rats, compared with the OVX group, the expression of TLR4 ($p<0.01$, Figure 5A and B, respectively), MyD88 ($p<0.01$, Figure 5A and C, respectively), P-P65 ($p<0.01$, Figure 5A and D, respectively) and P65 ($p<0.01$, Figure 5A and E, respectively) protein in the colons of both the OVX-E_2 group and the OVX-PB group were significantly decreased. Furthermore, the ratio of P-P65/P65 protein expression was significantly decreased ($p<0.05$, Figure 5A and F, respectively). These results suggested that estrogen or probiotics may effectively reduce intestinal inflammation by inhibiting the activation of the TLR4/NF-κB signaling pathway and reducing the expression levels of TLR4, MyD88, P-P65and P65 proteins in OVX rats.

Probiotics Can Improve the Blood-Brain Barrier and Neuronal Damage in the Hippocampus of OVX Rats

Studies have shown that gut microbes are involved in regulating the permeability of the blood-brain barrier (BBB).[28] In the present study, Western blot was used to detect the expression of tight-junction proteins ZO-1 and Occludin in the hippocampus of rats. As shown in Figure 6, compared with the Sham group, the expression levels of ZO-1 ($p<0.01$, Figure 6B and C, respectively) and Occludin ($p<0.05$, Figure 6B and D, respectively) in the hippocampus of OVX rats were significantly decreased. This indicated that estrogen deficiency can lead to hippocampal BBB damage. Estrogen or probiotic treatment significantly increased the expression levels of ZO-1 and Occludin in the hippocampus ($p<0.01$, $p<0.05$, Figure 6B–D,

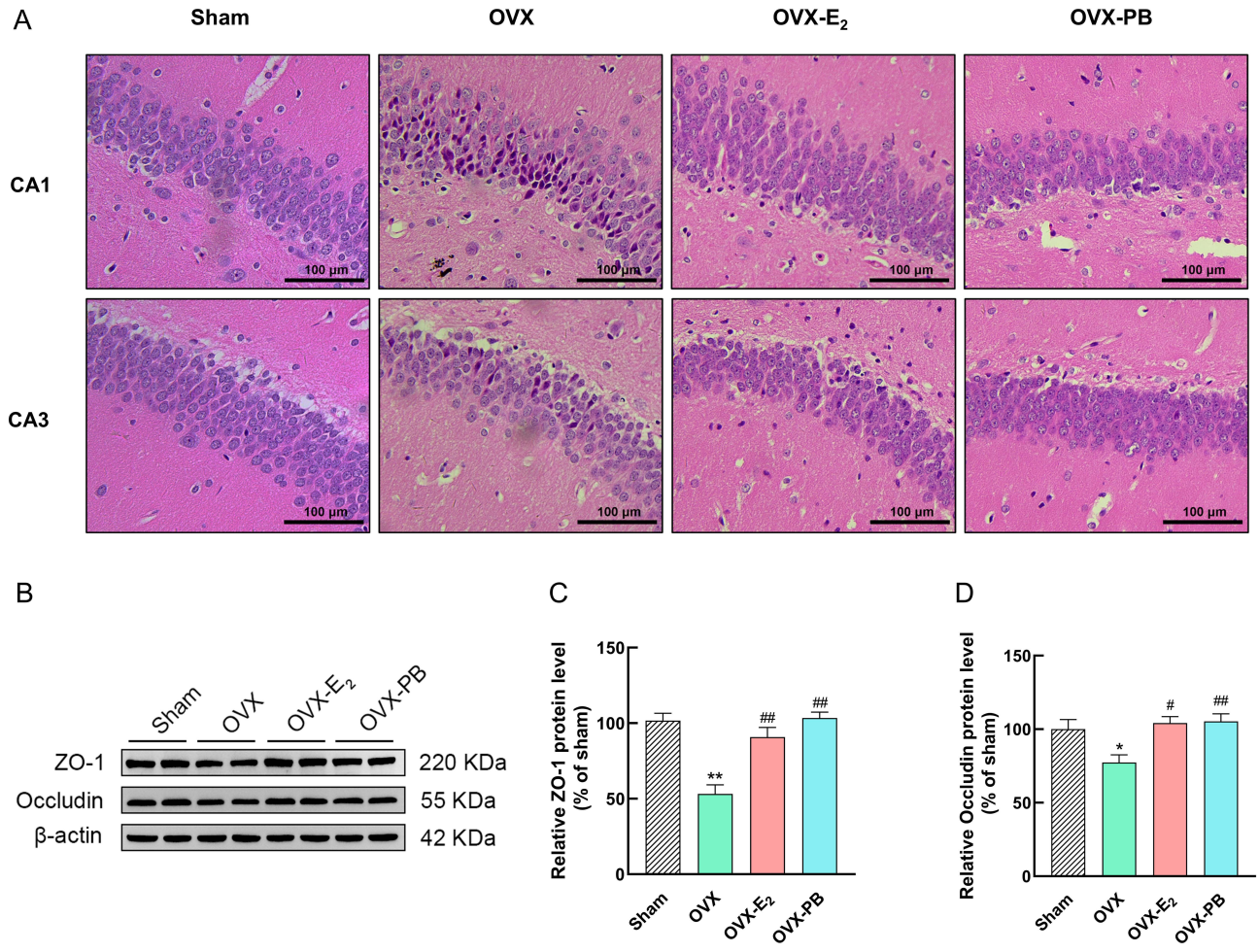

Figure 6 Assessment of the blood-brain barrier and neurons in the hippocampus of OVX rats. (**A**) HE staining. Magnification 20×. Scale bar = 100 μm. (**B**) Western blot protein bands; (**C**) ZO-1 protein level; (**D**) Occludin protein level. Data are presented as means ± SEM (n = 3 in each group). *$p<0.05$, **$p<0.01$ vs Sham, #$p<0.05$, ##$p<0.01$ vs OVX.

respectively). These results suggested that estrogen or probiotics can increase the expression level of tight-junction proteins in the hippocampal BBB, thereby effectively improving BBB damage in OVX rats.

Pyramidal neurons in the hippocampal CA1 and CA3 regions of the brain have been shown to be closely related to depression. The results of HE staining are shown in Figure 6A, pyramidal cells in the CA1 and CA3 regions of the hippocampus of rats in the Sham group were morphologically regular, with intact cells and clear nuclei. In the OVX group, the pyramidal cells in the hippocampal CA1 and CA3 regions were loose and disordered, the space around the cells was enlarged, the nucleus was pyknotic, and the cells were incomplete or even lost, indicating that ovariectomy can cause damage to the hippocampal brain area of rats. Compared with the OVX group, the phenomena such as disordered arrangement of pyramidal cells and increased intercellular spaces in the CA1 and CA3 regions of the hippocampus in the OVX-E_2 group and the OVX-PB group were effectively improved. These results suggested that estrogen or probiotics can effectively improve the damage to neurons in the hippocampus of OVX rats.

Probiotics Can Inhibit the Expression of NLRP3 Inflammasome-Related Protein in the Hippocampus of OVX Rats

Neuroinflammation is one of the most important risk factors for depression. Among them, the NLRP3 inflammasome plays an important role in depression. Western blot and ELISA were used to detect the expression of NLRP3 inflammasome-related proteins (NLRP3, ASC, Caspase-1, IL-1β and IL-18) and inflammatory factors (IL-6 and TNF-α) in the hippocampus.

Compared with the Sham group, the expression of NLRP3 ($p<0.01$, Figure 7A and B, respectively), ASC ($p<0.05$, Figure 7A and C, respectively), Caspase-1 ($p<0.01$, Figure 7A and D, respectively), IL-1β ($p<0.05$, Figure 7A and E, respectively) and IL-18 ($p<0.05$, Figure 7A and F, respectively) proteins in the hippocampus of the OVX group was significantly increased. These results indicated that estrogen deficiency can lead to activation of the NLRP3 inflammasome and up-regulation of the downstream inflammatory factors IL-1β and IL-18 in the hippocampus. After estrogen or probiotic therapy for 6 weeks, the protein expression levels of NLRP3, ASC, Caspase-1, IL-1β and IL-18 in the hippocampus of the OVX-E_2 and OVX-PB groups were significantly decreased ($p<0.01$, $p<0.05$, Figure 7A–F, respectively).

Compared with the Sham group, the expression levels of inflammatory factors IL-6 and TNF-α in the hippocampus of the OVX group were significantly increased ($p<0.01$, Figure 7G and H, respectively). After continuous treatment with estrogen or probiotics, the expression levels of IL-6 and TNF-α in the hippocampus of rats in the OVX-E_2 and OVX-PB groups were significantly lower than in the OVX group ($p<0.01$, Figure 7G and H, respectively).

These results suggested that estrogen or probiotics may reduce hippocampal inflammation by inhibiting the activation of the NLRP3 inflammasome and down-regulating the expression levels of IL-1β, IL-18, IL-6 and TNF-α, thereby improving the depression-like behavior induced by estrogen deficiency in rats.

Probiotics Can Inhibit the Activation of the TLR4/NF-κB Signaling Pathway in the Hippocampus of OVX Rats

TLR4 is an important factor in the development of neuroinflammation. TLR4 activation and its mediation of NLRP3 inflammasome activation are closely related to depression. As previously mentioned, probiotics were able to inhibit NLRP3 inflammasome activation in the hippocampus of OVX rats. Therefore, we hypothesized that probiotics could inhibit the activation of the TLR4/NF-κB signaling pathway upstream of the NLRP3 inflammasome in the hippocampus of OVX rats. To test this hypothesis, Western blot was used to further detect the expression levels of TLR4 and its downstream signaling pathway-related proteins (TLR4, MyD88, P-P65, P65) in the hippocampus of rats, and the ratio of P-P65/P65 protein expression was calculated.

Compared with the Sham group, the expression of TLR4/NF-κB signaling pathway-related proteins TLR4 ($p<0.01$, Figure 8A and B, respectively), MyD88 ($p<0.05$, Figure 8A and C, respectively), P-P65 ($p<0.01$, Figure 8A and D, respectively) and P65 ($p<0.01$, Figure 8A and E, respectively) protein in the hippocampus of the OVX group was significantly increased. The ratio of P-P65 /P65 protein expression also showed an upward trend ($p<0.05$, Figure 8A and F, respectively), indicating that estrogen deficiency could lead to activation of the TLR4/NF-κB signaling pathway in the hippocampus of OVX

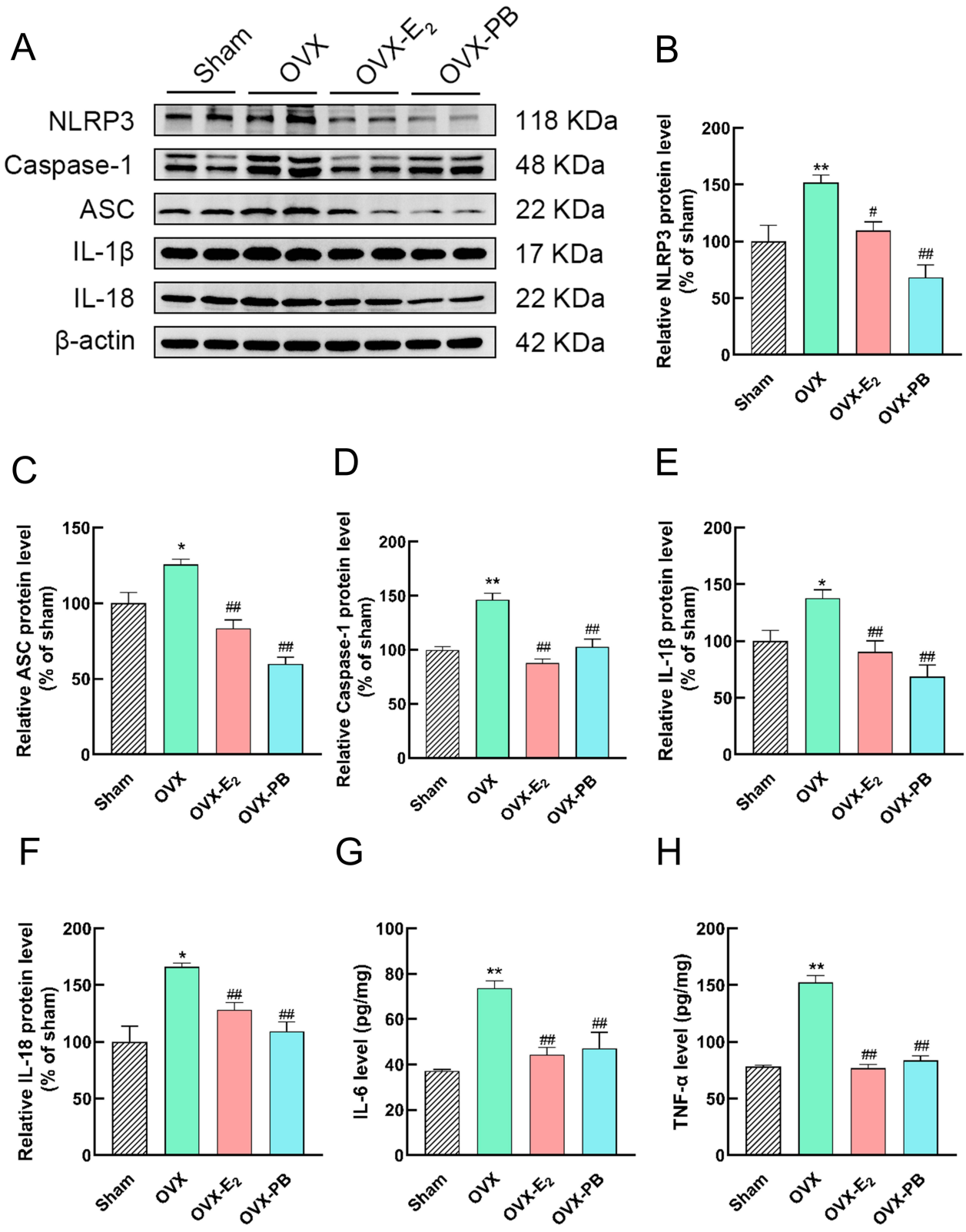

Figure 7 Assessment of NLRP3 inflammasome-related protein expression in the hippocampus of OVX rats. (**A**) Western blot protein bands; (**B**) NLRP3 protein level; (**C**) ASC protein level; (**D**) Caspase-1 protein level; (**E**) IL-1β protein level; (**F**) IL-18 protein level; (**G**) IL-6 protein level; (**H**) TNF-α protein level. Data are presented as means ± SEM (n = 3 in each group). **p*<0.05, ***p*<0.01 vs Sham, #*p*<0.05, ##*p*<0.01 vs OVX.

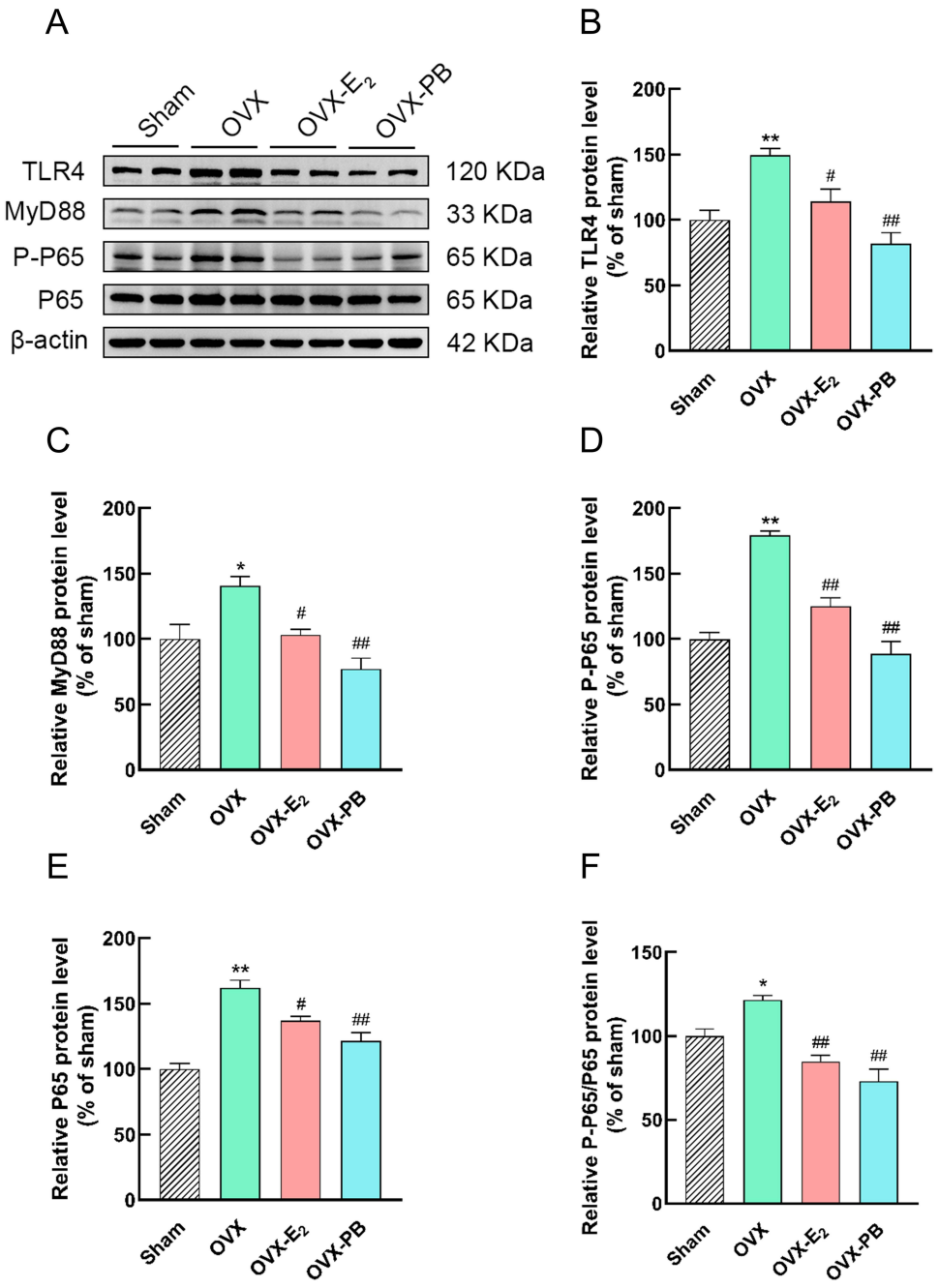

Figure 8 Assessment of the TLR4/NF-κB signaling pathway in the hippocampus of OVX rats. (**A**) Western blot protein bands; (**B**) TLR4 protein level; (**C**) MyD88 protein level; (**D**) P-P65 protein level; (**E**) P65 protein level; (**F**) P-P65/P65 ratios. Data are presented as means ± SEM (n = 3 in each group). *p<0.05, **p<0.01 vs Sham, $^{\#}p$<0.05, $^{\#\#}p$<0.01 vs OVX.

rats and trigger neuroinflammation. The results after estrogen or probiotics treatment are shown in Figure 8. Compared with the OVX group, the expression levels of TLR4, MyD88, P-P65 and P65 proteins in the hippocampus of the OVX-E_2 and OVX-PB groups were significantly decreased (p<0.01, p<0.05, Figure 8A–E, respectively). The ratio of P-P65/P65 protein expression also showed a downward trend (p<0.01, Figure 8A and F, respectively). These results suggested that estrogen or probiotics may inhibit the activation of the TLR4/NF-κB signaling pathway in the hippocampus of OVX rats and reduce the expression levels of TLR4, MyD88, P-P65 and P65 proteins, thereby reducing inflammation in the hippocampus.

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Discussion

Estrogen deficiency is a key risk factor for depression.[6] At the same time, low estrogen level can lead to gut microbiota disorder and NLRP3 inflammasome activation.[5,6,19,29] In recent years, it has been found that the MGB axis plays an important role in depression. Probiotics have been reported to improve severe depression and intestinal dysfunction.[30] The results of the present study show that ovariectomy leads to increased depression- and anxiety-like behaviors in rats. In addition, gut microbiota disorder and gut inflammation in OVX rats were observed, which triggered NLRP3 inflammasome-mediated neuroinflammation in the hippocampus. However, after treatment with probiotics Bifico, the depression- and anxiety-like behaviors of OVX rats were significantly improved, and the mechanism may be the inhibition of NLRP3 inflammasome-mediated neuroinflammation in the hippocampus through the MGB axis. These results suggest that the NLRP3 inflammasome mediated by the MGB axis may play a key role in the development of depression- and anxiety-like behaviors induced by estrogen deficiency.

Compared with healthy normal people, the gut microbiota of patients with depression is disordered, and the common feature is an increase in pro-inflammatory bacteria and a decrease in anti-inflammatory bacteria.[7,31–33] Our results showed that the abundance of *Lactobacillus sp., Barnesella* and *Desulfovibrio* in the intestine of OVX rats decreased, while that of *Porphyromonadaceae* increased, indicating that the depression- and anxiety-like behaviors caused by estrogen deficiency in rats were related to the disorder of gut microbiota. However, after probiotic treatment, the abundance of *Lactobacillus sp*. and *Desulfovibrio* increased in the guts of OVX rats, while that of *Porphyromonadaceae* and *Barnesella* decreased. Animal experiments and clinical studies have shown that *Lactobacillus* can reduce inflammatory response and improve anxiety and depression symptoms.[34–39] In addition, increased abundance of *Porphyromonadaceae* has been shown to cause brain inflammation in animals, leading to central nervous system (CNS) diseases.[40,41] The above findings are consistent with our experimental results. However, the changes in *Barnesella* and *Desulfovibrio* were less consistent with previous studies. For example, changes in the abundance of *Barnesella* species are different in IBD animal models and clinical studies of depression.[42] In addition, as a group of Gram-negative anaerobic bacteria that reduce sulfate and produce endogenous hydrogen sulfide (H_2S), the increase of H_2S can lead to inflammatory colitis and depression.[43] However, relevant studies have shown that H_2S has anti-inflammatory, antidepressant and anti-anxiety effects, and its mechanism of action may be related to H_2S-dependent regulation of N-methyl-D-aspartic acid receptor activity.[44] The above studies further indicated that there is no clear boundary between beneficial and harmful bacteria, and it is more important to focus on the balance of gut microbiota than on a single class of bacteria. In addition, the differences between the above studies may also be related to factors such as diet, environment, drug intervention time and dynamic changes of gut microbiota. At present, the functions of *Porphyromonadaceae, Barnesella* and *Desulfovibrio* are not clear, and whether they play an important role in the occurrence and development of PMD needs to be further explored. Our results suggest that probiotics can improve the depression- and anxiety-like behaviors of OVX rats by increasing the abundance of anti-inflammatory bacteria in gut microbiota and maintaining the balance of gut microbiota.

Recent studies have shown that the imbalance of intestinal flora can cause their metabolites and microbial cell components (eg, LPS and others) to pass through the damaged intestinal barrier (intestinal leakage), and then increase the levels of inflammatory factors such as IL-6, TNF-α and IL-1β, thus aggravating the systemic inflammatory response.[7,45,46] Importantly, tight junctions play a key role in the regulation of the intestinal mucosal barrier.[47] Animal experiments have shown that *Lacticaseibacillus rhamnosus* Fmb14 can improve colonic barrier damage and inhibit inflammatory response by increasing the expression levels of colonic tight-junction proteins ZO-1 and Occludin, thus reducing depression-like behavior in mice.[48] Our study also found that probiotics could improve the intestinal mucosal barrier damage by increasing the expression of ZO-1 and Occludin in the colon of OVX rats. These results suggest that the decreased expression of tight-junction proteins can cause intestinal barrier damage in OVX rats and may further trigger inflammatory response. Inflammatory response is important for the pathogenesis of depression,[4,49–51] in which the NLRP3 inflammasome plays a key role. The latest evidence shows that gut microbiota promotes the production of proinflammatory cytokines such as IL-1β by activating the NLRP3 inflammasome, leading to acute pancreatitis, colitis and depression.[52] In addition, TLR4 is a key sensor of intestinal microbial changes, and activation of the TLR4/NF-κB

signaling pathway promotes the activation of the NLRP3 inflammasome. In the present study, our results showed that probiotics could inhibit the activation of the TLR4/NF-κB/NLRP3 signaling pathway in the colon, reduce the expression levels of IL-1β and IL-18 in the colon and as well as IL-6 and TNF-α in serum, and reduce intestinal inflammation and systemic inflammation in OVX rats, thereby exerting antidepressant and anxiolytic effects. This is consistent with the results of previous clinical and animal studies.[28,52–54] For example, clinical studies have shown that decreased estrogen can increase the expression levels of inflammatory factors such as IL-6 and TNF-α in the serum of women with PMD,[53,54] and the increase of TNF-α may be related to the enhanced expression of membrane-bound TNF-α on CD14 monocytes.[53] Animal experiments showed that chronic unpredictable mild stress (CUMS) induced intestinal flora disorder in rats, increased expression levels of the NLRP3 inflammasome, IL-1β and IL-18 inflammatory factors in the colon, and then triggered neuroinflammation, leading to depression-like behavior in animals.[52] This suggests that inflammation is an important hub between gut microbiota and depression. Probiotics may reduce intestinal and systemic inflammation by inhibiting the activation of the NLRP3 inflammasome in the gut, and further affect the physiological activities of the brain, thereby improving the depression-like behavior and anxiety of OVX rats.

Further studies have found that the damage to intestinal epithelial cells leads to the migration of commensal bacteria into the blood, and the translocation of proinflammatory molecules through the intestinal barrier leads to a low-grade systemic inflammatory response, which in turn increases the permeability of the BBB,[55] leading to the recruitment of immune cells to the CNS, and ultimately causing neuroinflammation.[56] Increased permeability of the BBB has been linked to diseases of the CNS, such as Alzheimer's disease and depression.[57,58] Clinical studies have found increased bacterial populations in the brain tissue of patients with Alzheimer's disease, suggesting that microbial invasion of the CNS may significantly contribute to disease progression.[57] Accumulating evidence indicates gut microbiota and their metabolites SCFAs (propionate, butyrate) influence BBB permeability and tight-junction proteins expression.[59–64] Animal experiments showed that both broad-spectrum antibiotic-treated and germ-free (GF) mice have increased BBB permeability and dysregulation of endothelial-cell tight junctions.[60,61,65] For example: Braniste et al found that GF mice had increased blood-brain barrier permeability in different regions of the brain (including frontal cortex, hippocampus, and striatum) compared with mice with normal gut microbiota in specific pathogen free animals, and reduced the expression of the tight-junction proteins Occludin and Claudin-5.[60] However, GF mice receiving fecal microbiota transplantation from specific pathogen free mice or oral administration of sodium butyrate showed increased tight-junction proteins expression and restoration of BBB integrity. Clinical studies have shown that probiotics can reduce the levels of inflammatory factors and improve BBB function in patients with Alzheimer's disease.[66] Our study also found that probiotics could improve the BBB permeability by increasing the expression of tight-junction proteins Occludin and ZO-1 in the hippocampus of OVX rats. Notably, the BBB and the gut barrier share significant similarities at the cellular and molecular levels, making them susceptible to modulation by common signals, including those originating from gut microbiota.[63] These results suggest that gut microbiota may protect the hippocampal BBB by up-regulating the expression of tight-junction proteins in OVX rats, thereby improving the depression-like behavior of OVX rats.

In addition, the hippocampus as an important part of the limbic system, and NLRP3 inflammasome activation in the hippocampus has been confirmed to be associated with depression- and anxiety-like behaviors induced by estrogen deficiency in animals.[21,67] However, *IL-1β, IL-18, Caspase-1* and *NLRP3* inflammasome gene knockout can improve the depression-like behavior of mice.[17,68] The results of the present study showed that probiotics inhibited activation of the TLR4/NF-κB/NLRP3 signaling pathway in the brain hippocampus, down-regulating the protein expression levels of TLR4, MyD88, P-P65, P65, NLRP3, ASC, Caspase-1, IL-1β, IL-18, TNF-α and IL-6, thus exerting antidepressant and anxiolytic effects. Our experimental results are consistent with the previous results of our research group and that of others.[6,52,69] For example, Huang et al showed that after fecal transplantation, the expression levels of the NLRP3 inflammasome, IL-18 and IL-1β in the brain of CUMS rats decreased, and the depressive state of rats was significantly improved.[52] In addition, some researchers have found that *Prevotella histicola* (*P. histicola)* plays an antidepressant role by maintaining intestinal microecological balance and reducing hippocampal inflammation in OVX mice through the TLR4/MyD88/JNK/MAPK signaling pathway.[6] These results suggest that probiotics may inhibit the activation of the NLRP3 inflammasome in the brain and hippocampus through the MGB axis, thereby improving the depression- and anxiety-like behaviors of OVX rats.

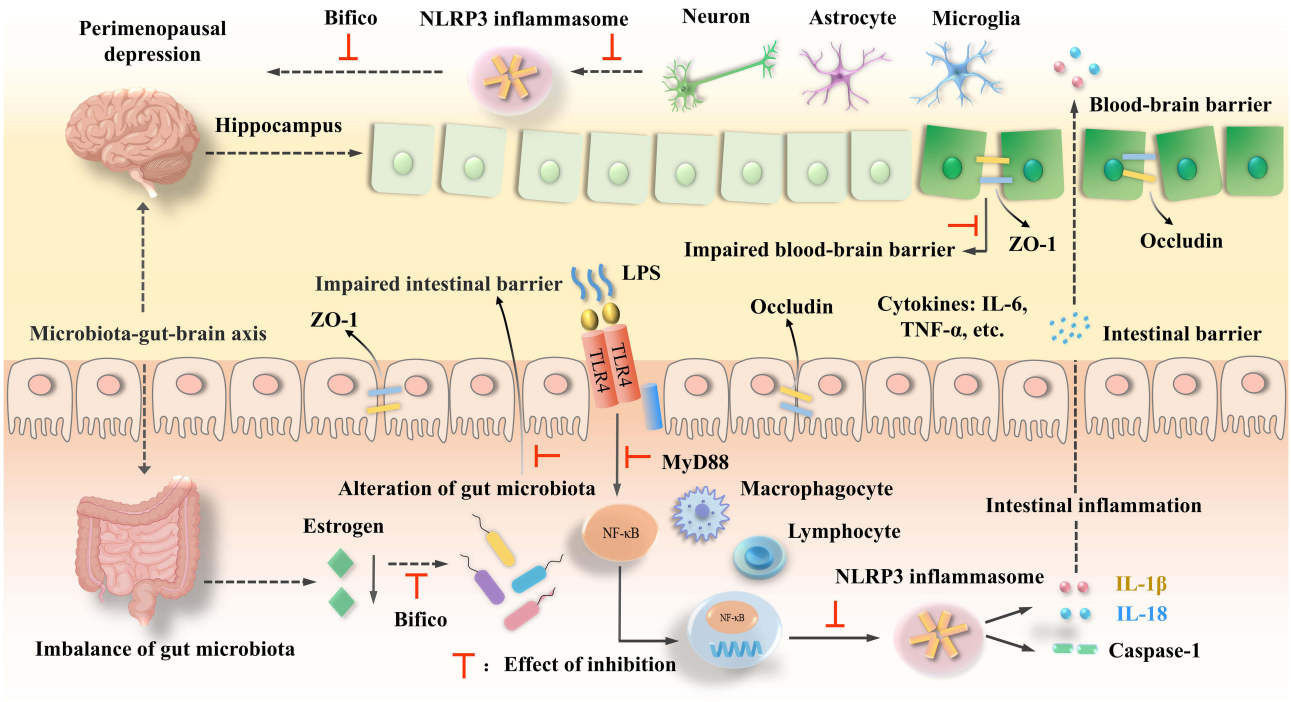

Figure 9 Mechanism of the NLRP3 inflammasome mediated by the MGB axis in PMD. Estrogen deficiency disrupts gut microbiota, which in turn disrupts the gut barrier and leads to the release of harmful substances (eg, LPS). TLR4 in intestinal epithelial cells and immune cells recognizes LPS and activates NF-κB and the NLRP3 inflammasome via MyD88 to promote Caspase-1 maturation and release of inflammatory factors, triggering intestinal and peripheral inflammation. Inflammation may increase the permeability of the hippocampal blood-brain barrier through the MGB axis, leading to activation of the NLRP3 inflammasome in the hippocampus, and finally triggering PMD. Probiotic Bifico may reverse the above phenomenon by maintaining intestinal microecological balance, thereby improving PMD.

There are some limitations of our study. First, we only observed the treatment effect after 6 weeks of probiotic intervention, and we did not examine longer periods of probiotic intervention, so we cannot know its effect and duration. Secondly, intestinal microecology is in a dynamic process of change, and the effect of changes in gut microbiota on depression-like behavior of OVX rats was not dynamically monitored in our study, so we cannot rule out the possibility of intestinal immune system changes.

In summary, this study found that estrogen deficiency causes gut microbiota disorder and activation of the NLRP3 inflammasome signaling pathway, which in turn causes gut inflammation and peripheral inflammation, and activates the NLRP3 inflammasome signaling pathway in the hippocampus through the MGB axis to trigger neuroinflammation, eventually leading to depression- and anxiety-like behaviors in OVX rats. Probiotic intervention could reverse this phenomenon (Figure 9). This study is the first to demonstrate that the probiotic Bifico improves estrogen deficiency-induced depression- and anxiety-like behaviors by inhibiting NLRP3 inflammasome. The results of the present study suggest that the NLRP3 inflammasome mediated by the MGB axis may be an important target for the treatment of affective disorders associated with estrogen deficiency. These findings provide an important reference for the development of novel MGB axis-based therapeutic strategies against PMD and provide insights into future research directions.

Abbreviations

ASC, apoptosis-associated speck-like protein containing a CARD; BBB, blood-brain barrier; CNS, central nervous system; CUMS, chronic unpredictable mild stress; E_2, estradiol; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; EPM, elevated plus-maze; FST, forced swimming test; GF, germ-free; HE, hematoxylin-eosin; H_2S, hydrogen sulfide; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-18, interleukin-18; LPS, lipopolysaccharide; MDD, major depressive disorder; MGB, microbiota-gut-brain; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear transcription factor-κB; NLRP3, NOD-like receptor protein 3; NMDA, N-methyl-

D-aspartic acid; OFT, open-field test; OVX, ovariectomized; PCR, Polymerase chain reaction; PMD, perimenopausal depression; SD, Sprague Dawley; SPT, sucrose preference test; TLR4, toll-like receptor 4; ZO-1, zona occludens 1.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare that they have no conflicts of interest in this work.

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