Probiotics alleviate depression-like behavior in mother-infant separation stress rats

Jinling Li[1], Lingyu LI[1], Wudian Xiao[2], Yong Tang[2], Wenbin Dong[1], Xiaoyu Liao[1], Wen Luo[1], Na Li[1], Yaxin Wang[1], Qing Lei[1], Guifang She[1], Peiyang Du[3], Xiaoping Lei[1], and Hongyan Sun[1]

[1]The Affiliated Hospital of Southwest Medical University
[2]Southwest Medical University
[3]Panzhihua University

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Abstract

Objective: To observe the changes of depression-like behavior in SD rats induced by maternal separation (MS) stress and to explore the effects of probiotics on antidepressant-like behavior and cAMP/CREB signaling pathway. Methods: Newborn SD rats were selected as experimental subjects and divided into MS+NS group, CON+NS group, MS+P group and CON+P group using random number table method, 12 rats in each group. At PND 22-49, the MS+P and CON+P groups were given 1×109 CFU (0.1 ml) of probiotic colonies by gavage daily and the corresponding dose (1 ml/100g) according to the change of body weight, and the MS+NS and CON+NS groups were given the corresponding saline dose (1 ml/100g). Behavioral tests were performed at PND50-56, and rats were executed at PND57 for laboratory tests. Results: FST increased, OPT increased and SPT decreased after probiotic intervention. It was suggested that the MS-induced depression-like behavior was improved to some extent. Compared with the model group, probiotic intervention increased the number of neurons in the CA1 region of the hippocampus, decreased serum-associated inflammatory factors, increased serum 5-HT concentration, and decreased CORT concentration in rats. In addition, the intervention increased the expression levels of cAMP, CREB and BDNF in the hippocampus of MS rats. Conclusion: Probiotics can alleviate anxiety/depression-like behavior in SD rats, which may be related to the activation of cAMP/CREB signaling pathway. The protective effect of probiotics as therapeutic food care in preventing or alleviating MS-induced depression-like behaviors provides an experimental basis for the application of probiotics to alleviate or improve anxiety/depression.

Probiotics alleviate depression-like behavior in mother-infant separation stress rats

Jinling Li[1,2], Lingyu Li[1,2], Wudian Xiao[3], Yong Tang[4], Dong Wen Bin[5], Xiaoyu Liao[1,2], Wen Luo[1,2], Na Li[1,2], Yaxin Wang[1,2], Qing Lei[1,2], Guifang She[1,2], Peiyang Du[6], Xiaoping Lei[5*] and Hongyan Sun[1,2,5*]

1. Department of Nursing, Affiliated Hospital of Southwest Medical University

2. School of Nursing, Southwest Medical University

3.Laboratory Animal Center, Department of Science and Technology, Southwest Medical University

4.Department of Science and Technology, Southwest Medical University

5.Department of Newborn Medicine, Affiliated Hospital of Southwest Medical University

6. Medical College of Panzhihua University

Abstract

Objective: To observe the changes of depression-like behavior in SD rats caused by Maternal Separation (MS) stress, and to explore the effects of probiotics on antidepressant-like behavior and on cAMP/CREB signaling pathway.

Methods: Newborn SD rats were selected as experimental subjects and divided into MS+NS group (mother-infant isolation + placebo group), CON+NS group (control + placebo group), MS+P group (mother-infant isolation + probiotic group), and CON+P group (control + probiotic group) by random number table method, 12 rats in each group. At PND22-49, probiotic colonies of 1×109 CFU (0.1 ml) were given by gavage daily in the MS+P and CON+P groups and the corresponding dose (1 ml/100g) was given according to the daily body weight change, and the corresponding saline dose (1 ml/100g) was given by gavage daily in the MS+NS and CON+NS groups according to the body weight change. The behavioral performance of the offspring rats was examined by sugar-water preference test, open field test and forced swimming test at PND50-56, and the rats were executed at PND57, blood was collected to isolate the serum, and brain tissue was taken for laboratory tests.

Results: After probiotic intervention, FST increased, OPT increased and SPT decreased. probiotics both improved maternal-infant separation-induced weight loss and depression-like behavior to some extent.Compared with the model group, the probiotic intervention resulted in an increase in the number of neurons in the CA1 region of the hippocampus, an improvement in morphological structure, a decrease in serum-related inflammatory factors, and an increase in serum 5-HT concentration and a decrease in CORT concentration in rats. In addition, the probiotic intervention significantly increased the expression levels of cAMP, CREB and BDNF in the hippocampus of mother-infant isolated rats.

Conclusion: Probiotics alleviate anxiety/depression-like behavior in SD rats, which may be associated with activation of the cAMP/CREB signaling pathway. The protective effect of probiotics, a therapeutic food care,on their prevention or alleviation of MS-induced depression-like behaviors provides an experimental basis for the application of probiotics to alleviate or ameliorate anxiety/depression.

Keywords: Maternal separation; depression; Brain; cAMP/CREB signaling pathway; Probiotics

Introduction

Depression is a growing health problem. It is a mental disorder with significant depressed mood, reduced mobility, and slowed thinking as the main symptoms[1, 2]. With the development of social economy and changes in social environment, mental disorders such as depression show a rising trend. According to the World Health Organization, more than 32 million people worldwide suffer from depression each year, Depression is expected to be one of the public health problems plaguing the world by 2030[3]. In China, there are more than 95 million people with depression, of which children and adolescents account for about 20%[4]. Currently, it is widely believed that depression is common in children and adolescents, but depressive symptoms in children and adolescents are not always similar to adult depression and can have some adverse consequences if left unrecognized and untreated.

That mother-infant separation is a model of early life stress and that this model of early life stress disrupts neurological development.To a large extent, this causes behavioral deficits, neuronal morphological damage, triggers dendrites to appear atrophy-like, reduces inter-synaptic connectivity, and ultimately leads to depressive-like behavior[5]。Mejia has confirmed that mother-infant separation can have some negative effects on the hippocampus of rats, which may lead to disruption of neurological development and even impairment of cognitive and emotional functions[6]. Petanjek has shown that the number of excitatory synapses in the brain during infancy and childhood has far exceeded that of adults by a factor of 2-3 and is the period when the brain is most sensitive to external environmental stimuli[7]. More than 60% of left-behind children have some psychological problems[8], mainly associated with the lack of maternal love and neglect during infancy or childhood[9]. This prolonged separation from the mother, or the presence of some disharmonious and unintimate parent-child relationship (parent-child conflict, poor parenting practices, abuse or neglect, etc.), causes children to have adjustment difficulties and increases the occurrence of depressive-like behaviors[10, 11]. These undesirable behavioral or psychological problems are key factors that induce a variety

of emotional dysfunctions in children such as depression and anxiety.This shows a close correlation between psychiatric disorders and pathological lesions or environmentaldamage to the brain during development, which is consistent with the study of Marín et al[12].

Since existing antidepressants generally have low cure rates, high relapse rates, and many side effects (gastrointestinal discomfort such as diarrhea and vomiting), and other disadvantages, Long-term use will bring financial and psychological burden and stress to families and individuals[13], It is important to develop complementary products that have a preventive effect and mitigate side effects, among which the use of probiotics may be an effective means of combating depression-like behavio[14]. It has been shown that lactobacilli can maintain intestinal flora homeostasis, produce beneficial immunomodulation, and relieve depression and anxiety in the host[15]. It hasbeen shown that the use of Bifidobacterium spp. can produce beneficial metabolites through the intestine, including: serotonin, histamine, and gamma-aminobutyric acid, improving depression-like behavior,social behavior[16]. It has been shown that Bifidobacterium shortumcan reduce blood creatinine and cortisol concentrations in the serum of mice in a chronic unpredictable stress model, restore normal function of the HPA axis, and improve depression[17]. Sun showed that Lactobacillus plantarum can alleviate anxiety and depression due to chronic stress[11]. Hazuki showed that after oral administration of Lactobacillus swiss, mice in a depression model improved social interaction behavior after gene expression in the vomeronasal nucleus, suggesting that this strain has an important role in relieving stressful emotions[18]. It is thus clear that probiotics, as active microorganisms, can interact positively with the host to alleviate mood disordersand prevent stress-induced changes in the intestinal flora, thus conferring health benefits to the host.

The pathogenesis of depression is unclear, with the main monoaminergic neurotransmission hypothesis[19]、Change in intestinal flora[20]、Immunoinflammatory response[21]、Hypothalamic-pituitary-adrenal[22]. However, more researchers are inclined to study antidepressant signaling pathways, analyze the association between their related signaling pathways, proteins, genes, etc., and actively search for potential, antidepressant-related mechanisms[13, 23]. Among them, the cAMP/CREB signaling pathway is regarded as an important player and regulator of depression pathogenesis, which can regulate synaptic plasticity, cytoplasmic division, transcription, and modulation of the HPAaxis[24, 25].cAMP is the most classical second messenger, a key factor in cell signaling, involved in the onset, development and regression of organismal physiopathology[24]. In the central nervous system, inhibition of the cAMP/PKA signaling pathway affects neuronal excitability, synaptic activity, and ultimately leads to the pathological outcome of depression[24]. In the presence of memory impairment, neurological disorders, addictive or emotional dysfunction, CREB-mediated transcription may be impaired, blocking downstream signaling pathways[26]. Inhibition of the cAMP/CREB signaling pathway affects neuronal excitation and synaptic plasticity, while activation of the cAMP/CREB signaling pathway will provide antidepressant effects[27]. Studies have shown that the pathogenesis of depression is related tothe fact that some antidepressants activate the PKA/CREB signaling pathway, which regulates the active expression of BDNF[28]. Therefore, this experiment intends to investigate and study the probioticsfrom cAMP/CREB signaling pathway to improve the depression-like behavior of mother-infant separated offspring rats.

2. Materials and Methods

2.1. Animals

Thirty-six newborn SD rats were selected. All neonatal rats were randomly assigned to three groups (12 SD rats in each group), That is, 1 control group and 2 mother-infant separation groups. Onegroup of mother-infant separated group was given normal drinking water and the other group of mother-infant separated group was given normal drinking water and probiotics, Provided by Laboratory Animal Center, Southwest Medical University, China. The final concentration of probiotics in drinking water was10^9 CFU/mL. The animal room is well ventilated, the environment is at 22-24℃ constant temperature, 55%-60% constant humidity, 12h/12h light and dark cycle, regular feeding, free diet. The date of completion of delivery of the female rat is 0 days after birth. In PND2-21, the offspring rats participating in the mother-infant separation were separated from their corresponding mothers for 3 hours per day (9:00-12:00), the offspring pups of

the mother-infant separation group were transferred toa new cage each day and returned to the original cage after 3 hours for 21 days. to establish a mother-infant separation model. The experimental animals used in this study were conducted in strict accordance with the standards of the Ethical Review of Animal Experiments at Southwest Medical University.

2.2. Behavioral Assessments

The SD rats were transferred to the experimental room 30 minutes before the formal testing in order to get familiar with the environment, and at the end of the experiment, the SD rats were put back into the cage and transferred to the breeding room. Behavioral tests were performed using the sugar water preference test, the open field test, and the forced swim test.

2.2.1 Sugar water preference test

Pleasure deficit is one of the main symptoms of depression, and we generally assess the symptoms of pleasure deficit in SD rats using sugar-water preference experiments[29]. Prior to the assay, the animals were trained in PND50 in a quiet environment to adapt to water containing sucrose, while two bottles of water containing 1% sucrose were placed in each cage; After 24 h, one bottle with 1% sucrose water and one bottle with distilled water were replaced. In order to avoid site preference of SD rats, the location of the sucrose water and distilled water bottles needed to be changed at 12 h intervals during the experiment; PND52 was given to SD rats with food and water fasting for 24h; The test was performed in PND53 by giving 1% sucrose water and distilled water, and the water bottle was removed after 2h. The intake of sucrose water and distilled water in 2h was weighed and recorded, and the sugar water preference index was calculated. Sugar water preference rate = sugar water consumption / (sucrose water consumption + distilled water consumption) × 100%.

2.2.2 Open field experiments

The open-field test, also known as the open-box test, is a classic method for detecting spontaneous activity, exploration, and anxiety-like behavior in mice and rats [30]. The device is composed of a 100cm x 100 cm x 100 cm open observation box with black surrounding walls and bottom. The experiment was conducted in PND54, and the experimenter grasped the SD rats' tails near the root third in a quiet environment and put them into the center of the open field. Using the video tracking system, the movement changes of each SD rat in 5 min were automatically recorded, including the totaldistance, the active distance in the central zone, and the active timein the central zone. Data analysis was performed by behavioral video software. Each SD rat was tested only once, and each SD rat was required to clean up the secretion in the field after the test was completed, and the alcohol wipe was cleaned to prevent the residual odor from affecting the action trajectory of the next SD rat.

2.2.3 Forced swimming test

Forced swimming experiment is an experiment to assess depression-like behavior in experimental animals[31]. In PND55 for acclimatization training, in a quiet environment the experimenter graspedthe SD rats by the tail near the root third, and put the SD rats into a transparent tall bottle of 50 cm height, 25 cm caliber, and 23-25°C water temperature, with the water depth of the SD rats just touching the bottom at the tip of the tail; after 15 min remove the rats, wipe them with a towel and then dry the hair of the SD rats with a hair dryer and put PND56 was used for formal testing, and the rest of the conditions were the same as before. Cell phone video recording of each SD rat swimming for 6 min, the main statistics in the test after 4 min SD rats stationary time, each SD rat test once, after the test need to change the water in the tall cylinder bottle.

2.3 Nissler staining

The fixed brain tissue was removed and placed in an embedding box, washed under running water and placed in 75% alcohol overnight. On day 2, they were dehydrated sequentially in different concentrations of alcohol, 85%-95%I-95%II-100%I-100%II, respectively. After transparency, paraffin embedding was performed.

After embedding, continuous sections were made by microtome, andthe section thickness was 4 μm. After conventional dewaxing, Nissl staining was performed with methyl violet staining solution, and conventional dehydration and sealing were performed after differentiation. The morphology of Nissl vesicles in the CA1 region of hippocampus was observed and photographed by light microscopy, and thenumber was counted.

2.4 Enzyme-linked immunosorbent assays

Blood was collected from the abdominal aorta, centrifuged at 3400 rpm for 20 min, and the supernatant was collected and stored at -80°C for further analysis. The levels of 5-HT, CORT, interleukin-1β, IL-6, and TNF-α were measured using a commercial ELISA kit (ab133053, Abcam, Cambridge, UK). Briefly, different concentrations of standard solutions and sample solutions were added to the ELISA plate. All ELISA measurements were performed in two replicates.

2.5 Immunohistochemistry

(1) Dewaxing of paraffin sections to water: the fixed brain tissue was removed and soaked in a cup with formalin, and the liquid was changed every 10 min, keeping the temperature at 37°C for 20minduring the process. alcohol dehydration was performed accordingto the gradient type: the sections were placed in xylene I, II andIIIin turn for 15 min each, anhydrous alcohol I and II for 5 mineach, 85% alcohol, the time takes 5min, 75% alcohol, time takes 5min, followed by distilled water to wash.

(2) Antigen repair: Place the sections in a box with buffer, heat for10 min, leave for 8 min, heat for 10 min, then leave until cool, drench the sections with buffer, hold for 5 min, and wash 3 times repeatedly.

(3) Blocking: put the section into a cup with hydrogen peroxide at room temperature, soak for 10 min, remove and dry the excess water, then rinse with PBS buffer 3 times, each time lasting 3 min.

(4) Closure: add closure solution dropwise and wait for 30min.?

(5) Dropwise addition of primary antibody: Drain excess solution from the sections, add primary antibody, and then place them in an incubation box overnight in a 4degC refrigerator.

(6) Dropwise addition of secondary antibody: Take out the box containing the sections from the refrigerator, put the sections on the slide stand, wait for rewarming, then wash with PBS for 3 min and repeat 3 times. Then put the sections back into the incubation box, add secondary antibody dropwise, cover the sections, and put them into a thermostat with 37 for 0.5h incubation time.

(7) DAB color development: take out the sections from the thermostat, wash them with PBS for 3 min, and repeat 3 times. Reconfigure the DAB solution, need to add the configured solution drop by drop on the tissue for color development, microscopic view and pay attention to the length of color development, if the appearance of the brownish yellow, considered positive, and finally wash with distilled water and stop color development.

(8) Re-staining cell nuclei: use hematoxylin staining for 3 min, rinse with water, and continue to rinse with running water after returning to blue.

(9) Dehydration sealing: The slices were placed in 75%, 85%, 95%,99.5% alcohol and xylene, respectively, and kept for 10 min, and sealed using neutral resin.

2.6 Protein Extraction and Western Blot

(1) Protein sample preparation ??

Protein extraction: take out the seahorse tissue sample, add the sample, 3mm steel beads and lysate in 2ml grinding tube in order (according to the size of the sample ratio: lysate = 1:10), debug the equipment, grind using the grinder, keep the temperature of the grinder at -20, grind 4 times for a total of 4min; take out from the grinder, put it into 4 refrigerator, wait for 30min and then take out and put it into the centrifuge instrument machine (4, 12000rpm centrifugation, 10min); after the operation, aspirate the upper layer, and then use the kit to measure the protein concentration of this sample.

Protein quantification (BCA method): Configure the protein standard solution according to the instruction, the amount is 1.2ml, adsorb it into the protein standard (30mg BSA) tube, shake it several times to make it homogeneous so that the final standard solution is 25mg/mL. Take the appropriate amount of standard solution and dilute it into 0.5mg/mL of protein solution. Add the protein standard to the 96-well plate and fill it with ultrapure water if it is less than 20ul. Prepare the appropriate amount of solution according to the size of the sample and in the ratio of 50:1 and mix thoroughly;add 200 μL of BCA solution to each well; shake and mix well, then place at 37°C for 0.5 h. Set well 0 as the control and measure the OD of the sample protein wavelength at 562 nm; according to the OD value, find out the protein content corresponding to it(μg)。

Sampling: Take 50μL amount of each group, add 5×Loding buffer according to certain ratio, shake well, set the temperature of thermal cycling machine to 95 for 15min, wait for cooling after the end, then centrifuge and sample, and finally store in -20 refrigerator for easy access.

(2) Glue making

Rinse the glass plate with water to keep it clean, then prepare the sample comb, glass plate, and glue filling equipment and other utensils, add ddH2O to observe whether there is water leakage, and use filter paper to save the water on the glass plate dry.

Mix TEMED and APS immediately, inject them into the glue-making glass plate, remove the air bubbles, and wait for some time to make sure the lower layer of glue is solidified.

Take equal volume of upper layer glue solution and upper layer glue buffer, stir well with pipette, add a certain proportion of coagulant solution and mix thoroughly again; slowly and smoothly beat into the glass plate of glue making and insert the comb teeth; after waiting for a period of time, after the upper layer glue solidifies, you can pull out the comb carefully.

(3) Sample loading, electrophoresis

When the upper layer of glue solidifies, hold both sides of the comb with both hands slowly and gently pull it upward, and then wait for 10min in the middle, while fixing the glass plate into the electrophoresis tank, and pour fresh buffer in the middle of the glass plate, and pour the old electrophoresis buffer in the surroundings of the tank, the solution should be on the marked line, and start to put on the sample.

Calculate the amount of sample to be added. The glass plate is fixed in the electrophoresis tank, add the appropriate amount of electrophoresis solution in the tank, and add the required protein samples of each group and Marker in order according to the grouping (during the process of adding samples, try to go faster so as not to spread the samples) of the loading solution, the samples need to wait for rewarming when they are taken out from the refrigerator,then stir well with the gun, and add samples in order with the small gun, when adding samples, it is necessary to replace the new gun to avoid cross-contamination.

Connect the power supply, keep the voltage at 100V for 15min. After the Marker dye runs to the separation gel, set the voltage to 180V and continue electrophoresis until the target strip is completely run, then you can turn off the power supply and stop the electrophoresis operation.

(4) Transfer film

According to the size of the separation gel, assemble the sponge, filter paper, protein gel, PVDF membrane, filter paper, and sponge in this order, in which the air bubbles on the surface need to be excluded. Then put it into the membrane transfer solution.

Subsequently, the membrane transfer apparatus is placed in an ice bath, connected to positive and negative terminals, and the power is turned on at 200mA for 1-2h.

(5) Closure

Equipped with 5% skim milk solution first, when the transfer is finished, the PVDF membrane is removed and placed in the incubation box with milk solution, and then transferred to a shaker for 2h.

(6) Antibody hybridization

Incubation of primary antibody: PVDF membrane was cleaned and put into the incubation box and added primary antibody (primary antibody concentration: BDNF 1:2000; CAMP 1:2000; CREB11:2000; β-actin 1:50,000), put the box on a shaker at 4 for overnight; after that, it was washed 3 times with TBST, each time lasting 5min. secondary antibody incubation: put the PVDF membrane into the incubation box with secondary antibody solution, put the box on a shaker and shake it for 2-3h; after that, wash it 3 times with TBST, each time lasts for 5min.

(7) development, fixation

The ECL luminescent solution is added dropwise in the box according to 1:1 equal volume, and the film paper is put into the box to make it mixed evenly for 60s, and then its film paper is laid flat on the exposure plate to ensure that the imager is carried out in the dark room, and the signal is waited for to be prompted for photo preservation.

(8) Image analysis

Using Image J software, graphical analysis was performed and the grayscale values of the target proteins were read and then compared with the internal reference to provide a basis for later data analysis.

2.7 Statistical Analysis

All data from this experiment were entered using excel sheets, and data analysis was performed using SPSS 23.0 statistical software, and Graphpad Prism 8.0 software was used for graphing. The data involved were measures and tested for normality using Shapiro-Wilk; all data were statistically described using the mean ± standard deviation (X±S) and analyzed using one-way ANOVA. If the differences between multiple groups were statistically significant, two-by-two comparisons would be made, and the LSD test would be used when the data met the chi-square, and the Dunnett-t3 test would be used if the chi-square did not, with α=0.05 as the test level in this study.

3.Results and Discussion

3.1 Behavioral test results

3.1.1 Effect of probiotics on the rate of sugar-water preference in mother-infant separated offspring of depressed-like rats

Pleasure deficit is one of the main symptoms of depression, and we generally assess the symptoms of pleasure deficit in SD rats using sugar-water preference experiments. The results of the sugar water preference experiment showed that the sugar water preference rate decreased in the MS+NS group compared with the CON+NS group, and the difference was statistically significant (P <0.01); after the intervention, the sugar water preference rate increased in the MS+P group compared with the MS+NS group, and the difference wasstatistically significant (P <0.01); compared with the MS+P group, there was no significant change in the sugar water preference rate of SD rats in the CON+NS group (P >0.05). Figure 1.

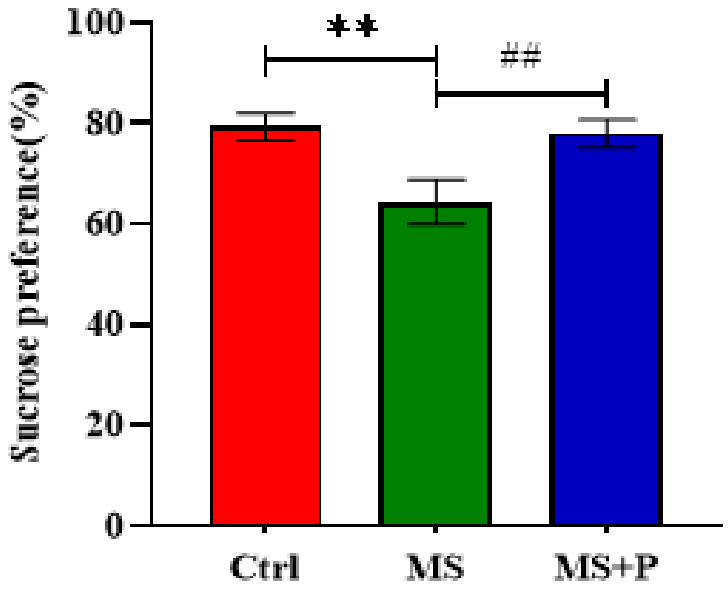

Figure 1 Sugar preference test

Note: Compared with the CON+NS group: $^{*}P$ <0.05, $^{**}P$ <0.01; compared with the MS+NS group:$^{\#}P$ <0.05,$^{\#\#}P$ <0.01.

3.1.2 Effect of probiotics on the autonomous activity of mother-infant separated offspring depression-like rats

(1) Central activity time

The open field experiment was to detect spontaneous activity, exploration, and anxiety-like behavior in rats. The results showed that the central activity time in the MS+NS group decreased comparedwith the CON+NS group, and the difference was statistically significant (P <0.01); the central activity time in the MS+P group increased compared with the MS+NS group, and the difference was statistically significant (P <0.05); the central activity time in the SD rats in the CON+NS group did not change significantly compared with the MS+P group (P >0.05). Figure 2(A).

(2) Central activity distance

The open field experiment was to detect spontaneous activity, exploration, and anxiety-like behavior of rats, and the results showedthat the central activity distance of MS+NS group decreased compared with CON+NS group, and the difference was statistically significant (P <0.01); the central activity distance of MS+NS group increased compared with MS+P group, and the difference was statisticallysignificant (P <0.01); compared with MS+P group, the central activity distance of CON+NS group There was no significant change in the central activity distance of SD rats compared with the MS+P group (P >0.05). Figure 2(B).

(3) Total distance travelled

The open field experiment was to detect spontaneous activity, exploration, and anxiety-like behavior of rats, and the results showed that the total distance traveled in the MS+NS group decreased compared with the CON+NS group, and the difference was statistically significant (P <0.05); the total distance traveled in the MS+P group increased compared with the MS+NS group, and the difference was statistically significant (P <0.05); compared with the MS+P group,the total distance traveled in the CON+NS group of SD rats There

was no significant change in the total distance traveled in the CON+NS group compared with the MS+P group (P >0.05). Figure 2(C).

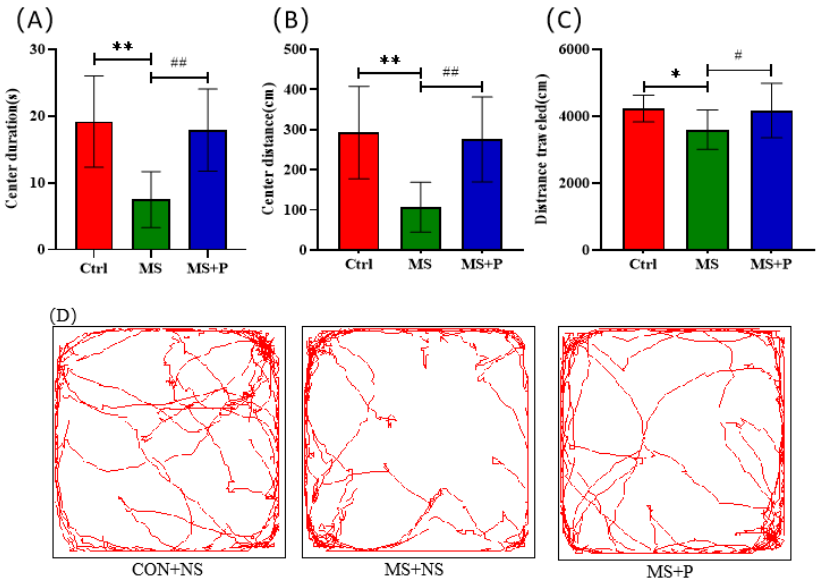

Figure 2 The open field experiment (n=12). (A) indicates the centraltime of each group of rats in the open-field experimental test; (B) indicates the central activity distance of each group of rats in the open-field experimental test; (C) indicates the total distance of each group of rats in the open-field experimental test; (D) indicates the schematic diagram of the trajectory of each group in the open-field experiment.

Note: $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the CON+NS group;$^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 compared with the MS+NS group.

3.1.3 Effect of probiotics on forced swimming experiment in mother-infant separated offspring of depressed-like rats

Forced swimming experiment tests immobilization time to reflect desperate behavior in rodents[46], The results showed that the floating immobility time was increased in the MS+NS group compared with the CON+NS group, and the difference was statistically significant (P <0.01); the floating immobility time was decreased in the MS+P group compared with the MS+NS group, and the difference was statistically significant (P <0.01); there was no significant change in the floating immobility time of rats in the CON+NS group compared with the MS+P group (P >0.05).Figure 3.

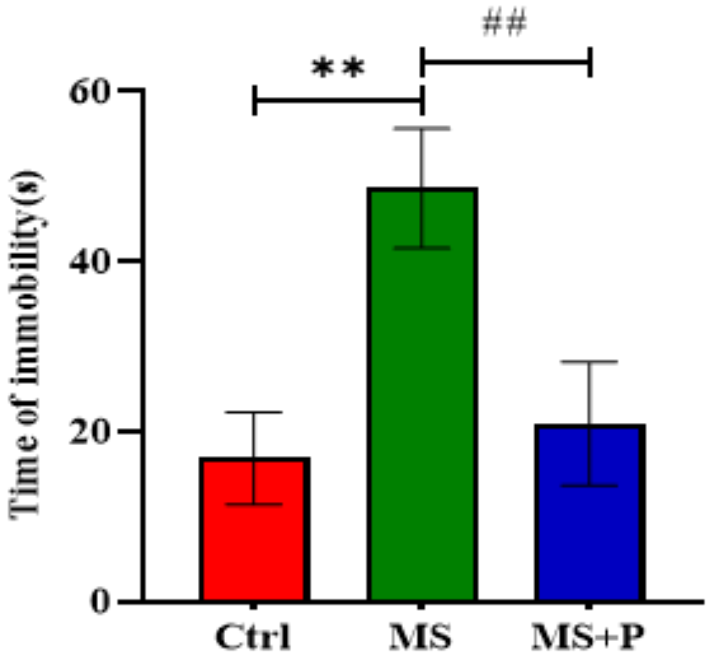

Figure 3 Forced swimming test

Note: $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the CON+NS group; $^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 compared with the MS+NS group.

3.2 Laboratory testing results

3.2.1 Effect of probiotics on neurons in CA1 area of hippocampus in mother-infant isolated offspring of depressed-like rats

The morphological and quantitative changes of neurons in the CA1 region of the hippocampus were detected using Nissler staining. Nissler staining results showed that, in terms of the number of neurons, the number of neurons in the CA1 area of hippocampus was reduced in the MS+NS group of rats compared with the CON+NS group, and the difference was statistically significant (P <0.05);After the intervention, the number of neurons in the hippocampal CA1 area of rats in the MS+P group increased compared with the MS+NS group, and the difference was statistically significant (P <0.05); the number of neurons in the hippocampal CA1 area of rats in the CON+NS group increased compared with the MS+P group (P >0.05). Morphologically, in the hippocampal CA1 area, neurons in the CON+NS group were neatly arranged, with intact cell structure and clear borders, and abundant intracellular niche; compared with the CON+NS group, the cells in the hippocampal CA1 area of the MS+NS group showed a disordered and sparse arrangement, the number of niche in the cytoplasm was reduced, the nucleus showed solidification, fragmentation, and lysis, and the neuron decreased in number;After the intervention, compared with the MS+NS group, the neuronal cells in the CA1 region of the hippocampus of the MS+P group were more neatly arranged, the clarity of the nuclei was improved, the Nisus vesicles were increased, the staining was relatively uniform, and the number of neurons was increased. Figures 4 and 5.

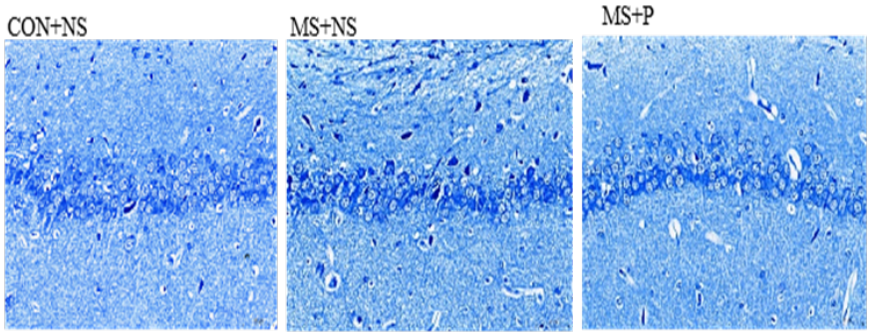

Figure 4 Nissl staining results on hippocampal CA1 area of SD rats in each group(Nissl,×400)

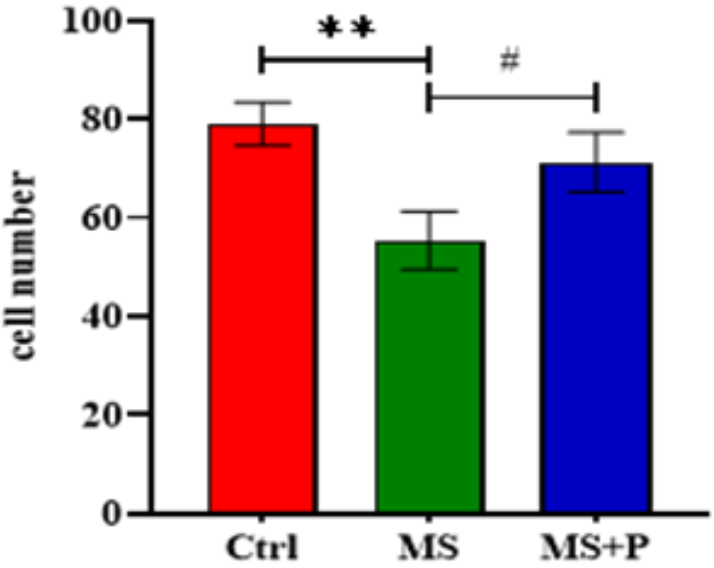

Figure 5 Effect of Nissl staining results on hippocampal CA1 area of SD rats in each group

Note: $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the CON+NS group; $^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 compared with the MS+NS group.

3.2.2 Effect of probiotics on inflammatory factors in the serum of mother-infant separated offspring depression-like rats

The levels of IL-1β, IL-6, and TNF-α inflammatory factors in rat serum were detected by ELISA. the results of Elisa assay showed that the concentrations of IL-1β, IL-6, and TNF-α in serum of rats in MS+NS group were increased compared with CON+NS group, and the difference was statistically significant (P <0.01); after the intervention, compared with MS+NS group, the The concentrations of IL-1β, IL-6 and TNF-α in serum of rats in MS+P group decreased, and the difference was statistically significant (P <0.05); compared with MS+P group, there was no significant change in the expression levels of IL-1β, IL-6 and TNF-α in serum of SD rats in CON+NS group (P >0.05). Figure 6 (A, B, C).

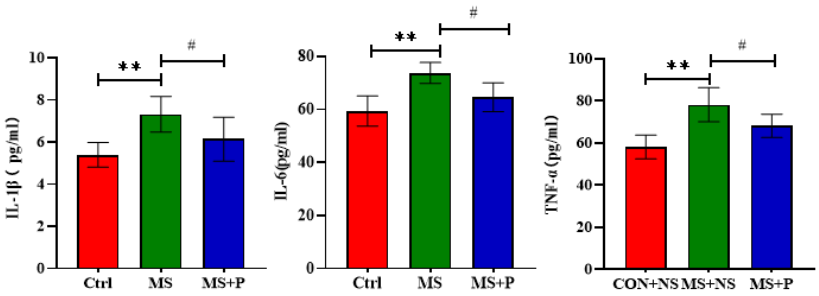

Figure 6 related inflammatory factors

Note: $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the CON+NS group; $^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 compared with the MS+NS group.

3.2.3 Effect of probiotics on the concentration of 5-HT and CORT inthe serum of mother-infant separated offspring depressed-like rats

The results of the Elisa assay showed that compared with the CON+NS group, the serum concentration of 5-HT and CORT in theMS+NS group decreased and the concentration of CORT increased, and the differences were statistically significant (P <0.01); compared with the MS+NS group, the serum concentration of 5-HT and CORT in the MS+P group increased and the differences were statistically significant (P <0.01 and P <0.05); compared with the MS+P group, the serum concentration of 5-HT and CORT in the SD rats increased and the differences were statistically significant (P <0.01 and P <0.05). The differences were statistically significant (P <0.01,P <0.05); compared with the MS+P group, the serum levels of 5-HT and CORT expression in SD rats in the CON+NS group did not change significantly (P >0.05). Figure 7 (A, B).

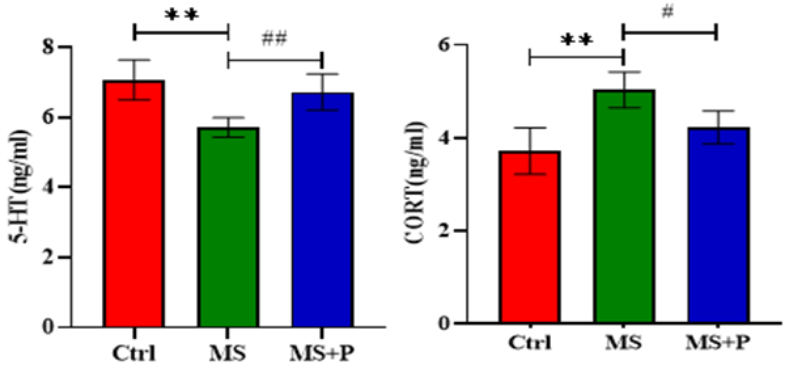

Figure 7 Concentration of 5-HT and CORT in serum

Note: $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the CON+NS group;$^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 compared with the MS+NS group.

3.2.4. Probiotics on hippocampal 5-HT and cAMP/CREB signaling pathways in mother-infant isolated offspring of depressed-like rats

(1) The immunohistochemical microscopic results showed that in the hippocampal region, the cells in the CON+NS group were structurally intact, with clear borders and neatly arranged; compared with the

CON+NS group, the hippocampal cells in the MS+NS groupwere structurally indistinct, poorly arranged and lax, with lysis and destruction, and the expression of 5-HT, cAMP, CREB, and BDNF was not obvious; compared with the MS+NS group, the MS+P group had relatively intact hippocampal cell edges, relatively neat arrangement, clearer structure, lysis and destruction, increased cell numbers, and more obvious expression of 5-HT,cAMP, CREB, and BDNF. Figure 8.

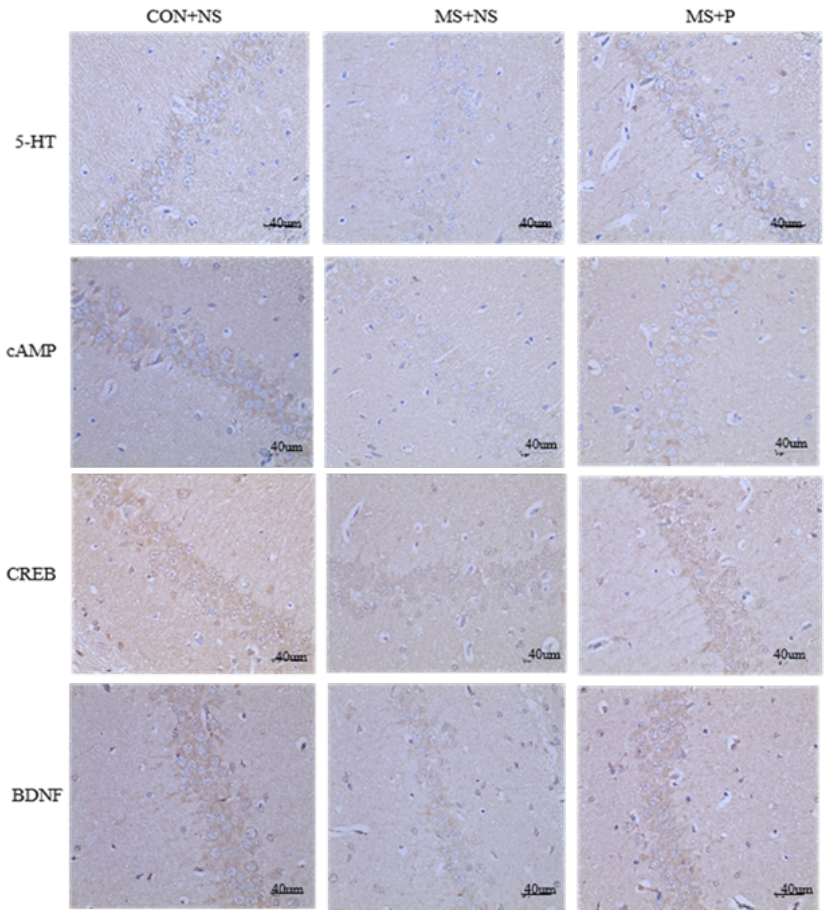

Figure 8 Expression of 5-HT,cAMP, CREB ,BDNFproteins in hippocampus of mice in each group (DAB staining, 40X)

(2) To further investigate whether probiotics activate the cAMP/CREB signaling pathway in the hippocampal tissue of mother-infantisolated rats, therefore, key molecules in the signaling pathway wereselected for detection in this experiment. WB technique showed thatthe cAMP, CREB, and BDNF protein expression levels were decreased in the MS+NS group compared with the CON+NS group, and the differences were statistically significant (P <0.01), compared with MS+NS group, cAMP protein expression level was increased in MS+P group, and the difference was statistically significant (P <0.01), CREB and BDNF protein expression was also increased (P >0.05), compared with CON+NS group, cAMP, CREB and BDNF protein expression level was decreased in MS+P group, and the difference was statistically significant (P <0.01, P <0.05,P <0.01). Figure 9.

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`image9.emf` available at https://authorea.com/users/626386/articles/647838-probiotics-alleviate-depression-like-behavior-in-mother-infant-separation-stress-rats

Figure 9 Western blot detection of cAMP, CREB, BDNF protein expression in rat hippocampus

Note: $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the CON+NS group; $^{\#}P$ <0.05 and$^{\#\#}P$ <0.01 compared with the MS+NS group.$^{\&}P$ <0.05 and$^{\&\&}P$ <0.01 compared with the MS+P group.

4.Discussion

This study showed that maternal-infant separation stress induceddepression-like behavior, led to emotional dysfunction, caused neurobehavioral changes in SD rats, triggered inflammatory responses in the body, and decreased the expression levels of related proteins in 5-HT and cAMP/CREB signaling pathways. Probiotics had antidepressant-like effects on the behavior of their mother-infant separation rats, modulated the inflammatory response of the body, and were involved in the regulation of 5-HT and cAMP/CREB signaling and thedevelopment and improvement of hippocampal neurons, providing an experimental basis for the application of probiotics in the treatment of depressive-like behavior.

In general, newborns will have early contact with their mothersafter birth in order to establish a good intimate bond, and the mother's touching, feeding and caring behaviors for her offspring are beneficial to the early neurological development of the newborn[32]. Walters pointed out that infancy and childhood are prime periods for the development of hippocampal brain regions, which are at important stages of behavioral development, cognitive development, emotional regulation, and character building[33, 34]. Adverse events experienced early in life may produce persistent emotional, cognitive, and behavioral deviations in infants and young children as they grow, providing a risk basis for the onset and development of depressive-like behaviors, and such changes have been demonstrated in rodent models[35, 36]. The mother-infant separation model can lead to emotional dysfunction in the offspring and the development of psychological disorders such as anxiety, depression or drug and alcohol addiction. This early stressful stimulus of mother-infant separation can affect the immune function of the organism and cause an inflammatory response, leading to the development of depression[37, 38]. Leon noted that the mother-infant separation model harms the normal development and maturation of the brain, has profound effects on its futureneuroendocrine system, and has been shown to induce depression, anxiety-like behaviors, and abnormalities in cognitive function[32]. Arborelius showed that if mother-infant separation stress is experienced early in life, rats have a reduced ability to recognize novelty anda reduced central activity walk in behavioral tests, and these results could suggest that the mother-infant separation model reduces socialinteraction, interaction behavior, and even depression and anxiety in offspring rats as adults[39]. Hao also showed that in an experimental test in the open field, mother-infant separated mice exhibited a highfrequency of grooming movements, preferring to move around and spending little time in the central area, and that this model of earlylife adverse stress may be related to the inhibition of the HPA axis,affecting the development and function of neurons, which in turn induces the onset and development of depression-like behavior[40]. We also observed a decrease in sugar-water preference, an increase in floating immobility time, and a decrease in central activity time, central activity distance, and total distance in MS rats, where a stressful event such as mother-infant separation leads to a lack of pleasure in the rats and behavior that shows despair, depression, or anxiety.

Probiotics are a simple and readily available complementary intervention that is involved in the development, growth and maturation of multiple systems, and many diseases have been shown to havea strong association with probiotics, For example, irritable bowel syndrome[41]、Metabolic syndrome[42]、Allergic diseases[43] etc, And probiotics have a positive effect on neurological development, cognitivefunction, behavior and emotional regulation[44]. A growing number of studies have shown that for infants and young children experiencing early life stressful events, the addition of probiotics from breast milk to formula mimics to some extent the nutritional supply of breast milk to infants and young children to achieve healthy growth[45, 46]. Breast milk flora mainly consists of Actinobacteria, Thick-walled Bacteria and Aspergillus, among which Lactobacillus and Bifidobacterium predominate in the intestinal tract of breastfed infants and children, and they play an important role in the homeostasis of the intestinal flora of infants and children[45, 47]. Leszek also noted that supplementation with Lactobacillus spp. increased bifidobacteria and Lactobacillus colonization, alleviated cognitive impairment caused bydepression, inhibited the growth of potential bacterial fungi, adhesion, contributed to short-chain fatty acid synthesis, modulated inflammatory factors, and improved depression[62].

It has been shown that Lactobacillus and Bifidobacterium can convert the amino acid glutamate to gamma-aminobutyric acid (GABA), and that disorders of the GABA receptor signaling pathway are associated with anxiety and depression[50]. Lebovitz showed that Lactobacillus rhamnosus GG modulates gastrointestinal metabolism, motility, and reduction of stress-induced visceral sensitization in mother and infant separation[48]. It has been shown that the mother-infant separation model can lead to increased colonic mucosal permeability and elevated inflammatory factors, while Bifidobacterium bifidum G9-1 can improve mucosal permeability, protect intestinal mucosal integrity and restore intestinal flora homeostasis[49]. Consistent with the results of previous studies, our findings suggest that the probiotic intervention resulted in an improvement in the behavioral expression of despair, depression or anxiety in mother-infant separated rats.

5-HT is a monoamine neurotransmitter that plays an important role in mood regulation and is closely related to the HPA axis, while CORT is a product secreted by the HPA axis, both of which areimportant substances that protect the nervous system[51]. Nisin vesicles in the hippocampus are unique to neurons and are distributed in the cytoplasm and dendrites, and their role is closely related to synthetic proteins and neurotransmitters, while 5-HT as a monoamine neurotransmitter has a positive effect on synaptic transmission and plasticity, neuronal repair and regeneration, and regulates mood, cognition, and autonomic neuron function[52]. Wu[53, 54] showed that oral administration of Lactobacillus casei reversed the elevation of 5-HT levels in the hippocampus of mice induced by chronic adverse stress, causing an increase in the number of neurons, improvement in neuronal morphology, clarity and integrity of cell structure, and inhibition of apoptosis or necrosis. Tian showed that oral administration of a subgenus of Bifidobacterium longum upregulated 5-HT concentration levels and was confirmed in behavioral assays, improving depressive, anxiety-like behavior in mice[55]. LI showed that probiotics have antidepressant effects and have improved efficacy in the regulation of 5-HT and CORT metabolism and have protective effects on neuronal regeneration and repair[56]. In this experiment, after the probiotic intervention, there was an increase in the number of Nisus vesicles, an increase in the number of neurons and an improvement in the morphology of neurons in the hippocampal CA1 region of MS rats; the serum 5-HT and CORT concentrations were detected by Elisa and it was found that the serum 5-HT concentration increased and the CORT concentration decreased in MS rats after the probiotic intervention; the results of 5-HT detection in the hippocampus of rats using immunohistochemistry found that After the probiotic intervention, the cell arrangement of 5-HT in the hippocampus of MS rats was neat, the cell structure was clear, the phenomenon of lysis destruction was alleviated, and the expression of 5-HT was more obvious. It can be seen that probiotics can improve the expression of 5-HT in the hippocampus of MS rats, and have a regulatory effect on the concentration of 5-HT and CORT in the serum, which in turn promotes the repair and regeneration of neurons in the hippocampus of MS rats, and has a certain protective effect on neurons.

Cellular inflammatory factors are often accompanied by alterations in the immune system, and once pro-inflammatory and anti-inflammatory factors are dysregulated[57], It accelerates oxidative stress, which affects neuronal repair or regeneration and plays a dangerous role in the pathogenesis of mental disorders[58]. It has been shown that immune inflammation is a crucial factor in the pathogenesis of depression, and that these inflammatory factors such as IL-1β, IL-6 and TNF-α are the most widely studied pro-inflammatory factors, are important indicators of the neuro-endocrine system and play a crucial role in the pathophysiology of depression[52, 59]. The beneficial effects of probiotics on anxiety and depression may be through competitive elimination of harmful intestinal pathogens, reduction of pro-inflammatory cytokines and communication with the central nervoussystem through vagal sensory fibers, leading to changes in neurotransmitter levels or function.Russo noted that Bifidobacterium longum was effective in alleviating gastrointestinal disorders in their infants and children, and the possible mechanism is related to the regulation of intestinal flora by probiotics, which produces resistance through mediated immune inflammatory signaling pathways and thus improves gastrointestinal discomfort[60]. Westfall showed that oral administration of probiotics or prebiotics, by alleviating the body's inflammatory response and improving the body's immunity, can reduce depression-like behavior in mice due to chronic stressful events[61]. Studies have shown that oral administration of probiotics or prebiotics, by alleviating the body's inflammatory response and improving the body's immunity, can reduce the depression-like behavior in mice due to chronic stressful events. It has been shown that

Bifidobacterium has an ameliorative effect on the pathological changes in the hippocampus of chronically stressed mice, and the results by Tunel staining showed that the number of positive cells was elevated and apoptosis was alleviated, and biochemically, the intervention of Bifidobacterium decreased the expression of inflammatory factors such as IL-1β and TNF-α levels in serum and down-regulated the content of pro-apoptotic proteins[55]. Maria showed that depression leads to systemic inflammation in the body and that this inflammation disrupts the blood-brain barrier in different pathways to reach the central nervous system, activating the central immune system and inducing an inflammatory response in the nervous system[63, 64]. Satya showedthat acetate-3 and acetate-5 inhibited monoamine oxidase-A enzymes,increased norepinephrine (NA) and 5-HT levels in rat brain, decreased plasma CORT levels, reduced TNF-α and IL-6 inflammatory factor levels, and improved depression-like symptoms[65]. It has been shown that in clinical practice, depressed patients receiving probiotic-assisted intervention therapy suppress inflammatory responses, reduce the release of inflammatory mediators, alleviate neurological damage,and reduce depression-like symptom[66]. It has been shown that in the acute phase, depressed patients have elevated serum levels of TNF-α and IL-1 inflammation, which can inhibit synaptic function and lead to anxiety and depression-like behaviors [52, 67]. Yoo showed that oral administration of Lactobacillus plantarum, which inhibited the expression of inflammatory factors such as IL-6 and TNF-α, attenuated the level of neuroinflammation in the brain of mice and led to the alleviation of their depression-like behavior[68]. In this experiment, we found that the levels of IL-1β, IL-6 and TNF-α inflammatory factors in the serum of MS rats were reduced after probiotic intervention by Elisa assay, and MS rats showed improvement in depression-like behavior, suggesting that probiotic intervention can inhibit the production of pro-inflammatory factor cells and reduce the neuroinflammatory response, and then improve the depression-like behavior of offspring rats due to maternal-infant separation, which is consistent with the findings of park[69].

The cAMP signaling pathway is one of the most classic and earliest studied, and plays an important role in neural cell and functional repair[70]. cAMP/CREB is involved in cell signaling and is closely related to neuronal growth and development, cell repair, and synaptic plasticity[71].BDNF is one of the most abundant and important neurotrophic factors in the body's brain, and is responsible for protecting neurons, participating in axonal growth, dendrite number, morphological regulation[72]. It is assumed that BDNF deficiency may lead to impaired neurological growth and development in the brain, which in turn makes it a risk factor for the pathogenesis of depression[73]. It has been shown that 5-HT can be regulated through the G protein-coupled cAMP/CREB signaling pathway, and that activation of the cAMP/CREB signaling pathway can in turn affect 5-HT production, thus inducing depression or anxiety-like disorders if the pathway is abnormal[74, 75]. Wu showed that the pathogenesis of depression is related to the fact that some drugs with antidepressant effects activate the PKA/CREB signaling pathway and modulate the expression of BDNF activity[28]. Li Zhiyong showed that Chai Shao An Shen Jie Yu granules could reduce the secretion of inflammatory factors in the body, upregulate hippocampal PKA/CREB-related proteins, activate anti-inflammatory factors, and help the effect of hippocampal neuron repair,thus improving post-stroke depression[76]. In thepresent study we evaluated the effect of probiotics on the cAMP/CREB signaling pathway in the hippocampal region of MS rats. We found that the cell structure of cAMP, CREB, and BDNF in the hippocampal region of MS rats was unclear, misaligned and lax, withsigns of lysis and disruption, and reduced protein expression, indicating a correlation between depression-like behavior and abnormal protein expression in hippocampal tissue. After probiotic intervention, the cell edges of hippocampal area in MS rats were relatively intact,relatively neatly arranged, with clearer structure, and the expression of cAMP, CREB and BDNF proteins were increased, indicating thatprobiotics would regulate the 5-HT content in hippocampus and serum, inhibit the inflammatory factors of the body, and then bind to the receptors, activate the cAMP/CREB signaling pathway, and improve the hippocampal related tissue protein levels and improve depression-like behavior in mother-infant separated offspring rats, which is consistent with the study of Mato[77].

5.Conclusion

In summary, probiotic treatment alleviates anxiety/depression-like behavior caused by maternal-infant separation stress, reduces changes in inflammatory factors in serum and also decreases the expression levels of related proteins in the cAMP/CREB signaling pathway,providing an experimental basis for its application

in the treatment of anxiety/depressive mood disorders.

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7.Declaration of Competing interest

The authors declare that no conflict of interest could be perceived as prejudicing the impartiality of the review.

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