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Maternal and filial consumption of probiotics protects from acute stress-induced anxiety and loss of benevolent gut microbes

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Abstract

The study aimed to determine the effects of probiotic consumption during pregnancy and lactation and post-weaning on acute stress-induced anxiety and gut beneficial microbiota of the female offspring mice.The female offspring mice were divided into several groups: intact, control (only stressed), PBS/dam (dams gavaged with PBS), PRO/dam (dams gavaged with probiotics), PRO/dam+off (both dams and offspring gavaged with probiotics), and PBS/dam+off (both dams and offspring gavaged with PBS)The probiotics chosen are mainly L. rhamnosus, B.breve, and B. longum (108 CFU/ml). Foot shock stress will be applied for one hour on the 43rd day after birth. Behavioral tests were conducted using the open field and elevated plus-maze. Corticosterone was measured by ELISA kit, and intestinal microflora with qPCR.The data showed that PRO/dam+off had more entries into open arms compared to the control group and decreased move distance and time spent in closed arms compared to the control group. However, there was no significant difference between the PRO/dam group and the control group. In the open field test, the control group spent less time in the inner zone compared to the intact group and in PRO/dam+off group. Corticosterone hormone was increased in the control group and was decreased in the PRO/dam+off. Bifidobacteria and Lactobacilli decreased in the control group in comparison to the intact group, and in the PRO/dam+off group increased compared with other groups. Maternal and filial supplementation with a multi-strain probiotic mixture increased levels of beneficial bacteria and reduced stress-induced anxiety in mice.

Keywords: Probiotic, Anxiety, Foot shock stress, Corticosterone, Gut micribiota.

1. Introduction

It is a consistent finding that the prevalence of anxiety disorders in women is approximately twice as high as in men [1]. During puberty, females undergo significant physical and emotional changes that can lead to increased levels of stress. Recent research suggests that heavy and acute stress during the puberty period of females can significantly alter the composition and function of the intestinal microbiota, potentially leading to adverse emotional health outcomes [2].

Electric foot shock stress is a widely used method for inducing acute stress in laboratory animals. This type of stress caused a decrease in the diversity of the gut microbiota in mice. This decrease was associated with an increase in the relative abundance of Proteobacteria and a decrease in the relative abundance of Firmicutes. These alterations in gut microbiota have been linked to the development of depressive/anxiety-like behavior in mice [3].

At a basic level, stress activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system, which can cause changes in gut motility, blood flow, and secretion. These changes can then lead to alterations in the composition and function of the gut microbiota, as well as increased intestinal permeability (i.e., leaky gut) [4].

There is also evidence that stress-induced inflammation and dysbiosis in the gut can directly affect the brain and contribute to the development of neuro-inflammation and associated symptoms such as anxiety. This is believed to occur through a variety of mechanisms, including direct neuronal signaling, activation of the immune system through the production of pro-inflammatory molecules by the gut microbiota, and alteration of neurotransmitter systems [5]. Certain types of bacteria in the gut are capable of producing lipopolysaccharides (LPS), which are potent activators of brain microglia. Activated microglia release more pro-inflammatory cytokines, creating a positive feedback loop of inflammation that can lead to anxiety-like behavior. In addition, neuro-inflammation can reduce the production of neurotransmitters like serotonin and dopamine, which are important for regulating mood and anxiety [6].

The relationship between stress, gut microbiota, and mental health is complex and multifaceted, but there is

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growing evidence to suggest that addressing these factors may be an important approach to preventing and treating a range of stress-related disorders. In this context, probiotics have gained significant attention as a potential solution to restore the gut microbiome's balance and improve mental health outcomes [7].

It is expected that the consumption of probiotics by mothers and offspring can reduce the impact of stress on the gut-brain axis and thus reduce the level of anxiety in females. Few studies have been done in this field. In one study, probiotics reduced LPS-induced sickness behavior in females at 12 hours and in males at 48 hours post-treatment. Probiotics also prevented increases in pro- and anti-inflammatory cytokines following LPS treatment, reduced cytokine mRNA expression in certain brain regions, and prevented LPS-induced changes in gut microbiota [8]. Exposure to LPS during puberty resulted in enduring depression-like behavior in female mice and anxiety-like behavior in male mice during adulthood. However, probiotics prevented these enduring behavioral changes in both sexes [6].

Results of our previous research, indicate that consumption by offspring in the post-weaning period has the ability to safeguard against anxiety caused by sub-chronic stress, disturbances in progesterone levels, and disruptions in the expression of certain genes related to anxiety [9].

The main hypothesis of this intergenerational study is that consuming probiotics during pregnancy and lactation for mothers with or without offspring during puberty, could effectively reduce acute stress-induced anxiety and promote the growth of beneficial intestinal microbiota.

2. Materials and Methods

2.1. Animal housing

All animals (n=40) will be housed under 12 h light–12 h dark cycle, lights on at 7 AM. Housing room temperature will be maintained at 22 ±2 ˚C and humidity at 60–70%.

Forty adult BALB/C mice will be purchased, including 5 males and 15 females (each cage includes 1 M: 3 F). Then those will be mated to take the offspring (n=48). The offspring will be 48 females which are selected randomly (in each group, n=8).

2.2. Grouping

The study involved four different groups of offspring: Intact group with no treatment, a group exposed to footshock stress (Control), a group whose mothers were treated with PBS and also exposed to footshock stress (PBS/dam), and a group whose mothers were treated with probiotics and also exposed to footshock stress (PRO/dam). Additionally, there was a group where the mothers were treated with PBS during pregnancy and lactation, and the offspring were also treated with PBS and exposed to footshock stress (PRO/dam+off), and another group where the pregnant and breastfeeding mothers were treated with PBS and their offspring were treated with PBS and exposed to footshock stress (PBS/dam +off) (Table.1).

Among the probiotics, bacterial strains were selected that, based on previous studies, had the greatest effect on increasing the beneficial strains of the intestinal microflora and reducing the harmful strains, as a result, strains of probiotics that were mainly L. rhamnosus, B.breve, B. longum (108 CFU/ml) were selected and tested. A mixture of these strains was gavage to mice as a probiotic.

2.3. Foot shock stress

Acute stress caused by shock was applied to the feet of mice with an intensity of 0.6 mA with a duration of 1 second with an interval between shocks of 30 seconds was applied for 1 hour. At this stage, 2% xylazine was used at the rate of 10 mg per kilogram of mouse weight, which was injected into the mouse body through peritoneal injection [10].

2.4. Behavioral tests

Firstly, the open field which is a low-stress test will be done only 1 h after inducing anxiety to the mice, and then by gapping 1 day, they will be tested by the elevated plus-maze.

2.5. Open-field test (OFT)

Mice will be assessed for their locomotor activity and response to a novel environment in the open field test, which is conducted as previously described [11]. Mice will be placed in an open arena (40 × 32 × 24 cm, L × W

Table 1. Animal grouping and study design.

Numbers	Groups	Foot-Shock Stress	PBS	Probiotic
1	Intact	—	—	—
2	Control	43rd day after birth	—	—
3	PBS/dam	43rd day after birth	Mother during pregnancy (3 weeks) and breastfeeding (3 weeks)	—
4	PBS/dam+off	43rd day after birth	Mother during pregnancy (3 weeks) and breastfeeding (3 weeks) + Offspring from 22nd day after birth, (3 weeks)	—
5	PRO/dam	43rd day after birth	—	Mother during pregnancy (3 weeks) and breastfeeding (3 weeks)
6	PRO/ dam+off	43rd day after birth	—	Mother during pregnancy (3 weeks) and breastfeeding (3 weeks) + Offspring from 22nd day after birth , (3 weeks)

× H) and will be allowed to explore the arena for 10 min. Animals will be habituated to the room 1 h before the test. Testing will be performed under dim light (60 lux). Experiments will be videotaped using a ceiling camera and will be analyzed for time spent in the virtual center zone (defined as 50% away from the edges).

2.6. Elevated plus-maze (EPM)

The plus-maze consists of two open arms (50 _ 10 cm) and two enclosed arms (50 _ 10 _ 38 cm) opposite each other at a height of 73 cm above the floor. Lighting in the open arms is 55 lux. At the beginning of each test, the mice will be placed in the center facing a closed arm.

During the 5-minute exposure, the number of entries into each of the arms and the time spent therein will be monitored by a video camera. An entry will be scored when two forepaws pass over the open or closed dividing line. Data will be processed to yield the ratio of time spent in the open arms versus total time and the number of entries into each arm of the maze. [9]

2.7. Hormone assay

Trunk blood will be collected from the site where the animal is decapitated and then centrifuged at 400 g for 20 minutes and will be stored at -80°C until the day of the analysis. Corticosterone will be measured by enzyme-linked immunosorbent assay (ELISA) kits.

2.8. Intestinal microflora quantification

To perform qPCR, a mixture of 1 µl forward and reverse primers from Sinacolon Co., Tehran, Iran (Table 2) 10 µl of 2X RealQ Plus Master Mix Green from Amplicon Bio, Seoul, South Korea, and 1 µl of extracted DNA was created.

The total volume of the mixture was 20 µl. The qPCR reaction was carried out using the quantitative Rotor-Gene Q real-time PCR system from QIAGEN GmbH, Hilden, Germany. The expression levels of transcripts were normalized by comparing them to the β-actin gene, and each sample was analyzed in triplicate. The software called REST 2009 was used to evaluate the expression fold change.

2.9. Data analysis

Graph Pad Prism version 8 was used to carry out data analysis. The analysis involved comparing two distinct groups using unpaired Student t-tests. Additionally, a one-way analysis of variance followed by Tukey posttest multiple comparisons tests was conducted. The data is presented as mean ± SEM, and any P value that was less than or equal to 0.05 was considered significant.

3. Results

An intergenerational study was conducted to investigate the effect of maternal with or without filial probiotics using on female puberty mice under footshock-induced stress.

The elevated plus maze and open field apparatus tests was used to assess the anxiety-like behavior of mice. Traces of mouse activity were found in both the open and closed arms of the maze (Figure1. A1, A2 and A3). The data showed that PRO/dam+off spent more time in the open arms compared to control group. and also PRO/dam+off group had a significant increase in the number of entries into open arms (Figure1, B1 and B2) and decreased moved distance and time spent in closed arms compared to the control group (Figure1, C2 and D2). However, there was no significant difference between the PRO/dam group and the control group.

In the open field test, the control group had decreased entries into the inner zone compared to the intact group and in PRO/dam+off group, this reduction was compensated (Figure2, A1, A2 and A3). The time spent in the inner zone was longer in the control group than in the intact group. This time increased in the PRO/dam+off group compared to the control group (Figure2, B1 and B2).

The increasing changes of corticosterone hormone in the control group compared to the intact group ($P<0.0001$) and its significant decrease in the PRO/dam+off group compared to the control group ($P<0.01$) were consistent

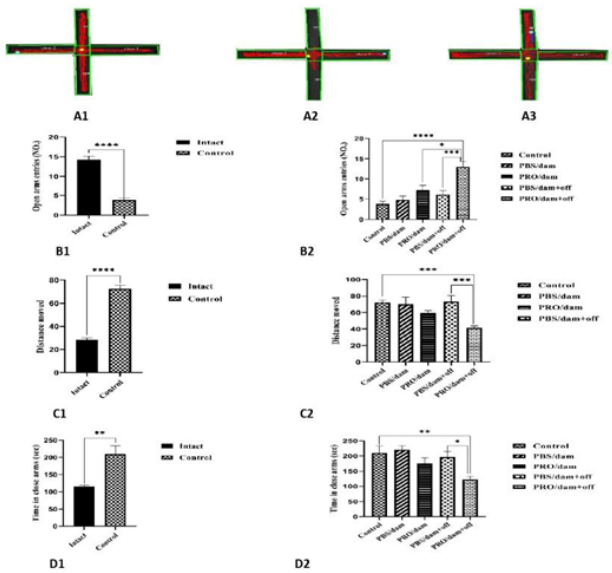

Fig. 1. Elevated plus maze test results A. Traces of mouse activity can be detected in both the open arms (vertical) and closed arms (horizontal) of the maze (A1: intact group, A2: control group and A3: PRO/dam +off group) B. Compare of open arm entries between B1: intact and control groups, B2: between control and other experimental groups C. Compare of distance moved between C1: intact and control groups, C2: control and other experimental groups D. Compare of time in close arms between D1: intact and control groups, D2: between control and other experimental groups.

Table 2. The primers used.

Genera	Primer	Sequences	Position in E. coli 16S rRNA
Bacillus	B-K1/F	5′-TCACCAAGGCRACGATGCG-3′	273-255
	B-K1/R1	5-′CGTATTCACCGCGGCATG-3′	1367-1350
Bifidobacterium	Pbi F1	5-CCGGAATAGCTCC-3′	156-144
	Pbi R2	5′-GACCATGCACCACCTGTGAA-3′	1058-1040

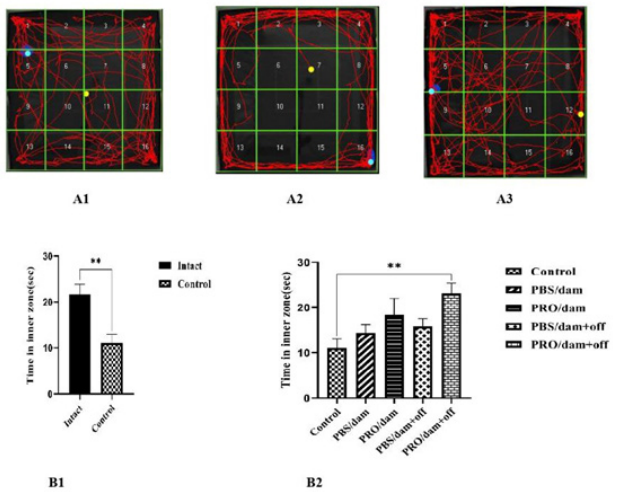

Fig. 2. Open field test results A. traces (A1: intact group, A2: control group and A3: PRO/dam group, A4: PRO/dam +off group) B. Compare of open arm entries (B1: between intact and control groups, B2: between control and other experimental groups).

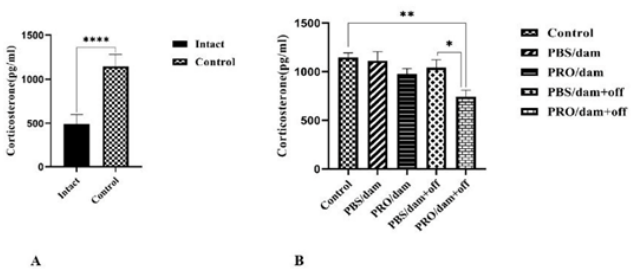

Fig. 3. Compare of serum corticosterone level (A) between intact and control groups (B) between control and other experimental groups.

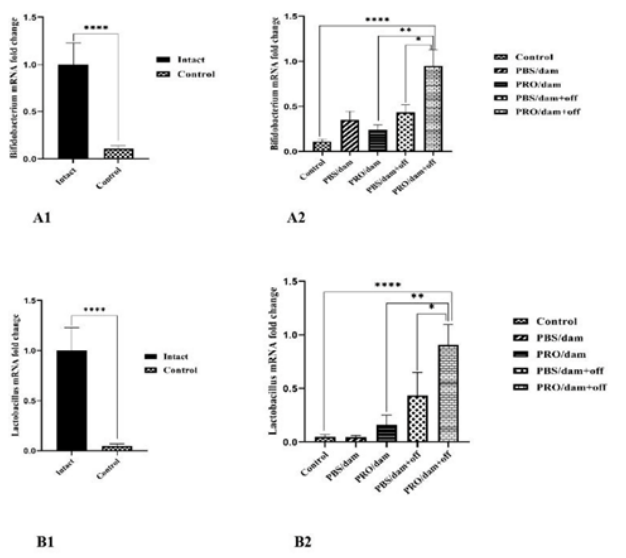

Fig. 4. Compare the mRNA fold change between A1: intact and control groups, B2: control and other experimental groups.

with the results of EPM and OFT and indicated the anti-anxiety effect of probiotic consumption by both mothers and children exposed to electric shock stress (Figure3, A1and A2).

Compare of the mRNA fold change between experimental groups indicated that this parameter for Bifidobacteria (Figure4, A1(and Lactobacilli (Figure4, B1 (decreased in control group in relation to intact group, which indicates the decrease in the population of these microbes in the intestine. Increase of these mRNA fold changes of these bacteria in the PRO/dam+off group compared with other groups (Figure4, A2 and B2 (suggest that population of these genera of bacteria has both increased significantly.

4. Discussion

The study focuses on maternal and filial consumption of probiotics by mice and its effects on their gut microbiota and behavior after exposure to acute stress. The findings of the study suggest that maternal and filial consumption of probiotics can indeed help restore the balance of beneficial bacteria in the gut and reduce anxiety-like behavior induced by stress. This has significant implications for human health, as stress-induced anxiety is a common problem worldwide, and finding natural solutions like probiotics could potentially improve the quality of life of acutely stressed adolescents.

Although the precise mechanisms through which intestinal microbiota dysbiosis induces anxiety are not yet fully understood, research has shown that disturbances in the gut microbiota can lead to alterations in this communication between the gut and the brain, resulting in changes in behavior, mood, and cognition [12]. Specifically, dysbiosis of the gut microbiota has been associated with increased levels of inflammation, oxidative stress, and neurotransmitter imbalances that can contribute to the development of anxiety symptoms through a complex network of neural, endocrine, and immunological pathways known as the gut-brain axis [13].

Anti-anxiety effects of probiotics and prevention of corticosterone increase due to stress can be caused by the following mechanisms:

Competitive exclusion with harmful bacteria, production of antimicrobial substances, Immunomodulation, metabolic activity (release of the beneficial metabolites such as short-chain fatty acids), Signaling to host cells via various pathways, including toll-like receptors, cytokines, and neurotransmitters, which can have downstream effects on multiple physiological systems [14]. They can also aid in the production of substances needed for proper gut-brain communication, like anxiolytic neurotransmitters (GABA, Serotonin and etc) [13].

Probiotics can also transmit signals through the vagus nerve associated with a normalized microbiota, leading to normalized signaling in the Hypothalamic Paraventricular Nucleus (PVN) and thereby HPA-axis activity [15]. By this, probiotics can help normalize neural processes that are important for emotional regulation and reward processing in areas like the amygdala, hippocampus, and PFC [13]. This may lead to more appropriate development of these areas during adolescence.

Probiotics can improve the function of tight junction proteins in intestinal cells, leading to a stronger intestinal barrier. This can help prevent inflammatory cytokines and microbial antigens from entering the bloodstream and causing neuro-inflammation [5].

Two-week probiotic supplementation before and after the birth of rats exposed to perinatal noise stress in the third trimester of pregnancy resulted in a two-fold increase in entries into the open arms of the EPM. The animals that were stressed before and after exposure to loud noises

had a reduction in their stress hormone levels in their bloodstream due to the addition of probiotics, which is in line with the results of the present study [16].

A different research project investigated how administering probiotics to mothers affected changes in the HPA-axis, immune system, and gut microflora of Wistar rats that were caused by stress during early life or adulthood. Contrary to the results of the previous studies, neonatal maternal separation induced alterations in ACTH but not corticosterone. This is probably due to the 10-day time lag between the cessation of neonatal maternal separation and blood collection. The common feature of the mentioned research results with the present results is that the natural composition of the microorganisms in the gut is changed in animals that are separated from their mothers, but giving probiotics to pregnant or new mothers seems to help restore the balance of Bifidobacteria in stressed adult animals to a level similar to unstressed animals. It has been argued that balance restoration was due to the increase in IgA in the digestive tracts of the rats that were induced by probiotics [17].

The results of another study suggested that administering Lactobacillus supplements had a significant positive impact on anxiety-related behavior in stressed rats, and also helped to normalize levels of ACTH, corticosterone, glucocorticoid receptor, serotonin, dopamine, and noradrenaline. Early life stress can potentially suppress the expression of miR-124a while increasing the expression of miR-132, which may disrupt the expression of glucocorticoid receptors. The decrease in anxiety behavior observed in probiotic-treated groups is thought to be enabled by the interaction between these miRNAs and the receptors. Additionally, there is mounting evidence indicating that early life stress can lead to lasting changes in the expression of AMPA and NMDA receptors, which can then cause anxiety. Probiotics may help balance the expression of these receptors by increasing GABA levels. Although the stress model and timing of it, differ with the present research, these mechanisms can be generalized to our study [18].

In a different study, during the prenatal period from day 10.5 of gestation until postnatal day 1, mouse dams were given probiotic Lactococcus lactis via drinking water. Once the pups reached 10 weeks old, they underwent various behavioral tests. The results showed that female pups who were exposed to the probiotic spent more time in the center of the open field, while there was no significant effect observed in male pups. It was concluded that probiotic treatment during the latter half of gestation alters cortical cytoarchitecture in postnatal day 1 offspring, suggesting that changes in cortical neuronal layer and vasculature protein expression at early postnatal stages may be associated with behavioral outcomes later in life. The mechanism that can justify the anti-anxiety effects of probiotics in the present study [19].

The use of probiotics while pregnant provided protection for the offspring against neurobiological and gastrointestinal disorders caused by separation from the mother, including depression-like behavior and delayed bowel movements. The transmission of *Bifidobacterium breve* CCFM1025 (a promising candidate psychobiotic strain) during pregnancy resulted in higher levels of 5-hydroxytryptamine in the colon and short-chain fatty acids in the cecum of the offspring. This suggests that the benefits of probiotics can be passed down through gut microbes, making it a promising method for reducing health risks during early life [20].

The present study results indicated that maternal probiotic supplementation during pregnancy and lactation, per se, did not result in any changes in the offspring's gut microbiota. During the period of 36 weeks of pregnancy until 3 months after giving birth while breastfeeding, women were given either probiotic milk or a placebo. The probiotic milk contained three types of bacteria - *Lactobacillus rhamnosus GG, L acidophilus La-5*, and *Bifidobacterium animalis subsp. lactis Bb-12.* However, only the *Lactobacillus rhamnosus GG* bacteria were found to be present in the infants at both 10 days and 3 months old, which is somewhat consistent with the results of the present study. It is worth noting that the study had relatively small sample sizes and different probiotic strains, doses, and durations of supplementation [21]. The more recent study in a significant population of mother-infant pairs (600 women and their newborns) showed that the composition of the vaginal microbiome during childbirth does not have an impact on the development and composition of the microbiome in a newborn's stool [22]. In other words, the composition of a mother's breast milk microbiota can be influenced by factors such as her diet, health status, and mode of delivery, and these factors can in turn affect the diversity and abundance of microbes in the infant's gut. One possibility to justify the results of the present research is that the consumption of probiotics by the mother has improved the intestinal microbiota and during breastfeeding, beneficial microbes have been transferred to the intestines of the offspring and left their own effects. However, this effect is enhanced in offspring who have taken probiotics post-weaning.

In none of the past studies, probiotics have not been given to both mother and offspring, their results cannot be completely compared with the current findings in terms of the probiotic mode of the function. Therefore, more supplementary and translational studies should be done in this field.

5. Conclusion

The consumption of probiotics may help reduce stress-induced anxiety through multiple mechanisms, including the regulation of gut microbiota composition, the promotion of gut health and immune function, and the modulation of stress hormone levels.

maternal and filial consumption of probiotics was shown to recover acute stress-induced anxiety and increase the abundance of benevolent microbes in mice. The study found that both maternal and filial supplementation with a multi-strain probiotic mixture improved gut microbiota composition, leading to increased levels of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. Maternal and filial consumption of probiotics may provide a novel strategy for restoring gut homeostasis and reducing stress-induced anxiety in offspring.

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Ethical statement
The National Institute of Health Guide for the care and use of laboratory animals was adhered to while carrying out all procedures.

Competing interests
The authors state that they have no conflict of interest.

Authors' contributions
The research study was designed by all the authors, while Abdulaziz Kadhim Alwali conducted the research and gathered as well as analyzed the data. Initially, he wrote the draft of the manuscript, which was later revised by all the authors. They discussed the findings and made contributions towards the final version of the manuscript.

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