Research Article

Neuroprotective Effects of Probiotic-Supplemented Diet on Cognitive Behavior of 3xTg-AD Mice

Chenxi Tan 1, Yang Liu 1, Huiyi Zhang 1, Cihan Di 2, Dechao Xu 1, Chunming Liang 3, Ningning Zhang 3, Bing Han 4, and Weiya Lang 5

1Ward 5, Department of Neurology, The Second Affiliated Hospital of Qiqihar Medical University, Qiqihar 161000, China
2Ward 1, Department of Neurology, The Second Affiliated Hospital of Qiqihar Medical University, Qiqihar 161000, China
3Department of Pathology, School of Basic Medicine, Qiqihar Medical University, Qiqihar 161000, China
4Department of Rehabilitation, The Second Affiliated Hospital of Qiqihar Medical University, Qiqihar 161000, China
5Department of Tissue Culture, School of Basic Medicine, Qiqihar Medical University, Qiqihar 161000, China

Correspondence should be addressed to Weiya Lang; langweiya@qmu.edu.cn

Received 23 November 2021; Revised 8 December 2021; Accepted 18 December 2021; Published 5 January 2022

Academic Editor: Bhagyaveni M.A

Copyright © 2022 Chenxi Tan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alzheimer’s disease (AD) is recognized as one of the most common types of senile dementia [1]. AD patients first suffer memory loss for recent events (short-term memory impairment). As the disease progresses, they are deprived of self-awareness [2]. Early onset of AD, old age, low education level, and some poor physical conditions affect the prevalence rate of the disease and the severity of cognitive disorder [3]. Alteration of intestinal microflora [4], cognitive disorder [5], and neuroinflammation [6] were reported to be associated with many central nervous systems (CNS) neurodegenerative diseases including AD. Experimental evidence showed that a change in the micronutrients is also a hazardous factor of AD [7]. At present, there is no definite therapy for AD. The therapies available can protect cognition and memory and delay the loss of function. These years, a growing number of researchers have been focusing on the role of intestinal flora in CNS disorders, especially the regulation over the gut-brain axis. Regulating gut microbiota

1. Introduction

Alzheimer’s disease (AD) is recognized as one of the most common types of senile dementia [1]. AD patients first suffer memory loss for recent events (short-term memory impairment). As the disease progresses, they are deprived of self-awareness [2]. Early onset of AD, old age, low education level, and some poor physical conditions affect the prevalence rate of the disease and the severity of cognitive disorder [3]. Alteration of intestinal microflora [4], cognitive disorder [5], and neuroinflammation [6] were reported to be associated with many central nervous systems (CNS) neurodegenerative diseases including AD. Experimental evidence showed that a change in the micronutrients is also a hazardous factor of AD [7]. At present, there is no definite therapy for AD. The therapies available can protect cognition and memory and delay the loss of function. These years, a growing number of researchers have been focusing on the role of intestinal flora in CNS disorders, especially the regulation over the gut-brain axis. Regulating gut microbiota
with probiotics has been proposed for the prevention and treatment of various diseases, including CNS disorders [8].

The microflora in human’s gastrointestinal tract is human’s biggest microorganism library which consists of at least 1,000 microorganism species and has a microbial population up to 10^{14}. Their diversity and stability vary from individual to individual and the quantity is 100 times that of human’s host cells [9]. The microbiota is a dynamic ecosystem subject to multiple factors including heredity, diet, metabolism, age, geography, antibiotic therapy, and stress [10–12]. Recent evidence indicated a clear link between the change in microbiota and the cognitive behaviors [13]. In addition, some animal researches have revealed the necessity of the microbiota-gut-brain axis for the optimal function of brain activities in behavior and electrophysiology [14]. The microbiota-gut-brain axis is a bidirectional communication system that is connected by the nerve, immune, endocrine, and metabolic pathways [15]. As symbiotic bacteria, probiotics can provide potential health benefits for the host [16]. When enough probiotics are offered, they will interact actively with the endogenous microbiota. Recent studies have shown that gut microbiota plays a key role in regulating the functions of the intestinal tract and the brain. There are preliminary studies that investigated the effects of probiotics on cognitive prognosis [17]. Although a growing body of evidence suggests a close correlation between the change in intestinal flora and the development and progression of AD, no evidence says whether adding probiotics can improve intestinal flora and the development and progression of AD, regardless of gender, were purchased from the Jackson Laboratory Animals formulated by the Ethics Committee of the Second Affiliated Hospital of Qiqihar Medical University. The laboratory mice were kept in plastic cages (33 cm × 15 cm × 13 cm, L × H × W) (Makronol, Covestro a.g., Filago, Italy) (four mice in each) at a temperature around (22 ± 1)°C and 50%–60% relative humidity in lighting conditions characterized by 12 h daylight as well as 12 h in dark (light is on at 7:00 and off at 19:00). All animals were allowed free access to water. Laboratory animals were placed in a room and separated from other animals. The experimental group and the control group were separated to avoid cross-contamination.

The 12-week-old AD mice (n = 24) and WT mice were divided into 2 groups, respectively: the WT + control group (n = 12), the WT + probiotic group (n = 12), the 3xTg-AD + control group (n = 12), and the 3xTg-AD + probiotic group (n = 12).

2.2. Probiotic Diet. The treatment started when the mice were 12 weeks old and lasted for 20 weeks. This experiment aimed at reducing the progression and functional impairment of AD. The probiotic diet-FREAMELIM® [18] (Jouxtens-Mézery, Switzerland) contains lysates of *Bifidobacterium* and *Lactobacillus acidophilus*, vitamins A and D, and w3 fatty acid from cod-liver oil, as well as vitamins B1, B3, B6, B9, and B12. It was given 5 times a week (120 mg/day) and consumed together with ordinary maintenance feed for 20 weeks. We monitored the food intake of all mice every day. The probiotic therapy would not change the animals’ intake of food and water.

2.3. Y Maze Test. After 20 weeks’ treatment, the animals underwent one-week cognitive function tests. During the test period, the animals were continuously given probiotic-supplemented diet. The Y maze (Wanlei Bio) was used to detect mice’s spatial memory. Three arms are at a 120° angle from each other and each arm was 28 cm × 6 cm × 18 cm (length × width × height). Both the underside and the walls of the maze were made of white PVC. The mice were first placed at one of the arms and over the next 8 minutes, the sequence and number of their entry into the three arms were monitored [19]. An alternation is defined when a mouse visits three consecutive arms (namely, ABC, BCA, or CAB, but not CAC, BAB, or ABA). Spontaneous alternation (%) = [(number of alternations)/(total number of arms−2)] × 100.

2.4. Morris Water Maze (MWM). The classical Morris water maze (MWM) experiment was conducted the day following the Y maze test. MWM is one of the classical behavioral methods used to measure spatial memory concerning brain diseases such as AD. The device is a grey round water tank with a diameter of 100 cm and a depth of 35 cm. The water there was kept at a temperature of 23 ± 2°C. The water tank was divided into four quadrants and an escape platform made of organic glass was placed 1 cm below the water surface of any of the quadrants (target quadrant). MWM consists of a 5-day place navigation test and a one-day space exploration test. During the place navigation test phase, each mouse underwent 4 tests to find the underwater platform every day. During the space exploration test phase, the underwater escape platform was removed and each mouse entered the tank randomly from two quadrants. A camera was placed at the top of the maze and connected with EthoVision software (Noldus Information Technology,
Wageningen, Netherlands) to record the movement trajectory of the mouse. In each trial of the place navigation test, the mouse had 60 seconds to swim freely. If the mouse cannot find the escape platform during that period, the experimenter needs to manually guide it to the platform and let it stay 15 s. In the space exploration test, two quadrants were selected randomly to put the mice into the maze and let them swim freely in 60 s. The incubation period, swimming speed, and the swimming time in the target quadrant of each group of mice were recorded.

2.5. 16S rRNA Gene Sequencing for Microbiome

2.5.1. Feces Collection. Feces were collected in a fume cupboard that was disinfected with ultraviolet light beforehand. Freshly collected feces were quickly put into sterile cryogenic vials and then stored in a cryogenic refrigerator.

2.5.2. 16S rRNA Gene Sequencing. Samples were sent to BMKCloud (Wuhan) for detection. The detection basically consisted of the following steps: extracting total DNA from the microbiome, amplifying the target segment, recovering and purifying the amplified products, fluorescent quantification of amplified products, and sequencing.

2.6. Western Blot Experiment. After all the behavioral tests were finished, the animals were anesthetized with pentobarbital sodium (50 mg/kg) before being decapitated for the brain. The hippocampi and the cortexes in the two hemispheres of the brain were separated on ice and then used for ELISA detection and the Western Blot experiment, respectively. The levels of tau (ab210703, 1:1000), p-tau (ab210703, 1:1000), GFAP (ab7260, 1:5000), Iba-1 (ab178846, 1:1000), and β-actin (ab6276, 1:5000) (all the antibodies were from Abcam company) in the hippocampus and the cortex were determined, respectively. The secondary antibodies were from Boster (bm3895 and bm3894). The homogenates from hippocampus and cortex samples of mice were separately added along with 30 mg total DNA to 10% SDS-PAGE gel for separation. They then were electrophorized under constant voltage, transferred onto a membrane, and incubated with specific antibodies for immunoblotting assays using the ECL immunoblotting analysis system. Each gel contains molecular weight markers (10–170 kDa). The band data obtained were processed with ImageJ (NIH) and the background mean and its standard error were calculated.

2.7. ELISA Detection. Aβ1-40 ELISA (Invitrogen, KMB3481) and Aβ1-42 ELISA (Invitrogen, KMB3441) kits for mice were used (according to the manufacturer’s instructions) to detect the concentrations of Aβ1-40 and Aβ1-42 in the hippocampus and cortex samples. A microplate reader was used to measure the optical density (OD value) at 450 nm in each well.

2.8. Statistical Analysis. Data are analyzed with SPSS 22.0 software. The Shapiro–Wilk test was used to evaluate the normality of data. For normally distributed data, nonparametric statistics were adopted. For duplicated data, two-way ANOVA was employed and a post hoc analysis (Bonferroni or Student–Newman–Keuls) was conducted. A P value <0.05 was considered statistically significant. Data are presented as mean ± SEM.

3. Results

3.1. The Probiotic-Supplemented Diet Can Alleviate the Spatial Memory Disorder of 3xTg-AD Mice. To explore the effects of the probiotic-supplemented diet on the spatial learning and memory of 3xTg-AD mice, we conducted the classical Morris water maze experiment and Y maze experiment. The Morris water maze is shown in Figure 1. Figure 1(a) stands for the escape latency of each group of mice in the place navigation test. It can be learned from the figure that the probiotic diet did not affect the escape latency of mice in the WT group. Compared with mice in the WT + control group, those in the 3xTg-AD + control group had an obviously longer escape latency and after the probiotic dietary therapy, the escape latency of 3xTg-AD rats became remarkably shorter (P < 0.05). Figure 1(b) stands for the swimming time of each group of mice in the target quadrant in the space exploration experiment. The swimming time of 3xTg-AD mice treated with the probiotic dietary therapy in the target quadrant was significantly increased (P < 0.05).

The spontaneous alternation accuracy of the mice in the Y maze reflected their spatial working memory. As shown in Figure 2, Figure 2(a) is the sketch map of the Y maze. It can be observed from Figure 2(b) that probiotics had no impact on the spontaneous alternation accuracy of mice in the WT group. Compared with mice in the WT + control group, those in the 3xTg-AD + control group had a lower spontaneous alternation accuracy (P < 0.05). After the treatment with a probiotic diet, the spontaneous alternation accuracy of mice in the 3xTg-AD + probiotic group increased (P < 0.05). Figure 2(c) shows that the total number of arm entries of each group of mice in the Y maze was not statistically different (P > 0.05). The above result suggested that the cognitive ability of the hippocampus in 3xTg-AD mice was severely damaged. The probiotic-supplemented diet can remarkably improve the spatial memory of 3xTg-AD mice and then change their cognitive function.

3.2. The Probiotic-Supplemented Diet Can Improve the Pathological Features of 3xTg-AD Mice. After the end of the behavioral tests, all animals were decapitated for brains and the hippocampus and the frontal lobes were separated quickly on ice. Sandwich ELISA kit was used to detect the levels of soluble and insoluble Aβ40 and Aβ42 in the cortex and the hippocampus. As shown in Figure 3, in the 3xTg-AD + control group, the levels of soluble and insoluble Aβ40 and Aβ42 in the cortex and the hippocampus increased significantly and after probiotics were added to the diet, the levels of Aβ40 and Aβ42 declined significantly (P < 0.05).
The probiotic-supplemented diet can inhibit the hyperphosphorylation of tau in the cortex and hippocampus of 3xTg-AD mice. Phosphorylated Tau S202 antibodies were used for Western Blots on the cortex and hippocampus of four groups of mice. As shown in Figure 4, compared with mice in the WT + control group and the WT + probiotic group, mice in the 3xTg-AD + control group had significantly increased level of Tau S202 in the cortex and hippocampus, and mice fed a probiotic-supplemented diet in the 3xTg-AD + probiotic group had a significantly reduced level of Tau S202 in the cortex and hippocampus ($P < 0.05$).

The probiotic-supplemented diet can inhibit inflammatory responses in the brain of 3xTg-AD mice. We used specific antibodies for astrocyte and microglia to conduct Western Blot experiments on the four groups of mice, respectively. As shown in Figures 5 and 6, compared with mice in the WT + control group and the WT + probiotic group, mice in the 3xTg-AD + control group had severe inflammatory responses in the cortex and hippocampus. The mice in the 3xTg-AD + probiotic group, after the treatment with the probiotic diet, experienced reduced inflammatory responses in the cortex and hippocampus ($P < 0.05$).

### 3.3. Intestinal Flora Change in 3xTg-AD Mice

To investigate the effects of probiotics on the enteric microbiome in 3xTg-AD mice and then change their cognitive function, the fecal samples of mice were measured. The fecal specimens underwent DNA separation and species identification. According to the results, compared with mice in the WT
group, those in the 3xTg-AD group exhibited a decreased diversity in intestinal flora. At the phylum level, as shown in Figure 7(a) and Table 1, compared with mice in the WT group, those in the 3xTg-AD group saw increases in the number of Helicobacteraceae, Desulfovibrionaceae, Coriobacteriaceae, Proteobacteria, Actinobacteria, and Firmicutes and a decrease in the number of Bacteroidetes. And as shown in Figure 7(b) and Table 2, at the genus level, compared with mice in the WT group, those in the 3xTg-AD group saw increases in the number of Odoribacter, Helicobacter, and Lactobacillus and decreases in the number of Ruminococcus, Prevotella, Bacteroides, and Sutterella.

4. Discussion

AD is a complex progressive cognitive disorder with cardinal symptoms including learning disorder, memory loss, and dysphasia [20]. Although many researches have focused on the pathogenesis of AD, the cause of AD remains unknown [21]. Probiotics are live microorganisms. Taking enough probiotics may benefit the health of the
host, especially in preventing and treating AD [22]. At present, the treatment of AD can only alleviate symptoms but cannot prevent or hinder this condition. The intestinal flora plays an important role in regulating multiple neurochemical pathways of the gut-brain axis [23]; therefore, this study is focused on the intestinal flora. Probiotic therapy is becoming a recognized strategy to preventing and treating many diseases including central nervous system diseases [24] and has been proposed to be able to lower the incidence rate of AD [25]. To verify this hypothesis, we added probiotics to the diet of 3xTg-AD mice, further observed changes in cognitive behaviors and pathological feature of AD, and explored the change in intestinal flora of 3xTg-AD mice and WT mice. This study can help offer new strategies for treating AD.

In this study, APP/PS1/tau triple transgenic (3xTg) mice with overexpressed mutants APP (APPSWE), PS1 (PS1M146V), and tau (tauP301L) were used to investigate the effects of a probiotic diet on 3xTg-AD. The research results showed that, compared with mice in the WT group, those in the 3xTg-AD group had a damaged spatial memory, increased levels of soluble and insoluble Aβ40 and Aβ42 in the hippocampus and cortex, and neurofibrillary tangles and inflammatory reactions. However, when given a probiotic diet, the above symptoms of the 3xTg-AD mice were mitigated.

To verify this hypothesis, 3xTg-AD mice underwent the probiotic-supplemented dietary therapy. The Morris water maze experiment showed that the probiotic diet can mitigate the damage of learning memory of 3xTg-AD mice. In the Y
The spontaneous alternation accuracy of mice in the WT group was improved compared to the 3xTg-AD mice. This finding indicates that probiotics have the potential to enhance memory function, which is a crucial aspect of neurocognitive health.

In summary, the study by Savignac et al. [26] demonstrated that daily oral administration of probiotics reversed the damage of working memory on APP/PS1 mice in the Y maze. The study by Bonfili et al. [27] showed that the probiotic-supplemented diet can significantly improve APP/PS1 mice’s spatial memory and then change their cognitive function. The study by Elmira Akbari, AD patients treated with probiotics showed some improvement in MMSE scores [29].

It is known that reducing or eliminating the production of Aβ is an important strategy for the treatment of Alzheimer’s disease [30]. According to research reports, one of the hallmark neuropathological features of AD is the senile plaques, a result of the accumulation of Aβ plaques, a result of the accumulation of Aβ plaques, a result of the accumulation of Aβ plaques, a result of the accumulation of Aβ plaques. As expected, we also found that the probiotic diet reduced the increase in phosphorylation of tau at S202.

Neuroinflammation is also a vital pathological hallmark of AD and is mediated by activated microglia and reactive astrocyte, with Iba1 and GFAP as the markers, respectively [40]. Some studies have shown that Aβ can induce oxidative stress in mice’s brains, activate microglia and astrocyte, and cause neuroinflammation, neuronal damage, and cognitive disorder [41]. As expected, we also found that the probiotic diet is a strong neuroprotective agent that can protect the neurons of 3xTg-AD mice from the erosion of neuroinflammation.

In recent years, a growing number of studies have reported that there exists a close link between intestinal flora and nerve system diseases and it is called the gut-brain axis. The intestinal flora of an AD patient is vastly different from that of a normal person in composition.
Table 1: Data of Figure 7(a).

|        | Helicobacteraceae (%) | Desulfovibrionaceae (%) | Coriobacteriaceae (%) | Proteobacteria (%) | Actinobacteria (%) | Fusobacteria (%) | Verrucomicrobia (%) | Firmicutes (%) | Bacteroidetes (%) | Others (%) |
|--------|-----------------------|-------------------------|-----------------------|-------------------|-------------------|-----------------|---------------------|---------------|-------------------|------------|
| WT     | 0.01                  | 0.05                    | 0.02                  | 0.002             | 0.01              | 0.001           | 0.001               | 0.5           | 0.22              | 0.31       |
| 3xTg-AD| 0.15                  | 0.15                    | 0.19                  | 0.04              | 0.1               | 0.002           | 0.001               | 0.15          | 0.35              | 0.01       |
which indicates that the AD patient’s composition of intestinal flora may be involved in the development of aβ amyloidosis in the brain.

5. Conclusion

In order to explore the change in and composition of the intestinal flora of the 3xTg-AD group, we determined the bacterial community in the fecal specimen with the 16S rRNA sequencing technique. The detection results showed a decreased α-diversity, a total statistical index of 3xTg-AD mice. Compared with mice in the WT group, those in the 3xTg-AD group had seen increases in the number of Helicobacteraceae, Desulfovibrionaceae, Coriobacteriaceae, Actinobacteria, and Firmicutes and a decrease in the number of Bacteroidetes; and increases in the number of Odoribacter, Helicobacter, and Lactobacillus and a decrease in the number of Prevotella. Those changes were consistent with what have been discovered in AD patients [42]. The change in intestinal flora may be linked with the cognitive impairment, pathological manifestations, and inflammatory responses observed in 3xTg-AD mice. It is worthy of further study.

In a word, the probiotic-supplemented diet can improve 3xTg-AD model mice’s cognitive function and reverse pathological manifestations of AD and the pathological changes of AD and the cognitive disorder are linked with the change in intestinal flora. The study reinforces the idea that the probiotic therapy can prevent and treat AD and is worthy of future investigation.

Data Availability

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethical Approval

The study was approved by the Ethics Committee of the Second Affiliated Hospital of Qiqihar Medical University.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

CT, YL, and WL led the conception and design of this study. CT, HZ, CD, DX, CL, NZ, and BH were responsible for the data collection and analysis. YL, HZ, DX, and CL were in charge of interpreting the data and drafting the manuscript. CT, CD, and WL made revision from critical perspective for important intellectual content. The final version was read and adopted by all the authors.

Acknowledgments

This study was supported by Joint Guidance Project, Qiqihar Science and Technology Plan (LHYD-202056), and Scientific Research Project of Heilongjiang Education Department (2016-KYYWF-0853).

References

[1] Y. Dong, X. Li, J. Cheng, and L. Hou, “Drug development for alzheimer’s disease: microglia induced neuroinflammation as a target?” International Journal of Molecular Sciences, vol. 20, no. 3, p. 558, 2019.
[2] F. Kumfor, D. Teo, L. Miller et al., “Examining the relationship between autobiographical memory impairment and carer burden in dementia syndromes,” Journal of Alzheimer’s Disease, vol. 51, no. 1, pp. 237–248, 2016.
[3] M. F. Mendez, “Early-onset alzheimer disease and its variants,” Continuum: Lifelong Learning in Neurology, vol. 25, no. 1, pp. 34–51, 2019.
[4] T. Tyler Patterson and R. Grandhi, “Gut microbiota and neurologic diseases and injuries,” Advances in Experimental Medicine & Biology, vol. 1238, pp. 73–91, 2020.
[5] C. de la Torre and V. Ceña, “The delivery challenge in neurodegenerative disorders: the nanoparticles role in alzheimer’s disease therapeutics and diagnostics,” Pharmaceutics, vol. 10, no. 4, p. 190, 2018.
[6] A. Bernaus, S. Blanco, and A. Sevilla, “Glia crosstalk in neuroinflammatory diseases,” Frontiers in Cellular Neuroscience, vol. 14, p. 209, 2020.
[7] M. Taghizadeh, S. A. Talaei, A. Djazayeri, and M. Salami, “Vitamin D supplementation restores suppressed synaptic plasticity in Alzheimer’s disease,” Nutritional Neuroscience, vol. 17, no. 4, pp. 172–177, 2014.
[8] V. Castelli, M. d’Angelo, M. Quintiliani, E. Benedetti, M. G. Cifone, and A. Cimini, “The emerging role of probiotics in neurodegenerative diseases: new hope for Parkinson’s disease?” NeuroRegen Res, vol. 16, pp. 628–634, 2021.
[9] W. J. Lukiw, “Gastrointestinal (GI) tract microbiome-derived neurotoxins-potent neuro-inflammatory signals from the GI tract via the systemic circulation into the brain,” Frontiers in Cellular and Infection Microbiology, vol. 10, no. 22, p. 22, 2020.
[10] M. R. Hufeldt, D. S. Nielsen, F. K. Vognesen, T. Midtvedt, and A. K. Hansen, “Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors,” Comparative Medicine, vol. 60, pp. 336–347, 2010.
[11] I. Cho, S. Yamanishi, L. Cox et al., “Antibiotics in early life alter the murine colonic microbiome and adiposity,” Nature, vol. 488, pp. 7413, pp. 621–626, 2012.
[12] D. Drago, M. Toscano, V. Rodighiero, E. De Vecchi, and G. Mogna, “Cultivable and pyrosequenced fecal microflora in...
