Gut Dysbiosis Activates C/EBPβ/AEP Pathway in Alzheimer's Disease Mouse Model

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Abstract. The mechanisms of developing Alzheimer's disease (AD) under the influence of gut microbial composition are intensively studied currently. However, the specific mechanism remains unknown. One recent study conducted by Chen et al. provided a breakthrough for the underlying mechanism of gut dysbiosis in contributing to the disease and showed the efficacy of two potential treatments in mouse models. Based on their study, gut dysbiosis occurred in transgenic mice increased the C/EBPβ/AEP signaling of the brain and the gut as aging, which increased the amount of truncated β-amyloid precursor protein and Tau. And the level of brain-derived neurotropic factor or BDNF was reduced significantly in the gut and the brain of aged transgenic mice. It implied that both genetic and gut microbial composition play roles in developing Alzheimer's disease. Besides, antibiotic treatments and prebiotic R13 were performed. Researcher mitigated the pathologies and phenotypical symptoms. But only R13 treatment upregulated BDNF in the brain. Some experimental methods of the study could be improved to provide more useful information including the effect in female mice. In general, the original paper provided a logical procedure or outline for the study and introduced promising treatments in AD. Some questions remain including the mechanism of gut microbiota alteration in mediating the gut and the brain BDNF production is unclear. Based on this, hypothetical experiments are designed in the review.

Keywords: Gut dysbiosis; Alzheimer's disease; C/EBPβ/AEP; BDNF; treatments.

1. Introduction

AD is one of the progressive neurodegenerative diseases. Two major pathological hallmarks have been identified in AD. They are aggregated polypeptide β-Amyloid 42 and phosphorylated Tau [1]. Both environmental and genetic factors can modify gut microbiota composition and raise the chance of getting AD [1]. However, the relationship between gut dysbiosis and the critical molecular mechanism remains unknown. Some scientists believed that specific microbes contribute to the progression of AD pathologies [2]. However, the potential pathological microbes are unclear. In Chen et al.'s paper, the researchers used transgenic mice to study the role of gut microbiota in developing AD [3]. Based on the stool samples, aged transgenic mice had gut dysbiosis and high C/EBPβ/AEP activities. After cohousing aged transgenic mice and young transgenic mice, the young transgenic mice showed less gut microbiota diversity and more C/EBPβ/AEP signaling than the controls. Besides, antibiotic and R13 treatments in aged transgenic mice altered gut microbiota composition and improved AD condition. Only R13 treatment induced Lactobacillus salivarius elevation and upregulated BDNF.

Daily diet, hygiene, and antibiotics can affect gut microbiota composition and influence the cognitive functions in human body. Some evidence implied that gut microbiotas contribute to AD pathologies. One study found reductions in Firmicutes and Actinobacteria in aged transgenic mice compared to the control, while the young transgenic mice remained unchanged [4]. Based on the study, amyloid-β levels were lower in germ-free transgenic mice compared to conventional raised transgenic mice. Another study used fecal samples from humans instead of rodents. This study showed that AD patients had lower gut microbial diversity, fewer Firmicutes, and more Bacteroidetes [5].

Besides studying the role of gut microbiota in developing AD, CCAAT-enhancer binding protein β (C/EBPβ) has also been studied in recent decades. C/EBPβ regulates inflammatory mediators when it's activated [3]. One previous study used immunoblot to show a higher increase of C/EBPβ level in

AD tissues compared to nondemented elderly control [6]. Similarly, another study observed a higher level of C/EBPβ in the patient's cortex and microglial cultures [7]. Besides, one study reported that C/EBPβ regulates asparagine endopeptidase (AEP) gene transcription [8]. And they found that AEP cleaved both APP and Tau, which contributes to AD pathologies.

Several studies have shown gut microbiota play roles in AD pathology. And several studies have discovered the levels of C/EBPβ elevated in AD. But there are no studies on the relationships between gut dysbiosis and C/EBPβ/AEP in AD. In the paper, Chen et al. provided evidence that gut dysbiosis is correlated with activations of the C/EBPβ/AEP pathway in aged transgenic mice. By using R13 and antibiotic treatments, they observed gut microbiota alteration and inhibition of the C/EBPβ/AEP pathway in transgenic mice. Antibiotic treatment improved cognitive functions, while R13 induced L. salivarius and alleviated gut leakage. This review summarizes the major findings and experimental methods of Chen et al.'s paper, analyzes the paper, and discusses future direction based on the study.

2. Methods and Major Results

2.1. Visual Summary

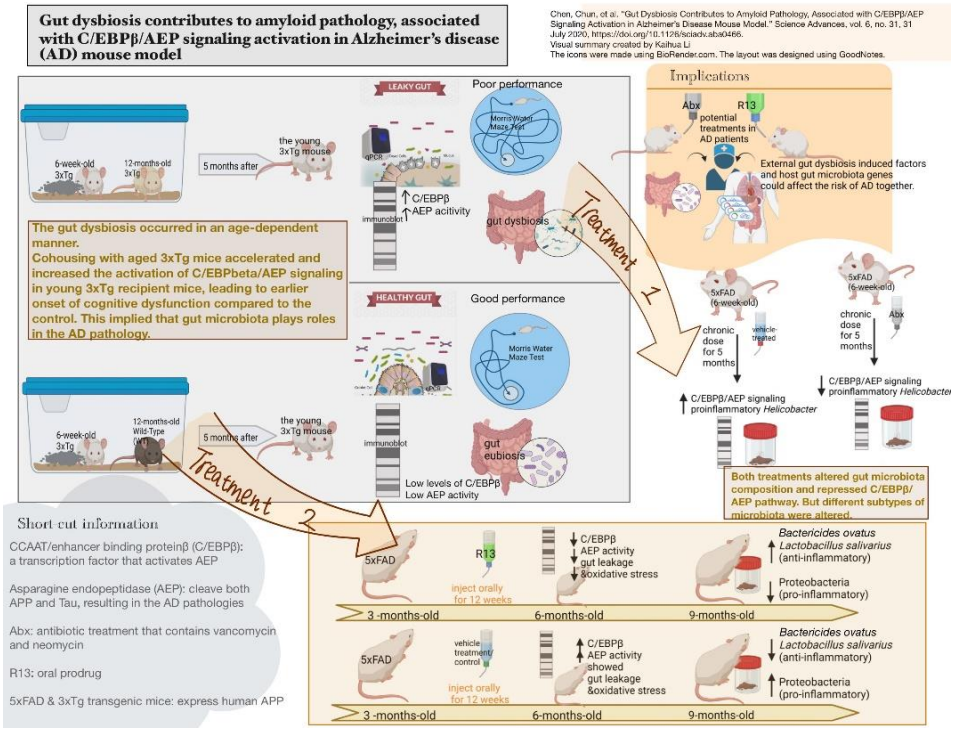


Fig. 1 The major experiments and findings of the study [3]

The top left panel of Figure 1 showed the effects of cohousing transgenic young mice with either wild type aged mice or transgenic aged mice. The top right panel showed the effects of transgenic young mice after receiving either chronic vehicle treatment or antibiotic treatment. The bottom panel showed the effects of young transgenic mice after receiving either vehicle treatment or R13 probiotic treatments.

2.2. Written Summary

2.2.1 Aging promoted the C/EBPβ/AEP signaling

The authors examined the large intestines of both 5xFAD and wild-type mice of different ages. By applying microtubule-associated protein 2 staining, they found that the green fluorescence signals decreased as mice aged, indicating loss of enteric neurons (see Figure 2). Besides, fluorescence signals for C/EBPβ and AEP increased as mice aged (see Figure 2).

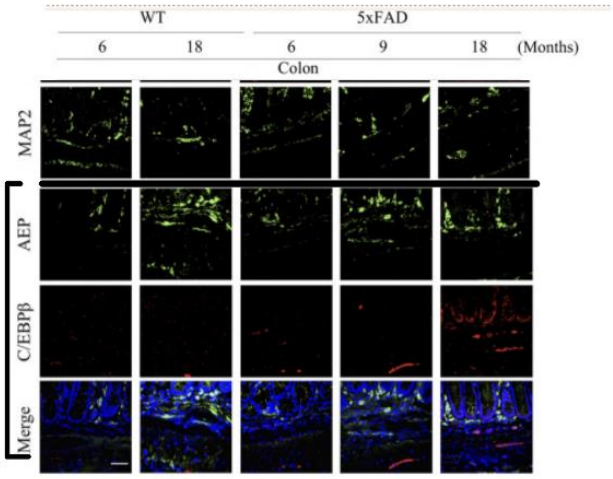
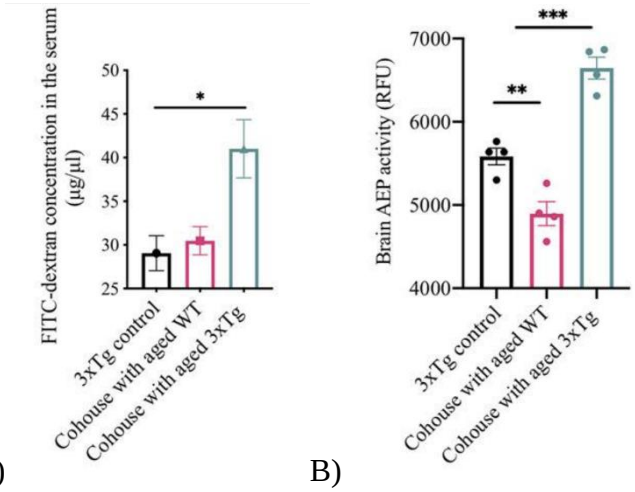


Fig. 2. Different immunofluorescence staining in 5xFAD and WT mice [3]

Three immunofluorescence staining were shown. The intensity of fluorescence indicated the number of proteins present [3].

2.2.2 Gut dysbiosis promote the C/EBPβ/AEP activity in the brains

Furthermore, they showed that the pathway was also activated in the mice's brains. The authors randomly picked young transgenic or 3xTg mice and put them to live either with aged 3xTg mice (experimental group) or aged wild-type mice (control). After 5 months, experimenter extracted DNA from young 3xTg mice's stool and used PCR to amplify the bacterial genes. Only in the experiment group, Firmicutes diversity was reduced, and Bacteroidetes diversity was enhanced. These mice also showed gut leakage due to higher fluorescein isothiocyanate (FITC)-dextran concentration than control (see Figure 3A). Because FITC-dextran can cross epithelium barrier of the gut, FITC-dextran fluorescence was performed to study gastrointestinal tract permeability. Higher FITC-dextran concentration means gut leakage as more FITC-dextran cross the barrier. Besides, western blot revealed higher levels of C/EBPβ, AEP (see Figure 3B), and truncated APP and Tau. The findings implied a higher C/EBPβ/AEP activity in young transgenic mice received dirty bedding from aged transgenic mice.



A) B)

Fig. 3. FITC-dextran concentration and Brain AEP Activity of Each Group [3]

A) FITC-dextran concentration in mice cohoused with aged transgenic or 3xTg was significantly higher (42 μg/μl> 31μg/μl) than mice cohoused with aged wild-type [3]. The difference between the control group and mice cohoused with aged wild type was not significant [3]. B) AEP activity is higher (6800 RFU> 4900 RFU) in young transgenic mice cohoused with aged transgenic mice [3].

In the Morris water maze (MVW) test, experimenters trained young 3xTg mice to find an invisible platform in a circular pool. After training, they presented a probe trial and calculated the percentage

of time. According to the data (see Figure 4), young 3xTg mice in the experimental group spent more time looking for the target than the controls. This observation implied severe cognitive defects including spatial memory and learning in these mice.

Results suggested that gut microbiota alteration in young transgenic mice promoted C/EBPβ/AEP activation, leading to earlier onset of cognitive defects.

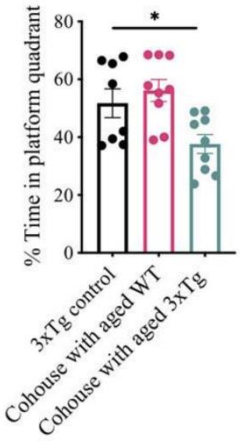


Fig. 4. The duration in platform quadrant of each group [3]

The percentage time in platform quadrant of mice cohoused with aged transgenic mice was significantly lower (35%< 55%) than mice cohoused with aged wild type mice [3].

2.2.3 Effects of antibiotic and prebiotic R13 treatments in C/EBPβ/AEP signaling

Antibiotic treatment or ABX contained ampicillin, vancomycin, and neomycin. In the experimental group, young 5xFAD male mice were treated ABX for 5 months. In the control group, young 5xFAD male mice drank water without ABX for 5 months. Immunoblotting showed reduced levels of C/EBPβ and AEP in the ABX-treated group (see Figure 5). Besides, anti-Aβ staining showed less fluorescence in ABX-treated mice (Figure 6), revealing that extracellular senile plaques were reduced. Results implied that the ABX decreased C/EBPβ/AEP activity, which reduced AD pathologies. In the MVW test, 5xFAD mice found the escape platform faster than the control, suggesting that the spatial memory and learning skills improved after ABX treatment.

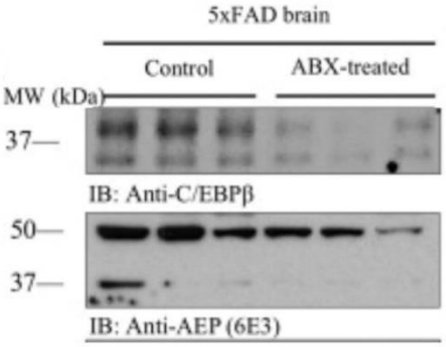


Fig. 5. Western blot analysis: comparison between ABX-treated and control group [3]

The intensity of anti-C/EBPβ and anti-AEP in the gel decreased significantly after ABX treatment compared to the control [3].

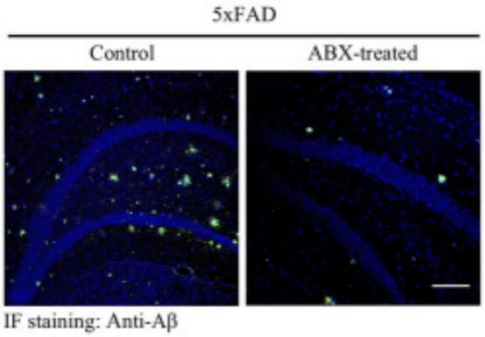


Fig. 6. Immunofluorescence co-staining with Anti-Aβ [3]

Figure 6 showed the intensity of fluorescence decreased significantly after ABX treatment compared to the control [3]. Previous studies showed 7,8-DHF against Alzheimer's disease [9]. 7,8-DHF has similar functions as brain-derived neurotrophic factor (BNDF). The researchers created a prodrug R13 that can be converted into 7,8-DHF. 3-month-old 5xFAD mice received R13 orally for 3 months in the experiment group. No R13 solution was treated in the control. Based on western blot and immunofluorescence staining assay, the amount of C/EBPβ, AEP, and truncated APP and Tau was reduced in R13-treated 5xFAD mice (see Figure 7). Besides, FITC-dextran concentration was decreasing in a dose-dependent manner (see Figure 8), meaning less gut leakage after treatment. Moreover, anti-BDNF staining revealed the escalation of BDNF in the mice's brains, implying BDNF upregulation by the treatment. Based on stool samples from 5xFAD mice, it revealed an increased abundance of anti-inflammatory Firmicutes and *L. salivarius,* and a reduction in pro-inflammatory *Helicobacter* in R13 treated mice.

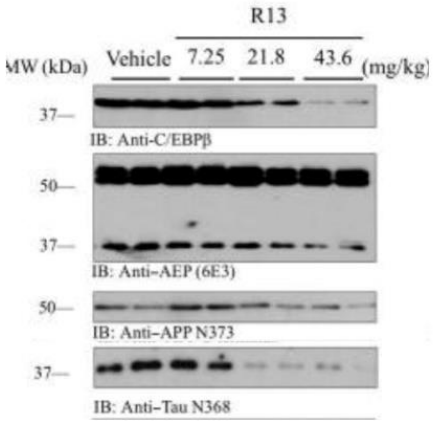


Fig. 7. Western blot analysis: comparison between R13-treated and control group [3]

The intensity of anti-C/EBPβ, anti-AEP, anti-truncated APP and anti-truncated Tau in the gel decreased significantly after ABX treatment compared to the control [3].

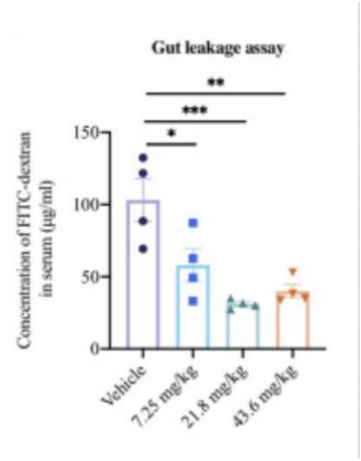


Fig. 8. FITC-dextran concentration of the control and R13-treated group [3]

FITC-dextran concentration was lower in R13-treated mice than the control. The level of FITC-dextran concentration reduced was positively correlated with the amount of R13 added [3]. Like some previous studies, the results showed gut microbiota alterations in AD. Moreover, the pattern of C/EBPβ was similar with previous studies. Studying the relationship between gut dysbiosis and C/EBPβ/AEP signaling provided a new horizon for future studies to confirm.

3. Discussion

In the gut of mouse model, C/EBPβ/AEP activity was increased as aging. Moreover, the pathway was accelerated by gut dysbiosis in the brain of transgenic mice, which caused cognitive dysfunctions. Antibiotic and R13 treatments can repress this pathway and mitigate AD pathology. R13 specifically increased the level of L. salivarius in the gut and upregulated brain BDNF. Human symbiotic microbes play roles in digestion, inflammation, and against infection [4-10]. Disruption of the human gut microbiome affects human health. Therefore, it is essential to understand how gut dysbiosis contributes to neurological diseases, especially AD. After finding the mechanism, studying ways to reverse the adverse effects is vital to treat AD patients in the future.

A past experiment showed that AD patients had lower gut microbial diversity, fewer Firmicutes, and more Bacteroidetes [5]. Another study found that gut dysbiosis is only present in aged transgenic mice, not the young [4]. Chen et al. combined them into one conclusion by examining fecal samples of transgenic mice of different ages. Most previous studies focus on gut dysbiosis and amyloid formation. For example, a study found that microbes can release many amyloids in aging people, contributing to the release of inflammatory cytokines [11]. However, Chen et al. opened a new field to study the relationship between gut microbiota and molecules beyond amyloid.

One study showed that ablation of the C/EBPβ-LIP upstream gene improved mouse models' health problems and extended lifespan [12]. Nevertheless, this study did not consider the role of gut microbiota. Chen et al. supported that the C/EBPβ/AEP pathway can be regulated by gut microbiota. They also applied treatments to prove the pathway can be eliminated, and AD pathology can be alleviated through changing gut microbiota composition. The observations could potentially be new breakthroughs. Besides, specific microbe L. salivarius and BDNF were found to play important roles in AD potentially. A review article mentioned Lactobacillus can convert glutamate into GABA [13]. And GABA signaling plays roles in neurogenesis. Its alteration may cause cognitive dysfunction and AD. In the R13 treatment experiment, Chen et al. found elevation of L. salivarius, which helped improve gut leakage. This finding suggested the positive role of L. salivarius in modulating AD pathology.

The authors combined two conclusions from two studies into one experiment. They proved the conclusions and added their new finding, C/EBPβ/AEP pathway [4, 5]. Furthermore, they used antibiotic and R13 treatments to show that the gut microbiota alteration causes C/EBPβ/AEP pathway changes. Chen et al. reported an increase diversity of Bacteroidetes and a decrease diversity of

Firmicutes in aged transgenic mice. However, one paper reported the opposite [14]. The exact reasons are unclear. A potential consideration could be different individuals have different gut microbial ratios and compositions [14]. And specific microbes may play a more prominent role than others in mediating AD pathologies. For instance, Chen et al. found L. salivarius is a subtype of Firmicutes, which is shown to mediate gut leakage and the C/EBPβ/AEP pathway.

In Chen et al.'s paper, some elements during experimental procedures may lead to controversy of their results. The incidence of developing AD is higher in females than in males. Females have estrogen compounds against mitochondrial toxicity, but it is declining with aging [15]. However, the authors only used male mice for antibiotic and probiotic treatments. This could show less efficacy and cause more adverse effects if the treatments are applied in females. One study indicated that long-term antibiotic treatment altered circulating peripheral inflammatory mediator levels and reduced Aβ deposition in only males [16]. Therefore, the researchers should use both sex mouse models to conduct the experiments. Besides, the experimenters only used the MVW test to evaluate cognitive function in transgenic mice. A journal article pointed out transgenic mice are more anxious than wild-type mice and lack orientation [17]. Thus, transgenic mice prefer to swim near the wall and have more physical contact, which is thigmotaxis. Prolonged training may not be sufficient to eliminate the effect [17]. Furthermore, swimming navigation behavior differs in different mice, affecting swimming speed [17]. The two noncognitive factors can affect the statistical data. Y Maze Spontaneous Alternation could be a better way to quantify cognitive deficits in transgenic mice. Typically, healthy mice prefer to walk around different new places. Based on the percentage of alternation, the cognitive functions of mice can be tested. In this test, the two noncognitive factors can be eliminated.

In general, Chen et al. reported different experiments logically. After the R13 treatment experiment, they found that BDNF levels increased in the brain. However, it remains unclear about the specific mechanisms of gut composition alteration mediating the brain BDNF levels, or which specific microbes cause the changes in BDNF levels in both the gut and the brain. The current study found that the levels of BDNF were reduced in both gut and the brain of the transgenic mice by gut dysbiosis. And R13 increased BDNF level in the brain but didn't mention the level changes in the gut. Gut microbiotas secrete amyloids and lipopolysaccharide, which modulates pro-inflammatory cytokines [3]. Pro-inflammatory cytokines including IL-6 and TNF-alpha may activate C/EBP family members [18]. Besides, one study found that the level of BDNF was inversely correlated with C/EBPβ levels in PD patients [19]. They showed that C/EBPβ repressed BDNF mRNA transcription by binding to the promoter of BDNF gene. But the experiment is done in PD mouse model instead of AD organism model. Presumably, gut dysbiosis can release specific proinflammatory cytokines, which activates C/EBPβ. It leads to the repression of BDNF transcription in the gut of AD patients. At the same time, the proinflammatory cytokines may travel through the vagus nerve and reduce BDNF transcription in the brain.

Based on this assumption, potential experiments could be conducted, and hypotheses could be made to study the mechanism. After R13 treatment, deduction of pro-inflammatory microbes reduces the level of pro-inflammatory cytokines including IL-6 and TNF-alpha that play roles in activating C/EBPβ in the gut and the brain of AD mouse model, which decreases the level of repression on the BDNF transcription. This hypothesis could be tested via several experimental methods. Create five transgenic AD mice of both sexes. Inject R13 into the gut of four transgenic female and male mice and phosphate-buffered saline in the gut of one female and male transgenic mouse. Collect fecal samples in both groups and extract DNA after purification. Then, use PCR and sequencing to analyze the specific changes in gut microbiota composition. Use immunofluorescence staining to observe and compare the level of IL-6 and TNF-alpha between the control and R13-treated group. Apply immunofluorescence staining for BDNF and C/EBPβ in the gut and the brain to observe the level changes. The most significant reduction of specific pro-inflammatory microbes and cytokines should be recorded. Expected results are reduction in the level of pro-inflammatory cytokines, C/EBPβ, and escalation in the level of BDNF in the gut and the brain of the transgenic mice after R13 treatment. Chromatin immunoprecipitation can be performed to study the C/EBPβ and BDNF gene interaction

in the gut and the brain before and after treatments in both groups. And perform PCR to observe the intensity of the band that indicates the level of BDNF transcription. If the level of BDNF transcription is low before the R13 treatment but increases after the treatments, it indicates the repression mechanism on BDNF transcription by C/EBPβ in AD mice model. The results between sexes should also be compared. If expected results are observed, the experiments can show that specific microbe's reduction that released specific pro-inflammatory can increase the BDNF production in the gut and the brain of AD transgenic mice after R13 gut injection.

4. Conclusion

In recent years, many scientists have been trying to figure out the mechanisms of gut microbiota alteration in developing AD. Although it remains unclear, much progress has been made. This review focuses on one of the newest published articles to summarize the major findings, compare the different results between different studies, discuss the significance of the study, and design potential experiments based on the study. Chen et al.'s study showed the gut dysbiosis contributes to the AD pathologies by mediating the C/EBPβ/AEP signaling. What's more, R13 probiotic treatment and antibiotic treatments were performed to study the effects on gut microbiota alteration in AD pathologies by repressing the C/EBPβ/AEP signaling. The study provided a significant relationship between gut dysbiosis and C/EBPβ/AEP pathway, which filled the gap of the previous studies. Besides, R13 probiotic treatments upregulated brain BDNF level and increased specific anti-inflammatory microbes such as L. salivarius compared to antibiotic treatments. However, the study didn't mention the BDNF level changes in the gut after R13 treatments. And the mechanisms of gut microbiota alteration in mediating gut and brain BDNF levels are unclear based on the results study. Some studies have studied the relationship between C/EBPβ and BDNF in PD mouse models. And some studies have studied the relationship between cytokines and C/EBPβ. But there are no previous studies that studied the specific mechanism of gut microbiota alteration in mediating BDNF level after R13 treatments. Therefore, experiments to test the hypothetical mechanism are designed in this review. Future studies could be conducted, which would help scientists to understand more about treating patients with Alzheimer's disease.

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