Exploring Probiotics and Fecal Microbiota Transplantation: Two Potential Therapeutics for Alzheimer’s Disease in a Mouse Model

OVERALL AIMS

Alzheimer’s disease (AD) is a neurodegenerative illness characterized by cognitive decline and memory loss. Pathologically, AD is characterized by amyloid (Aβ) plaques, formed by the abnormal cleavage of Amyloid Precursor Protein (APP) and neurofibrillary tangles. Current treatments do not target the underlying causes of AD, and thus are not highly effective in slowing disease progression. One promising therapeutic area is the gut microbiome, which consists of the microorganisms within the gastrointestinal tract. Recently, the gut-brain axis, a bidirectional signaling pathway between the central nervous system and enteric nervous system, has been implicated in brain diseases (Maiuolo et al., 2021). Several lines of evidence point to the role of the gut in AD. Namely, significant dysbiosis in microbial composition has been found in patients with AD, autism spectrum disorder, Parkinson’s disease, and Multiple Sclerosis (Umbrello and Esposito, 2016; Vogt et al., 2017). Similarly, reduced Aβ pathology was observed in germ-free transgenic mice, suggesting the involvement of the microbiota in Aβ buildup (Harach et al., 2017). Taken together, novel treatments that target the gut microbiome should be seriously considered.

Two potential microbiome-targeted interventions are probiotic administration and fecal microbiota transplantation (FMT). Aim 1 investigates the specific mechanisms of probiotics on AD in a mouse model. Multiple studies reveal beneficial effects of probiotics for AD, in both mouse models and clinical studies (Leblhuber et al., 2018; Xiao et al., 2020). We investigate *Bifidobacterium breve* (*B. breve*), as studies have examined its use in restoring cognitive impairment (Kobayashi et al., 2017; Xiao et al., 2020). However, not much is known about the mechanisms in which probiotic strains relieve disease progression. Thus, these experiments will help elucidate how probiotic bacteria affect three pathways: neurogenesis, neuroinflammation, and neurotransmitter production. Aim 2 explores the efficacy of FMT in an AD mouse model. FMT is a novel approach that has been investigated for psychiatric disorders and colonic diseases, but less so for AD (Chinna Meyyappan et al., 2020). Compared to probiotics, FMT takes a more holistic approach, as the gut is treated as a delicately balanced community of microorganisms. Stool samples from a healthy individual are transplanted into the intestines of a patient to restore gut balance. Overall, the proposed aims explore the relationship between the gut microbiome and AD, as well as investigate and compare the therapeutic effects of probiotics and FMT as potential treatments.

SPECIFIC AIMS

**Aim 1: To elucidate the therapeutic mechanisms of probiotics by administering *B. breve* in an AD mouse model.** This aim examines the avenues through which *B. breve* plays a role in AD pathology. These mechanisms include (1) neurogenesis, (2) neuroinflammation, and (3) neurotransmitter production. After oral administration of *B. breve* to transgenic mice, immunofluorescence staining of choline acetyltransferase (ChAT) and neuronal nuclear protein (NeuN) will quantify hippocampal neuronal growth. BDNF levels will be visualized using western blot analysis and immunofluorescence to measure synaptic plasticity. To explore neuroinflammation, interleukin-1β (IL-1β) and Tumor Necrosis Factor-alpha (TNF-α) will be measured using ELISA. γ-aminobutyric acid (GABA) levels in the brain will be measured using high-performance liquid chromatography (HPLC) to explore the role of probiotics in producing neurotransmitters. To quantify amyloid burden post-treatment, we will use Congo red staining. Lastly, various behavioral tests will assess cognitive function. Together, these experiments will elucidate possible mechanisms by which probiotic strains such as *B. breve* alleviate AD symptoms.

**Aim 2: To assess the effects of FMT on amyloid burden, microbial beta-diversity, and cognitive function in an AD mouse model.** This aim tests the hypothesis that FMT will interact in similar ways as probiotics and serve as a more effective AD treatment. Methods in Aim 1 will be replicated to facilitate direct comparison between probiotics and FMT. Additionally, 16s rRNA sequencing will be used to measure beta diversity of AD versus wild-type microbiota to explore which bacterial genera are up- and down-regulated in AD and which genera are directly affected post-FMT treatment.
RESEARCH STRATEGY

(a) Significance

AD is the leading cause of dementia, whose global prevalence is estimated to be 24 million and is expected to double every 20 years until 2040 (Mayeux and Stern, 2012). Thus, it is crucial to find an effective, targeted treatment for our aging population. Unfortunately, progress in drug development for AD is slow. In fact, today there is only one FDA-approved, disease-modifying therapy for AD called Aducanumab, a drug that targets Aβ plaques, but even so, its therapeutic effects are controversial (Knopman et al., 2021). Thus, novel therapeutic pathways must be explored.

Undeniable evidence of the gut microbiome’s role in AD is accumulating, but developing a treatment standard is difficult without fully understanding the mechanisms of certain bacteria in AD pathology. By evaluating the effects of *B. breve* on neurogenesis, neuroinflammation, and neurotransmitter production, this project aims to not only elucidate which pathways are being disrupted in AD, but also to guide future treatment development using this knowledge. While probiotic administration is one potential option, challenges in dosage, interactions between the probiotics and native flora, and strain type generally yield inconsistent efficacies in practice (Kothari et al., 2019). Moreover, probiotic ingestion may produce harmful side effects for those with medical conditions, such as excessive immune stimulation, horizontal gene transfer of antibiotic resistance to pathogenic bacteria, damaging metabolic activity, and even sepsis (Kothari et al., 2019). Nonetheless, probiotic administration research is important for understanding how certain strains affect specific pathways in AD pathology. Thus, the second half of the proposed research investigates FMT, which may lend itself to a more practical clinical solution for AD. This method is considered the most powerful intervention in regards to restoring the gut microbiome, since it undergoes stringent clinical trials and is disease-specific (Khoruts, 2018). Promisingly, FMT has recently been shown to yield a rapid improvement in AD symptoms in one AD patient who underwent FMT after a colonic infection (Hazan, 2020).

(b) Innovation

The applications of this research challenge the current paradigm of AD treatment development and may aid in diagnosis and preventative measures. Presently, treatments only manage AD symptoms rather than target the underlying causes. Since the gut is involved in several pathways that are disrupted in AD, intervening in the gut microbiome as a possible treatment appears promising. These pathways include neurogenesis, neuroinflammation, and neurotransmitter production (Cryan et al., 2019). Moreover, because gut dysbiosis is observed prior to the onset of typical pathological AD features, using the gut as a source of biomarkers for diagnosis can help early diagnosis that is less invasive than current diagnosis techniques (Chen et al., 2020). Specifically, since Aim 2 will allow us to identify specific bacteria that are overabundant or deficient in AD, quantifying these bacterial levels in patients could be used alongside other diagnosis methods to confirm the presence of AD.
(c) Approach

Background

Alzheimer’s disease. AD is a progressive, neurodegenerative illness characterized by Aβ plaques, neurofibrillary tangles, dementia, and cognitive impairment. Apart from the distressing emotional burdens of caring for those with AD, healthcare costs for AD patients total $355 billion in the United States alone (Alzheimer’s Association Report, 2021). With the unprecedented growth in the aging population, the need to find novel treatments is a top public health priority.

The gut-brain axis and AD. The gut microbiome consists of trillions of diverse microorganisms living in the gastrointestinal tract (Santiago and Potashkin, 2021). Bidirectional communication between the microbiome and the human host occurs through the autonomic, enteric, and neuroendocrine systems and has been implicated in neural pathway activation and brain chemistry (Santiago and Potashkin, 2021). Other than aiding digestion, the microbiome is involved with brain function, the immune system, and inflammation of the human host (Santiago and Potashkin, 2021). Because of this, the gut-brain axis is implicated in AD and other neurodegenerative diseases (Fung et al., 2017).

Accumulating evidence points to the connection between the microbiome and AD. For example, one study demonstrated that Escherichia coli produces amyloid fibers and can regulate the buildup of amyloid (Chapman et al., 2002). Other studies have highlighted that the use of antibiotics can provide neuroprotective, anti-inflammatory, and anti-amyloid effects, possibly due to reducing dysbiosis in the gut microbiome (Angelucci et al., 2019). Most notably, the taxonomic composition of gut microbiomes of AD patients is significantly different from non-AD individuals (Vogt et al., 2017). These differences include decreased microbial diversity and varying abundances of bacterial genera, like increased Bacteroides and decreased Bifidobacterium populations (Vogt et al., 2017). Because of the lower abundance of Bifidobacterium in AD, along with the beneficial cognitive effects of probiotic administration of Bifidobacterium in AD mouse models (Kobayashi et al., 2017; Kim et al., 2021), this proposal aims to investigate the specific mechanisms of Bifidobacterium using the species B. breve.

Neurogenesis and the gut. Adult hippocampal neurogenesis (AHN) is vital for many brain functions such as memory, plasticity, and learning (Kempermann et al., 2004). AD greatly affects the hippocampus, as AD causes its shrinkage and leads to memory impairment. Studies illustrate that AHN is severely disrupted in AD patients (Moreno-Jiménez et al., 2019). Interestingly, gut bacteria have been shown to be able to regulate adult neurogenesis. One study found germ-free mice that lack all gut microorganisms have increased AHN, suggesting the involvement of the microbiota in neurogenesis (Ogbonnaya et al., 2015). On the other hand, another study found that antibiotics decreased AHN, but this could be restored by probiotic treatment (Mohle et al., 2016). Thus, our experiments investigate if and how Bifidobacterium can rescue the neurogenesis deficits present in AD.

Neuroinflammation and the gut. While AD is generally thought of to be a neurological disease, its pathology has strong ties to the immune system and microglia. Over-activation of immune pathways
leads to sustained exposure to pro-inflammatory cytokines produced by microglia, which in turn results in neurodegeneration (Heneka et al., 2015). Several lines of evidence reveal the central role of the immune system in AD. First, network analysis found that the immune/microglia pathway had the strongest association with AD pathophysiology (Zhang et al., 2013). In addition, many AD risk genes are selectively and highly expressed by microglia, with aberrant microglial responses to Aβ plaques being linked to AD (Hansen et al., 2018). Various epidemiological studies also point to associations between inflammatory bowel disease (IBD) with dementia risk and AD development (Santiago and Potashkin, 2021). Lastly, overactivation of microglia results in greater levels of pro-inflammatory cytokines such as TNF-α and IL-1 (Heneka et al., 2015). This, in turn, disrupts the phagocytosis abilities of microglia and thus the clearance of Aβ plaques in AD (Heneka et al., 2015). Notably, one study found significantly elevated levels of IL-1 β and TNF-α in the hippocampus in an AD rat model, while probiotic intervention significantly decreased these levels (Mehrabadi and Sadr, 2020).

Because the gut microbiome plays a large role in regulating neuroimmune activities, the gut has been implicated in the immune response and thus AD (Kohler et al., 2016; Ticinesi et al., 2018). Therefore, manipulations of the gut microbiome may be useful in understanding its role in AD via neuroinflammation. Probiotic intervention has been shown to influence pro-inflammatory cytokine production and expression of inflammation-associated genes (Ohtsuka et al., 2012; Klemenak et al., 2015). Thus, we plan to explore the potential mechanisms of B. breve in modulating cytokine expressions of IL-1 β and TNF-α to examine the possibilities of a targeted treatment aimed at the neuroimmune dysfunction in AD.

**The production of neurotransmitters by the gut.** The third function of the microbiome we explore is the production of neurotransmitters by gut bacteria. Many native gut bacteria produce neuroactive metabolites, including neurotransmitters like GABA, acetylcholine, and serotonin (Santiago and Potashkin, 2021). These metabolites can circulate and directly enter the central nervous system (CNS), thus influencing neuroactivity (Fung et al., 2017).

One neurotransmitter of interest is GABA, which is an important inhibitory neurotransmitter whose dysfunction has been linked to various brain disorders (Cryan and Kaupmann, 2005). Abnormal GABA levels in the hippocampus of AD brains, as well as correlations between GABA levels and working memory capacity, strongly implicate GABA in AD etiology (Mandal et al., 2017). GABA also moderates immune responses and is being investigated for its potential use in treating IBD (Mittal et al., 2017), so its connection to inflammation is another compelling reason to explore its link to AD.

Since microbiome manipulations through probiotic interventions have been shown to regulate GABA pathways (Bravo et al., 2011), compounded by the fact that *Bifidobacterium* are high GABA producers (Yunes et al., 2016), we propose to explore the possibility that *B. breve* restores cognitive effects in AD through GABA production.
**Probiotics vs FMT as potential therapeutics.** Probiotic administration consists of supplements with live microbial strains, aimed to improve and restore balance in the gut microbiome (Mehrabadi and Sadr, 2020). In both mice and human clinical trials, probiotic intervention has been shown to rescue cognitive function in AD (Akbari et al., 2016; Kobayashi et al., 2017; Xiao et al., 2020; Cao et al., 2021). While positive outcomes of specific probiotic treatments have been established, further research is needed to understand which pathways are being affected and how these may affect AD patients with imbalanced microbiomes.

While probiotic administration is important for understanding targeted AD pathways, lack of knowledge regarding dosage, strains, and interactions with native flora yields high variability in its efficacy. Thus, FMT may be a more viable candidate for AD treatment. Currently, several challenges exist for developing safe and effective probiotic treatments. First, individual factors like age, sex, and underlying conditions lead to varying responses to the same probiotic treatment (Kothari et al., 2019). Second, host-microbe interactions highly influence the effects of probiotics, and may even cause detrimental effects such as horizontally-transferred antibiotic resistance and sepsis in at-risk populations (Kothari et al., 2019). Third, the lack of regulation in dosage, potency, and strains all contribute to the hesitancy of using probiotics as a drug (Kothari et al., 2019). Thus, the main goal of the proposed experiments in Aim 1 is to pinpoint specific mechanisms by which beneficial bacteria can alleviate AD symptoms, but not necessarily implicate probiotics as a therapeutic used clinically.

For the reasons stated above, a more plausible treatment option may be FMT. While research in FMT for AD is still in its early stages, its high efficacy in treating other diseases, along with the promising preliminary data, implicates FMT as a potential AD intervention. Originally designed for treating recurrent *Clostridioides difficile* (*C. difficile*) infections, FMT is expanding to psychiatric and metabolic disorder treatment (Chinna Meyyappan et al., 2020). In a recent study, AD transgenic mice treated with FMT showed cognitive improvements, reduced Aβ deposition, and increased synaptic plasticity (Sun et al., 2019). Therefore, our experiments will build on these past results by using Congo red staining to quantify Aβ deposition in the brain. Furthermore, a case study of an AD patient treated with FMT for a *C. difficile* infection found rapid symptom improvement after the procedure (Hazan, 2020). We will also utilize beta diversity measurements to compare bacteria abundance levels between healthy mice, AD transgenic mice, and AD transgenic mice post-FMT treatment to unravel potential bacterial species that are overabundant or deficient in AD. Along with Aim 1, Aim 2 will enable direct comparison between the effects of FMT and probiotics in terms of the specific mechanisms each treatment targets, as well as investigate how FMT specifically affects microbial communities.
Experimental Methods

General methods: In both aims, we evaluate the mechanisms of two potential therapeutics (probiotics and FMT) on neurogenesis, neuroinflammation, and neurotransmitter production. Experiments conducted in both aims are outlined below and in Figure 2.

A. APP/PS1 Double Transgenic mice. This widely-used AD mouse model contains mutations in both the APP and PSEN1 gene. APP encodes for the amyloid precursor protein which is enzymatically cleaved into smaller peptides; one of these is the Aβ peptide. On the other hand, PSEN1 encodes for the presenilin 1 protein, which cleaves APP into Aβ peptides. Taken together, APP/PS1 transgenic mice encapsulate characteristic AD hallmarks, including Aβ plaque deposition, neuronal loss, synaptic degeneration, and cognitive impairments (Gotz et al., 2018), and thus would be appropriate to use in both aims. Three experimental groups will be used: a wild-type control group (WT), an AD-transgenic mice group without treatment (Tg), and an AD mice group with treatment (Tg+P for probiotic treatment in Aim 1, and Tg+FMT for FMT treatment in Aim 2).

B. Behavioral Tests. Two behavioral tests will elucidate the clinical effects of these treatments on AD symptomology, specifically spatial working memory and learning.

Experimental Design: To test whether short-term spatial working memory is affected by these treatments, the Y-maze test will be used. Mice will be placed in a symmetrical Y-shaped maze for 5 minutes and can explore freely. While normal mice tend to explore new arms of the maze (Kraeutler et al., 2019), AD mice with hippocampal deficits cannot remember which arms have already been explored. To quantify this phenomenon, the percentage of alternations of visited arms is calculated throughout the trial. Thus, if the treatments improve spatial working memory deficits related to AD, the percent alternations should be greater in the treated group compared to the transgenic mice without treatment. The other behavioral test will be the passive avoidance test (Eagle et al., 2016), in which a mouse is placed in an apparatus chamber with a dark and a light compartment. During training, the mouse is put in the light compartment and gets an electric foot shock if they cross into the dark compartment. After 24 hours, they are tested to see if they retain the passive avoidance response. The paradigm tests learning and memory of the treated transgenic mice by evaluating if they can better learn the task and avoid the foot shock.

C. Quantifying Neurogenesis. In vitro experiments using tissue from post-treatment mouse brains will test the hypothesis that the treatments will restore AD-associated neurogenesis impairments. BDNF expression levels will be measured using western blot analysis, while localization of BDNF, ChAT, and NeuN within the brain will be visualized using immunofluorescence staining.
Rationale: BDNF is a neurotrophin that plays a vital role in learning, through its involvement in differentiation and survival of neurons during development (Miranda et al., 2019). Because BDNF promotes neurogenesis and synaptic growth, it is crucial for memory formation in hippocampal areas (Ng et al., 2019). Previous studies investigating its role in AD further supports BDNF’s relevance to this proposal. Weinstein and colleagues reported that higher BDNF levels yielded a protective effect against AD in older adults (Weinstein et al., 2014). Moreover, a recent meta-analysis found that AD patients have significantly lower BDNF levels than healthy controls (Ng et al., 2019). Lastly, interventions such as exercise, antioxidant diets, and environmental enrichment have been shown to increase BDNF levels (Ng et al., 2019). Because of its role in AD-targeted pathways, as well as its ability to be modulated by external treatments, we investigate both BDNF expression levels in the brain and its localization in the hippocampus with and without therapeutic intervention. Next, we also examine the differences in the number of ChAT-expressing neurons in the hippocampus. ChAT is the enzyme that synthesizes acetylcholine, and studies have shown that the cholinergic system in the hippocampus is indispensable for hippocampus-based memory and learning (Hawley et al., 2015). Furthermore, significant hippocampal neuronal loss in the CA1 and CA3 areas has been reported for AD patients (Padurariu et al., 2012). Taken together, we propose to use NeuN staining as a neuronal marker and ChAT staining to identify ChAT-expressing neurons in the hippocampus.

Experimental Design: To examine the effects of probiotics and FMT on neurogenesis, we will measure BDNF expression levels via western blot and immunochemistry (Kim et al., 2021). We will also assess the number of hippocampal neurons in the three experimental groups. Brain slices of 40 μm will be obtained using a microtome. After standard fixation and immunostaining techniques using ChAT antibodies (Millipore, AB144P), NeuN antibodies (Millipore, MAB377), BDNF antibodies (Invitrogen, OSB00017W), and Invitrogen fluorescent secondary antibodies, brain slices will be counterstained with DAPI to distinguish neurons of interest (Loesel et al., 2006). Samples will then be mounted, and images will be acquired using a confocal laser-scanning microscope. The cell counter software in ImageJ will be used to quantify the number of ChAT- and NeuN-expressing neurons in CA1, CA2, and CA3 regions of the hippocampus.

Preliminary Results: To determine the effects of AD on the cholinergic system of the hippocampus, we used immunofluorescent staining and quantification methods described above for the APP/PS1 transgenic mice. The significantly decreased amount of ChAT-expressing hippocampal neurons in our mouse model suggest that the APP/PS1 transgenic mouse is an appropriate model of AD (Fig. 1). In addition, these results posit that this deficiency in hippocampal neurogenesis may be the mechanistic target of probiotic/FMT treatments when rescuing cognitive impairments.
D. Measuring Neuroinflammation. The hypothesis that probiotics and FMT can reduce inflammatory cytokine levels in the brain will be tested using *in vitro* experiments that measure IL-1β and TNF-α levels.

**Rationale:** Recent studies have begun focusing on cytokine-moderated neuroinflammation as one of the main contributors to AD pathogenesis (Akiyama et al., 2000; McCaulley and Grush, 2015). TNF-α plays a major part in the cytokine cascade for neuroinflammatory responses (Chang et al., 2017). Thus, its role in AD pathophysiology has been investigated. Significant elevations of TNF-α levels were reported in both blood and CNS of AD patients, along with animal and clinical studies linking TNF-α abnormalities to AD (Chang et al., 2017). The mechanisms by which TNF-α contributes to disease progression include interference with Aβ clearance by microglia, upregulation of Aβ production, and increases in neuronal death (Chang et al., 2017). Another novel study found that two strains of probiotics (*Lactobacillus rhamnosus* and *Enterococcus faecium*) negatively modulated the production of TNF-α in a macrophage cell line (Divyashri et al., 2015).

IL-1β is another inflammatory cytokine worth exploring, since evidence links it to hippocampal-dependent memory impairments and AD (Rachal Pugh et al., 2001). Specifically, during long-term potentiation, elevated IL-1β levels were reported in *in vitro* and *in vivo* studies (Schneider et al., 1998). Another study observed increased IL-1β production most prominently in the hippocampus (Cacabelos et al., 1994). Moreover, IL-1β lies near the top of the cytokine signaling cascade that leads to neuroinflammatory responses in the brain (Basu et al., 2004). Because of IL-1β’s upstream position in this cytokine signaling cascade, it may serve as an efficient therapeutic target for treatments to stop the destructive, downstream consequences caused by neuroinflammation (Basu et al., 2004). One of the multiple cytokines that IL-1β modulates is TNF-α, as IL-1β induces greater levels of TNF-α expression in microglia (Shaftel et al., 2008). Therefore, we investigate fluctuations in IL-1β and TNF-α levels to help identify where in this pathway both treatments act. If both IL-1β and TNF-α levels decrease post-treatment, then the treatments might be affecting the more upstream IL-1β, with secondary effects on TNF-α levels. Alternatively, if only...
TNF-α levels are reduced, we can infer that the mechanism of the treatment is further downstream in the cytokine signaling cascade.

**Experimental Design:** In all three experimental groups, brain samples will be extracted and homogenized with a protease inhibitor cocktail to prevent protein degradation. After centrifugation, supernatants will be extracted for an ELISA assay to measure IL-1β and TNF-α levels.

**E. Measuring GABA Production.** HPLC will be applied with the goal of quantifying GABA production to explore if these treatments can modulate the production of neurotransmitters implicated in AD.

**Rationale:** We will measure GABA concentrations in the brain, as it is an important inhibitory neurotransmitter involved in AD. Accumulating evidence demonstrates that microbial GABA produced by gut bacteria can have a significant physiological impact on the host. Of relevance, bacterial GABA circulates throughout the body and gets transported to the brain through the vagus nerve (Altaib et al., 2021). In animal studies, ingestion of GABA-producing microbes has been shown to provide relief from various diseases such as depression and diabetes (Yunes et al., 2016; Pokusaeva et al., 2017; Patterson et al., 2019). Germ-free animals also have lower serum and luminal GABA levels (Matsumoto et al., 2013), which further highlights the role of the gut microbiome in modulating and producing neurotransmitters. The motivation for these studies is to demonstrate that these treatments may be able to ameliorate the GABAergic dysfunction linked to AD. Specifically, postmortem studies have revealed reduced GABA levels within various cortical areas and in the hippocampus (Lanctot et al., 2004). Moreover, the probiotic investigated in Aim 1, *B. breve*, is part of the *Bifidobacterium* genera, which is an established GABA producer (Barrett et al., 2012). Overall, we anticipate that increased GABA production by these treatments may relieve AD symptoms.

**Experimental Design:** As GABA is chemically classified as an amino acid, HPLC can be used to separate and quantify its levels. First, brain homogenate aliquots will be mixed with acidified methanol (8.4 ml 0.1 M HCl/100 ml methanol) as the organic solvent and then centrifuged. This derivatization process increases fluorescence detection sensitivity so that GABA can be detected. Next, free amino acid concentrations will be tested using a reaction between o-phtalaldehyde and mercaptoethanol, which are used for detecting fluorescent amino acids (Hung and Moon, 1983). These samples will then be injected into a column with a 50 mM phosphate buffer to obtain a chromatogram with peaks, which can be used to identify GABA levels. For full details, refer to the experimental design of (Zieminska et al., 2018).

**F. Measuring amyloid burden.** Congo red staining will be used to assess whether FMT can alleviate Aβ deposition in AD, as this stain indicates the presence of amyloid material in brain tissue.
Experimental Approach: Parasagittal brain sections 3 μm-thick from the midline will be deparaffinized, rehydrated, and counterstained. Brain slides will be fixated and then incubated with a polyclonal antibody specific to the Aβ peptide to allow for the immunodetection of plaques. Standard kits will be used to visualize the detection of these antibodies.

Figure 2. Outline of measurements in Aims 1 and 2. The effects of probiotic administration (Aim 1) and FMT (Aim 2) will be measured using behavioral tests, quantification of neurogenesis, neuroinflammation, GABA production, and amyloid burden.

Aim 1: To elucidate the therapeutic mechanisms of probiotics by administering *B. breve* in an AD mouse model.

Probiotic administration. *B. breve* will be orally administered to the Tg+P group to evaluate its potential mechanisms, outlined in the general methods above.

Experimental Approach: *Bifidobacterium* strain *B. breve* NCIMB 8807 will be grown in the culture conditions outlined in the study by (Pokusaeva et al., 2017). Tg+P mice were given 0.5 mL of 4 x 10⁸ CFU/mL of a suspended mixture of *B. breve* using the oral gavage technique to administer precise doses. WT mice will receive sterile phosphate-buffered saline (PBS). All mice will be 3-months-old, and treatment will continue for 30 days. The methods outlined in General Methods parts B-F will then be conducted to explore the specific mechanisms of probiotic administration on rescuing AD symptoms.

Aim 2: To assess the effects of FMT on AD and the gut microbiome in a mouse model.

Fecal Microbiota Transplantation. Transgenic mice in the FMT treatment group will receive stool from healthy WT donors transferred into their colons.

Experimental Approach: 0.2 ml of fecal solution from WT mice will be intragastrically administered to the Tg+FMT mice for 4 weeks. Before treatment, the mice will ingest triple antibiotics to eliminate native gut microbiota. To create the WT fecal solution that will be administered to the Tg+FMT mice, stool samples from WT mice will be collected and resuspended in PBS and saline, and then filtered to remove large particles. As a final step, this filtrate is centrifuged and then dissolved in saline at a concentration of 400 mg/ml. As a control, WT mice and Tg mice without treatment will be administered a saline treatment of the same dose. As done in Aim 1, the methods in General Methods parts B-F will be conducted, in addition to the methods described below.
16s rRNA sequencing. To determine the relative microbial abundances and compositional differences between WT, Tg, and Tg + FMT mice, 16s rRNA sequencing will be applied.

Rationale: 16s rRNA sequencing is a commonly used technique to identify the taxonomy of bacteria within a complex biological sample, providing resolution at both the species and strain level (Johnson et al., 2019). The 16s rRNA gene is highly conserved between all bacteria, so universal PCR primers that target these conserved regions of the gene can amplify genes from a multitude of microorganisms in one sample. Between these highly conserved regions in the 16s rRNA gene, there are variable regions that are unique to specific species, and this allows for identification and discrimination of different bacterial species in the sample. Thus, this fairly inexpensive, high-throughput method will be useful when comparing gut microbial composition of the three experimental groups.

Experimental Approach: Fresh fecal samples from the mice will be collected and stored at -80 °C. QIAGEN stool DNA extraction kits will be used to extract the DNA from the samples. Next, the Illumina MiSeq system will amplify the V3-V4 variable regions of the 16s rRNA gene. Then, QIIME, a bioinformatics platform, will be used to group bacteria into Operational Taxonomic Units (OTUs), which are collections of bacteria with DNA amplicons that are at least 97% identical. Essentially, each OTU represents a single genus of bacteria. From this data, an OTU table will be constructed, in which each row of the table represents an OTU, each column represents a sample, and the value in each entry is the frequency of the OTU in a particular sample. We will utilize the curated 16s database Greengenes Database to assign taxonomy to these OTUs. Finally, analysis will be done using the R package “betapart” (Baselga and Orme, 2012) to analyze and visualize beta diversity of samples. Linear discriminant analysis effect size will also be utilized to identify the OTUs that are statistically most likely to account for compositional differences between samples as done by (Sun et al., 2019).

Preliminary Results: Previous studies have established that AD patients have significantly different microbial compositions compared to healthy individuals (Vogt et al., 2017). Thus, we performed 16s rRNA sequencing on the APP/PS1 transgenic and wild-type mice. Consistent with past studies, we found significant differences in bacterial proportions at both the phylum and class level, indicating that our mouse model is representative of AD-specific effects on the gut microbiome (Fig. 3). The proposed studies will build on these experiments by further exploring differences in the Tg+FMT group at the order, family, and genus levels.
Figure 3. Differences in bacterial taxa proportions between fecal samples between wild-type and APP/PS1 transgenic mice on the (A) phylum level and (B) class level. All differences shown are statistically significant (p<0.05). Red bars represent wild-type samples, and blue bars represent transgenic samples.

Potential Pitfalls: We acknowledge several limitations of our study. First, the APP/PS1 mouse model does not mimic tau symptomology, so we are unable to explore the effects of these interventions on the neurofibrillary tangles. Nonetheless, the framework of experiments proposed could be easily replicated using another mouse model with these deficits. Next, other microbial mechanisms such as the production of neuroactive metabolites other than GABA, like short-chain fatty acids, may also prove to be important to understand the gut-brain axis’ role in AD. Finally, some of the effects measured, like cytokine production, could just be a side effect of an upstream process being targeted by the interventions. Regardless, identifying parts of pathways that are impacted is an important first step for treatment development.

Interpretation and Summary: While many microbiome studies focus on correlational effects, the results of Aims 1 and 2 will provide insight into the actual mechanistic interactions between specific bacteria and the host in relation to AD. Behavioral tests and amyloid burden quantification will measure the extent to which these treatments rescue AD symptoms. Recent literature has firmly established the beneficial effects of microbiome-based interventions, so we anticipate that these tests will proceed without any major impediments. The rest of the experiments will narrow down how these treatments fit into AD pathophysiology. Namely, if higher levels of BDNF and NeuN- and ChAT-positive neurons are observed in the treatment groups, this would suggest that the microbes are promoting neurogenesis. Alternatively, decreased cytokine levels in treatment groups would implicate the microbiome’s regulation of neuroinflammation. Lastly, increased GABA levels post-treatment would indicate that metabolite production from the bacteria could be relieving AD symptomology. These potential avenues are not mutually exclusive and are likely complementary, given the plethora of functions in which the microbiome is involved. Finally, in Aim 2, we explore how the overall bacterial composition in the gut community changes after FMT, to help elucidate potential bacterial species that are disrupted in AD. Together, these studies will enable a better understanding of the interactions between the gut microbiome and AD pathways, and will help guide the next generation of AD treatment development.
Bibliography


