The Gut-Brain Connection: Probiotic Supplementation to Alleviate Cognitive Decline and Inflammation in Rodent Models of Pretangle Tau and Stress

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Abstract

The microbiota-gut-brain axis is a crucial link to peripheral and central nervous systems, with gut health implicated in health and disease, including Alzheimer's disease (AD) and stress. The objective of this dissertation is to explore the role of strengthening the gut microbiota in brain health and early-AD pathologies.

First, I will investigate the effects of probiotic supplementation on cognitive function, brain inflammation, and gut microbiota composition. We employed a locus coeruleus hyperphosphorylated pretangle tau rat model, which closely resembles preclinical AD. Rats with pseudophosphorylated human tau in the LC showed deficits in spatial and olfactory learning, increased microglia and astrocyte activity, blood-brain barrier (BBB) leakage, and elevated peripheral inflammation. Probiotic supplementation increased gut microbiome diversity, optimized bacterial composition, and ameliorated cognitive deficits. A reduction in inflammation and glycogen synthase kinase 3 beta (GSK-3β) activity in the hippocampus of female rats was observed, suggesting gut health modulation as a potential therapeutic strategy in preclinical AD, and providing a possible mechanism underlying AD sex differences.

Second, I examined the effects of probiotics prior to chronic stress or enrichment on cognitive function and brain health. Probiotics prevented stress-induced spatial memory impairments and enhanced learning under enrichment conditions. We propose this is linked to increased gut microbiome diversity and eubiosis, which was observed in our animals. Probiotics prevented increased levels of the microglia marker ionized calcium-binding adaptor molecule 1 (Iba-1) found in stressed rats and showed differences in BBB integrity and tyrosine hydroxylase (TH)

levels in the hippocampus between stress and enrichment groups, with beneficial effects observed in enriched animals.

These findings highlight the potential of probiotics to enhance cognitive function and brain health through modulation of the gut microbiota, offering a non-invasive therapeutic approach for AD and stress-related cognitive decline.

General Summary

Probiotics are live microorganisms that confer health benefits when consumed in adequate amounts, primarily by modulating the gut microbiota. The field of gut microbiota research has rapidly evolved in recent years, highlighting the relationship between the gut and brain, known as the microbiota-gut-brain axis. This axis plays a pivotal role in various physiological processes including cognitive function, immune responses, and inflammation. As our understanding of this complex relationship deepens, probiotics have emerged as potential therapeutic agents for improving gut health and mitigating various diseases.

Alzheimer's disease (AD) typically manifests clinically at advanced stages, by which time substantial pathological changes have already occurred in the brain. Current therapies have failed to halt or reverse AD progression, possibly due to the late disease stage of these interventions. Our research utilizes a locus coeruleus hyperphosphorylated pretangle tau rat model which closely mimics the early, preclinical stages of AD. This model allows us to investigate the effects of interventions at a prodromal time point when pathological changes are still emerging, providing a crucial window for potentially effective treatments. Probiotics can modulate gut microbiota composition, reduce inflammation, and produce neuroactive substances that may improve cognitive functions, presenting a promising avenue for therapeutic intervention.

Chronic stress is a well-known contributor to inflammation, which is implicated in the pathogenesis of numerous diseases, including AD. Stress-induced inflammation can exacerbate neural damage and cognitive decline. By investigating the impact of probiotics on stress-induced inflammation, our research aims to uncover mechanisms through which gut health can be leveraged to ameliorate the adverse effects of stress on the brain.

Our findings reveal that probiotic supplementation can prevent stress-induced spatial memory impairments and reduce anxiety-like behaviors in rats. In the pretangle tau model, probiotics increased gut microbiome diversity, optimized bacterial composition, and ameliorated cognitive deficits. These benefits were associated with reduced brain inflammation and decreased activity of the tau-phosphorylation associated kinase, GSK-3β. Notably, probiotics also prevented increased microglia activity in stressed animals, suggesting a protective role against stress-induced brain inflammation. In conclusion, our research highlights the potential of probiotics as a non-invasive therapeutic approach to enhance cognitive function and brain health.

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Citations and Co-authorship Statements

I, Cassandra Flynn, hold first author status for the version of the manuscripts in Chapter 2-3 of this dissertation.

Chapter 2

Probiotic supplementation improves cognitive function and ameliorates inflammation in a pretangle tau rat model. This chapter is a version of a submitted manuscript. Currently in review. Cassandra M. Flynn, Tamunotonye Omoluabi, Alyssa M. Janes, Emma J. Rodgers, Sarah E. Torraville, Brenda L. Negandhi, Timothy E. Nobel, Shyamchand Mayengbam, Qi Yuan.

As the first author, I (C.M.F.) contributed in the project idea, experimental design, conducted the majority of experiments, analyzed data and wrote the first draft of the manuscript. T.E.N. and Q.Y. helped in conceiving the project idea. T.O., A.M.J., E.J.R., S.E.T, B.L.N. and T.E.N. contributed in the conducted research. Q.Y. contributed to the writing and refinement of the manuscript.

Chapter 3

Probiotic supplementation prevents stress-impaired spatial learning and enhances the effects of enrichment This chapter is a version of a submitted manuscript. Currently in review.
Cassandra M. Flynn, Lara M. Blackburn, Qi Yuan.

As the first author, I (C.M.F.) and Q.Y. conceived the project idea and designed the research. I conducted and analyzed the majority of the research and wrote the first draft of the manuscript.

L.M.B conducted a subset of IHC experiments. Q.Y. helped interpret the results and edited the manuscript.

Sections of the following published reviews have been adapted in Chapter 1 of this dissertation:

Flynn, C. M., & Yuan, Q. (2023). Probiotic supplement as a promising strategy in early tau pathology prevention: Focusing on GSK-3β?. *Frontiers in neuroscience*, *17*, 1159314. https://doi.org/10.3389/fnins.2023.1159314

As the first author and co-corresponding author of this review, I contributed significantly to the ideas, research and writing behind this work.

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As the co-first author and corresponding author of this review, I researched and wrote the microbiome sections of the manuscript (Sections 1, 2.3, 2.4, 3.3, 4, and 5). T.S.E and K.T.L contributed to environment sections (Sections 2.1, 2.2, 3.1, and 3.2). I edited and finalized the manuscript. Q.Y supervised this process and provided feedback.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

16SrRNA	16S ribosomal RNA
5HT	serotonin
AAV	adeno-associated virus
AD	Alzheimer's disease
Akt	Protein kinase B
APOE	apolipoprotein E
APP	amyloid precursor protein
Aβ	amyloid-beta
BACE1	β-secretase
BBB	blood-brain-barrier
BL	*Bifidobacterium lactis*
Ca^{2+}	calcium
CaM	calmodulin
CaMKII	calmodulin-dependent protein kinase II
CaN	calcineurin
CD68	Cluster of Differentiation 68
CFU	colony forming unit
CNS	central nervous system
CRF	corticotropin releasing factor
CSF	Cerebrospinal fluid
CUSP	chronic unpredictable stress paradigm
DAB	3,3′-Diaminobenzidine

DBH	dopamine β-hydroxylase
DIO	double inverted open reading frame
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EE	environmental enrichment
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
EOAD	early-onset Alzheimer's disease
ER	enrichment
fAD	familial Alzheimer's disease
FOS	oligofructose
GF	germ-free
GFAP	Glial fibrillary acidic protein
GFP	green fluorescent protein
GI	gastrointestinal
GPR	G protein-coupled receptors
GR	glucocorticoid receptor
GSK3β	glycogen synthase kinase 3 beta
GWAS	Genome-wide association studies
HPA	hypothalamic–pituitary–adrenal
HSV	Herpes Simplex virus
hTau	human phosphorylated tau
Iba1	Ionized calcium-binding adaptor molecule 1

IBS	irritable bowel syndrome
IDE	insulin degrading enzyme
IDT	Integrated DNA Technologies
IGF-1R	insulin-like growth factor 1 receptor
IgG	immunoglobulin G
IHC	immunofluorescence
IL	interleukin
IR	immunoreactivity
LC	locus coeruleus
LEfSe	Linear Discriminant Analysis Effect Size
LOAD	late-onset Alzheimer's disease
LPS	lipopolysaccharides
MAMPs	microbial-associated molecular patterns
MCI	mild cognitive impairment
MMP9	matrix metalloproteinase 9
MRI	magnetic resonance imaging
MS	maternal separation
Na^+	sodium
NE	Norepinephrine
NFT	neurofibrillary tangles
NIH	National Institutes of Health
ODAD	odor detection and discrimination task
P/P	probiotic + prebiotic diet

PBS	phosphate-buffered saline
PD	postnatal day
PET	position emission tomography
PI3K	Phosphoinositide 3-kinase
PKA	protein kinase A
PSEN1	presenilin 1
PSEN2	presenilin 2
pTau	phosphorylated tau
PVP	polyvinylpyrrolidone
*q*PCR	Quantitative polymerase chain reactions
ROD	relative optical density
ROS	reactive oxygen species
SCFA	short-chain fatty acid
SLR	spontaneous location recognition
SOD	standard object discrimination
STR	stress
TH	tyrosine-hydroxylase
TLR	toll-like receptor
TNF-α	Tumour Necrosis Factor alpha
TREM2	triggering receptors expressed on myeloid cells 2
Ucns	urocortin

List of Appendices

Chapter 1: Introduction

1.1 Overview

Alzheimer's disease (AD) remains a significant challenge as current treatments have been largely unsuccessful. This failure is primarily due to the late stage at which an AD diagnosis is typically made, long after the initial pathological changes have begun in the brain. These initial pathologies appear early, before entering a prolonged prodromal stage then finally progressing to irreversible AD progression (Braak and Del Tredici 2011).

Recent research has brought new hope by highlighting the gut microbiota's role in health and disease, particularly its connection with the brain, known as the microbiota-gut-brain axis (Mayer, Nance, and Chen 2022). Studies have demonstrated that a healthier gut microbiota composition can improve cognition and importantly, AD pathologies and symptoms (Kim et al. 2020; Grabrucker et al. 2023).

My research aims to explore the effects of a healthy gut microbiota, initiated through probiotic supplementation, on an early-stage pre-symptomatic, pre-tangle tau animal model, representative of early-stage AD (Ghosh et al. 2019). Additionally, I seek to understand the potential cognitive benefits of probiotic supplementation prior to environmental factors such as stress and enrichment. This work could provide new insights into early interventions that may alter the course of AD and improve patient outcomes.

1.2 Alzheimer's Disease

AD is a progressive neurodegenerative disorder recognized as a global public health priority by the World Health Organization. First reported by Alois Alzheimer in 1907, AD remains the leading cause of dementia in late adult life, with huge implications for both individuals and society (Alzheimer et al. 1995). The disease is characterized by a gradual decline in cognitive function, affecting episodic memory before progressing to impairments in language, decision-making, and daily tasks (Bäckman, Small, and Fratiglioni 2001; Frere and Slutsky 2018). A complex mix of genetic, environmental, and lifestyle factors are involved, leading to neuronal degeneration in specific brain regions such as the hippocampus, medial temporal cortex and additional cortical structures.

The AD disease presentation begins clinically with episodic memory problems in elderly individuals. As the clinical disease advances, cognitive difficulties begin to interfere with activities of daily living, and ultimately lead to a diagnosis. Diagnosis of AD is complex and historically has involved structured history reviews, along with an examination which focuses on cognitive testing. Cerebrospinal fluid (CSF) analysis and positron emission tomography (PET) have also helped in the diagnosis and management of AD. Recently, in June 2024, the National Institute of Aging and the Alzheimer's association have updated the criteria for diagnosis, placing a large weight on blood-based biomarkers (Jack et al. 2024). These biomarkers will focus on amyloid-beta (Aβ)-42 and phosphorylated tau isoforms in CSF or plasma (Jack et al. 2024). Despite significant advancements in understanding AD pathogenesis, and available therapeutics, there are currently no disease-modifying treatments available, and the etiology remains poorly understood.

1.2.1 Epidemiology and Etiology

AD is a major cause of dependence, disability, and mortality. It presents a significant and growing public health challenge, with the number of AD patients aged 65 and older projected to increase from 5.8 million to 13.8 million by 2050 ("2020 Alzheimer's Disease Facts and Figures" 2020; Hebert et al. 2013). This increase will be seen particularly in low and middle-income countries experiencing increased rates of cardiovascular disease, hypertension, and diabetes (World Alzheimer report 2018. London: Alzheimer's Disease International, 2018.). In the United States alone, the annual cost of dementia is predicted to exceed US$600 billion as the population ages (Prince et al. World Alzheimer Report 2014: Dementia and Risk Reduction an Analysis of Protective and Modifiable Factors, 2014.).

Despite affecting both sexes, AD demonstrates a notable sex difference, with more women living with a diagnosis than men. The estimated lifetime risk at age 45 is approximately one in five (20%) for women and one in 10 (10%) for men (Nebel et al. 2018). This is partly attributed to the longer life expectancy of women, as age is the strongest risk factor for sporadic AD (GBD 2016 Dementia Collaborators 2019). However, biological, psychosocial and cultural factors may contribute to this difference. For instance, hormonal changes in women, particularly post-menopause, have been linked to increased susceptibility to AD, as estrogen decline may influence amyloid-beta accumulation and tau pathology (Goodenough et al., 2005) Studies have suggested that these factors could influence not only disease prevalence but also the timing and progression of AD in a sex-specific manner, highlighting the need for further research into the epidemiology and risk factors associated with AD and its sex differences (Lopez-Lee et al. 2024).

There are two primary forms of AD, familial AD (fAD) also known as early-onset AD (EOAD), and sporadic AD. fAD accounts for less than 0.5% of cases and is characterized by early-onset symptoms that typically emerge between 30 and 50 years of age (Blennow, de Leon, and Zetterberg 2006). The etiology of fAD is better understood, with cases primarily caused by autosomal dominant mutations in genes such as amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2; (Bateman et al. 2011).

In contrast, sporadic AD, which constitutes the majority of cases, is influenced by a combination of genetic and environmental factors. Notably, the apolipoprotein E (APOE) gene with its ε4 allele, is the single most significant genetic risk factor for sporadic AD (Verghese, Castellano, and Holtzman 2011). Individuals carrying the ε4 allele have an increased risk of developing AD, although it is neither necessary nor sufficient for the development of AD (Qiu et al. 2004). Genome-wide association studies have also identified more than 20 genetic risk factors associated with inflammatory, cholesterol metabolism, and endosomal-vesicle recycling pathways, shedding light on the multifaceted nature of sporadic AD's etiology (Karch and Goate 2015).

Environmental factors also play a crucial role in AD risk, with epidemiological evidence highlighting the impact of certain lifestyle factors, notably gut health, stress, and enrichment. For example, studies indicate that higher levels of education and engagement in regular physical exercise may offer protection against AD (Xu et al. 2015). Additionally, familial aggregation of AD, where first-degree relatives of AD patients have a higher lifetime risk, suggests a

contribution from shared environmental factors along with genetic predisposition (Green et al. 2002).

1.2.2 Pathological Stages

AD is characterized by distinctive pathological features, including the presence of intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau proteins and extracellular amyloid plaques primarily composed of Aβ protein deposits (Hardy and Higgins 1992; Blennow, de Leon, and Zetterberg 2006). Downstream consequences including, but not limited to neuronal and synaptic loss, combined with these two main hallmark pathologies contribute to the neurodegeneration and pathology observed. Understanding these is crucial for the development of successful treatments.

1.2.2.1 Braak pretangle and NFT stages

AD is a complex neurodegenerative disorder that is believed to have a prodromal stage lasting for decades before clinical symptoms become apparent. A key pathological hallmark of AD is the presence of hyperphosphorylated tau protein, which progressively forms insoluble aggregates known as NFTs inside neurons. Physiologically, tau is a microtubule-associated protein that plays a crucial role in maintaining the structural integrity of neurons (Johnson and Stoothoff 2004). However, abnormal phosphorylation of tau disrupts its function and leads to the formation of NFTs (Wood et al. 1986). Hyperphosphorylation of tau is believed to be the start of the pathological form, which is initially soluble in nature (Braak et al. 2011; Harley et al. 2021). This

is known as the pretangle stage. Progressive entanglement makes it insoluble next, then finally creates intracellular NFTs, giving rise to the tangle stage (Braak et al. 2011).

Importantly, the early stages of this pathological process are asymptomatic, and the disease progression may begin as early as young adulthood or childhood (Braak and Del Tredici 2011). It is only much later, with advancing age, that some individuals cross a threshold into the symptomatic phase of the disease. Although tau aggregation is common in aging, the reasons why some individuals develop AD while others do not remain unclear. Factors such as genetic predisposition, lifestyle, and environmental influences likely play roles in determining susceptibility to AD.

Braak and colleagues conducted a post-mortem immunohistochemical study in 2332 brains over a large range of ages, from 1 to 100 years. They used the Gallyas silver method to identify argyrophilic NFTs and AT8 staining to identify abnormal hyperphosphorylation at serine 202 and threonine 205 residue. Their study helped characterize a comprehensive staging method for AD pathology, which outlines the progression of tau pathology from the pretangle to the tangle stage (Braak et al. 2011). These proposed stages will be detailed in the following subsections.

Pretangle Stages a-c:

The first stage, known as stage "a", marks the initial appearance of pathologically altered persistently phosphorylated tau in specific subcortical nuclei of the brain. This early phase suggests that the disease process may begin preferentially in the brainstem and more specifically,

in the locus coeruleus (LC) projection neurons. This is the first site where visible pathological changes occur, with initial alterations confined to proximal segments of long axons. These areas generate projections to various forebrain sites, particularly high-order association areas of the neocortex. This stage is mainly characterized by the continual formation of AT8 positive abnormally phosphorylated tau, a process that continues throughout the progression of AD. This is supported by showing that apart from the LC, none of the other central nervous system (CNS) regions known to become involved during AD display the presence of AT8-immunoreactive staining.

Stage "b" of AD progression involves the accumulation of pretangle material in the somatodendritic compartment of noradrenergic LC neurons. The hyperphosphorylated tau in this stage leads to secondary changes within the axonal microtubules, potentially resulting in their disintegration. As well, the increase in AT8-reactive tau results in visible cell bodies in the LC and spike-like structures throughout the somatic membrane (Harley et al. 2021). Initially, the pretangle material contains small amounts of newly formed, still-soluble tau. At later stages, neurons may convert this material into aggregates that push into the spaces between cell organelles becoming insoluble, fibrillar, and strongly argyrophilic. Interestingly, even severely altered axons can remain structurally intact for long periods.

In stage "c", the AT8+ material spreads beyond the LC to non-thalamic cortical projecting nuclei, such as the raphe nucleus. Subsequently, additional nuclei with diffuse cortical projections become involved.

These pretangle stages, a-c, typically occur in younger age groups and are characterized by the absence of cortical involvement. Despite the presence of abnormal tau, there is no observable dismantling of the tau or signs of cellular distress in the LC, such as swelling of the neuronal soma or displacement of the Nissl material or cell nucleus. Noradrenergic neurons bearing NFTs manage to survive for decades during the pathological process, with a remarkable number persisting even in the final stages, although the function of these neurons may become impaired. The toxicity of pretangle tau remains poorly understood as they co-exist with NFTs at later disease stages.

Pretangle Stages 1a & 1b:

In the stage known as Pretangle "stage 1a," the first cortical lesions appear as soluble condensates and non-argyrophilic material that can be identified by the AT8 antibody. These cortical changes start in the medial portion of the temporal lobe in the trans entorhinal cortex and appear as radially aligned thread-like neuronal processes, possibly representing the terminal portions of affected axons.

In the following stage, 1b, there is a noticeable presence of AT8-positive material in the somatodendritic compartment of pyramidal cells in the cerebral cortex. This is particularly prominent in the trans entorhinal region and specifically in cells projecting to the cortex.

Neurofibrillary Stages I-VI

In the progression of neurofibrillary stages I to VI, the trans entorhinal region stands out as the initial cortical site where argyrophilic NFTs develop. Stage I is marked by intraneuronal Gallyas-positive lesions, indicating the presence of NFTs, primarily localized in the trans entorhinal region and present in small numbers. Non-argyrophilic projection cells containing pretangle material are consistently detectable in this region, while the entorhinal region remains largely unaffected initially.

Advancing to stage II, additional lesions emerge in the entorhinal region, often accompanied by mild involvement in the hippocampus. The progressive involvement of pyramidal cells in the entorhinal layers leads to significant disruption in connectivity to and from the hippocampal formation, setting the stage for the cognitive decline seen in the clinical phase of AD.

Stage III is characterized by the appearance of intraneuronal material in the basal neocortical area of the temporal lobe, extending towards laterally adjacent high-order sensory association areas of the basal temporal neocortex. Subsequent stages (IV to VI) represent the further spread of pathology to additional high-order sensory association areas in the occipital and parietal lobes, the prefrontal neocortex, first-order sensory association areas, premotor fields, and eventually to primary sensory association areas, premotor and primary motor areas (Stage VI). It is notable that the severity of tau pathology in the LC increases with higher NFT stages.

A recent study employed a tau-PET ligand with affinity for NFTs, to assess tau pathology and CSF measures of phosphorylated tau (pTau) epitopes in relation to Braak stages (Therriault et al. 2022). The study confirmed that PET-based Braak stages correlate with distinct patterns of tau pathology and CSF pTau levels. Specifically, individuals at PET-based Braak stage I exhibited tau pathology limited to the trans entorhinal cortex, with no abnormal tau-PET detected

elsewhere. Progressing to stage II, abnormalities extended to the entorhinal cortex and hippocampus, in addition to stage I regions. Stages III and IV demonstrated increased involvement of earlier stages and greater occupation of the temporal neocortex. Stage V was characterized by extended involvement of association cortices, while stage VI exhibited tau abnormality extending into primary sensory areas. Interestingly, changes in CSF pTau epitopes were observed, with detectable differences in CSF measures of $pTau_{181}$, $pTau_{217}$, $pTau_{231}$, and $pTau_{235}$ emerging at PET-based Braak stage II, but not at stage I. Larger differences in CSF pTau levels for all phosphorylation sites were noted starting at stage III, when tau pathology began to accumulate outside of the medial temporal lobe and plateaued from stage IV onwards.

Bueicheku et al. developed advanced magnetic resonance imaging (MRI) techniques to assess LC integrity in vivo (Bueichekú et al. 2024). This approach addresses the limitations of PET in detecting tau pathology within the LC, given its small size and the off-target binding of tau tracers to neuromelanin. Their findings support Braak's findings, indicating that changes in LC integrity precede tau accumulation in the medial temporal lobe and are associated with lower cognitive performance. Additionally, Aβ appears to facilitate the spread of LC-related tau beyond the medial temporal lobe regions. These findings strengthen Braak staging in characterizing the progression of tau pathology in AD.

1.2.2.2 Amyloid-Beta Stages

The amyloid cascade hypothesis, formulated in the early 1990s, has been a focus of AD research, influencing drug discovery and clinical trials. It states that AD initiates with the accumulation and aggregation of Aβ peptides, leading to the formation of amyloid plaques (Hardy 2017). It is

supported by the genetic basis of early-onset familial AD, where mutations in the APP, PSEN1, or PSEN2 genes can alter Aβ peptide production or the Aβ42/40 ratio (Sun et al. 2017). Aβ42, a more hydrophobic peptide, is particularly prone to aggregation and amyloid formation. Braak et al., observed β-amyloid plaque deposition in humans between ages 30 and 40 years, a time point at which these cases already contained pretangle tau stage 1a or 1b (Braak et al. 2011).

The amyloid pathway in AD involves the production of Aβ, a 4 kDa fragment of APP. APP is widely produced in the brain by neurons, vascular and blood cells, and astrocytes. Two proteolytic cleavages of APP by β-secretase (BACE1) and γ-secretase generate Aβ (Blennow, de Leon, and Zetterberg 2006).

The deposition of Aβ follows a spatial-temporal pattern, starting with plaque-like deposits in regions with high metabolic activity rates like the association cortices (Phase 1). Subsequent accumulation is observed in allocortical regions, neocortical association areas and the midbrain (Phases 2 and 3). In later phases (4 and 5), accumulation extends to the cerebellum and brainstem, reflecting the continuous accumulation of Aβ throughout the disease progression, with continued worsening observed in earlier affected areas (Thal et al. 2002).

1.2.3 Pathogenesis

In past decades, the amyloid cascade hypothesis has been the central idea behind AD research. This hypothesis focuses on the idea that the disease initiates with the accumulation and aggregation of Aβ peptides, ultimately forming β-amyloid fibrils (Aβ42) in the brain. However,

clinical trials targeting Aβ peptide production and amyloid formation have not demonstrated efficacy in slowing cognitive decline or improving daily life for AD patients (Kurkinen et al. 2023). In response alternative hypotheses have emerged, including mainly the hyperphosphorylated tau hypothesis and the inflammatory hypothesis. In the following sections, we will explore the main pathology hypotheses and their relevance in current human and animal research.

1.2.3.1 Amyloid Hypothesis

Aβ is normally produced as a result of two successive cleavages of APP by β-secretase and γ-secretase enzymes. However, in AD, this process becomes dysregulated, leading to the accumulation of Aβ peptides (Hardy and Higgins 1992). These peptides can aggregate to form insoluble plaques, which are a hallmark pathological feature of AD. In this hypothesis, the aggregation of Aβ is believed to trigger a cascade of events, including the formation of NFTs composed of hyperphosphorylated tau protein, synaptic dysfunction, and ultimately neuronal death, leading to the clinical manifestations of AD.

Support for the amyloid hypothesis comes from various lines of evidence. First, mutations within and around the Aβ region of APP are linked to aggressive forms of fAD, indicating a direct role of Aβ in disease development (Goate et al. 1991). Additionally, the inheritance of a specific mutation in APP that reduces Aβ production is associated with a decreased risk of developing AD and age-related cognitive decline (Maloney et al. 2014). Missense mutations in PSEN1 or PSEN2, which are involved in Aβ production, are also known to cause early-onset AD (Scheuner et al. 1996). These genetic findings suggest a causal relationship between Aβ accumulation and

AD. Post-mortem studies consistently show Aβ plaques and tau tangles in the brains of AD patients, supporting the idea that these proteins play a central role in disease pathology. In addition, studies have shown that Aβ oligomers, which are small aggregates of Aβ peptides, can impair synaptic function and memory in animal models (Shankar et al. 2008; Rice et al. 2019). Strengthening this, imaging studies have demonstrated that Aβ deposition in the brain precedes the onset of cognitive symptoms in AD (Jagust 2018).

Even with this evidence, the hypothesis faces criticism and challenges. One argument is the correlation between Aβ plaque burden and cognitive impairment, with studies showing individuals with abundant Aβ deposits but no noticeable cognitive decline (Chételat et al. 2013). Additionally, clinical trials targeting Aβ have not consistently shown significant benefits in slowing cognitive decline in AD patients (Zhang et al. 2023).

1.2.3.2 Tau Hypothesis

The tau hypothesis offers a different perspective on the development of AD, emphasizing the role of tau protein pathology over Aβ accumulation, supported by various findings that challenge the amyloid hypothesis. Studies examining human brain tissues, notably those by Braak et al., have shown that tau pathology can emerge before the formation of Aβ plaques, indicating that abnormal tau accumulation might be an early trigger and primary driver of neuronal dysfunction and cognitive decline in AD (Braak et al. 2011) .

Tau is a crucial protein for stabilizing microtubules in neurons. However, in AD, tau becomes hyperphosphorylated, phosphorylated by a large number of kinases, including protein kinase A (PKA) and glycogen synthase kinase 3 beta (GSK3β; (Jicha et al. 1999). Tau detaches from

microtubules, and forms paired helical filaments, which eventually aggregate into neurofibrillary tangles- a hallmark of AD pathology associated with neuronal death (Kidd 1963; Schneider et al. 1999). The tau hypothesis is supported by neuropathological analyses showing a strong correlation between tau pathology and cognitive impairment in AD brains (Giannakopoulos et al. 2003). The tau hypothesis gains support from non-human primate studies, which demonstrate that the initial stages of tau pathology can be observed and that tau pathology correlates more closely with cognitive decline and neuronal loss than Aβ pathology (Braak et al. 2011; Giannakopoulos et al. 2003; La Joie et al. 2019).

Recent research suggests that soluble pretangle tau may play a more critical role in the disease process than NFTs (Congdon and Duff 2008; Brunden, Trojanowski, and Lee 2008; Spires-Jones and Hyman 2014). This idea is supported by several key pieces of evidence. Morsch et al. (1999) used computational modeling to demonstrate that neurons bearing NFTs can survive for decades, suggesting that NFTs may not directly cause cell death (Morsch, Simon, and Coleman 1999). Kril et al. (2002) found that although significant neuronal loss was present in AD brains, NFTs accounted for only a small percentage of total cell loss, indicating that neuronal death may occur prior to NFT formation (Kril et al. 2002). Animal models have further supported this notion, with studies showing that synaptic loss and dysfunction precede the formation of NFTs in some models (d'Orange et al. 2018; Yoshiyama et al. 2007). Additionally, Santacruz et al. (2005) reported that suppressing transgenic tau after NFT formation reversed neuronal loss and memory deficits in a mouse model, even though NFTs continued to accumulate (Santacruz et al. 2005).

Further evidence comes from studies in which the reduction of soluble tau and Aβ was sufficient to improve cognitive and behavioral deficits in mice, despite the presence of NFTs and amyloid

plaques (Oddo et al. 2006). Recently, a pretangle tau model developed in rats by our laboratory has provided additional insights into the early stages of tau pathology. In this model, human tau pseudophosphorylated at 14 sites (hTauE14) was seeded in the rat LC, leading to somatodendritic expression of the human tau, then showing spread to other neuromodulatory nuclei in the brainstem and the entorhinal cortex (Ghosh et al. 2019). This model resulted in impairment in olfactory associative discrimination 7-9 months post infusion, similar to the olfactory dysfunction seen in preclinical AD (Devanand et al. 2000; Sun et al. 2012). Additionally, LC fiber degeneration and neuronal loss were observed, correlating with the severity of behavioral deficits (Ghosh et al. 2019). Overall, these findings suggest that the degree of abnormal tau phosphorylation may be a crucial factor in tau pathology and the development of AD.

1.2.3.3 Inflammatory Hypothesis

The inflammatory hypothesis of AD focuses on neuroinflammation playing a pivotal role in the disease's development. The AD disease cascade involves chronic inflammation characterized by the activation of immune cells, including microglia (Hansen, Hanson, and Sheng 2018), astrocytes (Carter et al. 2019; Peters et al. 2009), and peripheral cytokines (Swardfager et al. 2010). This hypothesis is supported by elevated levels of inflammatory markers and the identification of AD risk genes related to innate immune function (Streit, Mrak, and Griffin 2004). Interestingly, more than 40 loci have been confirmed as target genes related to late-onset AD, many of which are concentrated in glial cells, particularly microglia.

Mainly, neuroinflammation in AD involves the production of proinflammatory cytokines and chemokines in response to pathological damage (Heneka et al. 2015). While acute inflammation in the brain serves as a defense mechanism, chronic inflammation can lead to neuronal damage over time. This can be seen in AD, with persistently activated microglia shown to produce high levels of proinflammatory cytokines, contributing to neuronal dysfunction. Microglia play a pivotal role in supporting the CNS (Solito and Sastre 2012; Fakhoury 2018; Sierra et al. 2014). They can mediate synapse loss (Sierra et al. 2014; Hong et al. 2016), exacerbate tau pathology (Cherry et al. 2016; Maphis et al. 2015), and secrete inflammatory factors that directly injure neurons or activate astrocytes (Rock et al. 2005).

The role of microglia-mediated inflammation in AD is complex, as microglia also have beneficial functions, such as phagocytosis of damaged cells and release of anti-inflammatory cytokines (Ueno et al. 2013; Streit, Mrak, and Griffin 2004). Microglial phagocytosis can eliminate Aβ peptides and injured cells, with early-stage Aβ pathology inducing Aβ clearance through the release of proteases such as insulin degrading enzyme (IDE) and matrix metalloproteinase 9 (MMP9) (El Khoury and Luster 2008). However, as AD progresses, Aβ accumulation continues despite microglial accumulation, potentially due to decreased Aβ phagocytosis and degradation (Hickman, Allison, and El Khoury 2008). Microglia have also emerged as key players in tau spread, with studies showing that their depletion suppresses tau propagation (Asai et al. 2015).

Genome-wide association studies (GWAS) have suggested a pivotal role for triggering receptors expressed on myeloid cells 2 (TREM2), a protein implicated in crucial inflammatory and neuroprotective processes, in AD susceptibility, with rare variants in the TREM2 gene increasing

the risk of sporadic AD (Guerreiro et al. 2013; Jonsson et al. 2013; Lue et al. 2015; Zheng et al. 2016). Under pathological conditions, TREM2 expression is typically upregulated, as observed in AD patients and mouse models with amyloid and tau pathology (Lue et al. 2015; Perez et al. 2017; Jay et al. 2017). This upregulation is believed to facilitate the recruitment of microglia to amyloid plaques. (Celarain et al. 2016; Ulrich et al. 2014). Notably, chronic inflammation, characteristic of conditions such as AD, is associated with increased TREM2 expression, while acute inflammation responses have previously shown conflicting effects (Lue et al. 2015; Perez et al. 2017).

Astrocytes, the most abundant glial subtype in the CNS, play crucial roles in regulating neuroinflammation and maintaining CNS homeostasis (Wyss-Coray and Rogers 2012; Carson, Thrash, and Walter 2006). While they typically perform various neuroprotective functions, upon activation by pathogens or injury, astrocytes can produce inflammatory cytokines contributing to the neurodegenerative processes in AD (Argaw et al. 2012; Bélanger and Magistretti 2009; Liddelow et al. 2017). Evidence implicating astrocytes in AD pathology includes their association with senile plaques and profound astrogliosis observed in AD animal models and patients (Matsuoka et al. 2001).

1.2.3.4 Environmental Factors in AD

The vast majority of AD, along with other neurodegenerative disorders like Parkinson's disease and amyotrophic lateral sclerosis, occur sporadically. These sporadic forms are likely the result of complex interactions between genetic predispositions and environmental risk factors.

Education is one factor that has been shown in multiple studies to show a positive impact on brain health. Both higher educational attainment and long-term education has been shown to delay the onset of AD by building cognitive reserve and brain volume (Wada et al. 2018; Andrews et al. 2021). Similarly, cognitive activities and bilingualism have also been found to preserve healthy cognitive functioning. Active engagement in cognitive activities has been associated with a 46% reduction in AD risk (Najar et al. 2019). Additionally, it was found that individuals who played more analog games experienced a reduced decline in memory ability and cognitive speed, potentially due to intensive connectivity between the hippocampus and frontal cortex (Altschul and Deary 2020).

New research approaches aiming to understand the role of environmental and dietary factors suggest that some of these factors may contribute to neurodegeneration through epigenetic modifications (Norton et al. 2014). These environmental agents and dietary factors can interfere with gene regulation over time, beginning at early developmental stages, though pathological results often do not manifest until much later in life.

Many longitudinal studies have identified various risks and protective factors for AD. One significant environmental modification that will be discussed in the coming chapters is diet and its impact on AD development and progression.

1.2.4 Animal Models

AD is typically categorized into three stages: the preclinical stage, mild cognitive impairment (MCI), and AD dementia. While it is possible that some animals in the wild such as non-human

primates and canines, can develop spontaneous AD-like dementia, the majority do not naturally develop the disease. The use of transgenic techniques has enabled researchers to utilize both invertebrate and vertebrate animals to investigate the underlying pathology of AD. However, it is important to note that there is currently no all-encompassing animal model for late-onset sporadic AD due to its multifaceted nature, which involves no direct genetic mutations and a mix of environmental influences. Additionally, widely used animal models often fail to fully replicate the pathological events seen in the MCI or preclinical stages of AD. While there is no "perfect" translational animal model for AD, many models that can be used to test therapeutic interventions at varying time points.

A comprehensive list of commonly used animal models that focus on specific main pathologies (ie. tau, amyloid, inflammation) are listed below in Table 1. Many of these models focus on the genetic mutations found in fAD (Oddo et al. 2003; Jawhar et al. 2012; Sturchler-Pierrat et al. 1997; Radde et al. 2006; Agca et al. 2016), while models focusing on other pathologies aside from Aβ show a wider range of pathologies to relate closer to clinical AD. For example, main models aim to recapitulate the multi-pathological features through the induction of multiple mutations, such as 3xTG and 5xFAD, which show cognitive impairment, inflammation and tau at later stages (Oddo et al. 2003; Jawhar et al. 2012). In contrast, models that focus on transgenic APP or PSEN1 mutations do not show tau as is seen in human AD (Sturchler-Pierrat et al. 1997; Radde et al. 2006). A main issue with many transgenic animal models is due to the fact that they are based on fAD, before being tested in sporadic AD, which significantly differs in disease progression and etiology.

Animal Model	Primary Pathology	Initiated By:	Other Pathologies:	References
3xTG (Mouse)	Aβ by 6 months	Transgenic fAD mutations; PSEN1, APP & MAPT	Cognitive impairment, decreased LTP, increased GFAP & Iba-1, tau in CA1 at 12 months	(Oddo et al. 2003)
5XFAD (Mouse)	Aβ at 1-3 months	Transgenic fAD mutations; PSEN1, 3x APP variations & MAPT	LTD and gliosis before 3 months, cognitive impairment and synaptic loss at 6 months	(Jawhar et al. 2012)
APP23 (Mouse)	APP (Aβ at 6 months)	7-fold overexpression of mutant APP	Cognitive impairment at 3 months, gliosis at 6 months and 14-28% neuronal loss at 14-18 months	(Sturchler-Pierrat et al. 1997)
APP/PS1 (Mouse or Rat)	Aβ at 6 weeks	Transgenic APP & PSEN1 mutations	Gliosis and synaptic loss around 3 months, cognitive impairment and impaired LTP at 8 months, modest neuronal loss at 17 months	(Radde et al. 2006)
htau (Mouse)	Human tau at 6 months	Transgenic MAPT mutation	Insoluble tau as early as 2 months but was hyperphosphorylated at 6 months. Cognitive impairment and neuronal loss at 9 months. High-frequency LTP abolished by 12 months	(Andorfer et al. 2003)
htau/P301S (Mouse)	NFT at 4 months	Transgenic MAPT with P301S mutation	Memory deficit and neuronal loss before 3 months, gliosis levels increased at 6 months	(Allen et al. 2002)
PolyI:C	Inflammation	Injection of Poly:I:C	Progressive neuroinflammation. Increased brain cytokine levels from 3 weeks of age. Tau hyperphosphorylation at 6 and 15 months, cognitive impairment at 20 months	(Krstic et al. 2012)
Okadaic acid (OKA)	Tau	Toxin to selectively inhibit phosphatases PP1 & 2A	Hyperphosphorylation of tau & apoptic cell death at 2 weeks, Aβ at 6 weeks	(Arendt et al. 1998)

p25	Inflammation; Activated microglia in week 1	Transgenic p25, or CDK5 activation	Hyperphosphorylation of tau and amyloid at 4 and 8 weeks, with cognitive impairment at 6 weeks	(Fischer et al. 2005; Sundaram et al. 2012)
LPS	Inflammation	LPS injection	Inflammation, impaired hippocampal-dependant spatial learning and memory.	(Shaw, Commins, and O'Mara 2001; Joshi et al. 2014; Zakaria et al. 2017)

Table 1.1. Animal models commonly used to induce AD-like pathologies in rodents.

1.3 Gut Microbiota Overview

1.3.1 Overview of Gut Microbiota System

The gastrointestinal (GI) tract is involved in many physiological roles, including mainly digestion and immunological defence. It maintains a balance for the need of nutrients, energy, vitamins, fluids and electrolytes while protecting against harmful microorganisms and toxins. Central to these functions is the enteric nervous system (ENS), often referred to as the "brain in the gut" (Furness 2008). The ENS is made up of a complex network of neurons and glia organized into ganglia, and regulates most of the local activity of the gut (Christensen and Rick 1987). These ganglia form multiple distinct enteric plexuses connected by neural pathways, which ensure extensive communication along the gut and between the myenteric and submucosal plexuses (Brehmer, Rupprecht, and Neuhuber 2010). With an estimated 200 million neurons and even more glial cells, the ENS is the largest in the peripheral nervous system (Furness 2008).

Advancements in microbiome research, notably through the Human Microbiome Project and the Metagenome of Human Intestinal Tract, have shed light on the intricate interactions between host and microbes. The makeup of the human gut bacterial species are collectively known as the “microbiome” and are 150 times larger than the human genome (Qin et al. 2010). The human gut microbiota comprising bacteria, archaea, eukarya, viruses, and parasites, is a dynamic community adapted to the GI environment (Bäckhed et al. 2005). Predominantly, the gut harbors bacteria from seven major divisions: Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia, and Cyanobacteria, with Firmicutes and Bacteroidetes making up over 90% of the population (Lagier et al. 2016). We will explore the gut microbiota system

and the critical role it plays in maintaining overall health and contributing to disease mechanisms.

Bacterial taxonomy is the classification system employed to organize bacterial specimens into taxonomic ranks (Ferraz Helene, Klepa, and Hungria 2022). This system mirrors that of other life forms such as plants and mammals and categorizes them into groups based on their genetic and phenotypic similarities. In this hierarchical structure, bacteria are classified starting from broader categories such as phyla and move towards more specific ones in the following order: class, order, family, genus, species and most specifically, strain (Maki et al. 2021). 16s rRNA sequencing techniques can effectively categorize down to the family level, and often genera and species level (Maki et al. 2021).

1.3.2 Gut Microbiota Changes Throughout Life

The composition of the gut microbiota varies significantly at different stages of life, with microbial colonization and development influenced by various factors, including maternal microbiota, mode of delivery, diet, genetics, health status, gestational age, and environmental factors. This microbiota colonization of the GI tract is essential, as microbiota-host interactions greatly influence human health and disease throughout one's life.

Microbial colonization of the infant gut is particularly significant, impacting immunological and metabolic pathways that influence health throughout life. During birth and infancy, the gut microbiota undergoes profound changes that are critical for health. Since the pioneering studies of Tissier (1866-1916) regarding the infant gut microbiota, it has been widely accepted that

fetuses are sterile in utero, and microbial colonization begins during and after birth (Van Gijsel-Bonnello 2015). During pregnancy, maternal microbiota, health status, and lifestyle can impact the initial colonization of the infant gut (Rodríguez et al. 2015). In infancy, this early colonization is influenced by various factors including the mother's microbiota, the method of delivery (vaginal vs. cesarian section), and the length of gestation. Breastfeeding has also been shown to play a crucial role in shaping the infant gut microbiota, along with other environmental factors such as geographical location and family environment.

Initially, early colonizers contribute to the low diversity of the neonatal gut microbiota, which is dominated by Proteobacteria and Actinobacteria. As time progresses after birth, the microbiota becomes more diverse, with Firmicutes and Bacteroidetes emerging as dominant phyla in this time period (Eckburg et al. 2005; Bäckhed 2011). The first year of life is a critical period for gut microbiota development and infants develop an individually distinct microbial profile. However, by the age of 2-5 years, the gut microbiota resembles an adults in terms of composition and diversity (Koenig et al. 2011; Yatsunenko et al. 2012). Dietary interventions during these formative years can have profound effects on host health and development.

In addition, the development of the immune system is closely related to the gut microbiota establishment, due to certain bacteria being crucial for parts of the immune system to mature (Gaufin, Tobin, and Aldrovandi 2018). The immune system of the infant undergoes rapid development in early life where is transitions from a suppressed state at birth to an active and responsive system. This immunological development is necessary for the infant to effectively respond to environmental antigens, many of which are also derived from the gut microbiota (Zhang, Zhivaki, and Lo-Man 2017). Additionally, the microbiota plays a vital role in training

the immune system to differentiate between commensal and pathogenic bacteria (Nakanishi, Sato, and Ohteki 2015).

In adulthood, the gut microbiota becomes stable, with a relatively consistent composition composed of members from the Actinobacteria, Proteobacteria, and primarily the Firmicutes and Bacteroidetes phyla (Claesson et al. 2011). Actinobacteria, particularly the genus Bifidobacterium, also play a significant role in this microbial community. This is based on extensive studies across developed countries, analyzing thousands of fecal samples and focusing on the 16S ribosomal RNA (16S rRNA) gene to identify microbial populations (Yatsunenko et al. 2012). Studies have shown that once this stable adult microbiota is established, it remains relatively consistent over time. For example, research by Faith et al. (Faith et al. 2013) followed 37 healthy adults over five years and found that 60% of the original strains were still present after this period, ensuring essential microbiome functions are consistently available. This stability serves to ensure essential microbiome functions are consistently available and may benefit the innate immune system as it recognizes the same microbial community and does not respond against it.

However, as individuals age the gut microbiota changes significantly. For individuals over the age of 70 this includes changes in digestion, nutrient absorption, and weakened immune activity which can further influence gut microbiota composition. Additionally, it has been shown that a loss of gut microbiota diversity can occur with age, including an effect observed in diversity-associated taxa, including Prevotella (Odamaki et al. 2016). In a study on 161 subjects aged 65 years and older, it was revealed that elderly individuals, particularly those who are more frail,

tend to have a gut microbiota population dominated by Bacteroidetes compared to younger individuals (Claesson et al. 2011). Typically, there is also a decrease in anaerobic bacteria observed such as Bifidobacterium and an increase in Clostridium and Proteobacteria (Odamaki et al. 2016).

The large variance in microbiota profiles among elderly subjects suggests that this trend alone is not highly predictive of the phenotype and the variability can be attributed to various external factors such as diet, exercise and mobility, medication, and co-habitation patterns. These changes are also observed in aging-related diseases, such as AD, where differences in gut microbiota have been noted, highlighting the potential role of gut health in the progression of age-related conditions.

1.3.3 Microbiota-Gut-Brain Axis

The gut is made up of millions of microbes, including bacteria, archaea, fungi and bioactive molecules (Adak and Khan 2019). These molecules can communicate with the brain and each other, and pathways such as blood circulation, blood-brain-barrier (BBB) communication, and the vagus nerve have been recognized as key players in this conversation (de Jonge 2013; Yano et al. 2015; Borovikova et al. 2000). Gut microbiota can produce multiple neuroactive metabolites, including neurotransmitters and their precursors, through a bottom-up pathway initiated by signalling to the brain (Valles-Colomer et al. 2019; Lai et al. 2022). Gut molecules can influence neurotransmitter synthesis, short-chain fatty acid (SCFA) production, and create the pathogenic movement of substances from the gut to the brain (Sochocka et al. 2019;

Albenberg and Wu 2014; Kaur et al. 2020; Kao et al. 2019). More recently, it has also been shown that neuroinflammatory processes, such as microglia activation, are under constant regulation by the gut microbiome (Erny et al. 2015). Many gut microbial signals can modulate these homeostatic reflexes by either acting locally on enteric neurons, on vagal and sympathetic afferent nerve terminals, which generate signals to the CNS, or by transmission to the brain via systemic circulation (Calabrò et al. 2023).

1.3.3.1 Microbiota-Gut-Brain Axis in AD

The microbiota–gut–brain axis represents the bidirectional communication between the gut and the brain. Gut dysbiosis is defined as the disruption of the microbiome makeup, which can contribute to a diseased state. In AD, the role of gut dysbiosis is becoming increasingly important due to the effects it can have on gut barrier function, BBB permeability, and neuroinflammation. Increased barrier permeability, as seen in AD studies, provides an opportunity for toxic amyloids, neuroinflammatory cytokines, lipopolysaccharides (LPS) to move into the brain and seed neurodegeneration (Varesi et al. 2022). The microbial community of the gut microbiota plays an active role in homeostasis and disease. In humans, aging contributes to the increase in pro-inflammatory bacteria, which has been found to induce systemic inflammation. Pro-inflammatory bacteria and, in turn, neuroinflammation has been found in amyloid-positive patients when compared to healthy elders (Cattaneo et al. 2017).

Human studies aiming to classify differences in bacterial species in the gut makeup of healthy vs. AD patients have provided insight into the relationship between human health and disease. Vogt

et al. discovered a significant decrease in the abundance of Firmicutes and Bifidobacteria in the fecal samples of AD patients, accompanied by an increase in Bacteroidetes species (Vogt et al. 2017). This was validated through 16s rRNA sequencing conducted by Zhuang et al. and Liu et al., finding similar results with the addition of the dysregulation of multiple other bacterial families (Zhuang et al. 2018; Liu et al. 2019). Interestingly, a difference between Enterobacteriaceae was also found between MCI and AD patients, indicating a change in gut composition with disease progression (Liu et al. 2019). A further understanding of the regulation of bacterial species through aging and disease is needed, as vast discrepancies can be found between patients, demographic regions, and ages.

1.3.3.3 Microbiota-Gut-Brain Axis in Environmental Factors

While AD has been shown to be highly heritable, with heritability estimates ranging from approximately 60–100% depending on the timing of disease onset, the prevalence of genetically linked AD cases remains very low, accounting for less than 0.5% of all cases (Blennow, de Leon, and Zetterberg 2006). This indicates that non-genetic, environmental risk factors play a significant role in the development of AD, and research has shown up to 40% of AD risk can be attributed to environmental factors (Sotiropoulos et al. 2011).

As mentioned above, the gut-brain axis is significantly influenced by environmental factors. These factors can include diet, lifestyle, stress, and exposure to toxins, all of which can impact the gut microbiota and, consequently, brain health. For example, diet influences the composition and function of the gut microbiota, which in turn affects the production of neurotransmitters and SCFAs that are crucial for brain function (Brüssow and Parkinson 2014). A diet high in

processed foods and low in fiber can lead to dysbiosis, which has been linked to neuroinflammation and increased AD risk (Brown et al. 2012).

Lifestyle as an environmental factor can include day-to-day activities such as physical activity, sleep, and social enrichment. Regular physical activity has been shown to promote a healthy gut microbiota composition, reduce systemic inflammation, and improve cognitive function (Monda et al. 2017; Zhang et al. 2022) Additionally, exposure to environmental toxins can negatively alter gut microbiota composition and function, leading to increased production of neurotoxic metabolites and inflammation (Chiu et al. 2020; De Filippis et al. 2024).

1.3.3.3.1 Stress as an Environmental Factor

Stress is widely recognized for its negative impact on various disorders, including cardiovascular disease, obesity, gastrointestinal issues, and psychiatric and neurodegenerative disorders (Torraville et al. 2023). Commonly, stress induces the release of hormones such as glucocorticoids and catecholamines which have been shown to modulate GI function and microbial growth (Ulrich-Lai and Herman 2009; Galley and Bailey 2014). Stress-induced changes along the vagus nerve have also been found to reduce digestive activity, likely altering substrate availability (Galley and Bailey 2014). It has been shown that stress-induced changes in gut microbiota in humans can degrade the physical gut barrier and increase gut permeability (van Wijck et al. 2012).

In students, it was found that fecal levels of lactic acid bacteria were lower during a high-stress situation, associated with an increase in cortisol concentration (Knowles, Nelson, and Palombo 2008). Strengthening this, higher levels of salivary cortisol, a well-known biomarker of the endocrinological stress response, were observed before an exam (Kato-Kataoka et al. 2016). The daily consumption of probiotic Lactobacillus casei significantly reduced gastrointestinal stress and symptoms and was found to preserve the diversity of the gut microbiota compared to non-probiotic-fed students (Kato-Kataoka et al. 2016).

Activation of the hypothalamic–pituitary–adrenal (HPA) axis causes a release of corticotropin releasing factor (CRF) from the hypothalamus, and CRF becomes one of the main stress-related neuropeptides involved with both the brain and gut (Barreau et al. 2007). Stress-induced activation of the HPA axis by maternal stress increases levels of corticosterone, CRF, and has been shown to also affect the permeability of the intestinal wall (la Fleur et al. 2005). Regulating the release of these peptides alters the inflammation in the GI tract and plays a role in increasing intestinal barrier leakage (Kohno et al. 2001; Bailey et al. 2011).

Circulating cortisol levels also appear to be affected in MCI and AD, with elevated levels detected in biological fluids such as plasma, saliva and CSF (Escher, Sannemann, and Jessen 2019; Caruso et al. 2018). These levels are correlated with the high prevalence of anxiety-related mental illnesses in AD patients (Caruso et al. 2018). Moreover, dementia patients exhibit impaired HPA axis feedback when given dexamethasone, indicating dysfunction in the HPA axis (Murialdo et al. 2000). Increased HPA activity leads to elevated cortisol release, which can accelerate and intensify AD progression.

Stress-induced increased inflammation and impaired cognition have been found in animal models of various neurodegenerative diseases, depression, and irritable bowel syndrome (IBS; Gareau et al. 2011; Lin, Zheng, and Zhang 2018). Specifically, the intestinal barrier has been found to be damaged in AD patients and animal models (Pellegrini et al. 2023; Bailey and Coe 1999). Gut microbes influenced by stress exposure can impact intestinal barrier function and ultimately lead to intestinal permeability (Dodiya et al. 2020; Xiao et al. 2020; Dandekar et al. 2022; Liang et al. 2015). Interestingly, when probiotic Lactobacillus was administered in rodents, this barrier leakiness was prevented, along with improved behavioral, cognitive, and biochemical parameters (Lin, Zheng, and Zhang 2018).

1.3.3.3.2 Enrichment as an Environmental Factor

Environmental enrichment (EE) in contrast has been shown to have a significant positive impact on the development and progression of dementia. Cognitive enrichment for example, which involved mentally stimulating activities of various kinds, has been shown to positively influence AD pathology among older adults. A longitudinal study on lifelong cognitive enrichment including education and other mentally engaging activities, revealed that high cognitive enrichment scores throughout life reduced the risk of cognitive impairment and delayed the progression from mild cognitive impairment to dementia (Xu et al. 2020).

A six-year multi-interview assessment with cognitively normal older adults examined the impact of frequent cognitive activities such as reading, watching television, listening to the radio, playing games, completing puzzles, and visiting museums (Wilson et al. 2010). It was found that

each additional point of cognitive activity reduced cognitive decline by 52% during follow-up assessments. Leisure activities that stimulate cognitive function, such as crossword puzzles, have also been associated with a reduced risk of dementia by improving cognitive reserve and promoting healthy behaviors (Pillai et al. 2011).

Psychological studies indicate that social conversation requiring attention, working memory, executive functions, and social cognition is highly stimulating. Isolation, particularly increased among older adults, has been consistently linked to cognitive decline and AD (Wilson et al. 2007). One study found that elderly individuals with no social connections were 2.37 times more likely to experience cognitive decline compared to those with five or six social connections (Bassuk, Glass, and Berkman 1999). Loneliness further increases the risk of AD by more than double and elevates levels of interleukin (IL)-6, an inflammatory agent associated with numerous age-related diseases including AD (Friedman et al. 2005).

Research on the relationship between EE and the gut microbiome is a relatively new field, with only a few studies examining the direct effects of EE on gut microbiome composition and diversity. One study by Kim et al., investigated the relationship between the gut microbiome and social exclusion (Kim et al. 2022). This revealed distinct gut microbial profiles between the those with and without social exclusion. The exclusion group exhibited a higher probability of having a Prevotella-enriched microbiome, a significantly reduced Firmicutes/Bacteroidetes ratio, and decreased abundance of Faecalibacterium.

To date, the effects for social enrichment have largely been attributed to shared diets, as members of the same household or social group tend to consume similar foods in similar

proportions (Kinross and Nicholson 2012). However, social relationships themselves could also shape gut microbiomes more directly, though further clarification is necessary.

1.3.3.4 Human Evidence of Microbiota-Gut-Brain Axis in AD

Recent advancements in DNA sequencing, metagenomic, and metabolomic analyses have aided in our understanding of the gut microbiota and its interactions with human health and disease. Abnormal changes in gut microbiota composition, called dysbiosis, has been implicated in the pathology of AD. Several human studies which provide evidence for the link between gut dysbiosis to AD will be discussed below (Vogt et al. 2017; Emery et al. 2017; Zhuang et al. 2018).

Using 16srRNA sequencing, fecal samples from AD patients and cognitively normal controls revealed significant differences in gut microbiota composition. AD patients exhibited variations in bacterial taxa such as Bacteroides, Actinobacteria, Ruminococcus, Lachnospiraceae, and Selenomonadales, suggesting that gut microbiota alterations may be involved in AD pathogenesis (Zhuang et al. 2018). Further analyses in AD patients have shown decreased microbial diversity and distinct compositional differences compared to controls (Vogt et al. 2017). Notably, decreased Firmicutes and Actinobacteria phylum, including Bifidobacterium, along with increased Bacteroidetes, were observed in the AD microbiome (Vogt et al. 2017). An additional study using 16srRNA on brain tissue samples revealed increased brain-bacterial populations in AD-affected brains compared to cognitively unimpaired individuals (Emery et al. 2017). In addition, studies on metabolic alterations linked to AD highlight the role of tryptophan metabolites produced by gut bacteria (Paley et al. 2018).

Research exploring the association between brain amyloidosis and the gut microbiota identified changes in bacterial taxa with pro- and anti-inflammatory roles. Specifically, an increase in the pro-inflammatory taxon Escherichia/Shigella and a reduction in the anti-inflammatory taxon E. rectale were linked to a peripheral inflammatory state in cognitively impaired patients with brain amyloidosis (Cattaneo et al. 2017).

The growth of human studies exploring the relationship of the microbiota and the brain has been driven by advancements in sequencing technologies. However, defining what constitutes a "normal" or "healthy" microbiome remains a key challenge.

1.3.3.5 Animal Model Evidence of Microbiota-Gut-Brain Axis in AD

Consistent with the gut microbiota alterations observed in AD patients, animal models of AD have shown significant differences in gut microbiota composition.

In 5XFAD mice, studies have reported an increase in Firmicutes and a decrease in Bacteroidetes at 9 weeks of age (Brandscheid et al. 2017). However, no significant changes were observed at 6 or 18 weeks in these mice compared to wildtype controls. Another study noted a decrease in Firmicutes and an increase in Bacteroidetes at 3 months of age in 5XFAD mice (Chen et al. 2020). By 6 months, these mice exhibited a marked increase in Bacteroidetes, Proteobacteria, and Deferribacteres. In APP/PS1 mice, researchers observed increases in Escherichia-Shigella, Desulfovibrio, Akkermansia, and Blautia starting from 1 month of age (Chen et al. 2020). At 8-12 months, these mice exhibited increased Verrucomicrobia and Proteobacteria, with significant decreases in Ruminococcus and Butyricicoccus (Zhang et al. 2017). Sex-specific differences

were also noted in APP/PS1 mice. Female transgenic mice were found with higher levels of Bacteroidetes compared to males (Cuervo-Zanatta, Garcia-Mena, and Perez-Cruz 2021).

A study in 2019 investigated the impact of gut microbiota on AD pathogenesis using a newly developed murine transgenic model, ADLP^{APT}, which exhibits both amyloid and tau pathologies in the brain (Kim et al. 2018; Kim et al. 2020). Notably, transfer and transplantation of fecal microbiota from healthy wild-type mice reduced amyloid plaque and tau tangle formation, mitigated memory deficits, and altered peripheral immune cell populations (Kim et al. 2020).

A novel study in 2023 investigated the involvement of AD patient gut microbiota by transplanting fecal microbiota from AD patients and age-matched healthy controls into microbiota-depleted young adult rats (Grabrucker et al. 2023). This study found that the rats receiving transplants from AD patients exhibited impairments in behaviors reliant on adult hippocampal neurogenesis, an essential process for certain memory functions and mood. The severity of these impairments also correlated with the clinical cognitive scores of the donor patients. Importantly, these findings suggest that symptoms can be transferred to healthy young organisms via the gut microbiota, confirming a causal role of gut microbiota in Alzheimer's disease.

1.3.4 Possible Mechanisms Underlying the Gut-AD Relationship

Several mechanisms have been supported to underly the gut-AD connection including gut and brain barrier dysfunction, neuroinflammation, peripheral inflammation, the effect of pathological molecules such as tau and amyloid, and the action of GSK3β kinase (Figure 1.1).

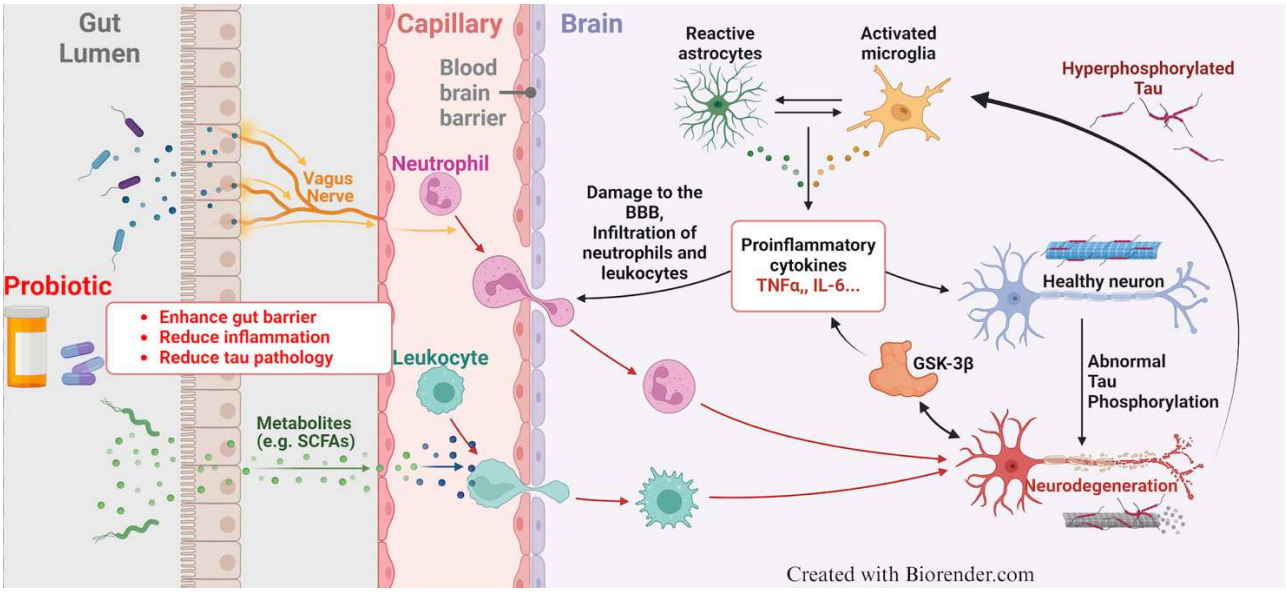


Figure 1.1 Possible Mechanisms behind probiotic supplementation and AD pathology amelioration. Mechanisms underlying the gut-brain connection in AD: gut and brain barrier dysfunction, neuroinflammation, peripheral inflammation, the effect of pathological molecules such as tau and amyloid, and the action of GSK3β kinase. As well as the possible effects of probiotics on these mechanisms.

Communication between the gut microbiota and the brain occurs through various pathways such as cytokine release by mucosal immune cells, hormone production by endocrine cells like serotonin (5HT), and directly through the vagus nerve. Stress-related efferent signals, including stress hormones and sympathetic neurotransmitters (e.g., GABA, 5-HT precursors) can also influence the gut microbial population, physiology, and gastrointestinal functions through these pathways.

One promising approach to modulating these mechanisms involves the use of probiotics. Probiotics are live microorganisms, including beneficial bacteria and yeast, that have shown positive effects on human health (Gibson and Roberfroid 1995). Recent studies have enhanced our understanding of probiotics actions on these underlying mechanisms, and specifically, have documented that probiotics can alter the intestinal microbiota, providing benefits in disease and enhancing overall wellness (Kim et al. 2019).

The following chapters will explore possible mechanisms, the role of probiotics on them, and their potential impact on AD.

1.3.4.1 Gut and Brain Barrier Dysfunction

The human intestinal mucosal barrier is made up of the mucus layer, epithelium, and lamina propria. Disruption of this barrier increases permeability, allowing bacteria and harmful substances into the bloodstream, a phenomenon known as atopobiosis. In the colon, the thick mucus layer is known to prevent bacterial localization (Paone and Cani 2020). Though certain bacteria can produce exotoxins that disrupt epithelial cell integrity and damage tight junctions.

This disruption leads to the migration of microbes and their products into the mucosal layer, subsequently triggering an immune response and the release of inflammatory mediators.

Research by Xie et al. demonstrated that mice lacking gut microbiota exhibit increased blood-CSF barrier permeability and disorganized tight junctions. This condition was ameliorated by supplementation of SCFAs (Xie et al. 2023). Other studies have shown there are many strains of probiotics known to play a direct role in SCFA production. Notably, Clostridium_sensu_stricto_1 produces SCFAs, particularly butyrate, which can enhance the expression of tight junction proteins in colon epithelia and strength the intestinal barrier, reducing mucosal permeability, and inhibiting inflammatory cytokines. Additionally, its decreased abundance in normal aging studies suggests a potential role in age-related gut health decline (Odamaki et al. 2016).

Dysfunction of the BBB is implicated in AD progression, triggering neuroinflammation and oxidative stress, which indirectly has been shown to enhance the activity AB & tau accumulation (Erickson and Banks 2013; Enciu, Gherghiceanu, and Popescu 2013; Blair et al. 2015). In AD models, gut barrier dysfunction is evidenced by microbial dysbiosis, including changes in species such as Lactobacillus, Bacteroides, Prevotella, and Bifidobacterium (D'Argenio et al. 2022). This dysbiosis is associated with altered gut permeability, as indicated by reduced expression of zonulin and occludin proteins, leading to increased circulating bacterial LPS and other pro-inflammatory markers (Cani et al. 2008; D'Argenio et al. 2022). Additionally, fecal calprotectin levels serve as a marker for intestinal inflammation, with experimental studies revealing increased calprotectin in the cerebrospinal fluid, brain, and fecal matter of AD patients (Leblhuber et al. 2015).

BBB dysfunction is increasingly recognized as an early event in AD pathogenesis (van de Haar et al. 2016; Montagne et al. 2020). For instance, in the APP/PS1 mouse model of AD, BBB dysfunction begins at around 4 months of age and becomes more severe by 9 months (Wang et al. 2022). Studies have also shown that age-dependent deterioration of the BBB occurs during aging, particularly in the human hippocampus (Montagne et al. 2015). In addition, evidence from in vitro studies and transgenic mouse models of tauopathy indicates that tau may contribute to BBB breakdown, with suppression of tau expression leading to a preserved BBB integrity (Blair et al. 2015).

Probiotic supplementation has been shown to benefit both the gut barrier and BBB. They are shown to enhance gut barrier directly through their surface components (flagella, pili, capsular proteins etc.), which constitute microbial-associated molecular patterns (MAMPs) (Liu et al. 2020). MAMPs can bind to receptors on the epithelial barrier and regulate cytokine production through protease-dependent signaling cascades. As a result, various anti-inflammatory cytokines are produced, which alleviate inflammation and enhance barrier function (Sharma, Young, and Neu 2010; Liu et al. 2020)

Probiotics and a healthy gut microbiome can also significantly influence the integrity and function of the BBB. The gut microbiota metabolize dietary components, including macronutrients, micronutrients, fibers, and polyphenols, into a variety of metabolites such as SCFAs, trimethylamines, amino acid derivatives, and vitamins (Parker, Fonseca, and Carding 2020). SCFAs, for instance, play multiple roles by stimulating colonic blood flow, influencing upper-gut motility, regulating water and salt uptake, and enhancing satiety. They can enter the bloodstream and positively impact the BBB's integrity and function. In germ-free (GF) mice,

colonization with the butyrate-producing bacterium Clostridium tyrobutyricum or the oral administration of sodium butyrate has been shown to decrease BBB permeability by upregulating tight junction proteins (Braniste et al. 2014). Multiple studies following traumatic brain injury, showed the administration of sodium butyrate and clostridium can prevent BBB breakdown and promote neurogenesis (Li et al. 2016; Li et al. 2018).

1.3.4.2 Neuroinflammation

Recent discoveries have expanded our understanding of immune-related mechanisms, including altered gut-brain axis, and microglial and astrocyte activation during AD pathogenesis (Cattaneo et al. 2017). In the gut, these cytokines exist in a balanced nature, but gut dysbiosis can lead to increased permeability of the intestinal barrier (Bischoff et al. 2014; Leblhuber et al. 2015), movement of pathogenic molecules (Leblhuber et al. 2015), and subsequent systemic inflammation. Recent studies demonstrate that altering the composition of gut microbiota via probiotics in AD animal models could lead to a reduction in levels of pro-inflammatory cytokines. Notably, feeding probiotic supplementation is known to enhance gut health, potentially attenuating inflammatory cytokine production within the gut (Milajerdi et al. 2020). Studies have shown bacterial species such as Rikenellaceae_Alistipes showed a significant correlation with inflammatory cytokines and is associated with an increase in SCFAs-producing bacteria (Li et al. 2016; Butera et al. 2018).

TREM2 is implicated as a potential key player in the systemic spread of inflammation and could serve as a crucial target for intervention. In conditions of gut-dysbiosis, compounds released by intestinal bacteria activate TREMs on macrophages, triggering an exaggerated pro-inflammatory

reaction that may compromise the integrity of the gut barrier (Park et al. 2009; Bonaz, Bazin, and Pellissier 2018). This disruption could facilitate the entry of TREM-positive activated macrophages, along with inflammatory mediators, into the brain via various routes, including the blood, lymphatic system, circumventricular organs, or the vagus nerve through the microbiota-gut-brain axis (Bonaz, Bazin, and Pellissier 2018). Additionally, TREM2-deficient dendritic cells exhibit diminished abilities to release pro-inflammatory cytokines, eliminate bacteria, and activate T cells in response to bacteria-associated antigens (Correale et al. 2013). These findings suggest that in the gut microenvironment, TREM2 may serve as an amplifier of inflammation.

One investigation using GF 3×Tg mice revealed a reduction in cerebral Aβ plaques and NFTs, corresponding with an increase in Bacteroides compared to pathogen-free mice (Chen et al. 2022). Following this increase, the mice showed altered microglial levels, inflammatory pathways and insulin/insulin-like growth factor (IGF-1) signaling in the absence of gut microbiota. Additional studies on GF mice and antibiotic-treated mice indicate that the gut microbiota also influences the development and maturation of the immune system (Khosravi et al. 2014; Kierdorf and Prinz 2013).

1.3.4.3 Peripheral Inflammation

In recent years, it has become evident that innate immune activation is crucial in the pathogenesis and progression of AD. It is well established that acute systemic bacterial or viral infections can affect brain function, with both preclinical and clinical AD studies showing that inflammatory mediators generated systemically can signal to the brain through neural and humoral pathways (Harrison et al. 2009).

Animal models frequently utilize peripheral injections of immune-stimulating molecules like LPS or pro-inflammatory cytokines to induce systemic inflammation (Seemann, Zohles, and Lupp 2017). A single systemic LPS injection can increase Aβ1-42 deposition and ptau levels in the brains of wild-type rodents (Wang et al. 2018). For instance, LPS-induced systemic inflammation leads to an increase in Aβ deposition in mice, and TNF-induced systemic inflammation in 15-month-old APP/PS1 mice enhances Aβ plaque formation (Krstic et al. 2012; Hennessy et al. 2017). However, there are contradictory results regarding the impact of systemic inflammation on Aβ burden in the mouse brain. Some studies on 3xTg-AD and 5XFAD mice show elevated C-terminal APP fragments (β-CTF) and tau hyperphosphorylation without changes in Aβ deposition.

Another study using AD mouse models demonstrated that changes in gut microbiota during AD progression lead to the peripheral accumulation of phenylalanine and isoleucine (Wang et al. 2019). These amino acids stimulate the differentiation and proliferation of pro-inflammatory Th1 cells, which infiltrate the brain and activate microglia thereby contributing to neuroinflammation. This phenomenon was also observed in patients with MCI, linking peripheral inflammation to brain inflammation (Wang et al. 2019). A study by Cattaneo et al., also provided a link between peripheral inflammation and gut microbiota changes. A positive correlation was observed in cognitively impaired patients between peripheral proinflammatory cytokines and the abundance of multiple inflammatory bacteria taxon such as Escherichia and Shigella (Cattaneo et al. 2017). Additionally, multiple studies have shown that diet manipulations such as the western diet, can induce systemic inflammation, impair the BBB and serve as a potential trigger for AD (Więckowska-Gacek et al. 2021). Sustained chronic inflammation from infections can disrupt the BBB, allowing peripheral mediators to enter the brain (Huang, Hussain, and Chang 2021). This

chronic brain inflammation can impair brain function, leading to cognitive decline in AD patients.

1.3.4.4 Pathological Molecules

Previously, the indirect effects of gut dysbiosis, such as inflammation exacerbating tau and amyloid presence in the brain was discussed. This chapter will focus on the direct mechanisms involving tau and amyloid in the gut. The ENS, a complex neural network within the gut wall, coordinates gastrointestinal functions and is newly emerging as a site for tau pathology, termed 'enteric tauopathy' (Benarroch 2007; Feng et al. 2018). While traditionally associated with synaptic plasticity in the CNS, tau's presence in the ENS was first noted in human post-mortem studies in the late 1980s and early 1990s (Trojanowski et al. 1989; Tam 1990). Subsequent research confirmed tau immunoreactivity in the gut of various species, including humans and rodents. One study performed IHC staining of intestinal and colon samples from infants and children and found intense tau immunoreactivity in cell bodies and processes in both myenteric and submucosal enteric neurons (Tam 1990). Following studies have also shown tau presence along the entire GI tract of mice (Feng et al. 2018; Lionnet et al. 2018).

Notably, only a subset of the six tau isoforms expressed in the human CNS are found in the gut. Studies by Dugger et al. using HT7 antibody and western blot revealed different tau isoforms in the gut compared to the brain, with colonic tau being physiologically phosphorylated at specific residues (Dugger et al. 2016). This phosphorylation in the adult colon was found to occur at serine 202, 231, 262, and 396 along with threonine 181 and 205 (Dugger et al. 2016; Lionnet et al. 2018; Chiocchetti et al. 2021; Pellegrini et al. 2020). Interestingly, treatment of broad-spectrum phosphatase had no effect on tau immunoreactivity with antibodies specific to tau

phosphorylation, showing a resistance to dephosphorylation in sharp contrast with the brain (Lionnet et al. 2018).

In addition to tau, Aβ has also been implicated in gut-brain interactions. Although the exact source of Aβ in the AD brain is unclear, evidence suggests peripheral Aβ can cross the BBB or travel via the vagal nerve to the brain (Zlokovic et al. 1994; Sun et al. 2020; Chen et al. 2021). Studies with APP/PS1 transgenic mice have shown that their gut microbiota can significantly increase cerebral Aβ pathology, compared to GF mice (Jin et al. 2023). Aβ is detected throughout the gut in both mice and humans, with levels rising with age and enhanced by gut microbiota from APP/PS1 mice (Jin et al. 2023). Additionally, soluble Aβ forms are found in plasma and at the BBB (Chen et al. 2017). Research indicates that Aβ and/or precursor protein is present in the small intestine's epithelial cells, influenced by dietary factors like high-fat feeding (Galloway et al. 2007). These findings highlight the gut as a significant source of Aβ peptides, linking gut health directly to amyloid pathology in the brain, while causative pathology of tau from the ENS remains poorly understood.

1.3.4.5 GSK3B Kinase

A key feature in tau hyperphosphorylation is GSK-3, a proline-rich serine/threonine kinase (Sayas and Ávila 2021). GSK-3 is physiologically present in two isoforms GSK-3α and GSK-3β. The field has largely focused on the role of GSK-3β in tau pathology. Excessively activated GSK-3β contributes to the abnormal phosphorylation of tau, leading to the destabilization of microtubules, as seen in AD pathogenesis (Morris et al. 2011; Sayas and Ávila 2021). In addition to this, GSK-3 is a downstream regulator of other tau kinases and phosphatases, such as cyclin-

dependent kinase 5 and protein phosphatase 1 and 2A (Bennecib et al. 2000; Plattner, Angelo, and Giese 2006).

GSK-3β expression is up-regulated in the hippocampus of AD patients (Pei et al. 1999). The active GSK- 3β is initially found in pretangle neurons in the entorhinal cortex and extends to other brain regions in the same spatial sequence as tau pathology (Pei et al. 1999). Overexpression of GSK-3β in mice results in tau hyperphosphorylation, prevents induction of LTP (Hooper et al. 2007) and impairs spatial learning (Hernández et al. 2002). Normalizing GSK-3β restores normal phosphorylated tau levels, reduces neuronal loss and cognitive deficit (Hernández et al. 2002). Interestingly, GSK-3β overexpression is associated with tau hyperphosphorylation but not tangles in the hippocampus (Hernández et al. 2002). Lithium, an inhibitor of GSK-3β, effectively reduces tau hyperphosphorylation (Muñoz-Montaño et al. 1997).

Probiotic therapy has the potential of correcting tau hyperphosphorylation through GSK-3β suppression (Hooper, Killick, and Lovestone 2008; Lin et al. 2020). Lactobacillus plantarum DP189 (Song et al. 2022) and Bifidobacterium Breve (Abdelhamid et al. 2022) strains of probiotics inhibits tau hyperphosphorylation in mouse models of AD. At mechanistic level, probiotic supplementation could exert its effect on GSK- 3β and tau phosphorylation through PI3K/Akt signaling. SCFAs such as butyrate, produced by gut bacteria and subsequently released in the bloodstream, enhances gut barrier function and free fatty acid receptor FFA2/GPR43-mediated PI3K/Akt signaling in muscle cells (Tang et al. 2022). Probiotics or SCFAs can also act on PI3K/Akt signaling via other receptors such as insulin-like growth factor 1 receptor (IGF-1R) or TLRs (Larraufie et al. 2017; Dang et al. 2018; Mohseni et al. 2021; Pavelješek et al. 2021). In the brain, Lactobacillus plantarum gut administration results in an increase in Akt

phosphorylation at S473, causing an elevated level of phosphorylated GSK-3β at S9 and subsequent inactivation of GSK- 3β (Song et al. 2022). The inactivation of GSK-3β decreases tau phosphorylation at numerous proline-rich and non-proline sites (Hanger, Anderton, and Noble 2009; Sayas and Ávila 2021).

The precise route and mechanism of how gut probiotic supplement influences PI3K/Akt/GSK-3β signaling in the brain is not clear. However, Lactobacillus plantarum has been shown to increase the abundance of butyrate-producing bacteria Anaerotruncus and Faecalibacterium (J. Wang et al. 2018). Therefore, it could mediate the brain effect through SCFAs circulating in the blood and binding to GPR43 receptors (Brown et al. 2003; Barki et al. 2022; Tang et al. 2022). TLR (Dang et al. 2018; Mohseni et al. 2021; Paveljšek et al. 2021), or IGF-1R *via* elevated serum IGF-1 (Endo et al. 2013; Yan et al. 2016; Mohseni et al. 2021). IGF-1R is widely expressed in the brain such as the hippocampus (Lin et al. 2020). TLR is abundantly expressed in microglia, and to a lesser degree, neurons (Tang et al. 2022; Fiebich et al. 2018). G protein-coupled receptors (GPR)-43 are expressed in multiple tissues including neurons (Kimura et al. 2020; Barki et al. 2022). In another study, two strains of Lactobacillus Acidophilus treatment in mice down-regulates GSK-3β gene expression (Yan et al. 2019). These studies suggest that probiotics can directly act on the GSK-3β pathway and alleviate tau hyperphosphorylation. Furthermore, probiotic has been proven effective in treating gastric infection caused by *H. Pylori* (Aiba et al. 2015), which induces tau hyperphosphorylation in mouse hippocampal tissue (Wang et al. 2014; Uberti et al. 2022), via the GSK-3β pathway (Wang et al. 2014). Dysregulation of gut microbiota via gut- brain axis is associated with AD and probiotic supplement has the potential of correcting tau hyperphosphorylation through GSK-3β suppression. More extensive future research is needed to characterize the relationship between gut microbiota and tau hyperphosphorylation,

especially in suitable animal models with GSK-3β induced tau hyperphosphorylation as a key feature.

1.4 What is Missing?

As mentioned, current therapies and AD research resources have focused on a later time point in the disease, when Aβ is a prominent pathology. Our pretangle tau animal model addresses the need for a model that targets a preclinical time point in the disease. This time point matches disease pathology and clinical decline as seen in humans and mimics the prodromal stage and multifaceted factors that have been widely reported. With the microbiota-gut-brain axis emerging as a crucial link between peripheral health and CNS function, the focus on gut dysbiosis as a possibly pathological driver in AD becomes a promising avenue for potential therapeutics.

Gut dysbiosis is increasingly implicated in AD pathogenesis through mechanisms such as inflammation, however the role of the gut microbiota in the early preclinical stages of AD remains poorly understood. This leaves a significant gap in current research concerning the impact of the gut microbiome and probiotic interventions during this stage of disease development. While probiotics have shown promise in enhancing gut health and reducing inflammation, the potential to alter the course of AD when administered early is yet to be explored. Similarly, chronic stress is a well-documented risk factor for cognitive decline and brain health. Stress-induced inflammation and subsequent neuronal damage represent another critical area where early intervention could yield significant benefits.

By examining the gut microbiome composition, peripheral and brain inflammation and cognitive outcomes, this study seeks to provide a comprehensive understanding of how probiotics might

modulate the gut-brain interactions and offer a viable, non-invasive therapeutic strategy for slowing AD progression and mitigative stress-induced cognitive impairments.

Chapter 2: Probiotic supplementation improves cognitive function and ameliorates inflammation in a pretangle tau rat model

This chapter is a version of a submitted manuscript. Currently in review.

2.1 Introduction

AD presents a significant and debilitating challenge. Despite its considerable impact on individuals and society, much about its biological progression remains poorly understood. While the accumulation of Aβ plaques and NFTs has traditionally been emphasized as pivotal in AD's pathogenesis (Hardy and Higgins 1992; Long and Holtzman 2019) recent research indicates that the disease process may commence much earlier than previously recognized. The long prodromal period of AD offers an optimal window for AD prevention.

Braak and colleagues have identified pretangle tau, a soluble, persistently phosphorylated protein precursor of NFTs, as an early marker of AD pathology. Pretangle tau is found in the LC as early as childhood (Braak et al. 2011). Pretangle tau spreads to other regions in the brain stem and trans-entorhinal cortex over decades, eventually evolving into six stages of NFTs. Clinical symptoms typically emerge around NFT stage III/IV. To study pretangle tau-mediated pathology, we developed a rat model mimicking Braak's pretangle stages of preclinical AD (Ghosh et al. 2019; Omoluabi et al. 2021). By introducing a human tau pseudophosphorylated at 14 sites (htauE14; mimicking pretangle tau) into the rat LC, we observed a somatodendritic

expression pattern of htauE14 in LC neurons, subsequently spreading to other neuromodulatory nuclei of the brainstem and the entorhinal cortex (Ghosh et al. 2019; Omoluabi et al. 2021). Notably, even in the absence of NFTs, htauE14 rats displayed impairments in associative olfactory discrimination and spatial learning, mirroring deficits observed in pre-clinical AD (Devanand et al. 2015). Furthermore, LC fiber degeneration and neuronal loss correlated with the severity of behavioral deficiencies, mirroring human observations (Gulyás et al. 2010; Theofilas et al. 2017). Therefore, our model serves as a valuable tool for studying tau pathology associated with preclinical AD.

Environmental factors play a significant role in AD onset, with the gut-microbiota-brain axis emerging as a crucial link. Dysbiosis in the gut microbiome, characterized by compositional alterations and reduced biodiversity, has been observed in AD patients (Vogt et al. 2017; Sochocka et al. 2019) as well as AD rodent models (Nimgampalle and Kuna 2017; Lee et al. 2019; Z. Li et al. 2020). In AD, gut dysbiosis contributes to impaired gut barrier function, BBB integrity, and neuroinflammation (Vogt et al. 2017; Sochocka et al. 2019). Increased barrier permeability, as observed in AD studies (Stadlbauer et al. 2020), facilitates the entry of toxic amyloids and neuroinflammatory cytokines into the brain, precipitating neurodegeneration (Lin, Zheng, and Zhang 2018; Pellegrini et al. 2018; Kowalski and Mulak 2019). Differences in gut microbiota composition have been noted between healthy individuals, those with mild cognitive impairment, and AD patients, highlighting its importance in disease progression (P. Liu et al. 2019).

Probiotics, defined by the World Health Organization as live microorganisms conferring health benefits on the host, offer a potential therapeutic avenue for addressing gut dysbiosis (Gibson and Roberfroid 1995; Krumbeck et al. 2016). Probiotic supplementation holds promise in restoring a healthy gut microbiome composition (Vogt et al. 2017; Lin, Zheng, and Zhang 2018; Peterson 2020). However, animal studies have largely focused on how gut microbiota alterations affect Aβ pathology, the effects of probiotic supplementation on preclinical stages of AD and associated animal models have not been studied. The present study aims to elucidate the relationship between pretangle tau pathology in our model, and improved gut health through probiotic supplementation. We seek to explore potential mechanisms linking preclinical AD and gut health, including inflammation, GSK-3β enzyme activity, and BBB integrity.

2.2 Material and Methods

2.2.1 Subjects & Ethics Statement

Offspring of both sexes from homozygous TH-CRE male breeders (Sage Labs) and Sprague-Dawley female rats were used. Rats were kept in a standard 12-hour light-dark cycle, with food and water *ad libitum* except during the probiotic feeding stage where water was replaced by dissolved probiotics. The regular water was filtered three times (0.2 microns) and the diet (Teklad 2018) was irradiated. Experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and followed the Canadian Council's Guidelines on Animal Care and the National Institutes of Health (NIH) guidelines.

2.2.2 Experimental Design

Figure 2.1A shows the flow of the experiment. Rats were infused with adeno-associated virus (AAV) carrying htauE14 (AAV9-rEF1a-DIO-EGFP-htauE14) or control AAV (AAV9-rEF1a-DIO-EGFP) in the LC at 3 months of age. The EGFP-htauE14 expression cassette was placed under a double inverted open reading frame (DIO) for CRE-dependent expression in noradrenergic LC cells in TH-CRE rats. Subsequently, at 9 months of age (6 months post-infusion), animals were divided into groups receiving either probiotic or control diets for a period of 3 months. Following probiotic feeding, at 13-14 months of age, animals underwent a battery of behavioral tasks to assess general behavior and cognitive function. Following the behavioral assessments, at 15 months, animals were euthanized for assessment of brain inflammation, BBB leakage and GSK-3β activation. This time point was established for the implementation of our htauE14 model (Ghosh et al., 2019), as well as the establishment of probiotic effects. Immediately before and after the probiotic feeding, blood and fecal samples were collected from all groups. Gut microbiota was measured by 16S rRNA gene sequencing analysis and qPCR. Peripheral inflammation markers were measured by enzyme-linked immunosorbent assay (ELISA).

Rats were randomly assigned to four conditions, (1) Green fluorescent protein (GFP) + control diet, (2) GFP + probiotic diet, (3) htauE14 + control diet, or (4) htauE14 + probiotic diet. Groups were sex balanced.

2.2.3 AAV infusion surgeries

Under isoflurane anesthesia, rats were placed in a stereotaxic apparatus and infused with either AAV9-rEF1a-DIO-EGFP-htauE14 (2.26E+ 13 vg/ ml, MIT) or control AAV9-rEF1a-DIO-EGFP (2.35E+ 13 vg/ml) into the LC. Each LC received 1 µl of AAV solution mixed with 0.4 µl blue fluorescent beads (0.1%; ThermoFisher Scientific) via an infusion pump and a guide cannula angled at 20° parasagittal, caudal to the coronal plane. The coordinates for the LC infusion were 11.8–12.2 mm posterior, 1.3 mm bilateral, and 6.3 mm ventral with respect to the bregma (Ghosh et al. 2019).

2.2.4 Probiotic Diet Supplementation

ProBiotic-4, comprised of *Bifidobacterium lactis* (50%), *Lactobacillus casei* (25%), *Bifidobacterium bifidum* (12.5%), and *Lactobacillus acidophilus* (12.5%), well documented for their anti-inflammatory and beneficial gut-health properties, were purchased from Swanson (Fargo, ND, USA). Rats received ProBiotic-4 (3 x 109 colony forming unit (CFU) in water) once daily for three months between 9 and 12 months of age (Yang et al. 2020), mixed with prebiotic oligofructose/(FOS) Orafti® P95 powder, to enhance the overall effect of probiotics (200 mg/kg; Quadra Chemicals). The probiotic+prebiotic mixture was dissolved in drinking water and replaced regular water supply to ensure consumption. Initially, probiotics are inactive in water because of their formulation, which includes a carrier compound that binds to the probiotics and allows their activation in the gut (Azm et al. 2018). Control rats were given regular water only.

2.2.5 Blood and Fecal Collection

Blood and fecal samples were collected before and following the probiotic feeding. Blood was collected from the saphenous vein of each animal, in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged for 10 min at 1500×*g* and 4°C to obtain the plasma, which was then immediately frozen at -20°C until use. For fecal samples, animals were placed in clean autoclaved cages, where freshly voided fecal material was collected in sterile centrifuge tubes before being placed immediately on dry ice. The samples were then stored at -80°C until use.

2.2.6 ELISA

In obtained plasma samples, two proinflammatory cytokines, TNFα, IL-6, and one anti-inflammatory cytokine, IL-10, were measured. Levels of TNF-α were determined using commercially available Quantikine ELISA kits (Cat # R1000, R & D Systems, Inc., Minneapolis, MN, USA). The concentration of IL-6 was determined using the Rat IL-6 Quantikine ELISA Kit (Cat # R6000B, R & D Systems, Inc., Minneapolis, MN, USA), and the concentration of IL-10 was determined using the Rat IL-10 Quantikine ELISA kit (Cat # R1000, R & D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instruction.

2.2.7 DNA extraction from Feces

Isolation of microbial genomic DNA from each animal's stool sample was performed using the QIAamp® PowerFecal® Pro DNA Kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction.

Prior to storage, quality control measures were implemented to evaluate DNA purity via the Thermo ScientificTM NanoDropTM One Spectrophotometer (Thermo ScientificTM 840274100). The DNA concentration from 1µl of each sample was determined by absorbance at

260nm (A260), and the purity was estimated by determining the A260/A280 ratio with the Nanodrop spectrophotometer. Acceptable purity ranges for inclusion were required between 1.80-2, which was met by all samples used. Following this purity checkpoint, the DNA extractions were stored in elution tubes provided in the kit in a -20°C commercial freezer.

2.2.8 Microbiome Analysis

Quantitative polymerase chain reactions (*q*PCR) were performed using the 'Real-Time PCR System-Applied Biosystems ViiA7' system. The PCR supermix SsoAdvanced Universal SYBR Green Supermix (BioRad, Future Medicine Ltd, London, UK) was used according to the manufacturer's instructions. Primers were selected to analyze the total *Bifidobacterium lactis* (BL) stool DNA transcripts involving the following primer sequences: *FW: 5'-ACCTCACCAATCCGCTGTTC-3' and RV: 5'-GATCCGCATGGTGGAACTCT-3'* obtained from Integrated DNA Technologies (IDT) (Kim et al. 2020).

16S rRNA sequencing was performed at the Integrated Microbiome Resource (Dalhousie University, Halifax, Canada). Only samples from female rats were included. The V6-V8 bacterial region of 16S rRNA genes was analyzed. Amplicon fragments are PCR-amplified from the DNA in duplicate using separate template dilutions (1:10) using the high-fidelity Phusion Pluspolymerase. A single round of PCR was done using fusion primers (Illumina adaptors + indices + specific regions) targeting various sub-regions of the 16S gene. Specific primers B969F = (5'- ACGCGHNRAACCTTACC-3') and BA1406R = (3'-ACGGGCRGTGWGTRCAA-5') with the barcodes were applied (Comeau et al. 2011). The library was sequenced on an Illumina MiSeq platform.

2.2.9 Behavioral Testing

2.2.9.1 Exploratory, Anxiety and Depressive Behavior

Rats were given one 10-minute trial to explore an open field (60 × 60 × 40.5 cm^3) and recorded with ANY-Maze software (Stoelting). Distance traveled, time spent rearing (including free and supported rearing), and time spent freezing were recorded and analyzed offline. Freezing was counted as no body movement except breathing. Rearing was defined as the two front paws being lifted above the ground.

Anxiety was measured by the open field behavior, a 5 minute Elevated Plus Maze trial (50 × 10 cm2 /arm with an 11 × 11 cm2 central platform, 38 cm walls on the closed arms), where time spent inside closed arms vs. open arms were analyzed, and a marble burying test (number of 16 marbles buried in 30 minutes). Depressive behavior was assessed by calculating the percentage of 24-hr sucrose (0.75%) consumption relative to total liquid intake, with rats having access to both water and sucrose bottles.

2.2.9.2 Spatial Memory Assessments

The Y-maze test assesses short-term spatial memory. Animals explored a black opaque Plexiglas Y-shaped maze with three arms 120 ° apart (50 cm x 16 cm x 32 cm) in a training phase, and four hours later in a testing phase. For the training phase, animals were placed facing away from the experimenter at the end of the “start” arm for a 15-minute trial in which they were allowed to freely explore two of three arms. Which of the arms was closed for this training phase was counterbalanced between groups and animals. For the testing phase (4 hours later), animals were re-placed into the same “start” arm for another 15-minute trial and allowed to explore the full

maze with all three arms open, with the previously closed arm considered to be the “novel” arm. Number of entries into the arms and duration in each arm was recorded for analysis.

In the spontaneous location recognition (SLR) task assessing long-term memory, rats were given 10 min in an open arena (60 × 60 × 40.5 cm3) with three identical objects (1, 2, and 3) placed at specific positions. Objects 2 and 3 were placed 20 cm apart. During testing (24 hrs later), rats were placed in the same arena with two identical objects, one in the same position as Object 1 (a familiar location), the other midway between previous Objects 2 and 3 (a novel location). The discrimination ratio was the difference between time spent at the novel and familiar objects over the total time spent on both objects (Omoluabi et al. 2021).

2.2.9.3 Odor Discrimination Task

Discrimination of similar odors was tested with an odor detection and discrimination task, using perforated micro-centrifuge tube containing filter paper with 60 µl of odorant or mineral oil. The first three trials used odorless mineral oil, the next three trials used odor 1 (O1, 1-heptanol, 0.001%), and the last trial used an odor mixture that had a similar smell to O1 (O2, 1-heptanol and 1- octanol in a 1:1 ratio, 0.001%). Each trial lasted 1 min with a 1-min interval between trials, with the trial starting once the first sniff is made. The tests were videotaped, and sniffing time within a 1 cm radius around the odor tube was measured offline. The discrimination index was the ratio of the sniffing time difference between the O2 and the third presentation of O1 to the total sniffing time (tO2 -tO1-3)/(tO2 +tO1-3).

2.2.10 Histology, Immunohistochemistry (IHC) & Imaging

Whole brain and LC tissue were extracted after decapitation and stored in 20% sucrose in 0.1 M phosphate-buffered saline (PBS). Twenty-five micrometers of coronal sections cut by a cryostat (HM550, Thermoscientific) were mounted on chrome-gelatin coated slides, and 50 µm sections at 150 µm intervals were collected in polyvinylpyrrolidone (PVP) cryoprotectant (1% polyvinylpyrrolidone, 30% sucrose, 30% ethylene glycol in 0.1 M PBS) for free-floating IHC.

All histology and IHC followed established procedures (Ghosh et al. 2019; Omoluabi et al. 2021). Primary antibodies used included: dopamine β-hydroxylase (DBH) (MAB308, Millipore-Sigma, 1:2000), Ionized calcium-binding adaptor molecule 1 (Iba1) (019-19741, Wako, 1:2000), Cluster of Differentiation 68 (CD68) (MCA341GA, Bio-Rad, 1:1000), Glial fibrillary acidic protein (GFAP) (MAB3402, Millipore-Sigma, 1:500), and Albumin (16475-l-AP, ProteinTech, 1:1000) in PBS with 0.2% Triton-X and 2% goat serum.

After primary antibody incubation at 4°C over 1 to 3 nights, tissue was washed and incubated with Alexa Fluor secondary antibodies (Invitrogen, 1:1000) at room temperature for 2 hrs before being cover-slipped with Fluoroshield Mounting Medium with 4′,6-diamidino-2-phenylindole (Invitrogen). Non-fluorescent stained sections underwent a 90-min incubation in 1% A+B (PK-6101, Avidin-Biotin-complex, Vector Laboratories). Sections were then washed before reacting in a 3,3′-Diaminobenzidine (DAB)-tetrachloride solution (50 ml Tris buffer, 50 ml water, 50 mg DAB, 30 µl hydrogen peroxide) for 5 to 20 minutes.

Bright-field and fluorescence microscopy used an Olympus BX53 (Olympus) and EVOS M5000 imaging system (Thermo Fisher Scientific), respectively. Image analysis was conducted with ImageJ. The light intensity and exposure parameters were standardized across all captured

images. In the LC, the numbers of positive staining for all antibodies were manually counted and normalized to the region of interest (/mm^2). Albumin expression was measured as the mean density of the LC, normalized to the background level in the lateral vestibular nucleus (Kelly et al. 2019). Three sections of each marker within the same rostral to caudal range were used from all animals and counts from the two hemispheres were averaged. Analysis was conducted by experimenters that were blind to the experimental conditions.

2.2.11 Western Blotting

Hippocampal tissue was extracted from whole brains after decapitation and stored frozen. Brain tissue processing followed established protocols (Morrison et al. 2013). Total protein concentration was quantified by standard Pierce BCA protein assay kit (Thermo Scientific, 23225). Equal amounts of protein (20 μg) were separated by SDS-PAGE on 10% gels and were then transferred to Immobilon-P Transfer PVDF membranes (Merck Millipore, IPVH00010). Following transfer, the membranes were briefly rinsed with 1x low salt TBS-T (containing 1.5M NaCl, 1M Tris Base and 0.1% Tween 20) and blocked for 1 hr with 5% nonfat skim milk at room temperature. They were then incubated for 2 hrs at room temperature with the following antibodies: pTau S262 (AB92627, Abcam, 1:2000), pTau S356 (AB75603, Abcam, 1:5000), pGSK-3β (sc-373800, Santa Cruz, 1:1000) and GSK-3β (sc-53831, Santa Cruz, 1:1000). The membranes were rinsed in 2 x 5 min high salt TBS-T (containing 5M NaCl, 1M Tris Base and 0.1% Tween 20) and in 1 x 5 min low salt TBS-T and incubated for 1.5 hrs at room temperature, with either horseradish peroxidase–labeled anti-rabbit immunoglobulin G (IgG; 31460, 1:4000) or anti-mouse IgG (31430, Thermo Fisher Scientific, 1:4000). The protein bands were visualized

using chemiluminescent substrate (ThermoFisher Supersignal West PICO, 34577) on a digital image scanner (ImageQuant LAS 4000) and quantified with the ImageJ software.

2.2.12 Statistical Analysis

All data are shown as mean ± standard error of the mean. Statistical analysis was conducted with OriginPro 2022b software. 16S rRNA data (female samples only) were analyzed by Chao1 indices (alpha and beta diversity), Wilcoxin statistics (heat-tree), 2-way ANOVAs and Linear Discriminant Analysis Effect Size (LEfSe; single-factor univariate statistics; microbiomeanalyst.com). The pTau expression levels in the LC were analyzed using two sample t-tests. All other results were analyzed by three-way ANOVAs comparing AAV x Diet x Sex and *post-hoc* Tukey tests were used for group comparisons.

2.3 Results

2.3.1 Probiotic Supplementation Rescues the Spatial Learning Deficiency in htauE14 Rats

We found no effects of AAV phenotypes, diet or their interaction in all the tests related to exploratory behavior and anxiety level, including the open field maze (Fig. 2.1B), marble burying task (Fig. 2.1C), or elevated plus maze (Fig. 2.1D). There was a moderate increase of the number of buried marbles by the htauE14 rats compared to GFP rats, however, it did not reach statistical significance ($F_{1,39}$= 3.762; p = 0.060).

The sucrose preference test assessing the level of anhedonia showed significant differences between the probiotic and control diet groups ($F_{1,39}$= 23.063, p = 2.33E^{-5}; Fig. 1E). Post-hoc analysis revealed the animals fed with probiotic supplement consumed higher amount of sucrose solution ($p < 0.01$) compared to control diet fed animals, suggesting increased hedonic level. No sex differences were observed due to AAV or diet. However, males in general consumed more sucrose solution ($p < 0.01$).

Spatial memory was affected by AAV x diet interactions in both Y-maze (Fig. 2.1F) and SLR (Fig. 2.1G) tests. In Y-maze, there was a significant AAV x diet interaction in the percentage of time spent in the novel arm ($F_{1,39}$= 4.228, p = 0.046; Figure 2.1F). The htauE14 + control diet group spent significantly less time in the novel arm than the GFP + control diet group ($p < 0.01$). While probiotic feeding increased the time spent in the novel arm in htauE14-infused animals, it did not reach statistical significance (p = 0.06). In the SLR task, there was a significant AAV x diet interaction in the discrimination ratio ($F_{1,39}$= 6.240, p = 0.017; Figure 2.1G). The htauE14 + control diet group spent significantly less time in the novel object location compared to the two GFP groups ($p < 0.01$), and this deficit was restored by probiotic supplementation ($p < 0.05$). No sex differences of AAV, diet or their interactions were observed in either test.

Olfactory discrimination impairments were observed in htauE14 animals ($F_{1,39}$= 7.521, p = 0.009; Figure 2.1H), consistent with preclinical AD symptoms in human studies (Devanand et al. 2015a). There was neither diet effect nor diet x AAV interaction effect. Interestingly, in general, male rats performed better than female rats ($F_{1,39}$= 7.367, p = 0.010).

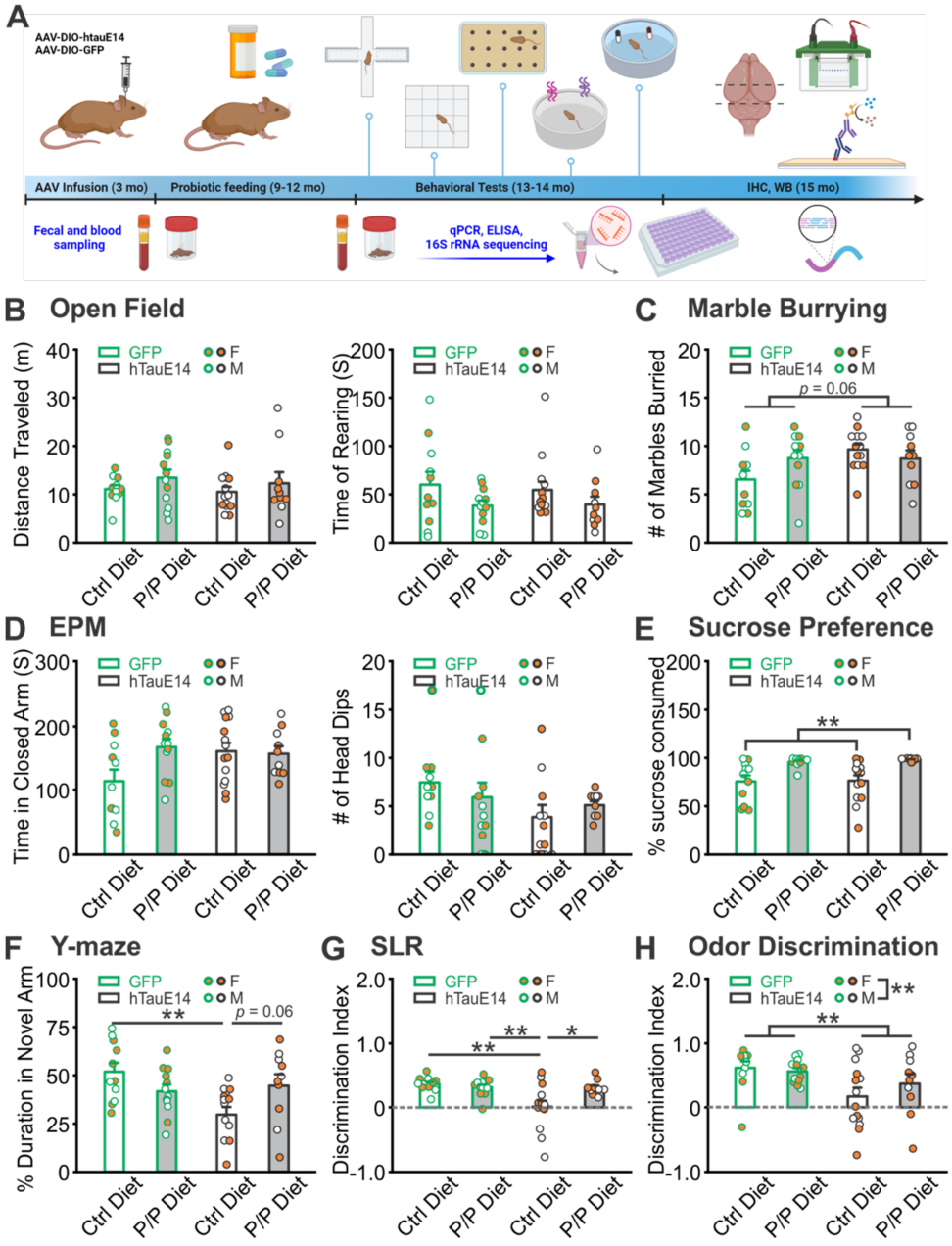


Figure 2.1. The Effects of probiotic supplementation on behavioral tasks. A. Schematic of experimental design, detailing timeline from AAV infusion to tissue processing. **B.** Distance traveled and time spent rearing in an open field maze test. **C.** Number of marbles buried in a marble burying task. **D.** Time spent in the closed arm and number of head dips over the edge in

an elevated plus maze (EPM) test. **E.** Percentage of sucrose solution consumption over a 24 hr period. **F.** Percentage of time spent in the novel arm in a Y-maze test. **G.** Discrimination index in a spontaneous location recognition (SLR) task. **H.** Discrimination index in an odor discrimination task. Ctrl: control. P/P: probiotic+prebiobiotic. N: GFP + Ctrl Diet: 6F/5M; GFP + P/P Diet: 6F/6M; htauE14 + Ctrl Diet: 6F/8M; htauE14 + P/P Diet: 6F/4M. *$p < 0.05$, **$p < 0.01$.

2.3.2 Probiotic Supplementation Enhances short-chain fatty acid (SCFA) Associated Bacteria in the Gut and Enriches Microbiome Diversity

Rats were fed probiotic-4 supplement once daily for 3 months. We measured the levels of *Bifidobacterium lactis,* which comprises 50% of probiotic supplement mixture, using qPCR analysis. Fold change between diets was significant ($F_{1,33}$= 6.223, p = 0.018; Fig. 2.2A) with the presence of *Bifidobacterium lactis* significantly increased in probiotic fed groups ($p < 0.01$). There were no significant differences in fold change based on AAV, sex, and their interaction with diet.

Levels of alpha diversity and beta diversity were obtained from 16S rRNA sequencing using the Chao1 index. Alpha diversity level, a metric measuring the richness (number of taxa), or evenness (relative abundance of those taxa) was analyzed. A significant increase in microbiome diversity richness was observed in the probiotic diet groups compared to control diet groups ($F_{1,31}$ = 8.065, p = 0.007; Figure 2.2B). No significant difference was found between AAV groups. Levels of beta diversity, representing the diversity and variability of community composition, showed no significant differences between AAV groups ($F_{1,31}$= 0.7311, p = 0.774; Figure 2.2C). However, a significant increase in beta diversity was observed in the probiotic diet groups, compared to control diet groups ($F_{1,31}$= 3.4867, p = 0.001; Figure 2.2D).

Changes in the taxa species induced by Probiotic-4 mixture feeding were further assessed. At the genus level, bacterial taxa were analyzed for abundance levels between groups to observe any effect from the introduction of probiotics (Fig. 2.2E, Table 1 and Supplementary Figure 2.2). The heat map in Fig. 2.2E shows average levels of each taxa and significance levels of the probiotic diet groups comparing to the control diet using Wilcoxon test. A labelled taxa is indicative of a

significant difference between groups. Overall, six taxa genuses were upregulated in abundance in the probiotic diet groups, and 6 others were downregulated, compared to control. Table 1 shows single-factor univariate comparison of diet or AAV, obtained from LEfSe statistical analysis to observe which individual taxa resulted in a significant difference between groups. Fifteen taxa were significantly different between the htauE14 and GFP groups, with five species higher in htauE14 rats. Thirty-seven taxa were different between probiotic and control diet, with 19 of these higher in the probiotic diet rats. We then compared the nine Taxa that were significantly different between diet groups from both Wilcoxon (Fig. 2.2E) and univariate ANOVA test (Table 1), in a 2-way ANOVA comparing AAV and diet effects and their interaction (Supplementary Fig. 2.1). Multiple gut microbiome differences were observed between diet groups. Bacterial taxa Clostridium_sensu_strico_1 ($F_{1,35}$= 11.191, p = 0.002), Bacerteroids_dorei ($F_{1,35}$= 7.989, p = 0.008), and Lactococcus_formosensis ($F_{1,35}$= 11.048, p = 0.002), were significantly increased in probiotic supplemented animals. Taxa Bacteroids_caecimuris ($F_{1,35}$= 5,598, p = 0.023), Rikenellaceae_Alistipes ($F_{1,35}$= 7.752, p = 0.009), and UCG-010 ($F_{1,35}$= 5.323, p = 0.027) were significantly higher in control diet animals compared to probiotic diet animals. There were significant AAV x diet interaction effects on Bernesiellacaea_barnesiella ($F_{1,35}$= 4.788, p = 0.035) and Ruminococcus ($F_{1,35}$= 5.856, p = 0.021) taxa, with GFP + probiotic group showed significantly higher level of these taxa compared to control diet groups ($p < 0.05$ or $p < 0.01$).

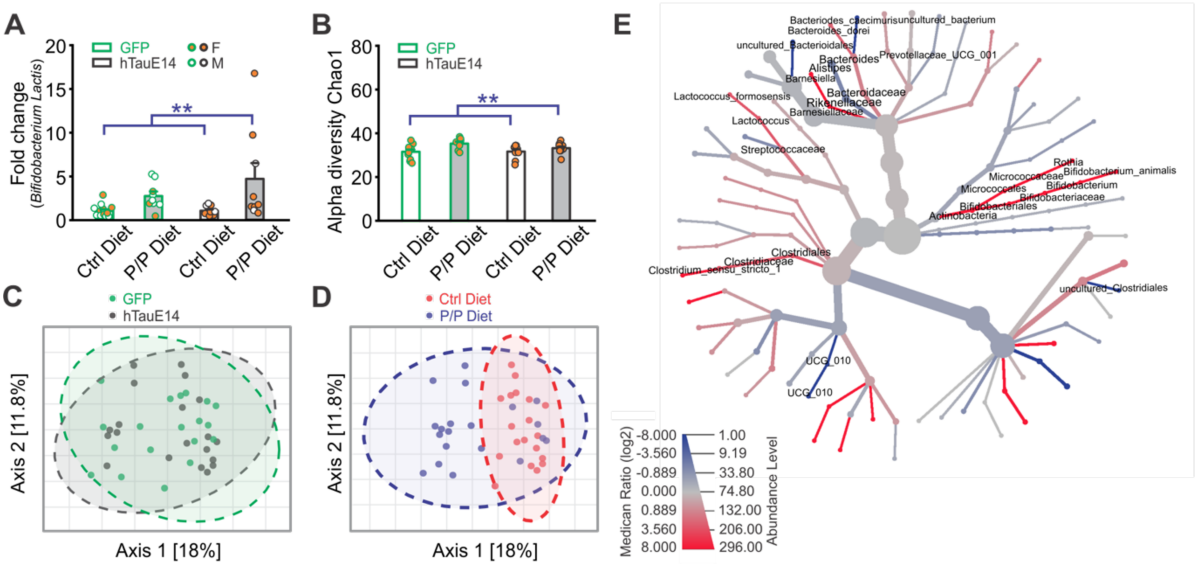


Figure 2.2. Alterations of microbiota in diversity and abundance following probiotic supplementation. A. Fold change of *Bifidobacterium lactis,* a main bacteria in Probiotic supplement, as quantified by qPCR. N: GFP + Ctrl Diet: 7F/6M; GFP + P/P Diet: 3F/6M; htauE14 + Ctrl Diet: 6F/4M; htauE14 + P/P Diet: 7F/2M. **B.** Chao1 analysis of Alpha Diversity levels. **C.** Ordination plot of Beta Diversity index between AAV groups, using Bray-Curtis index distance method. There was no significant difference in Beta Diversity between GFP and htauE14 animals. **D.** Ordination plot of Beta Diversity index between diet groups, using Bray-Curtis index distance method. A significant difference between community composition was found between probiotic and control diet groups. **E.** A heat tree demonstrating bacterial abundance differences between diet groups. Labeled branches represent a significant abundance level between groups. Ctrl: control. P/P: probiotic+prebiobiotic. N: GFP + Ctrl Diet: 11F; GFP + P/P Diet: 8F; htauE14 + Ctrl Diet: 9F; htauE14 + P/P Diet: 11F. *$p < 0.05$, **$p < 0.01$.

TAXA/Bacteria Name	AAV (p-value)	Higher	Diet (p-value)	Higher
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae	0.003934	CTRL		
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium	0.009394	E14		
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium	0.024439	CTRL		
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium	0.028355	CTRL		
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae	0.032864	CTRL		
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Ruminococcus; s__Ruminococcus_flavefaciens	0.007651	E14		
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Ruminococcus	0.043645	CTRL	0.023978	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides	0.033771	CTRL		
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides	0.03782	E14		
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.014743	CTRL		
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.045895	CTRL		
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.047166	CTRL		
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae	0.047821	CTRL		
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__Clostridium_sensu_stricto_1	0.022832	E14	0.00301	PRO
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__Oscillibacter; s__unidentified	0.030466	E14		
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			2.57E-05	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae			3.84E-05	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae			0.000469	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_organism			0.001239	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			0.001689	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae			0.002203	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			0.002291	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_organism			0.004931	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae			0.006189	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_Bacteroidales			0.008643	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_organism			0.010384	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			0.012587	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae			0.021365	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			0.023645	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae			0.024397	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_organism			0.033736	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			0.037296	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium			0.044825	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_NK3B31_group; s__uncultured_bacterium			0.001105	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_NK3B31_group; s__uncultured_bacterium			0.002572	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_UCG-001; s__uncultured_bacterium			0.00269	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_Ga6A1_group; s__uncultured_bacterium			0.034552	CTRL
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__[Eubacterium]_xylanophilum_group; s__uncultured_bacterium			0.007253	CTRL
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae			0.025958	PRO
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__[Eubacterium]_ventriosum_group			0.038955	CTRL
p__Firmicutes; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae			0.043206	PRO
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae			0.016081	PRO
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__Oscillibacter			0.025078	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__Bacteroides_caecimuris			0.012056	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__Bacteroides_dorei			0.025973	PRO
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Lactococcus; s__Lactococcus_formosensis			0.001367	PRO
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus			0.012035	PRO
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes			0.001396	CTRL
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Barnesiellaceae; g__Barnesiella			0.005195	PRO
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__UCG-010; g__UCG-010			0.026838	CTRL
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Ruminococcus			0.035302	PRO

Table 2.1. Bacteria significantly altered by htauE14 or probiotic feeding. LEfSe analysis of significant bacteria between main effects. Highlighted taxa indicate significance in both single-factor univariate statistics and LEfSe analysis.

2.3.3 htauE14 infusion increases LC tau phosphorylation and GSK activity in the LC target area hippocampus

AAV infusions in the bilateral LC (Fig. 2.3A) resulted in expression of htauE14 or GFP genes in the LC neurons (Fig. 2.3B). Here and previously we have reported >80% expression rates of htauE14 in the LC with this approach (Ghosh et al. 2019; Omoluabi et al. 2021). We measured phosphorylated tau at two microtubule binding sites in the LC and revealed increased phosphorylation of tau at S262 (t_3 = -4.834, p = 0.017; Fig. 2.3C) and S356 (t_3 = -3.258, p = 0.047) (Fig. 2.3D).

We measured the level of GSK-3β and its phosphorylated form (S9) in the hippocampus. GSK-3β is a key enzyme that phosphorylates tau including S262 and S356 sites (Morris et al. 2011; Sayas and Ávila 2021). Phosphorylation of GSK-3β at S9 site (pGSK-3β) negatively regulates its activity (Sayas and Ávila 2021). There were significant effects of diet on pGSK-3β ($F_{1,24}$= 4.381, p = 0.047) and diet x sex interaction ($F_{1,24}$= 4.918, p = 0.036) (Fig. 2.3E-G). Probiotic-fed female rats showed significantly higher pGSK-3β compared to control diet-fed female rats ($p < 0.01$) and probiotic-fed males ($p < 0.05$), corresponding to less GSK-3β activation in female rats supplemented with probiotics. Total GSK-3β levels were not different among groups (Fig. 2.3H-I). However, the ratio of pGSK-3β over total GSK-3β were significantly lower in the htauE14 animals, compared to the GFP rats ($F_{1,24}$= 10.530, p = 0.003; Figure 3J). The relative higher activation of GSK-3β in the htauE14 rats is consistent with elevated level of tau phosphorylation in these rats.

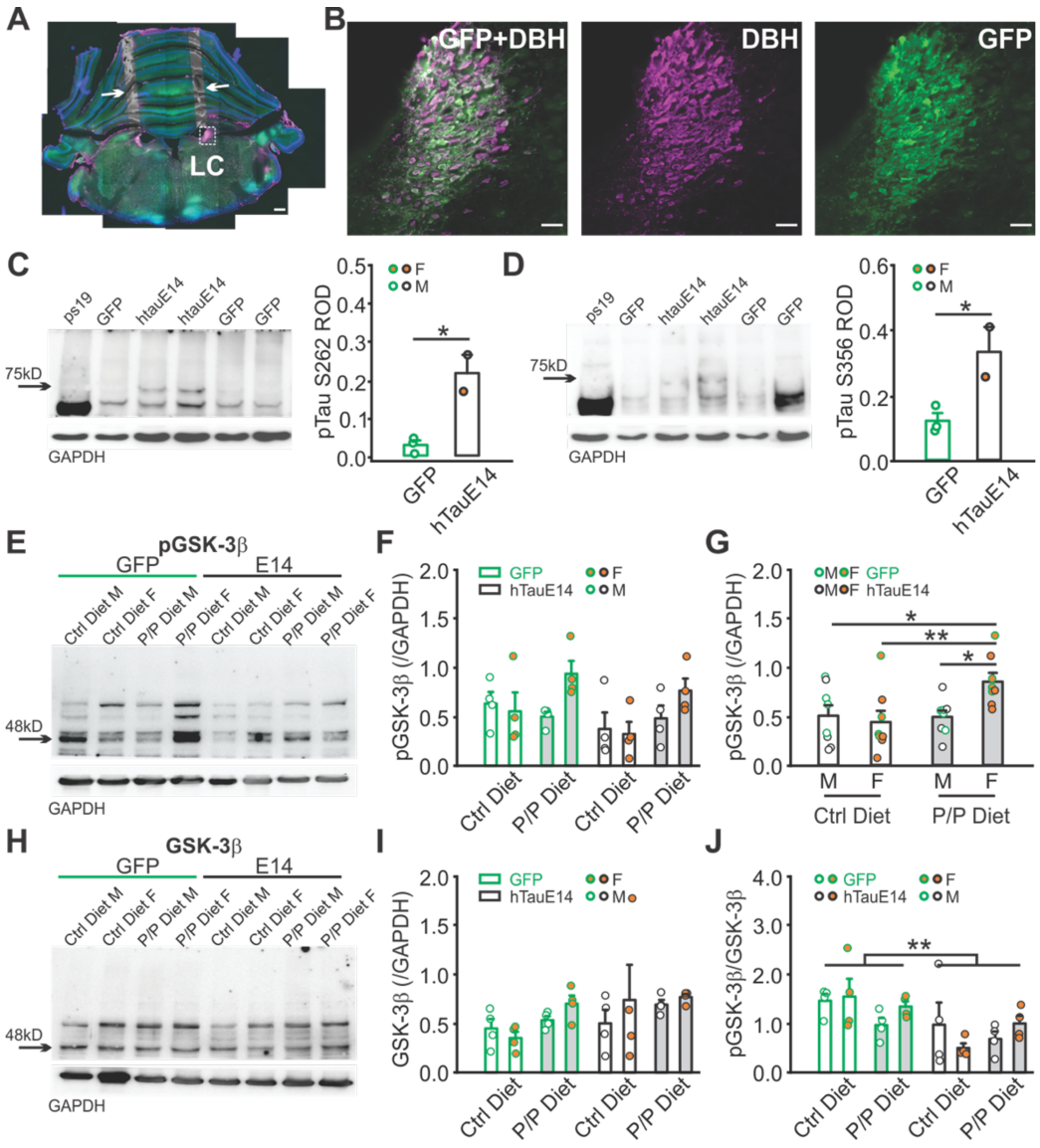


Figure 2.3. The effects of htauE14 and probiotic feeding on tau phosphorylation in the LC and levels of associated GSK-3β activation in the hippocampus. A. An example image of bilateral AAV infusion sites in the LC. **B.** Co-expression of htauE14 (GFP; green) and dopamine β-hydroxylase (DBH; red) in LC neurons. Scale bars: 50 µm. **C.** Representative blot of pTau S262 in LC tissue (left) and quantification of relative optical density (ROD; right). **D.** Representative blot of pTau S356 in LC tissue (left) and quantification of relative optical density (ROD; right). N: GFP: 3M; htauE14: 1F/1M. **E.** Representative blot of pGSK-3β across groups

in hippocampal tissue. **F.** RODs of pGSK-3β levels. **G.** Comparison of pGSK-3β levels based on diet x sex interaction. **H.** Representative blot of pGSK-3β across groups in hippocampal tissue. **I.** RODs of GSK-3β levels. **J**. Ratios of pGSK-3β/GSK-3β. N: GFP + Ctrl Diet: 4F/4M; GFP + P/P Diet: 4F/4M; htauE14 + Ctrl Diet: 4F/4M; htauE14 + P/P Diet: 4F/4M. $*p < 0.05$, $**p < 0.01$.

2.3.4 Increased Peripheral and Neuronal Inflammation in htauE14 Animals are Ameliorated by Probiotic Supplementation

Levels of peripheral inflammation markers were measured using ELISA in blood following probiotic feeding. Concentration of IL-6 was significantly different between AAVs ($F_{1,32}$= 6.171, p = 0.018), and with AAV x diet interaction ($F_{1,32}$= 4.692, p = 0.038; Fig. 2.4A). The htauE14 + control diet group showed higher level of IL-6 compared to the two GFP groups ($p <$ 0.05 or $p <$ 0.01), while probiotic supplementation significantly lowered levels of IL-6 in htauE14 rats compared to the htauE14 rats fed with control diet ($p < 0.05$).

Levels of TNFα showed a significant increase in htauE14 groups (both probiotic and control) compared to GFP groups ($F_{1,40}$= 5.848, p = 0.020; Fig. 2.4B). No significant effect was observed by probiotic supplementation.

IL10, a cytokine that acts as both proinflammatory and anti-inflammatory, showed significant effects of AAV ($F_{1,28}$= 20.586, p = 9.818E^{-5}) and AAV x diet x sex interaction ($F_{1,28}$= 5.443, p = 0.027; Fig. 2.4C). Male htauE14 + probiotic diet group exhibited higher level of IL-10 compared to both GFP male groups ($p < 0.01$). Interestingly, probiotic feeding reduced the level of IL-10 in htauE14 infused rats ($p < 0.05$ compared to the control diet group).

In the brain, levels of microglia were measured in the LC with Iba1 (total microglia) and CD68 (active microglia) markers. For Iba1, a significant effect of AAV x diet interaction was observed ($F_{1,25}$= 5.284, p = 0.030; Fig. 2.4D), with the htauE14 + control diet group showing higher level of Iba1 than the GFP + control diet group ($p < 0.01$). Similarly, CD68 exhibited significant effects of AAV x diet interaction ($F_{1,26}$= 8.441, p = 0.007; Fig. 2.4E); with htauE14 + control diet

showing higher level of CD68 than the two GFP groups ($p < 0.05$ or $p < 0.01$). However, probiotic supplementation significantly lowered the levels of CD68 present in the htauE14 rats ($p < 0.05$).

Astrocyte levels were measured using GFAP-immunoreactivity (IR) as a marker. A significant interaction of AAV x diet x sex was observed ($F_{1,19} = 5.099$, $p = 0.036$; Fig. 2.4F). Female htauE14 + control diet rats showed higher level of GFAP than the two GFP female groups ($p < 0.05$). However, probiotic feeding did not reverse the increase of GFAP in the htauE14 rats.

Finally, we found increased BBB leakage in htauE14 rats, using albumin as a marker. There was a significant effect of AAV ($F_{1,25} = 7.134$, $p = 0.0139$; Fig. 2.4G). The htauE14 rats showed overall higher expression of albumin ($p < 0.01$), suggesting a high level of BBB leakage associated with these rats. However, probiotic feeding did not alter the albumin level in the LC of the htauE14 rats.

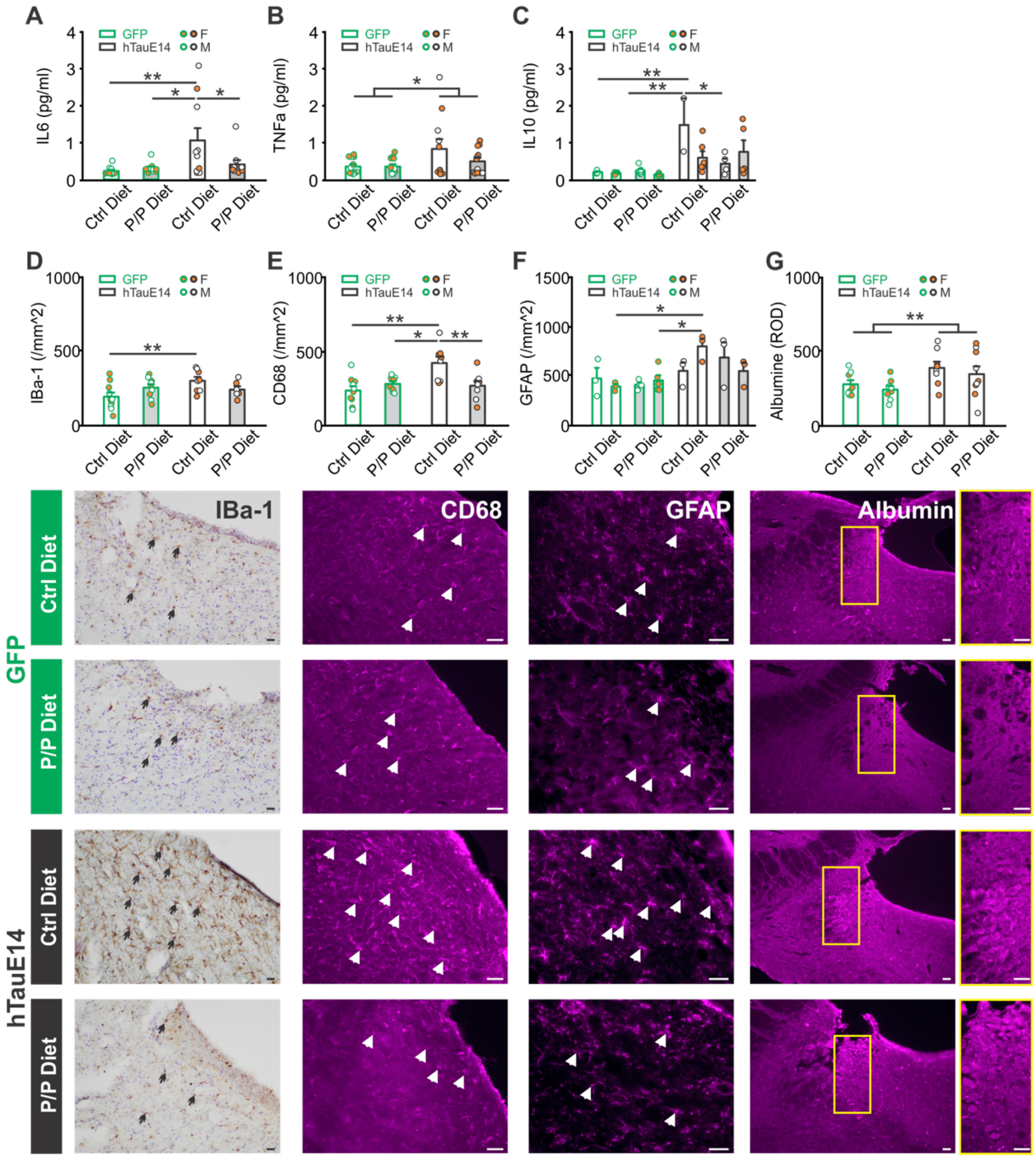


Figure 2.4. The effects of probiotic supplementation on peripheral and central inflammation. A. Concentration of IL6 (mg/ml) from serum at 12-months of age. N: GFP + Ctrl Diet: 4F/6M; GFP + P/P Diet: 4F/6M; htauE14 + Ctrl Diet: 8F/2M; htauE14 + P/P Diet:

6F/4M. **B.** Concentration of TNFα (mg/ml) from serum at 12-months of age. N: GFP + Ctrl Diet: 6F/7M; GFP + P/P Diet: 6F/6M; htauE14 + Ctrl Diet: 9F/2M; htauE14 + P/P Diet: 7F/5M. **C.** Concentration of IL10 (mg/ml) from serum at 12-months of age. N: GFP + Ctrl Diet: 5F/3M; GFP + P/P Diet: 5F/6M; htauE14 + Ctrl Diet: 6F/2M; htauE14 + P/P Diet: 5F/4M. **D.** Number of Iba1 cells per mm^2 in the LC. N: GFP + Ctrl Diet: 6F/4M; GFP + P/P Diet: 5F/4M; htauE14 + Ctrl Diet: 4F/4M; htauE14 + P/P Diet: 3F/3M. **E.** Number of CD68 cells per mm^2 in the LC. N: GFP + Ctrl Diet: 6F/4M; GFP + P/P Diet: 4F/4M; htauE14 + Ctrl Diet: 4F/4M; htauE14 + P/P Diet: 4F/4M. **F.** Number of GFAP cells per mm^2 in the LC. N: GFP + Ctrl Diet: 3F/3M; GFP + P/P Diet: 5F/4M; htauE14 + Ctrl Diet: 3F/3M; htauE14 + P/P Diet: 3F/3M. G. Mean intensity of Albumin staining in the LC. N: GFP + Ctrl Diet: 4F/4M; GFP + P/P Diet: 4F/4M; htauE14 + Ctrl Diet: 4F/4M; htauE14 + P/P Diet: 5F/4M. Example images of Iba-1, CD68, GFAP, and Albumin, respectively, in the LC across all groups were shown in the lower panels. Scale bars: 50 μm. Arrows indicate positively stained cells. *$p < 0.05$, **$p < 0.01$.

2.4 Discussion

In this study, we replicated the effect of pretangle tau on cognitive function and brain inflammation. We observed impaired spatial and olfactory discrimination learning in rats infused with pretangle tau. Building upon our prior research demonstrating elevated levels of CD68 and TREM2 in the LC target structure dentate gyrus of the hippocampus (Omoluabi et al. 2021), we examined the LC itself. Our findings revealed increased microglia activity (Iba1 and CD68) overall and GFAP, strongly expressed in astrocytes, in female rats. We also detected tau phosphorylation at two microtubule binding sites S262 and S356 (Ando et al. 2016) within the LC, which aligned with increased GSK-3β activity in the hippocampus. These pathological changes were accompanied by increased BBB leakage in brains infused with pretangle tau. Furthermore, we observed increased expression of multiple peripheral inflammation markers in htauE14 animals, including proinflammatory markers IL6 and TNFα, as well as anti-inflammatory marker IL10.

Based on these results, we propose that abnormally phosphorylated pretangle tau, present in the preclinical stage of AD, is sufficient to induce neuronal degeneration and inflammation. Our model has previously demonstrated LC axonal degeneration (Ghosh et al. 2019; Omoluabi et al. 2021). Neuronal degeneration and subsequent release of pretangle tau could activate microglia and astrocytes, triggering neural inflammation and the release of proinflammatory cytokines such as TNFα and IL-6 (Argaw et al. 2012; Michalicova, Majerova, and Kovac 2020). This neural inflammation damages the BBB, allowing infiltration of neutrophils and leukocytes from the peripheral into the brain. The increase in anti-inflammatory cytokine IL-10 may suggest an initial protective immune response. Both proinflammatory (such as IL-6) and anti-inflammatory

cytokines (such as IL-10) are known to be increased in AD (Papassotiropoulos et al. 1999; Strle et al. 2001).

GSK-3β, a key serine/threonine kinase involved in tau phosphorylation (Morris et al. 2011; Sayas and Ávila 2021), exhibited increased activity in the target structure of htauE14 brains, as indicated by reduced phosphorylation of S9, an inhibitory site of GSK-3β. Elevated GSK-3β activity may further promote tau hyperphosphorylation, directly engage inflammatory signaling (Martin et al. 2005; Forlenza et al. 2011), and consequently, exacerbate BBB damage. Increased GSK-3β has been reported in platelets of AD patients (Forlenza et al. 2011). Peripheral GSK-3β also directly regulates inflammatory cytokines (Martin et al. 2005), thus reinforcing its effects in the brain to exacerbate tau pathology.

Alternately, LC-norepinephrine (NE) signaling is believed to regulate neurovascular coupling and play a role in BBB maintenance (Raichle et al. 1975). LC degeneration results in BBB breakdown, as evidenced by prominent albumin extravasation, in a Tg344-19 rat AD model (Kelly et al. 2019) which is also accompanied by increased inflammation and cognitive deficits.

Probiotic supplementation for three months led to a significant enhancement in gut microbiome diversity and optimized bacteria composition. Our analysis of gut microbiome composition following probiotic supplementation revealed notable changes in specific bacterial taxa compared to control groups. Three taxa, Clostridium_sensu_stricto_1, Bacteroides dorei, and Lactococcus formosensis, were found to be significantly higher in probiotic-fed groups. These taxa are associated with distinct processes that may contribute to the observed effects of probiotic supplementation.

Clostridium_sensu_stricto_1 is known for its ability to produce SCFAs, particularly butyrate. This production could enhance the expression of tight junction proteins in colon epithelia, thereby strengthening the intestinal barrier, reducing mucosal permeability, and inhibiting inflammatory cytokines (Monda et al. 2017). Additionally, its decreased abundance in normal aging studies suggests a potential role in age-related gut health decline (Odamaki et al. 2016). Bacteroides dorei, on the other hand, has been shown to reduce gut microbial lipopolysaccharide production, leading to the suppression of proinflammatory immune responses and lower intestinal permeability in patients with coronary artery disease (Yoshida et al. 2018). Studies have also shown that Bacteroides dorei treatment results in lower levels of proinflammatory cytokines and alterations in gut microbiota (Song et al. 2021). While research on Lactococcus formosensis is limited, various strains of Lactococcus have been associated with AD, underscoring the necessity for further investigation into its specific role in probiotic-mediated effects (Sun et al. 2023).

Our study also identified three bacterial taxa, namely Bacteroides caecimuris, Rikenellaceae_Alistipes, and UCG-010, which exhibited a reduction following probiotic supplementation. Bacteroides caecimuris, although not extensively characterized, has been associated with severe type-2 diabetes (Debédat et al. 2022), a condition strongly linked to AD (Biessels et al. 2006). Insulin resistance in the brain, observed in severe type-2 diabetes, can lead to impaired synaptic formation, neuronal plasticity, and mitochondrial metabolism, all of which are implicated in AD pathogenesis (Petersen et al. 2003; Messier 2005). Rikenellaceae_Alistipes, on the other hand, has shown conflicting evidence regarding its pathogenicity and protective effects against various diseases. While some studies suggest a protective role against several diseases (Moschen et al. 2016; Zuo et al. 2019), others have shown a significant correlation with

inflammatory cytokines (Butera et al. 2018), depression, and mood disorders (Naseribafrouei et al. 2014). Interestingly, Rikenellaceae_Alistipes has been associated with an increase in SCFAs-producing bacteria, similar to taxa observed to be increased in probiotic-fed animals, indicating a complex role in gut health (Li et al. 2016). Lastly, UCG-010 remains poorly characterized, with limited information available regarding its specific functions. These findings highlight the intricate relationships between specific bacterial taxa and their potential roles in gut dysbiosis and AD pathogenesis, warranting further investigation into their mechanisms of action and interactions with probiotic supplementation.

Overall, these findings suggest that probiotic supplementation may modulate gut microbiota composition to favorably influence intestinal barrier function and immune responses, potentially contributing to the overall health benefits observed in AD animal models.

In line with this, probiotic feeding rescued spatial learning deficiency in htauE14 rats and enhanced hedonic behavior in rats. Concurrently, probiotic feeding reduced peripheral and brain inflammation. Furthermore, it enhanced the phosphorylation at the inhibitory S9 site thus lowered GSK-3β activity in the hippocampus of female rats. However, our study found no significant effect of probiotic supplementation on the levels of TNFα, suggesting that this inflammatory marker may not be influenced by gut manipulation in this context. This lack of response aligns with the intricate and multifaceted nature of neuroinflammation in AD, highlighting the complexity of targeting specific inflammatory pathways in therapeutic interventions.

The main mechanisms thought to underlie the regulation of the brain function by gut microbiota include targeting inflammation, barrier dysfunction (gut and blood-brain) and GSK-3β action

(Flynn and Yuan 2023). Probiotic supplementation has been shown to enhance gut barrier directly through their surface components (flagella, pili, capsular proteins etc.), which constitute MAMPs (Liu et al. 2020). MAMPs can bind to receptors on the epithelial barrier and regulate cytokine production through protease-dependent signaling cascades. As a result, various cytokines are produced, which alleviate inflammation and enhance barrier function (Sharma, Young, and Neu 2010; Liu et al. 2020). This decrease in gut-derived inflammation may interrupt the inflammatory cascade that propagates systemically. Indirectly, through probiotic metabolites such as SCFAs, primarily butyrate, probiotics strengthen tight junction proteins and increase barrier integrity (Liu et al. 2020). Enhanced barrier integrity at both gut-blood and blood-brain interfaces could slow and ameliorate system and brain inflammation, consequently, reduce abnormal tau associated neurodegeneration in the LC and its target areas. Interestingly, a recent study showed that gut microbiome ablation (through methods such as GF condition and short-term antibiotic treatment) also enhances gut barrier function, decreases neuroinflammation and reduces tau pathology, in an APOE dependent manner (Seo et al. 2023).

Probiotic therapy also has the potential to rectify tau hyperphosphorylation through GSK-3β (Hooper, Killick, and Lovestone 2008; Lin et al. 2020). Several probiotic strains have been shown to inhibit tau hyperphosphorylation in rodent models, by increasing Akt phosphorylation in the brain, leading to inactivation of GSK-3β (Song et al. 2022). The inactivation of GSK-3β could decrease tau phosphorylation at numerous sites (Hanger, Anderton, and Noble 2009; Sayas and Ávila 2021). In this study, lower relative levels of pGSK-3β over total GSK-3β were observed in htauE14 animals, corresponding to higher GSK-3β activity in htauE14 brains, and consistent with other animal and human studies.

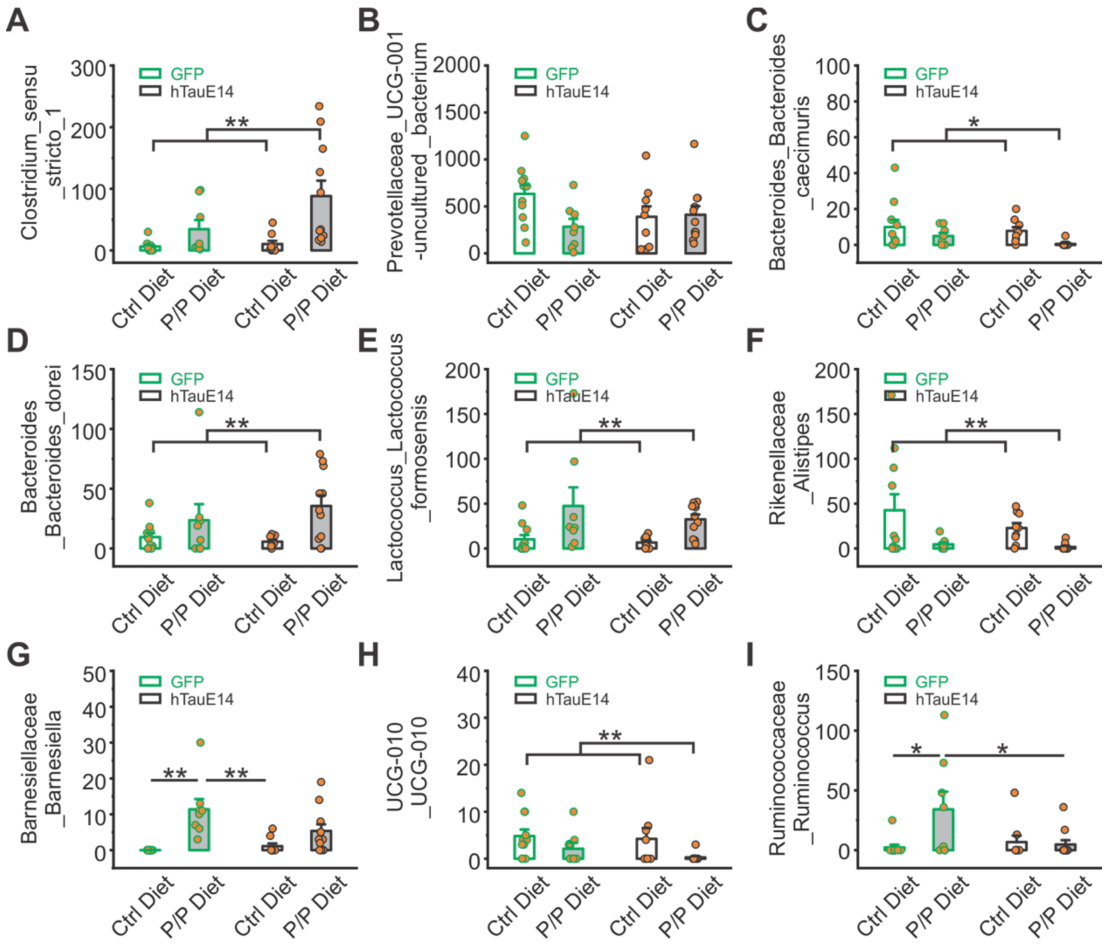
Sex differences are often overlooked in preclinical AD studies despite being evident in tau models (Buccarello et al. 2017) and across various parameters in human AD (Podcasy and Epperson 2016; Grimm and Eckert 2017; Mosconi, Berti, Quinn, McHugh, Petrongolo, Varsavsky, et al. 2017; Mosconi, Berti, Quinn, McHugh, Petrongolo, Osorio, et al. 2017; Laws, Irvine, and Gale 2018; Yanguas-Casás et al. 2018). Women face a twofold increased risk of developing AD compared to men (Podcasy and Epperson 2016). Perimenopause emerges as a stage at which women exhibit vulnerability to AD (Brinton et al. 2015; Mosconi, Berti, Quinn, McHugh, Petrongolo, Osorio, et al. 2017; Neu et al. 2017; Pike 2017). Studies have linked significant reductions in Aβ accumulation and hyperphosphorylated tau levels to the activation of 17β-estradiol, which inactivates the GSK-3β pathway (Goodenough et al. 2005). Female P301L tau mutant mice also exhibit more pronounced pathological changes (Buccarello et al. 2017). Our research findings reveal that female rats performed worse in the olfactory discrimination task compared to their male counterparts. Additionally, female htauE14 rats on a control diet showed elevated level of astrogliosis in comparison to the GFP groups. Notably, in female rats, probiotic supplementation led to increased levels of pGSK-3β, indicating a positive impact of probiotics on GSK-3β activity levels. However, no discernible sex-based differences were observed in spatial learning, or levels of peripheral and central inflammation with probiotic feeding. A limitation of the current study is the relatively small sample size per sex, which may reduce the power to detect subtle sex-specific effects. Future studies should consider larger cohort sizes to provide a more thorough analysis of potential sex differences in response to interventions

In summary, our investigation into the mechanism underlying the beneficial effects of probiotic supplementation in a pretangle tau model reveals a multifaceted interplay between gut

microbiota, systemic inflammation, and central nervous system pathology. Notably, our study demonstrates significant changes in cognitive performance, inflammatory cytokines and brain markers, and the level of GSK activation following probiotic intervention. These effects are a consequence of an increase in the abundance of specific bacterial species within the gut microbiota following probiotic supplementation, many of which are known for their involvement in SCFA production. SCFAs, such as butyrate, play a crucial role in strengthening the gut barrier, thus mitigating gut barrier breakdown and reducing peripheral inflammation. Additionally, certain gut microbiota species exhibit direct anti-inflammatory properties. The improved gut microbiomes and decrease in overall peripheral inflammation have profound implications at the brain level, as evidenced by our study's findings. This intricate interplay underscores the potential of gut health modulation as a promising intervention strategy during preclinical AD.

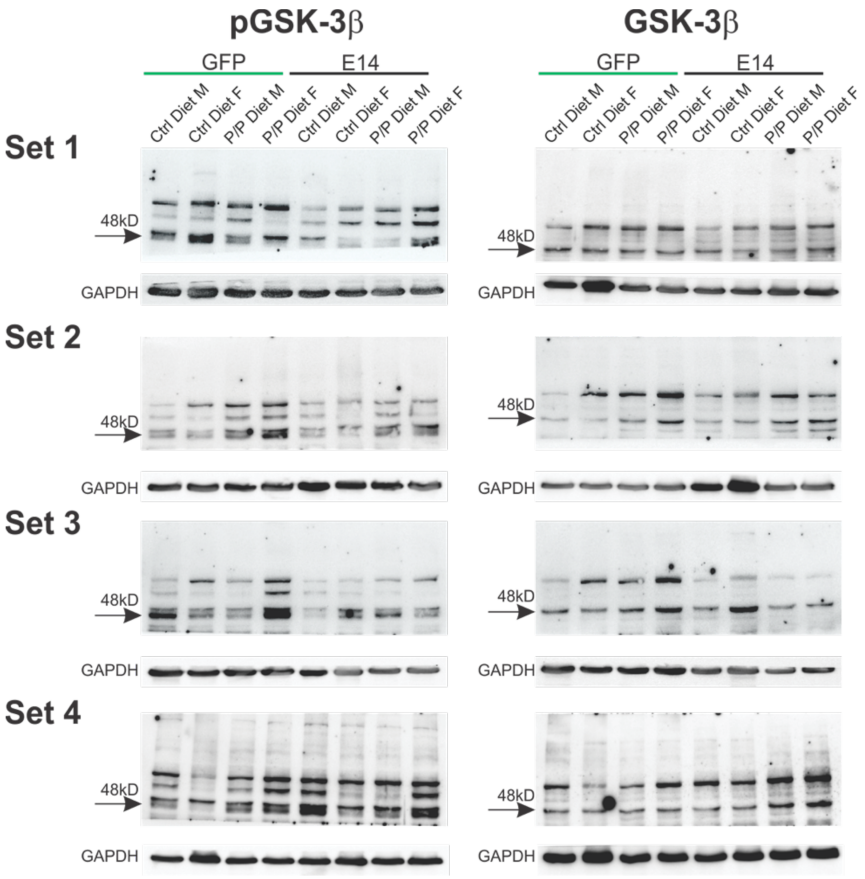
2.5 Supplementary Figures

Supplementary Figure 2.1



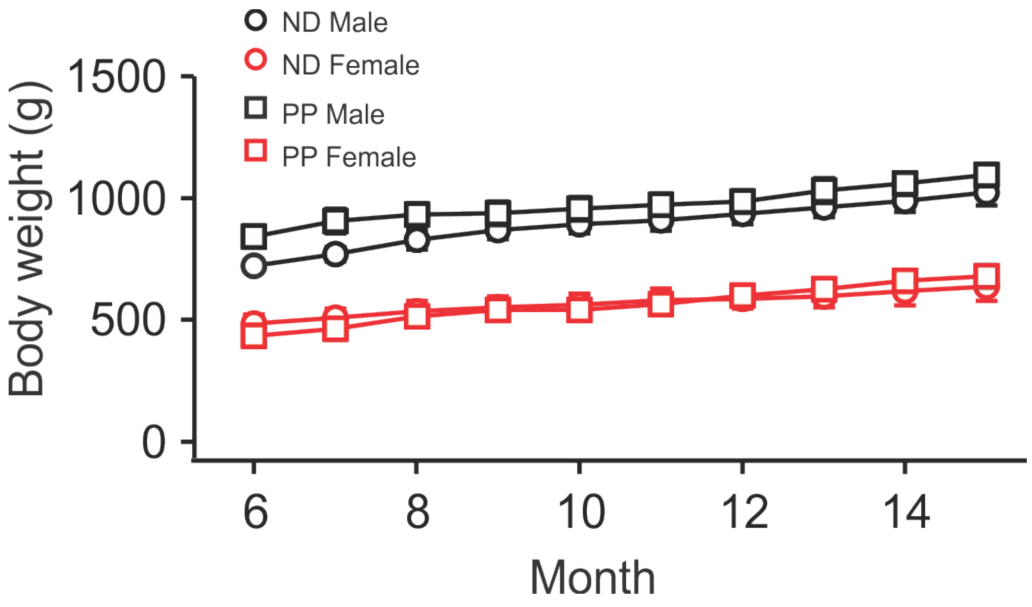
Supplementary Figure 2.1. Single-factor Univariate Taxa comparison between groups. Relative abundance of nine taxa selected by being significant in both Wilcoxin and single-factor univariate tests.

Supplementary Figure 2.2



Supplementary Figure 2.2. Full sets of pGSK-3β and GSK-3β Western Blot.

Supplementary Figure 2.3



Supplementary Figure 3.1 Body weights of normal diet and probiotic-fed animals throughout the project. A three-way ANOVA (AAV X diet X sex) showed a significant AAV X sex interaction (F_{39}=6.578, p =0.014), with no same-sex significance. A significant main effect of diet was observed (F_{39}=5.083, p =0.029), with probiotic animals having a higher body mass than normal diet animals ($p < 0.05$). A significant main effect of sex was observed with females having a lower body mass compared to males, regardless of diet ($p < 0.01$). To further understand diet effect on growth, we analyzed AAV X diet X time for each sex. No significant effect was shown in males, but females showed a significant main effect of diet X AAV (F_{225}=27.821, p =3.12E^{-7}), with probiotics showing an increased weight gain in htauE14 animals ($p < 0.01$). N: Ctrl Diet: 12F/13M; P/P Diet: 12F/10M.

Chapter 3: Probiotic supplementation prevents stress-impaired spatial learning and enhances the effects of enrichment

This chapter is a version of a submitted manuscript. Currently in review.

3.1 Introduction

Chronic stress negatively impacts learning and behaviour, and increases susceptibility to age-related diseases, including AD, by disrupting cognitive processes, healing mechanisms, coping abilities, and overall quality of life (Polsky, Rentscher, and Carroll 2022; Sotiropoulos et al. 2011; Torraville et al. 2023). It is implicated in the pathophysiology of various disorders such as cardiovascular diseases, obesity, gastrointestinal disorders, psychiatric conditions, and neurodegenerative diseases (Torraville et al. 2023). Individuals with high neuroticism, characterized by elevated distress and negative emotions, are particularly vulnerable to conditions like depression, anxiety disorders, and post-traumatic stress disorder (Escher, Sannemann, and Jessen 2019). Activation of neurobiological stress responses, such as the sympathetic nervous system and the HPA axis, contributes to higher morbidity and mortality rates, highlighting the profound impact of chronic stress on health and aging (Schneiderman, Ironson, and Siegel 2005; Shields and Slavich 2017). The LC-noradrenergic system is critically involved in stress-related disorders, with its dysregulation negatively impacting health and cognition (Suárez-Pereira et al. 2022). Noradrenergic neurons project to the hypothalamus and

key structures involved in learning and memory, therefore, stress-induced alterations in LC health and norepinephrine release can significantly impair brain function (Wang et al. 2017). Additionally, stress hormones impair hippocampal function via glucocorticoid receptors, affecting various types of memories (Lupien et al. 2018). Chronic stress also disrupts microglial function, potentially compromising brain homeostasis and contributing to anxiety phenotypes (Chen et al. 2024).

In contrast, environmental enrichment (EE) promotes cognitive health and resilience by providing cognitive, sensory, and motor stimulation that enhances brain plasticity and cognitive reserve (Mandolesi et al. 2017; Torraville et al. 2023). EE fosters neurobiological adaptations, such as improved learning and memory, increased neurotrophic factors, enhanced hippocampal neurogenesis, and improved synaptic connections, (Mora, Segovia, and del Arco 2007; van Praag, Kempermann, and Gage 2000; Segovia, del Arco, and Mora 2009; Leggio et al. 2005), which collectively fortify brain health against age-related cognitive decline and neurodegenerative diseases (Rolland, Abellan van Kan, and Vellas 2008; Marx 2005).

Emerging research highlights the interaction between gut microbiota and stress. The gut microbiota, comprising trillions of microorganisms in the gastrointestinal tract, plays a critical role in nutrient metabolism, immune modulation, and overall health (Thursby and Juge 2017). Maintaining a balanced gut microbiome is essential for health, as disruptions, known as microbial dysbiosis, can lead to inflammation and accelerated aging (Thevaranjan et al. 2017). Stress alters gut microbiota composition and function by affecting gastrointestinal motility, increasing gut permeability, and influencing microbial growth (Ulrich-Lai and Herman 2009; van Wijck et al. 2012; Galley and Bailey 2014).

Probiotics, live microorganisms conferring health benefits, have gained attention for their potential to modulate gut microbiota composition, enhance gut health, and improve immunity (Liu, Wang, and Wu 2021). Lactobacillus and Bifidobacterium species, extensively studied for their stress-alleviating, gastrointestinal barrier-enhancing, and anti-inflammatory effects, may also impact neurobiological pathways involved in stress resilience and cognitive function (Arseneault-Bréard et al. 2012; Moya-Pérez et al. 2017; Liu et al. 2020), though the exact mechanisms remain unclear.

Our study investigates whether probiotic supplementation can alter the brain's response to chronic stress and amplify the positive effects of environmental enrichment on cognition and brain health. By exploring these interactions, we aim to uncover mechanisms through which probiotics may mitigate stress-induced effects and enhance the beneficial impacts of environmental enrichment on cognitive resilience and overall brain health.

3.2 Materials and Methods

3.2.1 Subjects and Ethics Statement

Sprague-Dawley rats of both sexes were used. Rats were kept in a standard 12-hour light-dark cycle, with food and water *ad libitum* except during the probiotic feeding stage. The regular water was filtered three times (0.2 microns) and the diet (Teklad 2018) was irradiated. Experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and followed the Canadian Council's Guidelines on Animal Care.

3.2.2 Experimental Design

Figure 3.1A shows the flow of the experiment. Rats underwent diet supplementation at 6 months of age, for 3 months. Following that, animals were randomly assigned stress and enrichment, or cage control (no stress/enrichment) paradigms daily for 6 weeks, from 9-10 months of age. Animals then underwent a battery of behavioral tasks to assess general behavior and cognitive function before being sacrificed for Immunohistochemistry and Western Blot assays. A subset of animals had fecal collection following probiotic supplementation, for 16srRNA sequencing and gut microbiome analysis. Surfaces were sterilized with 70% ethanol, cages and toys were autoclaved.

Rats were randomly assigned to five conditions, (1) Cage (control without stress or enrichment manipulation + control diet), (2) Stress (STR) (stress paradigm + control diet), (3) STR + P/P (stress paradigm + probiotic/prebiotic diet), (4) Enrichment (ER) (enrichment paradigm + control diet), or (5) ER + P/P (enrichment paradigm + probiotic/prebiotic diet). Groups were sex balanced.

3.2.3 Probiotic Diet Supplementation

ProBiotic-4, comprised of *Bifidobacterium lactis* (50%), *Lactobacillus casei* (25%), *Bifidobacterium bifidum* (12.5%), and *Lactobacillus acidophilus* (12.5%), were purchased from Swanson (Fargo, ND, USA). Rats received ProBiotic-4 (3 x 109 CFU) once daily for three months at 6-9 months of age, dissolved in 30ml water (Yang et al. 2020), mixed with prebiotic oligofructose/FOS Orafti® P95 powder (200 mg/kg; Quadra Chemicals), to improve the effectiveness of probiotics (Roy and Dhaneshwar 2023). Regular water was provided only after

probiotic mixture was consumed. Control rats received regular water only. Body weight was measured bi-weekly.

3.2.4 Stress Paradigms

Stress paradigms were implemented using a chronic unpredictable stress paradigm for six weeks from 9-10 months of age following a protocol similar to those previously described, and frequently used to induce a depressive-like phenotype (Yalcin, Aksu, and Belzung 2005; Zhou, Shi, and Zhang 2019, Strekalova et al. 2022). Stressors were applied for the same 2 hours per day for the duration, including restraint, wetted bedding, tilted cage, and an irregular light cycle.

3.2.5 Enrichment Paradigms

Enrichment paradigms were implemented by placing 4-5 animals together in a 60x60x50cm Plexiglas play arena for 2 hours per day for six weeks, at 9-10 months of age (Leggio et al. 2005). The play arena contained toys, chews, exercise equipment, and treats. Males and female animals were separated into different arenas.

3.2.6 Fecal Collection

Fecal samples were collected following probiotic supplementation from a subset of animals. Animals were placed in clean autoclaved cages, where freshly voided fecal material was collected in sterile centrifuge tubes before being stored at -80°C until DNA extraction.

3.2.7 DNA extraction from Feces

Isolation of microbial genomic DNA from each animal's stool sample was performed using the QIAamp® PowerFecal® Pro DNA Kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction.

Prior to storage, quality control measures were implemented to evaluate DNA purity via the Thermo ScientificTM NanoDropTM One Spectrophotometer (Thermo ScientificTM 840274100). The DNA concentration from 1µl of each sample was determined by absorbance at 260nm (A260), and the purity was estimated by determining the A260/A280 ratio with the Nanodrop spectrophotometer, then samples were stored in -20°C until shipment.

3.2.8 Microbiome Analysis

16S rRNA sequencing was performed at the Integrated Microbiome Resource (Dalhousie University, Halifax, Canada). Only samples from female rats were included. The V6-V8 bacterial region of 16S rRNA genes was analyzed as previously described (Comeau et al. 2011). The library was sequenced on an Illumina MiSeq platform.

3.2.9 Behavioral Testing

3.2.9.1 Exploratory, Anxiety and Depressive Behavior

Rats were given one 10-minute trial to explore an open field ($60 \times 60 \times 40.5$ cm^3) and recorded with ANY-Maze software (Stoelting). Distance traveled, time spent rearing (including free and supported rearing), average speed, and time spent in center (indicating the level of anxiety) were recorded and analyzed offline as previously described (Omoluabi et al. 2021).

Anxiety was measured by the open field behavior, a 5 minute Elevated Plus Maze trial (50 × 10 cm2 /arm with an 11 × 11 cm2 central platform, 38 cm walls on the closed arms), where time spent inside closed arms vs. open arms as well as head dips over the open arms were analyzed. Depressive behavior was measured by 24 hours sucrose (0.75%) percentage consumption.

3.2.9.2 Spatial Memory Assessments

To assess short-term spatial memory, the Y-maze was used and to assess long-term spatial memory, animals underwent the SLR task.

In the Y-maze, animals explored a black opaque Plexiglas Y-shaped maze with three arms 120 apart (50 cm x 16 cm x 32 cm3). For the training phase, animals had a 15-minute trial in which they were allowed to freely explore two of three arms. Which of the arms was closed for this training phase was counterbalanced between groups and animals. For the testing phase (4 hours later), animals were re-placed into the same "start" arm for another 15-minute trial and allowed to explore the full maze with all three arms open, with the previously closed arm considered to be the "novel" arm. Number of entries into the arms and duration in each arm was recorded for analysis (Dellu et al. 1992).

For the SLR task, rats were given 10 minutes in an open arena (60 × 60 × 40.5 cm3) with three identical objects (1, 2, and 3) placed at specific positions. During testing (24 hours later), rats were placed in the same arena with two identical objects, one in the same position as Object 1 (a familiar location, F), the other midway between previous Objects 2 and 3 (a novel location, N). The discrimination ratio was the difference between time spent at the novel and familiar objects over the total time spent on both objects (Bekinschtein et al. 2013).

3.2.9.3 Odor Discrimination Task

Discrimination of similar odors was tested with an odor detection and discrimination task (ODAD), using perforated micro-centrifuge tube containing filter paper with 60 µl of odorant or mineral oil. The first three trials used odorless mineral oil, the next three trials used odor 1 (O1, 1-heptanol, 0.001%), and the last trial used an odor mixture that had a similar smell to O1 (O2, 1-heptanol and 1- octanol in a 1:1 ratio, 0.001%). The discrimination index was the ratio of the sniffing time difference between the O2 and the third presentation of O1 to the total sniffing time (tO2 -tO1-3)/(tO2 +tO1-3).

3.2.9.4 Odor Associative Learning

Rats were food restricted for 3–6 days before the onset of the experiments and food deprivation continued during the experiment. Rats were placed in an open arena (60 × 60 × 40.5 cm3) with 2 scented sponges, and Reese's puff cereal was used as a food reward. This procedure consisted of a habituation phase, followed by an associative training phase. In the habituation phase, rats were exposed to an unscented sponge placed in random locations, baited with food.

In the associative training phase, rats were placed in a designated home corner and presented with 2 scented sponges (locations varied each trial pseudo-randomly and given a maximum of 300 s to retrieve the cereal pellet from a retrievable center hole in one scented sponge. Percentage of correct responses was counted as the number of correct responses over the number of total nose pokes.

3.2.10 Histology, Immunohistochemistry (IHC) & Imaging

LC tissue was extracted after decapitation and stored in 4% paraformaldehyde before being transferred to 20% sucrose in 0.1 M PBS. Fifty μm sections at 150 μm intervals were collected in PVP cryoprotectant for free-floating IHC.

All histology and IHC followed established procedures (Ghosh et al. 2019; Omoluabi et al. 2021). Primary antibodies used included: Ionized calcium-binding adaptor molecule 1 (Iba1) (019-19741, Wako, 1:2000), and Albumin (16475-l-AP, ProteinTech, 1:1000). Alexa Fluor secondary antibodies (Invitrogen, 1:1000) were used.

Bright-field and fluorescence microscopy used an Olympus BX53 (Olympus) and EVOS M5000 imaging system (Thermo Fisher Scientific), respectively. Image analysis was conducted with ImageJ. The light intensity and exposure parameters were standardized across all captured images. In the LC, the numbers of positive stained cells for Iba-1 were counted and normalized to the region of interest (/mm^2). Albumin expression was measured as the mean density of the fluorescence in the LC, normalized to the background level in the lateral vestibular nucleus (Kelly et al. 2019). Three sections of each marker, distributed evenly within the same rostral to caudal range were used from all animals and counts from the two hemispheres were averaged. Analysis was conducted by experimenters that were blind to the experimental conditions.

3.2.11 Western Blotting

Hippocampal tissue was extracted after decapitation and stored frozen. Brain tissue processing followed established protocols (Morrison et al. 2013). Total protein concentration was quantified by standard Pierce BCA protein assay kit (Thermo Scientific, 23225). Equal amounts of protein

(20 μg) were separated by SDS-PAGE on 10% gels and were then transferred to Immobilon-P Transfer PVDF membranes (Merck Millipore, IPVH00010). Following transfer, the membranes were briefly rinsed with 1x low salt TBS-T (containing 1.5M NaCl, 1M Tris Base and 0.1% Tween 20) and blocked for 1 hr with 5% nonfat skim milk at room temperature. They were then incubated for 2 hrs at room temperature with the following antibodies: Glucocorticoid receptor (GR; AB92627, Abcam, 1:2000), TNFα (AB6671, Abcam, 1:2000), TH (MAB318, Millipore Sigma, 1:2000). The membranes were rinsed in TBS-T (containing 5M NaCl, 1M Tris Base and 0.1% Tween 20) and incubated for 1.5 hrs at room temperature, with either horseradish peroxidase–labeled anti-rabbit IgG (31460, 1:4000) or anti-mouse IgG (31430, Thermo Fisher Scientific, 1:4000). The protein bands were visualized using chemiluminescent substrate (ThermoFisher Supersignal West PICO, 34577) on a digital image scanner (ImageQuant LAS 4000) and quantified with the ImageJ software.

3.2.12 Statistical Analysis

All data are shown as mean ± standard error of the mean. Statistical analysis was conducted with OriginPro 2022b software. The 16S rRNA data were analyzed using statistical tools provided by MicrobiomeAnalyst.ca. Alpha diversity was assessed using the Chao1 index, and differences between the two groups were evaluated with a t-test. Beta diversity was quantified using the Bray-Curtis index, and statistical significance was determined via PERMANOVA. Additionally, heat tree analysis, which leverages the hierarchical structure of taxonomic classifications, was used to visualize and compare taxonomic differences between microbial communities. The differences were quantitatively represented using median abundance and statistically evaluated using the non-parametric Wilcoxon Rank Sum test. Behavioural results were analyzed by two-way ANOVA (group x sex). IHC and Western blotting results were analyzed by three-way

(treatment x diet x sex) ANOVAs. *Post-hoc* Tukey tests were used for group comparisons. Homogeneity of variance was assessed with Levene's test. Normality of the data was assessed with Shapiro-Wilk test and met before t-tests or ANOVAs. One outliner in one experiment in Fig 4C (> mean ± 2SD) was removed from the final analysis.

3.3 Results

3.3.1 Enrichment Increased Exploratory Behavior while Stress Induced Anxiety

The open field task revealed significant differences between groups in distance traveled ($F_{4,41}$ = 4.732; p = 0.0031; Figure 3.1B1), rearing ($F_{4,41}$ = 3.781; p = 0.01; Figure 3.1B2), speed ($F_{4,41}$ = 5.119; p = 0.0019; Figure 3.1B3), and time spent in center ($F_{4,17}$ = 8.439; p = 6.09E-4; Figure 3.1B4). Both ER groups regardless of diet type traveled significantly longer distance than the STR group ($p < 0.01$). In terms of exploration, ER animals spent significantly more time rearing compared to cage control animals ($p < 0.05$). Similarly, the ER groups had higher travel speed compare to cage ($p < 0.05$), and STR ($p < 0.01$) groups. A sex difference was observed ($F_{1,41}$ = 4.395; p = 0.042), with females moving at a higher speed than males. STR animals showed a significantly lower time spent in the center compared to cage groups ($p < 0.05$), regardless of diet type.

Anxiety levels were then measured using the elevated plus maze, with a significant main effect observed between groups for time spent in the closed arm ($F_{4,41}$ = 5.394; p = 0.0014; Fig. 3.1C1), no significance was observed between sexes. ER animals spent significantly less time in the

closed arm than caged animals regardless of diet type (control diet, $p < 0.05$; probiotic diet, $p < 0.01$). When measuring head dips, another indicator for anxiety level, a significant main effect was found between groups ($F_{4,41} = 35.7789$; $p = 7.18E^{-13}$; Fig. 3.1C2). Similarly, ER and ER + probiotic groups showed significantly higher levels of this behavior over cage ($p < 0.01$). Interestingly, probiotic supplementation significantly increased this exploratory behavior in STR animals compared to control diet-fed ones ($p < 0.01$). No sex differences were observed.

The sucrose preference test assessed the level of anhedonia, as animals with anhedonia are less interested in palatable food. Our result revealed no significant effects in the consumption of sucrose water between groups, or sexes (Fig. 3.1D).

3.3.2 Probiotic Supplementation Rescued the Spatial Learning Deficiency in STR Rats, and Enhanced Memory of ER Rats

In the Y-maze task, a group x sex interaction was observed in duration of time spent in the novel arm ($F_{4,41} = 2.908$, $p = 0.033$; Fig. 3.1E1), although no significant difference was found in the post-hoc Tukey test. However, there was a significant main effect of groups for the number of entries in the novel arm ($F_{4,41} = 6.441$, $p = 4.06E^{-4}$; Fig. 3.1E2). Following stress, animals displayed a deficit compared to cage control groups ($p < 0.01$) and ER groups ($p < 0.05$ or $p < 0.01$), and this was prevented by probiotic feeding ($p < 0.01$).

In the SLR task, there was a significant difference between groups ($F_{4,41} = 14.508$, $p = 1.83E^{-7}$; Fig. 3.1F). A memory deficit was observed for STR animals compared to cage control animals ($p < 0.01$). Like the Y-maze task, this deficit was restored, with a significant increase in discrimination observed in STR + probiotic group ($p < 0.01$). Probiotic feeding in ER animals

led to a significantly better discrimination ability than cage ($p < 0.05$) and STR ($p < 0.01$) animals.

Olfactory impairments were tested using a similar odor discrimination task. No differences were observed between groups. Interestingly, in general, male rats performed better than female rats ($F_{1,41} = 4.2$, $p = 0.046$; Fig 3.1G). Additionally, an olfactory dependent rewards association task revealed no differences between groups or sexes (Fig. 3.1H).

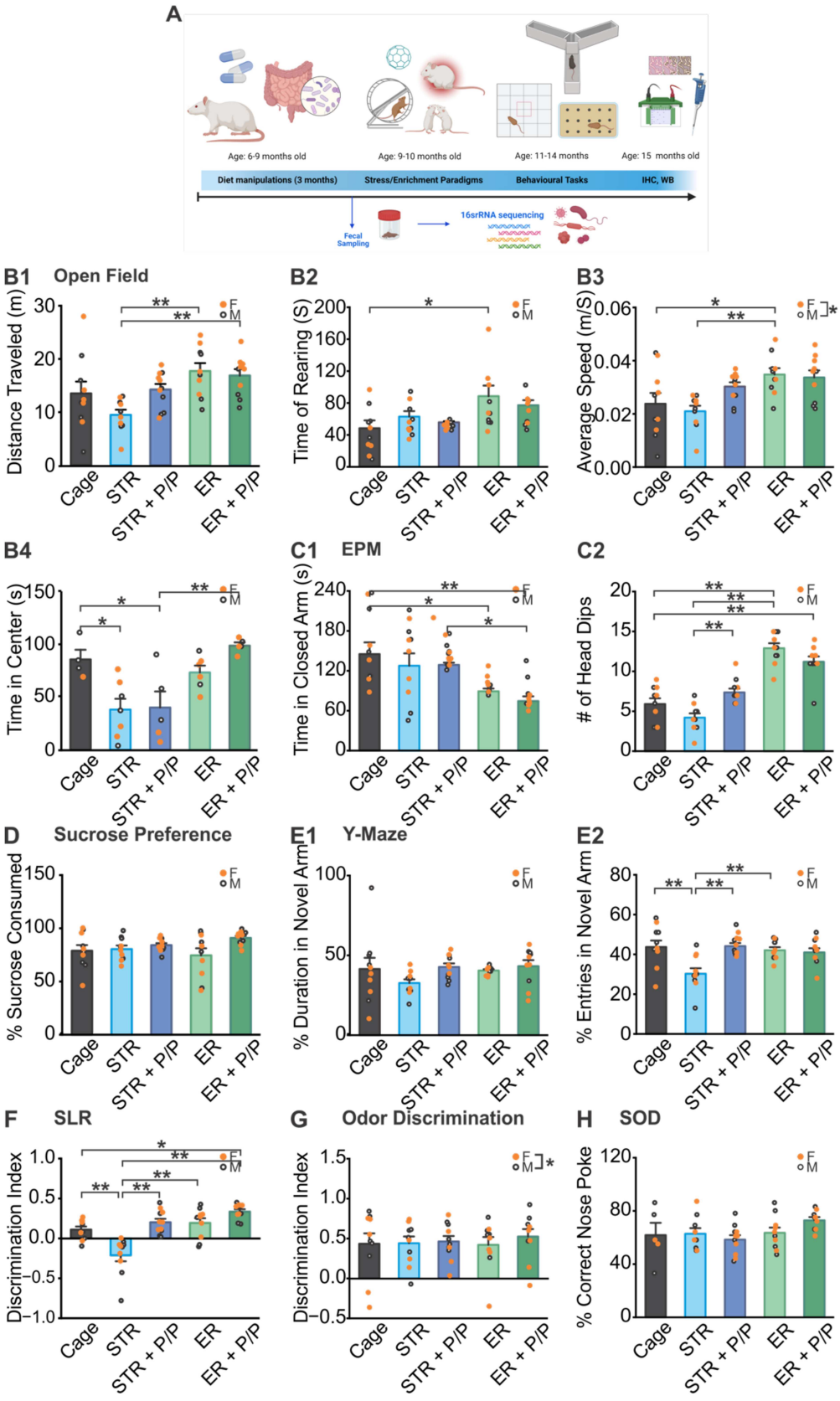

A
Age: 6-9 months old
Age: 9-10 months old
Age: 11-14 months
Age: 15 months old
Diet manipulations (3 months)
Stress/Enrichment Paradigms
Behavioural Tasks
IHC, WB
Fecal Sampling
16srRNA sequencing
B1 Open Field
Distance Traveled (m)
B2
Time of Rearing (S)
B3
Average Speed (m/S)
B4
Time in Center (s)
C1 EPM
Time in Closed Arm (s)
C2
# of Head Dips
D Sucrose Preference
% Sucrose Consumed
E1 Y-Maze
% Duration in Novel Arm
E2
% Entries in Novel Arm
F SLR
Discrimination Index
G Odor Discrimination
Discrimination Index
H SOD
% Correct Nose Poke
F
M
Cage
STR
STR + P/P
ER
ER + P/P

Figure 3.1. The effects of probiotics in combination with stress and enrichment on behavioral tasks. A. Schematic of experimental design timeline. **B1-B4.** Distance traveled (B1), time spent rearing (B2), average speed (B3) and time spent in center (B4) measurements in an open field maze test. **C1-C2.** Time spent in the closed arm (C1) and number of head dips over the edge (C2) in an elevated plus maze (EPM) test. **D.** Percentage of sucrose solution consumption over a 24 hr period. **E1-E2.** Percentage of time (E1) and number of entries (E2) in the novel arm in a Y-maze test. **F.** Discrimination index in a spontaneous location recognition (SLR) task. **G.** Discrimination index in an odor discrimination task. **H.** Percentage of correct nose poke in standard object discrimination (SOD) task. STR: stress. ER: enrichment. P/P: probiotic + prebiotic. N (except B4): Cage: 5F/5M; STR: 5F/5M; STR + P/P Diet: 6F/5M; ER: 5F/5M; ER + P/P Diet: 6F/4M. N (B4): Cage: 1F/3M; STR: 4F/3M; STR + P/P Diet: 2F/3M; ER: 4F/2M; ER + P/P Diet: 3F/2M *$p < 0.05$, **$p < 0.01$.

3.3.3 Probiotic Supplementation Enriches Microbiome Diversity in the Gut

Levels of alpha diversity and beta diversity were obtained from 16S rRNA sequencing. Using the Chao1 index, alpha diversity level, a metric measuring the richness (number of taxa), or evenness (relative abundance of those taxa) was analyzed. A significant increase in microbiome alpha diversity was observed in the probiotic diet groups compared to control diet groups (F_{17} =5.783, p = 0.027; Fig. 3.2A). Levels of beta diversity, representing the diversity and variability of community composition, showed an increase in the probiotic diet groups, compared to control diet groups (F_{17}=1.566, p =0.066; Fig. 3.2B).

Changes in the taxa species induced by Probiotic-4 mixture feeding were further assessed. At the genus level, bacterial taxa were analyzed for abundance levels between groups to observe any effect from the introduction of probiotics (Fig. 3.2C). The heat map in Fig. 3.2C shows average levels of each taxon and significance levels of the probiotic diet groups compared to the control diet using the Wilcoxon test. A labeled taxa is indicative of a significant difference between groups. Overall, five taxa genera were upregulated in abundance in the probiotic diet groups, and one was downregulated, compared to control, demonstrating a significant difference in gut microbiome makeup from specific bacterial taxonomic groups. This includes the upregulation of Actinobacteria (including Bifidobacterium & Rothia species), Ruminococcaceae, Monoglobales, Streptococcaceae, and Barneseillaceae, as well as a downregulation of Lachnoclostridium.

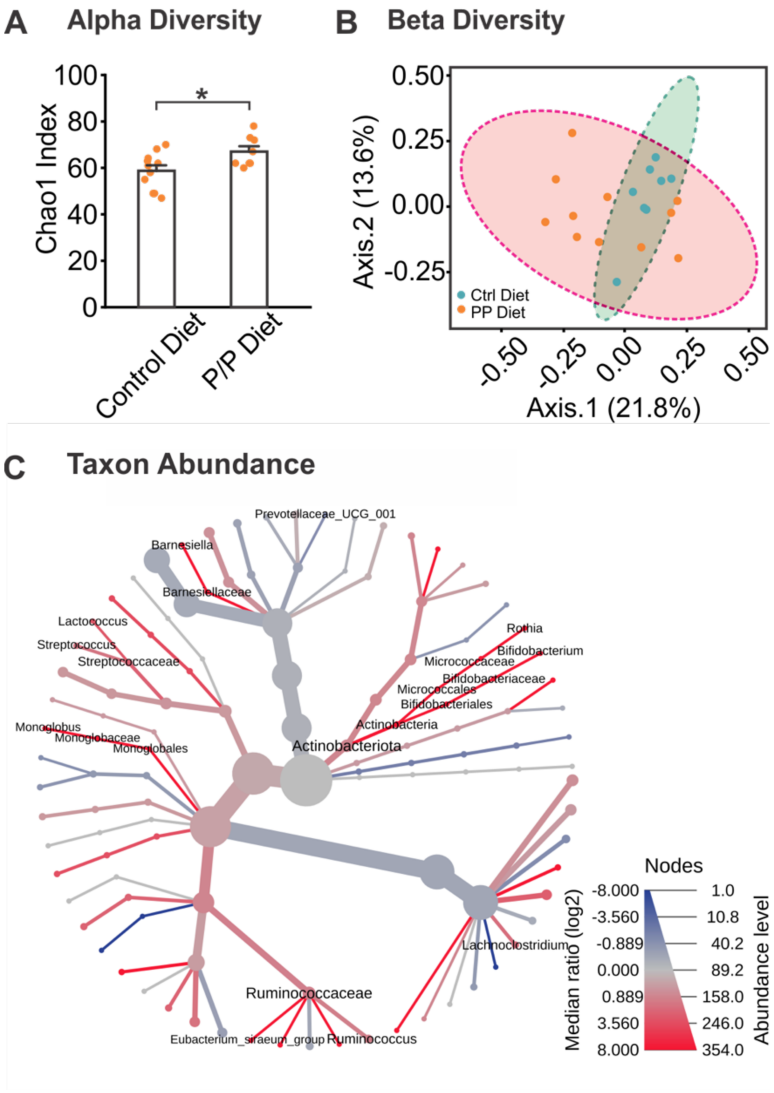


Figure 2. Alterations of microbiota in diversity and abundance following probiotic supplementation. A. Chao1 analysis of Alpha Diversity levels between control diet and probiotic-fed groups. **B.** Ordination plot of Beta Diversity index between diet groups, using Bray-Curtis index distance method. **C.** A heat tree demonstrating bacterial abundance differences between diet groups. Labeled branches represent a significant abundance level between groups. N: Control diet: 11F; Probiotic diet: 8F. *$p < 0.05$, **$p < 0.01$.

3.3.4 Stress Increased Levels of Microglia in the Locus Coeruleus Compared to Enrichment, which was Reversed by Probiotic Supplementation

LC is critically involved in stress response and novelty-associated enrichment (Prokopiou et al. 2022; Suárez-Pereira et al. 2022). We therefore tested LC inflammation levels using Iba-1 microglial marker. We also measured BBB integration with albumin staining. Stress has been shown to impair BBB and probiotic supplementation has been associated with improved BBB (Torraville et al. 2023).

For Iba-1 levels, there was a significant treatment x diet interaction ($F_{1,29} = 4.866$, $p = 0.035$; Fig. 3.3A1-3.3A2), with stress treated animals showing a significant increase compared to animals in enrichment groups ($p < 0.05$ or $p < 0.01$) which was reserved by probiotic supplementation ($p < 0.05$). Albumin levels showed a significant difference between treatment groups ($F_{1,29} = 4.811$, $p = 0.036$; Fig. 3.3B1-3.3B2), with significantly higher levels in STR animals compared to ER animals ($p < 0.05$). A sex difference was also observed in albumin levels ($F_{1,29} = 4.788$, $p = 0.037$), with females showing higher levels of albumin than males.

Furthermore, measurements of GR, involved in stress regulation (Fig. 3.4A), inflammation marker TNFα (Fig. 3.4B), and TH for noradrenergic levels (Fig. 3.4C) were conducted in the hippocampus. There were no significant differences among groups in the levels of GR and TNFα. TH levels were higher in the enrichment treated rats than the stressed rats ($F_{1,30} = 8.941$, $p = 0.006$; Fig. 3.4C).

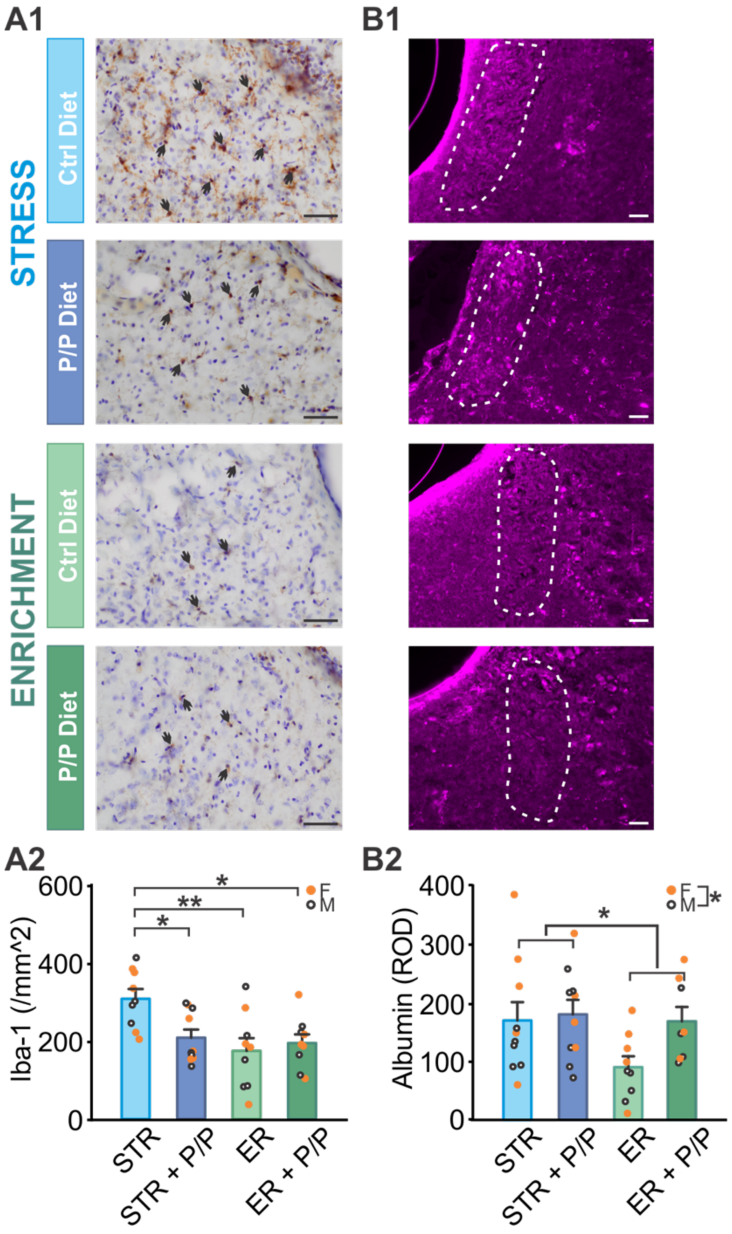


Figure 3.3 The effects of stress, enrichment and diet manipulation on microglia and albumin levels in the locus coeruleus (LC). A1. Example images of Iba-1 staining in the LC. Arrows indicate positively stained cells. **A2.** Number of Iba1 cells per mm^2 in the LC. N: STR: 5F/4M; STR + P/P Diet: 5F/5M; ER: 4F/5M; ER + P/P Diet: 5F/4M. **B1**. Example images of albumin staining in the LC. Whilte circles indicated the LC region. **B2.** Mean intensity of albumin staining in the LC. N: STR: 5F/5M; STR + P/P Diet: 4F/5M; ER: 5F/4M; ER + P/P

Diet: 5F/4M. STR: stress. ER: enrichment. P/P: probiotic + prebiotic. Scale bars: 50 μm. *$p <$ 0.05, **$p < 0.01$.

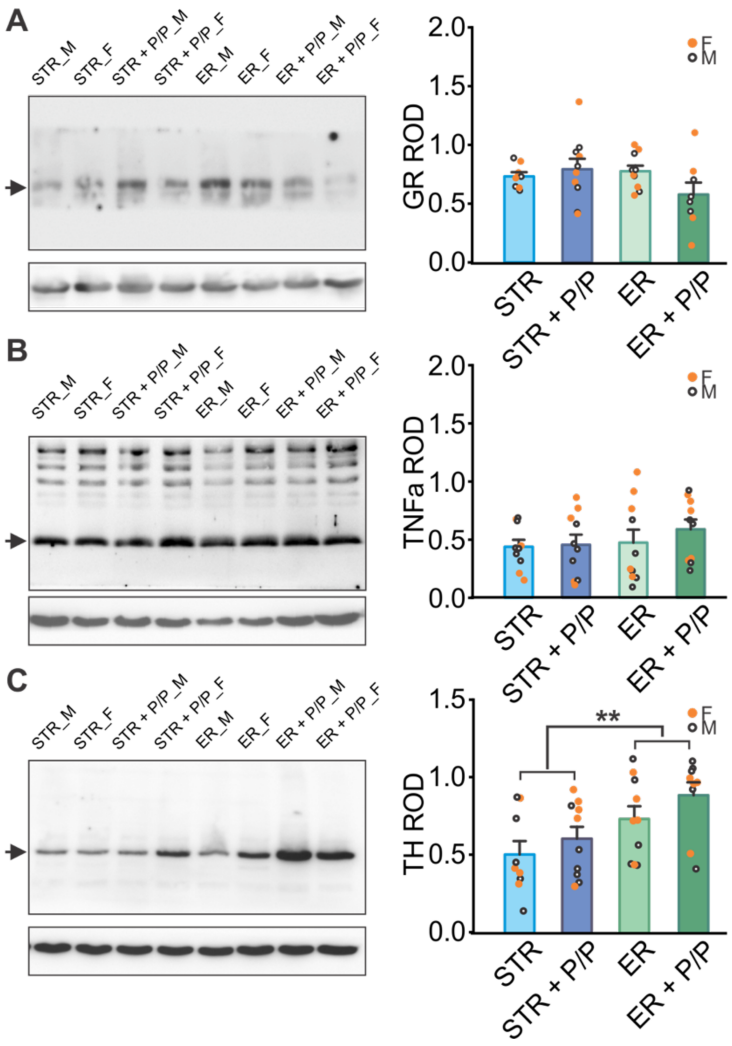


Figure 3.4. The effects of stress, enrichment and diet manipulation on hippocampal markers. A. Representative blot and quantity of glucocorticoid receptor (GR) normalized to beta-actin levels in the hippocampus. N: STR: 4F/4M; STR + P/P Diet: 5F/5M; ER: 5F/5M; ER + P/P Diet: 4F/4M. **B.** Representative blot and quantity of Tumour Necrosis Factor aloha (TNFα) normalized to beta-actin levels in the hippocampus. N: STR: 4F/5M; STR + P/P Diet: 5F/5M; ER: 5F/5M; ER + P/P Diet: 5F/5M. **C.** Representative blot and quantity of tyrosine hydroxylase (TH) normalized to beta-actin levels in the hippocampus. N: STR: 5F/4M; STR +

P/P Diet: 5F/5M; ER: 5F/5M; ER + P/P Diet: 5F/4M. STR: stress. ER: enrichment. P/P: probiotic + prebiotic. **$p < 0.01$.

3.4 Discussion

Current research highlights the intricate relationship between stress and the gut microbiota, with probiotic interventions emerging as a promising strategy to modulate these interactions. This study investigated the effect of probiotics combined with stress or enrichment on cognition and inflammation. We observed increased anxiety in animals that underwent chronic stress, while enrichment paradigm led to increased exploration and reduced anxiety. Probiotic supplementation increased gut microbiome diversity and health, ameliorated anxiety, prevented spatial learning impairment in stress-treated rats, and enhanced learning in the enrichment group. Chronic stress increased microglia activity (Iba-1) in the LC, which was prevented by probiotics. Blood-brain barrier integrity was lower in stressed animals compared to enriched rats. Higher TH levels in the hippocampus of enriched groups may correlate with better LC function and axonal release. These results suggest a mechanistic connection between reduced stress and improved gut health through decreased brain inflammation.

Chronic stress can significantly impact the immune system through the microbiota-gut-brain axis, as evidenced by systemic inflammatory increases coinciding with stress and microbiota barrier and makeup changes (Pasiakos et al. 2016; De Palma et al. 2015; Moya-Pérez et al. 2017). In addition, this response triggers a dysregulated HPA axis, which results in increased inflammation and altered immune responses, shown through an upregulation of corticosterone and adrenocorticotropic hormone in GF mouse studies (Ackerman, Hofer, and Weiner 1978). This stress-induced inflammation can exacerbate psychiatric disorders like depression and anxiety, which in turn can further disrupt gut microbiota composition, creating a vicious cycle of stress and dysbiosis (Li et al. 2019). This cycle is associated with increased intestinal

permeability, often referred to as "leaky gut," and increased BBB permeability, which we observed in our study. These barrier weakening can lead to systemic inflammation and contribute to various health issues (Chatzaki et al. 2003; Dodiya et al. 2020; Welcome and Mastorakis 2020). The lack of significance observed for TNFα and GR expression suggests that the inflammatory and stress response markers may not be acutely impacted under the conditions and duration of probiotic and stress interventions used in this study.

Probiotics, beneficial live microorganisms, can positively influence the gut microbiota and, by extension, the gut-brain axis (Arseneault-Bréard et al. 2012; Moya-Pérez et al. 2017). They help restore the balance of gut microbiota, which is often disrupted by stress, by competing with pathogenic bacteria and enhancing the growth of beneficial microbes, ultimately enhancing the overall makeup (Dimidi et al. 2017). This restoration can reduce inflammation and improve both gut and mental health (Arseneault-Bréard et al. 2012; O'Sullivan et al. 2011; Bravo et al. 2011). Interestingly, here we demonstrate that prior probiotic supplementation can enhance animals' resistance to chronic stress. As well, probiotics have been shown to enhance the production of anti-inflammatory cytokines and reduce the levels of pro-inflammatory cytokines, modulating immune responses and reducing chronic inflammation (Virk et al. 2024). Additionally, probiotics can increase the expression of tight junction proteins, reducing intestinal permeability and preventing systemic inflammation (Gou et al. 2022). Specific probiotic strains have been shown to produce neurotransmitters like serotonin and GABA, which can improve mood and reduce anxiety (Duranti et al. 2020; Akram et al. 2024). Importantly, SCFA producing bacteria are found to be in higher levels following probiotic feeding, which can in turn act on anti-inflammatory cytokine release and tight junction integrity (Markowiak-Kopeć and Śliżewska 2020).

Dietary interventions involving probiotics have shown promise in clinical settings. They can be particularly effective as adjunctive treatments for managing chronic stress, improving mental health, and enhancing overall immune function (Den et al. 2020; Mazziotta et al. 2023). This approach is beneficial for conditions characterized by both psychological and gastrointestinal symptoms. In this study, animals are fed probiotics prior to a significant environmental manipulation. Prolonged probiotic supplementation can prevent stress-induced learning deficiencies and neuroinflammation. Therefore, probiotic supplementation can serve as a preventive strategy against stress-induced physiological and psychological disorders.

In summary, the relationship between the gut microbiome and stress can be regulated by the immune response and reduced inflammation through probiotic supplementation. Incorporating probiotics into the diet is a viable strategy to mitigate the adverse effects of chronic stress on the immune system. This approach promotes a healthy gut microbiota, supporting better mental and physical health. One limitation of our study is the small sample size for sex-dependent analysis. Additionally, further research is needed to characterize the gut microbiome in male animals. Another limitation is we did not include separate probiotic or prebiotic only controls. Despite these limitations, our study underscores the importance of the gut-brain-immune axis in developing therapeutic interventions, offering a holistic approach to health management.

Chapter 4. Summary of Major Findings

4.1 Gut Diversity and Abundance Shift from Probiotic Supplementation

Our studies demonstrate that a three-month course of probiotic supplementation led to significant enhancements in gut microbiome diversity and optimized bacterial composition. This was evidenced by a marked increase in alpha diversity and a notable shift in beta diversity between groups post-feeding.

Analyzing the gut microbiome composition post-probiotic supplementation revealed significant changes in specific bacterial taxa compared to the control groups (chapter 2). Notably, Clostridium_sensu_stricto_1, Bacteroides dorei, and Lactococcus formosensis were significantly elevated in the probiotic-fed groups. These taxa play essential roles in gut health, with Clostridium_sensu_stricto_1 known for producing SCFAs like butyrate, which enhances tight junction protein expression in colon epithelia . This action strengthens the intestinal barrier, reduces mucosal permeability, and inhibits inflammatory cytokines (Monda et al., 2017). Interestingly, the decreased abundance of Clostridium_sensu_stricto_1 in normal aging suggests its role in age-related gut health decline (Odamaki et al., 2016).. Bacteroides dorei was shown to reduce gut microbial LPS production, suppressing proinflammatory immune responses and lowering intestinal permeability (Yoshida et al., 2018).

The importance of alpha and beta diversity in gut and brain health is supported by numerous studies. For instance, a review of nine studies reported a significant decrease in Shannon (alpha) diversity among AD patients, indicating a moderate reduction in the number of species (Jemimah

et al. 2023). Another study investigating individuals with MCI due to AD found a significant reduction in alpha diversity, as assessed using the Shannon index, in the amyloid + MCI group relative to the non-amyloid group (Kim et al. 2024).

Probiotics have been used to modulate diversity levels in different disease models. A study in piglets showed an increase in diversity and richness due to probiotic feeding (Shin et al. 2019). Human studies have shown similar results, with a maintained microbiome diversity in probiotic-fed depressed patients, compared to a lower diversity index in placebo (Schaub et al. 2022). Although probiotics can change the makeup and abundance of specific bacteria in the microbiome, research on alpha and beta diversity levels following probiotics in AD is limited (Naomi et al. 2021).

In htauE14 animals, no change in diversity was observed, however a slight shift in individual bacterial strains was observed in htauE14 animals compared to controls. The shift in composition and presence of specific bacterial species in our htauE14 rats suggests that there is a possible contribution to the pathogenesis of AD. This could be possible through the interaction of these bacteria with mechanisms such as enhanced gut permeability, systemic inflammation, and direct neuroinflammatory processes. Previous studies have shown that following gut dysbiosis, the translocation of bacterial products and inflammatory mediators can move into the bloodstream and influence brain function and neurodegenerative conditions (Akira, Uematsu, and Takeuchi 2006). This agrees with current research at later AD timepoints, exploring positive effects through the introduction of probiotics and specific beneficial bacteria in both animal and human models of AD (Naomi et al. 2021; Torraville et al. 2023).

Overall, these findings suggest that enhancing gut microbiome diversity through probiotic supplementation may play a critical role in maintaining gut health and potentially mitigating the progression of neurodegenerative diseases like AD. The specific effects of probiotic supplementation shown in this project such as effects on cognition, inflammation, and GSK3B kinase will be discussed below.

4.2 Probiotic Supplementation on Cognitive Performance in pretangle tau model

AD is characterized by a progressive decline in cognitive functions that significantly impact daily life. Both long-term and short-term memory tasks show impairments in AD animal groups, mirroring the cognitive deficits seen in humans. This decline is particularly evident in hippocampus-dependent working memory, as demonstrated in animal models using tests like novel object recognition (Cohen et al. 2013; Morrone et al. 2020). AD progression can be characterized by the progression of memory function decline, with 1) preclinical stages having neuropathological changes and the absence of cognitive impairment (Sperling et al. 2011), 2) the MCI stage showing deficits in both memory and non-memory functions (Albert et al. 2011), and 3) the AD dementia stage where cognitive impairment is poor enough to severely interfere with functional abilities (McKhann et al. 2011). Among the cognitive deficits, episodic memory impairment stands out as an early marker of the disease (Mistridis et al. 2015).

Olfactory identification and memory deficits are another common symptom, present in 85–90% of AD patients (Doty, Reyes, and Gregor 1987; Marine and Boriana 2014). A review of twelve studies examining 563 patients with MCI and 788 patients with AD revealed that olfactory identification was more profoundly impaired in AD patients than in those with MCI (Jung, Shin,

and Lee 2019). In a study by Audronyte et al., the “Sniffin' Sticks” test showed that odor discrimination is impaired early in AD and continues to deteriorate as the disease progresses (Audronyte et al. 2023). This was evident in a sample of 30 patients with mild dementia due to AD, 30 with MCI due to AD, and 30 elderly controls (Audronyte et al. 2023).

Our pre-tangle tau model exhibits behavioral deficits, particularly in spatial memory and olfactory functions. This was shown in this project, as well as previous projects from our lab. Probiotic supplementation was found to restore these spatial learning deficits, suggesting a beneficial effect on cognitive performance for the first time in this model. This improvement could be attributed to the reduction in inflammation, which directly impacts amyloid and tau levels, key factors that target memory systems in AD pathology.

Spatial memory deficits in AD models are commonly reported. For instance, our pre-tangle tau model has shown spatial memory impairments (Omoluabi et al. 2021). Additionally, in a study involving a cross between 5xFAD mice and JNPL3 mice, which express the P301L tau mutation, researchers observed cognitive impairments in behavioral tests such as the Y-maze and Morris water maze (MWM) from as early as two months of age (Kang, Kim, and Chang 2021). This onset of cognitive deficits occurred earlier than in the 5xFAD model alone, highlighting the significant impact of tau pathology on spatial memory.

The reduction of tau pathology in critical early brain regions may directly contribute to the observed improvements in spatial memory. Mechanistically, this could be due to a decrease in peripheral inflammation or GSK3B kinase regulation, as will be discussed in the following chapters.

4.3 Effect of Probiotic Supplementation on Systemic Inflammation in htauE14 Animals

Systemic inflammation is a well-documented feature in AD, particularly at later stages. However, research at early sporadic AD time points focusing on systemic inflammation is sparse due to the lack of animal models representing this time point. One aim of my study was to fill that gap by exploring the impact of probiotic supplementation on systemic inflammation in a pre-tangle tau model of AD, representing an early stage of tau pathology and disease.

Our research supports the critical role of chronic inflammation in the pathogenesis and progression of AD, characterized by the increased presence of immune cells such as microglia, astrocytes, and peripheral cytokines (Hansen, Hanson, and Sheng 2018; Carter et al. 2019; Swardfager et al. 2010). Recent findings suggest that modifying the gut microbiota composition with probiotics in AD models can reduce pro-inflammatory cytokine levels (Zhu et al. 2023; Seo and Holtzman 2024) Additionally, Cattaneo et al. found a positive correlation between peripheral pro-inflammatory cytokines and the abundance of multiple inflammatory bacteria taxa in cognitively impaired patients (Cattaneo et al. 2017). This gut-derived inflammation reduction may disrupt the inflammatory cascade that propagates systemically and reaches the brain, potentially exacerbating systemic inflammation.

In AD, neuroinflammation increases with disease progression, with peaks during the early stages and the transition from mild cognitive impairment to dementia (Fan et al. 2017). Previous studies have highlighted over-activation of microglia (Schafer et al. 2012), and increased cytokine production as potential diagnostic biomarkers (Swardfager et al. 2010). Interleukin (IL)-6, expressed by various cell types emerges as a pivotal player in neuroinflammation (Tanaka,

Narazaki, and Kishimoto 2014; Wu et al. 2015). An existing body of research suggests a potential link between elevated IL-6 levels and cognitive deficits (Bauer et al. 1991; Swardfager et al. 2010). Our main findings revealed increased overall microglial activity overall (Iba1 and CD68) and GFAP-IR in female rats. We also observed elevated levels of multiple peripheral inflammation markers in htauE14 animals, including pro-inflammatory markers IL-6 and TNFα, and the anti-inflammatory marker IL-10. These results suggest that abnormally phosphorylated pretangle tau in the preclinical stage of AD is sufficient to induce neuronal degeneration and inflammation. The increase in anti-inflammatory cytokine IL-10 may indicate an initial protective immune response, as both pro-inflammatory and anti-inflammatory cytokines are known to be elevated in AD.

Lowering systemic inflammation could potentially alleviate tau and amyloid levels, as shown in numerous studies. As mentioned, a single systemic LPS injection has been shown to increase Aβ1-42 deposition and p-tau levels in the brains of wild-type rodents (Wang et al. 2018). Additionally, a study using Rutin, a natural flavonoid with anti-inflammatory properties, lowered the production of proinflammatory cytokines, and in turn inhibited tau aggregation both in vivo, and in a Tau-P301S mouse model (Sun et al. 2021).

Here, this is a novel finding due to the early stage of pre-tangle tau that our animals represent. It highlights the potential of probiotic supplementation as a therapeutic option to slow inflammation and consequently, AD progression. By targeting systemic inflammation at an early stage, we may be able to prevent or mitigate the neural inflammation that damages the BBB and exacerbates AD pathology.

4.4 GSK3B as Mechanism for Probiotic Supplementation in AD

Our results revealed that GSK-3β, a critical kinase involved in tau phosphorylation, exhibited increased activity in the hippocampus of htauE14 brains. This was indicated by the reduced phosphorylation of S9, an inhibitory site of GSK-3β. Additionally, we detected tau phosphorylation at two microtubule binding sites, S262 and S356, within the LC, correlating with increased GSK-3β activity in the hippocampus.

Excessive activation of GSK-3β contributes significantly to the abnormal phosphorylation of tau, destabilizing microtubules and advancing AD pathogenesis (Morris et al. 2011; Sayas and Ávila 2021). Studies have shown that GSK-3β expression is up-regulated in the hippocampus of AD patients and is initially found in pretangle neurons in the entorhinal cortex before following a similar sequence to tau pathology (Pei et al. 1999). Overexpression of GSK-3β in mice not only results in tau hyperphosphorylation but also leads to impaired spatial learning, similar to our observations (Hernández et al. 2002).

Our findings align with previous studies suggesting that probiotics can directly impact the GSK-3β pathway, alleviating tau hyperphosphorylation. One study demonstrated that Lactobacillus prevented cognitive dysfunction and AD pathology in a D-galactose and AlCl3-induced AD model by regulating the PI3K/Akt/GSK-3β pathway (Song et al. 2022). However, our study is novel in its use of a pre-clinical animal model to show this correlation, particularly at an early pre-tangle tau stage, which has not been extensively explored in combination with probiotics previously.

The novelty of our findings is further highlighted by the lack of studies that investigate this mechanism at early AD stages. Previous research primarily focused on later stages of the disease, overlooking the potential of early intervention through probiotic supplementation. Our study provides crucial insights into the mechanistic link between probiotics and GSK-3β activity, offering a promising avenue for early therapeutic intervention.

This mechanism is also relevant in the context of inflammation. One study demonstrated that systemic exposure to Porphyromonas gingivalis increases GSK-3β activity by reducing its phosphorylation at S9, promoting neuroinflammation through elevated levels of pro-inflammatory cytokines such as IL-1β and TNFα, while decreasing anti-inflammatory cytokines like IL-10 (Jiang et al. 2021). Additionally, GSK-3β can modulate inflammatory responses and has been shown to downregulate microglial induced inflammatory responses (Wang et al. 2010). The activated state of GSK-3β promotes the activation of nuclear factor kappa-light-chain-enhancer of activated B cells and leads to a pro-inflammatory response, while activation at multiple TLRs reverse this activation, and lead to a downregulation of GSK-3β and subsequent anti-inflammatory response (Ko and Lee 2016; Cortés-Vieyra et al. 2021).

In conclusion, our study establishes a mechanistic link between GSK-3β activity and inflammation in a pre-tangle tau model, demonstrating that probiotic supplementation can ameliorate both pathways. This dual benefit provides a compelling case for using probiotics as a therapeutic strategy to reduce AD progression by targeting interconnected mechanisms of tau pathology and inflammation.

4.5 Barrier effects in AD

Our study identified increased BBB leakage in htauE14 rats, using albumin as a marker. This finding indicates a high level of BBB disruption in these rats. Dysfunction of the BBB is implicated in AD progression, triggering neuroinflammation and oxidative stress, which has been shown to indirectly enhance Aβ and tau accumulation (Erickson and Banks 2013; Blair et al. 2015). LC degeneration also results in BBB breakdown, as evidenced by prominent albumin extravasation, increased inflammation, and cognitive deficits in AD models (Kelly et al. 2019).

The novelty of our finding lies in its timing within the disease progression. For the first time, increased BBB leakage has been demonstrated in the LC, the starting point of pathology, in a pre-tangle tau animal model. This suggests that BBB breakdown may occur earlier in the disease progression, possibly exacerbating other pathologies such as tau accumulation and neuroinflammation.

Interestingly, probiotic feeding did not significantly impact the albumin levels in the LC of htauE14 rats. This may indicate that the direct passage of pathological molecules through the BBB could be a mechanism behind AD progression and not be mitigated by probiotics. It is also important to note that probiotic feeding ameliorated cognitive and inflammatory deficits. This could suggest that BBB dysfunction could be a downstream effect of AD, and not a causative mechanism. This finding suggests that while probiotics have beneficial effects on other aspects of the disease, including gut barrier function, they may not address all underlying mechanisms, such as direct BBB permeability changes.

4.6 Sex differences in gut-brain-axis

Sex differences in the gut-brain axis are a critical area of research, given the sexually dimorphic patterns in energy and nutritional requirements observed across the lifespan (Bolnick et al. 2014). These differences may play a significant role in various biological processes, including the development and progression of diseases like AD. A wealth of research supports this notion, highlighting critical sex differences in the gut microbiome and brain interactions, caused primarily through environmental factors, epigenetics, and hormones (Jašarević, Morrison, and Bale 2016). Our findings contribute to this body of knowledge by revealing that female rats performed worse in general in the olfactory discrimination task compared to their male counterparts. Additionally, female htauE14 rats on a control diet showed elevated levels of astrogliosis compared to the GFP groups.

Studies in mice have demonstrated that the female-biased risk for autoimmune disorders is significantly influenced by sex differences in the gut microbiome. For instance, the adoptive transfer of male microbiota to recipient females delayed the onset and reduced the severity of autoimmune diseases (Markle et al. 2013). This suggests that sex-specific gut microbiota compositions contribute to disease susceptibility and progression. Hormones such as estrogen and progesterone play crucial roles in the sex-specific morphology and function of the brain, likely interacting with various neurotransmitter systems, including glutamate, GABA, dopamine, and serotonin (McEwen et al. 2012). Changes in synaptic density, volume, and spine formation are shown in response to these hormones in areas like the hippocampus, prefrontal cortex, and amygdala (McEwen and Woolley 1994; Woolley et al. 1990). As mentioned, women face a two-fold increased risk of developing AD compared to men (Podcasy and Epperson 2016). Research

has linked significant reductions in Aβ accumulation and hyperphosphorylated tau levels to the activation of 17β-estradiol, which inactivates the GSK-3β pathway (Goodenough et al. 2005). Moreover, sex differences in the gut microbiota become pronounced after puberty, supporting the idea that sex hormones significantly influence gut microbiota composition (Kim et al. 2020).

Notably in our findings, female rats with probiotic supplementation led to increased levels of pGSK-3β, indicating a positive impact of probiotics on GSK-3β activity levels, similar to the effects seen from 17β-estradiol in females (Goodenough et al. 2005). However, no sex-based differences were observed in spatial learning or levels of peripheral and central inflammation with probiotic feeding.

In contrast to our findings, existing research suggests that sex differences have been found in inflammatory processes. Sex hormones, mainly estrogen (17β-estradiol) and testosterone, play a neuroprotective role through various mechanisms such as neurogenesis, immune response, and regulation of microglia function and excitotoxicity (Céspedes Rubio et al. 2018). In inflammatory conditions, estrogen is known to promote the phosphorylation of annexin A1, reinforcing tight junctions and mitigating inflammation (Cristante et al. 2013; Maggioli et al. 2016). Estrogens also activate macrophages, modulating levels of inflammatory cytokines (Ritzel, Capozzi, and McCullough 2013; Conway et al. 2015). These findings suggest that sex differences in the gut-brain axis and immune responses may significantly impact the progression and treatment of diseases like AD.

4.7 Beneficial Effect of Probiotic Supplementation following Stress Paradigm Deficits on Cognition and Behaviour

Our study aimed to investigate the beneficial effects of probiotic supplementation in preventing stress-induced cognitive deficits. In an SD animal model, we observed that probiotic feeding successfully prevented stress-induced spatial memory impairment and anxiety effects. These findings align with our hypothesis that probiotic supplementation could provide protective effects against cognitive and emotional disturbances induced by chronic stress. The precise mechanisms underlying these benefits remain unclear in the existing literature, and our study aimed to address this gap.

One potential mechanistic explanation we found was the increase in microglia in stressed animals compared to those fed with probiotics. Probiotics have been shown to reduce inflammation through the strengthening and health of the gut barrier, providing beneficial effects on intestinal inflammation and secondary downstream effects on brain inflammation (Cristofori et al. 2021). This was evidenced in our earlier study on a pretangle tau animal model discussed above, where probiotic supplementation led to reduced markers of peripheral and neuroinflammation.

Supporting studies further corroborate our findings. Bifidobacterium probiotics, for instance, have been established to reduce gut inflammation and protect gut barrier functions (Cao et al. 2023). These probiotics act as guardians, protecting encased microbes from oxidative damage in inflamed environments, thereby rapidly redirecting the barrier functions and gut microbiome towards a beneficial state (Cao et al. 2023; Martorell et al. 2021). Another study tested the effects of a probiotic product containing four strains of Bifidobacterium species and one

Lactobacillus strain on extremely premature infants (Samara et al. 2022). This study found that Bifidobacterium-driven microbiome maturation was linked to an anti-inflammatory intestinal immune milieu, highlighting the probiotics' role in fostering a healthy gut environment that could translate to cognitive and emotional benefits. It has also been shown that stress can allow entry of pathogenic bacteria into the gut, due to a stress-induced barrier permeability, and induce local inflammation, which can induce systemic inflammation (Zeng, Inohara, and Nuñez 2017; Vanuytsel, Tack, and Farre 2021).

Overall, our findings suggest that probiotic supplementation can effectively counteract the detrimental cognitive and emotional effects of chronic stress in SD animals, potentially through mechanisms involving the reduction of gut and brain inflammation. This novel insight emphasizes the importance of gut health in maintaining cognitive function and emotional stability, particularly under stress, and continues to support therapeutic interventions using probiotics at any stage in life.

Future directions could focus on the effect of stress and gut microbiota manipulation in our pretangle tau model. Research using animal models has shown that altering the glucocorticoid balance can lead to behavioral, molecular, and cellular changes (Budas et al. 1999). Specifically, stress or glucocorticoid administration increases amyloid-beta precursor protein and tau phosphorylation, both of which are linked to synaptic dysfunction and neuronal death in AD (Green et al. 2006). Research has directly connected stress to AD pathology. Sotiropoulos and colleagues found that stress leads to abnormal tau phosphorylation, upregulating tau epitopes strongly implicated in AD (Sotiropoulos et al. 2011). A study tracking over thirteen thousand patients for fifty years discovered that those with depression had a two-fold greater risk of developing dementia later in life (Barnes et al. 2012). Data from the Rush Memory and Aging

Project also revealed that individuals with high "distress proneness" were three times more likely to develop dementia over three years (Wilson et al. 2006).

It has been well documented that stress exposure can have a direct influence on the gut microbiota makeup and can damage the balance between microbes. A plethora of Bacteroides has been found to be regulated through chronic stress paradigms in animal models. Gareau et al. (Gareau et al. 2007) showed increased adhesion and penetration of total bacteria in the gut, along with significantly reduced levels of Lactobacillus species from stress. Notably, gut bacterial profiles, specifically SCFA-producing genera can be dysregulated due to MS. For instance, significantly higher levels of SCFA-producing genera such as Fusobacterium and Clostridium have been found in the gut, and this was shown to be positively correlated with the degree of visceral hypersensitivity (Zhou et al. 2016; Lingpeng et al. 2021).

With stress having a lasting impact on gut health and contributing to gut dysbiosis, many physiological and behavioral consequences have also been highlighted. Stress-induced increased inflammation and impaired cognition have been found in animal models of various neurodegenerative diseases, depression, and IBS (Gareau et al. 2011; Lin, Zheng, and Zhang 2018). Specifically, the intestinal barrier has been found to be damaged in AD patients and animal models (Pellegrini et al. 2023; Bailey and Coe 1999). Gut microbes influenced by stress exposure can impact intestinal barrier function and ultimately lead to intestinal permeability (Dodiya et al. 2020; Xiao et al. 2020; Dandekar et al. 2022; Liang et al. 2015). Interestingly, when probiotic Lactobacillus was administered in rodents, this barrier leakiness was prevented, along with improved behavioral, cognitive, and biochemical parameters (Lin, Zheng, and Zhang 2018).

A correlation between increased dysbiosis and stress in the gut was shown to be related to the PI3K/Akt/mTOR pathways (Li et al. 2022). Chronic stress decreased phosphorylation of this pathway in microglia and enhanced LPS-induced microglial activation (Li et al. 2022). This is consistent with AD research, in that the activation of this pathway subsequently produces an increase in GSK-3B-induced tau phosphorylation (Kesika et al. 2021).

4.8 Advantages and Limitations of the Study

A significant advantage of our study is the use of a pre-tangle tau model to represent an AD-like pathology and phenotype in animals, established by Ghosh et al. in our lab (Ghosh et al. 2019). Although our model does not exactly replicate human AD, this model is one of the earliest stage representations that still induces tau spread, paralleling the stages of tau in human preclinical AD and cognitive decline as defined by Braak (Braak et al. 2011). Focusing on tau pathology is crucial because it strongly correlates with cognitive decline in AD. Tau also plays a central role in several key pathophysiological features of AD, including microtubule destabilization, disruption of axonal transport, dysregulation of intracellular calcium, mitochondrial dysfunction, oxidative stress, proteasome dysfunction, promotion of neuroinflammation, degeneration of microglia, synaptic dysfunction and loss, altered neuronal activity, and neuronal loss. Notably, all features and behavioral deficiencies observed in our model occur in the absence of tangles, highlighting the pathogenicity of soluble abnormal tau. Additionally, tau pathology can be targeted through various therapeutic modalities, making it a strong model for potential therapeutic implications.

Our model also demonstrates a long prodromal period before cognitive deficits become apparent, mirroring human AD progression. The extended window before deficits emerge makes this model ideal for testing the efficacy of drugs and therapies aimed at the prodromal stage of the disease, such as our probiotic intervention. As well, the use of MAPT as a mutation in other models showing tau propagation as a major pathological driver serves as a disadvantage in comparison to our model, as sporadic AD does not arise from one mutation (Jawhar et al., 2012; Andorfer et al., 2003).

Another advantage is the ease of probiotic use, considering factors like cost, availability, and simplicity of administration. Furthermore, we could monitor probiotic bacterial strains both before and after supplementation, as each animal was kept in a controlled environment, caged individually, with no other dietary manipulations, allowing us to observe the effects of probiotics alone.

However, one limitation also stems from the controlled environment in which probiotics were used. Translationally, human gut microbiota composition varies between and within individuals over time. Although the general makeup remains consistent, this study does not account for the human impact of varying diets, antibiotics, and environmental toxins, which would likely have implications in disease and gut makeup. While probiotics were administered, the interaction with pre-existing microbiota in non-germ-free animals may have affected the outcomes. Additionally, baseline microbiota composition was not measured prior to intervention, which could provide insight into any inherent microbiota variations among groups. Additionally, the absence of male gut microbiota samples is a limitation. Sex differences in the gut microbiome are significant, and gut-related changes in diversity and abundance shown in this study are concluded only from female samples. Literature suggests that similar changes may be observed in male samples,

though they will likely differ due to hormonal influences. Another limitation involves the discrepancy between the behaviors of biologically phosphorylated tau and pseudophosphorylated hTauE14. AT8 and other phosphorylation-targeted antibodies do not recognize pseudophosphorylation sites, but despite this human tau expression in hTauE14 tissue was successfully demonstrated in this study.

4.9 Therapeutic Implications

Stress is increasingly recognized as a significant risk factor for AD, with chronic stress leading to neuroinflammation, blood-brain barrier disruptions, and altered gut microbiota—all factors relevant to AD pathology. By examining probiotics' potential to mitigate both AD-like pathology and stress-induced cognitive impairment, this work highlights the interconnectedness of stress and neurodegenerative disease processes, reinforcing the relevance of the gut-brain axis as a therapeutic target.

As discussed, current AD treatments and therapeutics primarily target Aβ pathology and have a high clinical failure rate. This is likely due to the late stage of intervention when a diagnosis of AD can be made. By this time, symptoms have already manifested and are indicative of advanced disease progression. Our study provides evidence that probiotics, introduced at a pre-tangle tau stage, can offer substantial benefits such as improved memory and cognition, which are critical symptoms in AD. Our findings therefore highlight the significant therapeutic potential of probiotics in the early-pretangle stage of AD, a stage where symptoms are not yet

present and it is uncertain whether an individual will develop sporadic AD. Introducing probiotics at this early stage could potentially slow disease progression, intervening before irreversible damage occurs.

Similarly, in our SD model of chronic stress and enrichment, probiotics demonstrated protective effects on cognition, inflammation, and anxiety-like behaviors. Stress and enrichment are parts of daily human life and chronic stress has significant implications for health and disease. Our research indicates that even before stressors are introduced, probiotics can provide a protective barrier, enhancing resilience against cognitive and emotional challenges.

In conclusion, probiotics emerge as a promising, cost-effective, and non-invasive therapeutic approach for healthy aging and cognitive preservation. Their early introduction, whether in the context of potential AD or daily stress, could offer substantial benefits in maintaining brain health and possibly delaying the onset of debilitating symptoms. This strategy represents a simple yet powerful tool in the broader effort to mitigate the impacts of aging and stress on cognitive function.

4.10 Future Directions

The main findings of this study open several promising avenues for future research. One of the primary directions involves the classification and analysis of tau isoforms. Notably, ptau isoforms such as ptau181, ptau217, and ptau231 in CSF and plasma have shown potential in detecting the AD continuum in comparison to healthy individuals (Janelidze et al. 2023). Expanding on our findings, future studies could aim to assess peripheral blood biomarkers in our

htauE14 model, with particular focus on measuring phosphorylated tau in plasma. Utilizing the probiotic intervention in our htauE14 model, it would be critical to observe and understand peripheral inflammatory interactions with tau isoforms. Additionally, longitudinal studies using htauE14 model could assess systemic inflammation, BBB integrity and cognitive decline at various time points. As well, correlation studies between these factors could provide further clarity on any causative effects or benefits derived from gut health improvements.

Secondly, it would be both feasible and essential to study the longitudinal effects of probiotics in humans at a pre-AD stage. A meta-analysis of studies involving adults with AD or MCI who consumed probiotics showed positive effects on cognitive performance, inflammation, and oxidative biomarkers (Den et al. 2020). Therefore, a future study could focus on observing humans with pre-clinical tau levels and the impact of probiotics on the incidence and severity of AD. Such research could elucidate the potential of early probiotic intervention in delaying or mitigating the progression of AD.

Another valuable direction could be the use of targeted manipulations on the gut microbiota to assess the role of specific bacterial taxa in AD pathology. Specifically, techniques such as selective bacterial depletion, fecal microbiota transplants, or targeted bacterial supplementation could be used.

Thirdly, understanding the effect of probiotic supplementation on lipidomics in our model presents another valuable direction. AD disrupts the blood lipidome, although the effect of probiotics in pre-clinical disease stages on lipids remains to be fully understood (Su et al. 2021). Preliminary studies in our lab assessed blood lipid profiles via electrospray ionization-mass spectrometry in pre-tangle animals with probiotics. Blood lipid markers suggested successful

reparation of intestinal barrier integrity. Specifically, pre-tangle pathology resulted in a decrease in anti-inflammatory oleic acid and an increase in pro-inflammatory lipids. Probiotic supplementation, in turn, was associated with favorable changes to the blood lipid profile, including an increase in phosphatidylcholines and a decrease in their precursors, potentially indicating improved intestinal barrier integrity. Future projects will expand sample sizes to observe these effects at both pre- and post-feeding time points in our htauE14 animal model, providing a comprehensive understanding of probiotics' impact on lipid metabolism in the context of early AD.

Following our studies on the effect of probiotics in stressed SD animal models, we set the foundation to explore the effects this might have in our htauE14 model in combination with stress. Future studies regarding this can explore the question of the similarities and relationship between stress and AD cognitive decline, and the combined effect of probiotics in this sense.

These future directions stemming from the current study's findings, aim to further elucidate the therapeutic potential of probiotics in both preventing and mitigating AD, particularly when administered at early stages of the disease continuum.

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Ethics Approval

Subject: Your Animal Use Protocol has been renewed

ambakwe@mun.ca
to Qi.Yuan, ambakwe

Fri, Jun 7, 3:41 PM

MEMORIAL UNIVERSITY
Animal Care Committee (ACC)
St. John's, NL, Canada A1C 5S7
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Dear: Dr. Qi Yuan, Faculty of Medicine\Division of BioMedical Sciences

Researcher Portal File No.: 20250175
Animal Care File: 18-01-QY
Entitled: (18-01-QY) Locus coeruleus norepinephrine modulation in learning and Alzheimer's Disease
Status: Active
Related Awards:

Awards File No	Title	Status	
20190269	Material Transfer Agreement - Not Publishable	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20191784	Locus coeruleus NE modulation in learning and Alzheimer's disease	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20200652	MTA for cis p-tau antibody from Harvard University	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20200817	Renewal of (18-01-QY) Locus coeruleus norepinephrine modulation in learning and Alzheimer's Disease	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20220448	Material Transfer Agreement	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20220620	The Role of Hippocampal LTCCs in Synaptic Plasticity During Aging and in Pretangle Tau Pathology	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20230984	Pre-tangle tau in the hippocampus impairs synaptic plasticity and spatial memory via L-type calcium channels: A hypothesis	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses

Ethics Clearance Terminates: May 01, 2027

Your Animal Use Protocol has been renewed for a three-year term. This file replaces previous File ID [[20220211]], but the Animal Care ID [[18-01-QY]] remains the same. Please note the new file ID (if required) when referring to this protocol.

This ethics clearance includes the following Team Members: Dr. Qi Yuan (Principal Investigator)
Dr. Susan Walling (Co-Investigator)

This ethics clearance includes the following related awards:

Awards File No	Title	Status	
20190269	Material Transfer Agreement - Not Publishable	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20191784	Locus coeruleus NE modulation in learning and Alzheimer's disease	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20200652	MTA for cis p-tau antibody from Harvard University	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20200817	Renewal of (18-01-QY) Locus coeruleus norepinephrine modulation in learning and Alzheimer's Disease	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20220448	Material Transfer Agreement	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20220620	The Role of Hippocampal LTCCs in Synaptic Plasticity During Aging and in Pretangle Tau Pathology	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20230984	Pre-tangle tau in the hippocampus impairs synaptic plasticity and spatial memory via L-type calcium channels: A hypothesis	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses

An Event [Annual Report] will be required following each year of protocol activity.

Should you encounter an unexpected incident that negatively affects animal welfare or the research project relating to animal use, please submit an Event [Incident Report].

Any alterations to the protocol requires prior submission and approval of an Event [Amendment].

NOTE: You can access a copy of this email at any time under the "Shared Communications" section of the Logs tab of your file in the Memorial Researcher Portal.

Please note that approval of the protocol or amendment does not guarantee space for animal housing or procedures. Coordination with Animal Care & Veterinary Resources is required prior to ordering animals.

Sincerely,

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