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Alzheimer's Disease genetics and short-chain fatty acid treatment in studies of the murine gut microbiome

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ALZHEIMER'S DISEASE GENETICS AND SHORT-CHAIN FATTY ACID TREATMENT IN STUDIES OF THE MURINE GUT MICROBIOME

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

ALZHEIMER'S DISEASE GENETICS AND SHORT-CHAIN FATTY ACID TREATMENT IN STUDIES OF THE MURINE GUT MICROBIOME

Elucidating the relationship of the gut microbiome in Alzheimer's Disease (AD) risk and pathogenesis is an area of intense interest. Since 60 to 80% of AD risk is related to genetics and *APOE* alleles represent the most impactful genetic risk factors for AD, their mechanism(s) of action are under intense scrutiny.

First, I conducted a study on *APOE* targeted replacement mice to investigate the impact of *APOE* alleles on the murine gut microbiome. The relative abundance of bacteria from the family *Ruminococacceae* and related genera increased with *APOE2* status. The relative abundance of the class Erysipelotrichia increased with *APOE4* status, a finding that extended to humans. Since *Ruminococacceae* have been associated with increased SCFA production, these findings suggest that SCFA-producing bacteria are increased in the AD-protective *APOE2* positive mice.

Next, I compared the effects of short-chain fatty acid (SCFA)- vs. saline-treated water on APPswe/PSEN1dE9 mice maintained under standard laboratory conditions. I found that SCFA treatment increased alpha-diversity and impacted the gut microbiome profile by increasing the relative abundance of the genera *Bifidobacterium* and *Lactobacillus*, which are known to produce SCFAs and SCFA precursors. Although gut microbiome changes in SCFA-treated mice were robust, SCFA treatment did not significantly affect behavior, cortical or hippocampal astrocyte activation, or soluble and insoluble amyloid levels.

In conclusion, there is robust evidence of an *APOE* allelic effect on the murine gut microbiome that implies an AD-relevant genetic impact on the gut microbiome. The gut microbiome can be modulated by SCFA supplementation, revealing a potential therapeutic for AD prevention. These pioneering studies represent the medical importance of gut health on disease prevention and treatment.

KEYWORDS: Alzheimer's Disease, Genetics, Neurodegenerative disease, Gut microbiome, Short-chain fatty acids, Apolipoprotein E

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04/27/2023

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Alzheimer's Disease genetics and short-chain fatty acid treatment in studies of the murine gut microbiome

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DEDICATION

To Babcia Marysia and Dziadek Emil – My grandmother, Maria Zając, and my grandfather, Emil Zając

I'm sorry for your suffering from Alzheimer's Disease, and I thank you for your love and inspiration. You live on in our hearts, and in my research, every day.

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The journey to a PhD often starts sooner than the graduate school program. For me, I had embarked on this path my senior year of high school, although I was not yet aware of the arduous road ahead of me. To get where I am today earning my PhD, I want to thank my family, friends and many mentors that guided me and supported me along the way.

When I got accepted into the PhD program at University of Kentucky, I attended the Physiology Department's research posters of available PIs. I came in with a goal: to study Alzheimer's Disease. I came in with inspiration: the profound impact of the gut microbiome on human physiology. I honestly had no real knowledge depth about the relationship of the two, but when I met Dr. Steve Estus and he told me about the preliminary study his lab had done investigating Alzheimer's Disease genetic influence on the gut microbiome, I was immediately hooked. He was surprised that I already had an *a priori* interest in the topic, but it wasn't until I joined his lab and started on the work that I really gained an understanding of the field. To me, I had just manifested one of the dreams on my subconscious vision board, and I felt absolutely blessed and lucky. It really felt like it had all aligned, because out of 16 graduate school applications, several interviews and acceptance letters, the lab of Dr. Steve Estus at University of Kentucky was exactly what I had been looking for. Since that first meeting, I knew I wanted to join the lab, and from then on Dr. Estus has been an amazing mentor and role model for me. We have celebrated many publications together, as well as mutually venting our frustrations during times of stress or challenge. I learned so much from Steve about a

career in academia as a PhD. Steve has held and holds many positions in academia that involve research, teaching and mentorship, and has been the best role model I could have wished for. For his guidance, mentorship, wisdom, and support, I thank him with a full heart. Thank you for welcoming me into your lab, and for teaching me what it means to be a PhD. Thank you as well to Jim and Ben in the lab for their friendship and mentorship. I am honored to have been a part of the Estus Lab and am grateful for all of our time together.

For the five years in the PhD program and the several years leading up to it I would like to thank my parents Roman and Kasia Zajac. Getting a PhD isn't a financially fruitful path, and it is time-consuming and stressful. They have supported me with their unconditional love, their belief in me, their financial support, and their understanding when our family holidays had to be cut short or saved for another year. It hasn't been easy, and yet they never gave up on me. When I stubbornly decided that I wanted to pursue a career in research in high school, they constantly checked in to make sure I was certain of my decision. They definitely knew before I did how difficult it would be. Even more constantly, they have been my biggest supporters. My father would read every paper as soon as it was published and discuss it with me, having no background in science research himself, and my mother always made sure my health and my home were taken care of, even when I was too strained to do so myself. I love them both and can't thank them enough. Equally so, I thank my brother, Robert Zajac, for being my biggest and proudest supporter and the best brother I could ever have. I love you, bro! Additionally, from my family, I want to thank my grandparents, those who have passed and those still with me. They all believed deeply in me, and in my academic endeavors,

and wished upon me all the joy, love and success a girl could dream of. They are always with me.

Along the same lines, I want to thank my closest friends. I can't name them all, as the list is long, but whether I've known you since childhood or only the last few years, I have the best support group in every one of you and I am so grateful. There were times when I would fall and weep, and you pulled me out of those depths every time. Even if we live far away, your love has crossed stateliness and countries. Thank you so much for your love and for always cheering for me. I want to also say something special about my friends who are on this journey with me, especially those I've grown closest to, who will soon be getting their own PhDs, which they very much deserve. Thank you Shadan Hadi and Nick Devanney for being great friends embarking together on this impressive path. Thank you to all of the other students in our program, I love our community and am so grateful for it. Thank you as well to those who entered the Integrated Biomedical Sciences program with me the first year, studying together and struggling together, that first year with you all is what made sure I would not quit. And thank you to the IBS program in general, to Dr. Brett Spear and Jennifer Kennedy, for getting us excited to be here, knowing we are supported throughout the whole process.

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along the way. Second, I want to thank Dr. Jamie Sturgill, for not only being an amazing role model and mentor but also a friend that sparked my creative pursuit of knowledge and reminded me of how my work can make a difference in patients' lives. Third, thank you to Dr. Donna Wilcock for her support, always offering me such positivity and excitement that made me feel so confident and sparked my drive to pursue my own path in research. Fourth, I want to thank Dr. Lance Johnson for his mentorship and support in collaboration on this dissertation work and throughout my time as a graduate student. Lastly, I want to thank the various PIs who have taught and mentored me in IBS, advanced physiology courses, and teaching courses, you have all been amazing role models, inspirations, and believers in me. Thank you for all your support during my graduate career and for pushing me to greater heights. Thank you as well to the department and to all of the staff that support the students and see us grow, your support means the world.

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With my PhD coming to a close, I realize all of the people in my life who have had a positive impact, those who have been mentors and role models, friends and fellow researchers, and how grateful I am for their parts in my journey. The list of all those I want to thank is too lengthy, even for a dissertation, but even if I did not name you on

paper, I did so in my heart. Thank you. It's been ten years since I had started this pursuit, and many times I wanted to give up. I remember that every time I wanted to wave that white flag, I had someone who would not let me go down without a fight, who bet on me. They reminded me how to bet on myself in the face of possible failure, and that the feeling of giving up is worse than that of trying, failing, and getting back up to try again. They taught me to truly believe in my potential and how to create my reality. I'm a wild dreamer, so of course I had to learn how to manifest these ambitious dreams. I have grown and matured so much, learned a lot about myself, and gained a new level of self-confidence that I did not know I could have. Thank you all so much for creating the Diana you know today, the one who didn't give up, the one who made this dream come true. Here is to the relentless pursuit of our dreams and the unfolding of our wings.

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CHAPTER 1. INTRODUCTION

1.1 Alzheimer's Disease: A brief overview of epidemiology and pathophysiology

1.1.1 Epidemiology of Alzheimer's Disease

Alzheimer's Disease (AD) is an aging-related disease and the most common form of dementia, affecting over 58 million people worldwide and 6 million in the United States [1]. AD affects the aging population and incidence rates increase with age, where about 10% of the population aged 65 years and older is diagnosed with AD, and 33% of the population aged 85 years and older is diagnosed with AD. Along with the increase in prevalence, mortality rates increased 146.2% from 2000 to 2018, making AD the fifth-largest cause of death in elderly Americans [2]. AD also disparately affects women, where the estimated lifetime risk for AD at age 45 is 20% in women and 10% in men, and increases in both at age 65, although recent studies suggest that selection bias contributes to the reports of these sex differences [1]. Similarly, Blacks and Hispanics seem to be at a higher risk for AD and AD-related deaths, in part due to genetic risk factors that differ by race, but mostly due to historical socioeconomic disparities that influence lifestyle, healthcare and knowledge of risk factors in these populations [1]. In addition, mortality rates from AD increased in 2020 by 17% due to the neurological effects of COVID-19 and the increased the risk of cognitive impairment following critical illness, mechanical ventilation, and stay in an Intensive Care Unit (ICU) [1]. For additional information regarding AD prevalence, risk, care and more, see the Alzheimer's Association Fact and Figures 2022 [1].

1.1.2 Discovery of Alzheimer's Disease

AD was first discovered and characterized by Alois Alzheimer when he reported abnormal anatomical findings in a patient that showed early clinical symptoms of cognitive decline that were unlike the classified clinical patterns known at the time ([3] and translated to English in [4]). At 51-years of age, the patient suffered from memory impairments, auditory hallucinations, perception disorders and delusions, and delirium. Alzheimer noted that his patient "showed a complete helplessness," "was disoriented as to time and place," and "often would scream for hours and hours in a horrible voice." However, the patient did not show any signs of motor impairment or cardiac hypertrophy. The mental regression advanced steadily until the patient had lost all means of self-reliance and passed away after four and half years of illness. Autopsy and brain staining by the neurologist, and his pupil, Perusini revealed an "evenly atrophic brain" with "characteristic changes in neurofibrils", where about one-quarter to one-third of the cerebral cortex showed signs of neurofibrillary tangles (NFTs) that formed inside neurons [5]. In addition, throughout the entire cortex were "miliary foci" which represented sites of deposition of what is now known to be amyloid beta peptides, now referred to as amyloid beta (Aβ) plaques or Aβ accumulation. Alois Alzheimer concluded that he is reporting on a little-known disease and states that "we must reach the stage in which the vast well-known disease groups must be subdivided into many smaller groups, each one with its own clinical and anatomical characteristics."

1.1.3 Pathophysiology and Genetics of Alzheimer's Disease

1.1.3.1 Amyloid and tau pathology

AD is characterized by the accumulation of Aβ plaques and NFTs in the brain. Aβ was first isolated as the component of amyloid plaques in 1984 [6]. A few years later, the amyloid precursor protein (APP) was identified through genetic linkage analysis ([6,7] and cloned by [7]), and an AD-causing missense mutation identified [8]. In 1995, familial AD (FAD) mutations were discovered in presenilin1 (PSEN1) [9] and presenilin2 (PSEN2) [10] were discovered, both of which are part of the enzyme γ-secretase that is involved in APP processing. These discoveries supported the later proposed "amyloid cascade hypothesis" [11] which suggests that Aβ, a product of APP processing, aggregated into plaques, leading to neuronal death and the formation of NFTs. In the 1980s, the diagnostic criteria for AD were the presence of amyloid plaques and NFTs at autopsy [12]. Since then, clinical biomarkers of AD have been identified that allow for a clinical prognosis before death: (i) abnormally phosphorylated tau proteins from the brain can be detected in the cerebrospinal fluid (CSF) of AD patients [13,14], (ii) amyloid protein can also be detected in the CSF of AD patients and Aβ42 begins to decline in the CSF as it accumulates in plaques [15], and (iii) radiotracers for positron emission tomography (PET) were developed to visualize NFT and amyloid deposits in the brains of living patients [16]. More recent studies employ big data analysis with the aim of identifying further CSF biomarkers of AD [17].

1.1.3.1 Genetics

Lastly, with the advancement of sequencing technologies and big data analysis, AD research is identifying disease-associated single nucleotide polymorphisms (SNPs) in multiple genes that are becoming the latest targets of AD studies (reviewed in [18]). Importantly, family and twin studies of AD estimate that up to 80% of AD risk is genetic

[19]. Of the genes with identified AD-associated SNPs, several additional phenotypes are recognized to modulate disease progression, including cholesterol metabolism and neuroinflammation [20]. Furthermore, mutations in APP , PSEN1 and PSEN2 are causative for Familial Alzheimer's Disease (FAD), which typically has an early onset by the age of 40-50yo [8-10,21,22]. Late Onset Alzheimer's Disease (LOAD) occurs later in life, typically around the age of 65-80yo, and SNPs identified by Genome Wide Association Studies (GWAS) modulate AD risk for LOAD [23-26]. Tanzi et al. provides an excellent review of the history of identified FAD and LOAD SNPs [27], while Jayadev et al. and Andrade-Guerrero et al. provide more recent overviews of the genetics of AD including risk loci identified by GWAS [28,29].

1.1.3.1 Neuroinflammation

Several common variants in genes that are associated with immune response and neuroinflammation have been identified that are associated with AD risk in GWAS: *CR1, CD33, MS4A, CLU, ABCA7, TREM2,* and *INPP5D* [20]. Some of these AD-associated genes—*TREM2* [30] and *CD33* [31]—encode receptors on microglia, the resident macrophages of the central nervous system (CNS), impacting immune signaling in the brain. Microglia act as sentinels in the brain pruning unused synapses for cell turnover and surveilling the environment for lesions and neurotoxic debris, such as protein aggregates, dead cells, and microbes (reviewed in [32]). In AD, a subset of microglia shift into a Disease Associated Microglia (DAM) transcriptional profile that is associated with inflammation and increased functions of motility, proliferation and phagocytosis [33-35]. Microglia respond to Aβ plaques in the brains of AD patients and in AD murine models, where they associate with the plaques and act to compact and phagocytose them, thereby

reducing amyloid burden [33]. In addition, microglia release cytokines and chemokines that modulate the immune response in the CNS (reviewed in [32]). It is now well established that along with amyloid and tau pathology, dysfunctional neurons, activated astrocytes and microglia, the AD brain is also prone to high levels of neuroinflammation ([36-41] and reviewed in [42,43]), and therapeutic targets for AD are also targeting to reduce this neuroinflammation [37,44-46].

1.1.4 Apolipoprotein E and Alzheimer's Disease Risk

Apolipoprotein E (APOE) is one of the genes identified to have polymorphisms that significantly associate with AD risk [20]. The most common APOE allele is APOE3, with an allele frequency of 78% (reviewed in [47]). The APOE4 allele has a frequency of 14% and increases AD risk up to four-fold with one copy, and up to twelve-fold with both copies of the allele relative to APOE3. On the other hand, the APOE2 allele is protective for AD, and has a frequency of 8% ([48-51] and reviewed in [47,52]). APOE, along with TREM2, has also been studied regarding neuroinflammation and microglial activation, and is required for the shift of homeostatic microglia to the DAM state [33]. In AD murine models, targeting of the APOE-TREM2 pathway restored the homeostatic microglial phenotype and reduced neuronal loss [33]. In postmortem AD brains, APOE4 is associated with increased Aβ plaque accumulation and increased severity of cerebral amyloid angiopathy (CAA), whereas APOE2 is associated with reduced neuritic plaques (reviewed in [52]).

1.2 The Gut-Brain-Axis

Neurological and neurodegenerative disorders are CNS diseases, yet there is extensive evidence of periphery-CNS cross-talk in many of these diseases (reviewed in [53,54]). Specifically, multiple lines of evidence point to the involvement of the gut microbiome in the development of CNS disorders, including stress [55,56], autism [57-59], depression [60,61], Multiple Sclerosis (MS) [62], Parkinson's Disease (PD) [63-65] and AD [66]. The gut microbiome is made of up a diverse variety of microbiota living in the human gut, which is the entire gastrointestinal tract, including the esophagus, stomach, small intestine and colon. These microorganisms live in the human gut in a host-specific symbiosis, where they regulate digestion, immune, metabolic and neurological functions (reviewed in [67]). Of these microorganisms, bacteria in the gut are involved in the digestion and absorption of food sources for the production of metabolites essential for host function, such as the bacterial fermentation of complex carbohydrates for the synthesis of short-chain fatty acids (SCFAs) [68]. These types of bacterial metabolites are involved in the cross-talk between the gut, the periphery and the CNS [68-72]. This bacterial cross-talk with the brain is mediated by key pathways involving the vagus nerve [73], the immune and neuroendocrine system [74,75], and the neurotransmitters and metabolites of the gut microbiota (reviewed in [53,73]). With the rise in big data analysis and sequencing technologies, several metabolites have been identified as potential therapeutic targets for AD [76-81].

1.2.1 Gut Microbiome and Alzheimer's Disease

Gut microbiome studies in the context of AD pathology are of growing interest. Initial studies identified significant differences in the gut microbial compositions of AD

patients versus individuals without amyloid pathology (reviewed in [82]). In one study, some of the bacteria that were increased in the AD patients were previously associated as pro-inflammatory taxa, whereas anti-inflammatory taxa were increased in the non-AD individuals [83]. Another study showed that bacterial diversity appeared to be decreased in AD patients compared to controls [84]. Additional studies have examined gut microbiome differences between AD and non-AD aged adults [83,85-90], as well as altered microbiome profiles in mouse models of amyloidosis [66,91,92].

Here, I summarize the findings from a few of the relevant gut microbiome studies done in AD patients. First, One study found that AD individuals exhibited profiles with increased genera belonging to the families *Lachnospiraceae* (genera *Agathobacter, unclassified_f_Lachnospiraceae, Eubacterium_ventriosum_group, Lachnospiraceae_NC2004 and Coprococcus_1*), *Ruminococcaceae* (genera *Faecalibacterium and Ruminococca-ceae_UCG-007*), *Prevotellaceae* (genus *Alloprevotella*), *Atopobiaceae* (genus *Atopobium*), *Clostridial* (genus *Parvimonas*), *Synergistaceae* (genus *Cloacibacillus*), *Erysipelotrichaceae* (genus *Solobacterium*), and *Pseudomonadaceae* (genus *Pseudomonas*). In contrast, AD individuals showed a decrease in genera belonging to families *Lachnospiraceae* (genus *Tyzzerella*) and *Erysipelotrichaceae* (genus *Erysipelatoclostridium*) [89]. Second, a study of AD patients in Kazakhstan identified bacteria taxa that were differential for AD, including increased relative abundance of genera *Prevotella, Alloprevotella, Ruminococcus,* and *Akkermansia,* and decreases in *Roseburia, Tyzerella, Erysipelotrichaceae UCG-003,* and *Lactobacillaceae* in AD patients [90]. Third, further studies have shown a gut microbiome-inflammation relationship that is associated with AD pathology in humans.

Cattaneo et al. showed that amyloid-positive patients had increased levels of inflammatory taxa Escherichia/Shigella and a reduction in anti-inflammatory taxon E. rectale, as well as higher levels of inflammatory cytokines and reduced anti-inflammatory cytokines in the blood compared to healthy controls [83]. Fourth, another study comparing mild cognitively impaired (MCI) and AD adults to healthy controls found that patients with AD or MCI had increased abundance of the family *Erysipelotoclostridiaceae* and order Erysipleotrichales, which were also positively correlated with APOE4 status [93]. Lastly, Vogt et al. analyzed the gut microbiome profiles of AD patients compared to cognitively healthy controls and found that AD patients had decreased microbial diversity, including decreases in Firmicutes, increases in Bacteroidetes, and decreases in Bifidobacterium, which correlated with CSF biomarkers of AD [84].

1.2.1.1 Gut microbiome alterations in AD murine models

In addition to evidence of gut microbiome differences between AD and healthy adults, studies in murine models of AD corroborate that there are distinct differences in gut microbiome profiles in disease versus health [94-96], and that modulation of the gut microbiome can have direct impact on disease pathology. Several reports have found that Aβ burden in murine models is reduced in gnotobiotic mice or mice treated with antibiotics [97-102]. In addition to antibiotic knockdown of the gut microbiome causing shifts in AD-like pathology in these mouse models, treatment with pre-/pro-biotics to alter the gut microbiome also modulates disease pathology [100].

1.2.1.2 ApoE Effects on The Gut Microbiome

ApoE is a lipid transporter, contributing to lipid homeostasis in the periphery and in the brain. ApoE binds lipoproteins and lipid complexes in the plasma for transport to specific cell-surface receptors [103]. In the brain, ApoE modulates several pathways including lipid transport, glucose metabolism and neuroinflammation (reviewed in [52]). In the periphery, *APOE2* is associated with decreased low-density lipoprotein (LDL) cholesterol, whereas *APOE4* is associated with increased LDL cholesterol, relative to *APOE3* [47]. The *APOE* effects on lipid metabolism include *APOE4*-associated risks of increased heart disease, and that *APOE4* carriers may benefit from dietary intervention with omega fatty acids (reviewed in [104]). Although ApoE in the brain is separate from that in the periphery, many studies show a diet-dependent effect of ApoE in the brain (reviewed in [105]). Furthermore, more recent studies in mice and humans have shown differential effects of *APOE* on the gut microbiome (reviewed in [105]). The first study that caught the attention of my lab and inspired our gut microbiome studies was done by Dr. Richard Guerrant's lab. In his studies of children in Northeast Brazil, Guerrant found that children who were carriers of the *APOE4* allele had better defense against childhood diarrheal diseases [106-108]. In my studies of *APOE* effects on the gut microbiome, I observe increased gut microbial diversity in *APOE4* carriers, which is associated with protection from gut dysbiosis ([109,110] and reviewed in [111,112]) and I suspect explains the *APOE4* protective mechanism against gastrointestinal (GI) insult.

Several studies in *APOE* mice observed significant differences in gut microbiome profiles related to APOE status. First, *APOE*-deficient mice display microbiome differences relative to wild-type mice [113]. Second, *APOE4*-targeted replacement (TR) mice were more resistant to gastrointestinal Cryptosporidium infection than *APOE3* mice

[114]. Lastly, we and others have recently reported microbiome differences in a comparison of *APOE3* and *APOE4*-TR mice [94,115-118]. In addition, a study done by Hou et al. genotyped 30 AD and 47 control patients, paired with 16S rRNA microbiome sequencing, and observed differential taxa associated with the *APOE4* variant [119].

1.2.1.3 Gut microbiome effects on microglia

Earlier I introduced microglia as the resident immune cells of the brain responsible for the compaction and phagocytosis of Aβ plaques in the brain. In AD, microglia shift more towards a DAM transcriptomic profile, which causes a functional shift from homeostatic, sentinel microglia to that of increased motility, proliferation and phagocytosis. Modulation of these microglial functions has an impact on AD pathology and neurodegeneration [120-122]. Recent studies have investigated how gut microbiome modulation impacts microglial functions ([101,123] and reviewed in [124-126]). Erny et al. demonstrated that the lack of a gut microbiome lacking in germ free (GF) mice causes defective microglia lacking functional responsiveness and an impaired innate immune response and that recolonization of the gut microbiome restored microglial functions [123]. In addition, Erny et al. determined that short-chain fatty acids (SCFAs) contribute to regulating microglial function because mice deficient for the SCFA receptor *FFAR2* displayed the same defective microglial phenotype as GF mice.

1.2.1.4 Gut microbiome metabolites and short-chain fatty acids

SCFAs are a major microbiota metabolite that have been suggested to mediate gut microbiome effects in the brain (reviewed in [127,128]). The major source of SCFAs in the body is microbial digestion of resistant starch. Recently, SCFA treatment was reported to

increase amyloid burden in specific-pathogen-free (SPF) APP/PS1 mice [101] while butyrate treatment was reported to decrease amyloid burden in SPF 5xFAD mice [44] and in 5xFAD mice maintained on a conventional microbiome [129]. We refer to a conventional microbiome as the microbiome of mice that were conventionally raised, as opposed to germ free (GF) or SPF mice, which have laboratory controlled/limited microbiomes. In an *APOE*-taupathy model for AD, male GF *APOE4* mice treated with SCFAs showed an increase in reactive astrocytes and microglia, as well as an increase in phosphorylated tau pathology [118]. These studies provide evidence that SCFA supplementation has direct effects on AD pathology, although it is unclear if the overall effects will be beneficial or detrimental to disease. Current evidence suggests that SCFA supplementation may also depend on the mouse model, administration method, duration of treatment, conventional versus GF microbiomes, sex, strain, and other possibly unknown factors. Further studies are needed to evaluate the efficacy of SCFA treatment as a potential dietary therapeutic for MCI and AD. In this dissertation, I present my study investigating SCFA treatment on APP/PS1 mice, using the same treatment protocol of Colombo et al. and a similar mouse model of amyloidosis, and the effects of treatment on the gut microbiome, cognition, and amyloid pathology. We chose the APP/PS1 mouse because it is well characterized as a model of amyloidosis and cognitive impairment, differing from the APPPS1 mouse used by Colombo et al. mostly in that it is a less aggressive model of amyloidosis, taking several months to develop significant amyloid deposition and to present with cognitive deficits.

1.3 Summary

The gut microbiome changes throughout life and is modulated by most environmental factors, including inoculation at birth, pets in the home, diet, age and hormonal changes, exercise, medical conditions, and medical treatments (reviewed in [130-133]). The impact that these environmental and lifestyle factors have on disease risk is in part due to these gut microbiome changes, and evidence shows that modulation of the gut microbiome with antibiotic knockdown and fecal transplants can immediately reduce disease pathology for a number of conditions [134-137], including neuro-cognitive disease such as autism [57,58,138] and Parkinson's Disease [139-141]. Interestingly, modulation of the gut microbiome may also have a profound impact on AD pathology as supported by studies in AD murine models [101,118,129,142]. *APOE* genetics are known to impact AD risk and also seem to be differential for altered gut microbiome profiles in humans [108,119,143], and murine models [94,115-117]. Here, I investigate gut microbiome changes associated with *APOE* status in *APOE* targeted replacement (*APOE*-TR) mice. The findings from this study lead me to conduct a second study wherein I investigate the effect of SCFA treatment on the gut microbiome and AD pathology of APP/PS1 mice. The findings from these studies support the hypothesis that *APOE* genetics and SCFAs affect gut microbiome diversity, which could potentially impact disease pathology.

CHAPTER 2. APOE GENETICS INFLUENCE MURINE GUT MICROBIOME

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2.1 Introduction

Apolipoprotein E (APOE) alleles impact multiple facets of the human condition, ranging from Alzheimer's Disease (AD) to cardiovascular disease, metabolic syndrome, obesity, fertility and longevity (reviewed in [144]). The three primary *APOE* alleles include *APOE3*, which has a 78% minor allele frequency, as well as *APOE4* and *APOE2*, with minor allele frequencies of 14 and 8%, respectively. Regarding AD, *APOE2* reduces AD risk while APOE4 strongly increases AD risk, both relative to *APOE3* (reviewed in [47]). This association has prompted intense evaluation of possible mechanism(s) underlying *APOE* effects in AD, resulting in *APOE* allelic association with amyloid-beta (Aβ) clearance, Aβ aggregation and astrocyte stress [145-150]. In the periphery, *APOE2* is associated with decreased low-density lipoprotein (LDL) cholesterol, whereas *APOE4* is associated with increased LDL cholesterol, relative to *APOE3*. While this may account for *APOE* association with cardiovascular disease, the mechanisms underlying *APOE* allelic effects on glucose metabolism, inflammation and innate immunity are unclear [47]. Elucidating these differential actions of *APOE* alleles may provide insights to these processes.

Several studies have suggested a relationship between *APOE* status, the gut microbiome and AD neuropathology. First, *APOE*-deficient mice display microbiome differences relative to wild-type mice [113]. Second, *APOE4*-targeted replacement (TR) mice were more resistant to gastrointestinal Cryptosporidium infection than *APOE3* mice [114]. Third, the *APOE4* allele in humans was associated with better defense against childhood diarrheal diseases in lower income countries [106-108]. Fourth, we and others have recently reported microbiome differences in a comparison of *APOE3* and *APOE4*-TR mice [94,115,116]. Lastly, several reports have found that Aβ-burden in murine models is reduced in gnotobiotic mice or mice treated with antibiotics [97-100]. The mechanism(s) whereby *APOE* alleles influence the gut microbiome are unclear, although *APOE4* has been associated with a greater inflammatory response to lipopolysaccharide (LPS), a microbiome product common to all gram-negative bacteria, in both humans and mice [151,152].

To begin to evaluate whether *APOE* allelic effects are dominant, co-dominant, or recessive, we compared animals heterozygous and homozygous for *APOE* alleles. Additionally, we improved our study design for rigor and reproducibility by backcrossing the *APOE*-TR mice to obviate possible genetic drift, maintaining mice with mixed genotypes in the same cages to minimize possible cage effects, and mixing used bedding between cages to ensure a homogenous microbial environment among cages.

2.2 Materials and Methods

2.2.1 Mice

APOE3-TR [153,154] male mice were crossed to *APOE4* and *APOE2* female mice to produce *APOE2/E3* and *APOE3/E4* heterozygous offspring. These mice were then crossed to generate 76 experimental mice that included *APOE2/E2* (N=6), *APOE2/E3* (N=12), *APOE3/E3* (N=5), *APOE3/E4* (N=8), and *APOE4/E4* (N=4) female mice and *APOE2/E2* (N=5), *APOE2/E3* (N=7), *APOE3/E3* (N=13), *APOE3/E4* (N=11), and *APOE4/E4* (N=5) male mice. Genotypes were determined by TaqMan SNP assays (Thermo). At weaning, mice were separated by sex and housed as mixed genotypes, 2-5 mice per cage (average of 3.7 ± 1.4 (mean ± SD)). Mice were maintained on Teklad Global 18% Protein Rodent Diet. To minimize potential confounding effects of coprophagy (mice feeding partially on their feces) [155], approximately 20% of the new bedding was a mixture of used bedding from all the cages. Feces were obtained from this cohort of mice at three-, five- and seven-months of age. To obtain feces, mice were temporarily removed from their cage and placed into a clean Styrofoam cup. Fresh fecal pellets were stored at -80˚C until DNA isolation. All methods were approved by University of Kentucky Institutional Animal Care and Use Committee. This study was carried out in compliance with ARRIVE guidelines.

2.2.2 Microbiome Analysis

Fecal DNA was isolated by using a QIAamp PowerFecal Pro DNA Kit (QIAGEN). Genomic DNA was polymerase chain reaction (PCR) amplified with primers CS1_515F and CS2_806R (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and

GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal RNA genes. Amplicons were generated using a two-stage PCR amplification protocol as described previously [156]. The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2). First stage PCR amplifications were performed in 10 microliter reactions in 96-well plates, using MyTaq HS 2X mastermix (Bioline). PCR conditions were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30", 55°C for 45" and 72°C for 60".

Subsequently, a second PCR amplification was performed in 10 µl reactions in 96-well plates. A mastermix for the entire plate was made using MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). Cycling conditions were: 95°C for 5 mins, followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". Samples were then pooled, purified, and sequenced on an Illumina MiniSeq platform employing paired-end 2x153 base reads. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Genome Research Core (GRC) within the Research Resources Center (RRC).

Forward and reverse reads were merged using PEAR [157] and trimmed based on a quality threshold of p =0.01. Ambiguous nucleotides and primer sequences were removed and sequences shorter than 225 bp were discarded. Chimeric sequences were identified and removed using the USEARCH algorithm with a comparison to the Silva 132_16S reference database [158,159]. Amplicon sequence variants (ASVs) were

identified using DADA2 [160] and their taxonomic annotations determined using the UCLUST algorithm and Silva 132_16S reference with a minimum similarity threshold of 90% [158,159]. Sequence processing and annotation was performed by the Research Informatics Core (RIC) within the RRC.

This sequencing effort yielded 10,162,042 reads. Raw sequence data files were submitted in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI). Two samples with fewer than 30,000 reads each were discarded. Since *APOE* effects may be sex dependent [94], microbiomes from male and female mice were analyzed separately. Average read counts per sample for the three-month males was 48,725, three-month females was 49,499, five-month males was 45,985, five-month females was 49,939, in seven-month males was 50,975, in seven-month females was 51,931. Using MicrobiomeAnalyst [161] (updated version February 2021), samples were rarified to the minimum library size, which for three-month males was 36,421, three-month females was 37,069, five-month males to 30,738, five-month females to 35,467, seven-month males to 35,452, and seven-month females to 36,730. Low abundance ASVs were removed, *i.e.*, ASVs with < three counts in > 90% of the samples were removed, and low variance ASVs were also removed, *i.e.*, ASVs whose inter-quantile range was in the lowest 10% [161]. These corrections reduced the number of ASVs from 263 to 63 ASVs in three-month males and females, 67 in five-month females, 69 in five-month males, 68 in seven-month females and 66 in seven-month males. Count data was normalized with a centered log-ratio transformation. Regarding *APOE* genetics, the results were analyzed as separate genotypes or as pooled alleles, *i.e.*, *APOE2/E3* heterozygous mice were grouped with *APOE2/E2* mice while *APOE3/E4* mice were

grouped with *APOE4/E4* as described in other *APOE* studies [162,163]. This resulted in 12 *APOE2*, 13 *APOE3* and 16 *APOE4* male mice and 18 *APOE2*, five *APOE3* and 12 *APOE4* female mice.

Bacteria associated with *APOE* were identified by a linear discriminant analysis effect size (LefSe) approach [164]. Significance thresholds were set to 0.05 for the alpha values for Kruskal-Wallis/Wilcoxon tests and 2.0 for the logarithmic linear discriminant analysis (LDA) score, using a one-against-all multi-class analysis approach. These results were then plotted as a cladogram to document the phylogenetic relatedness of *APOE* allelic associations with the bacteria at each taxonomic level. Results are also presented as an LDA histogram.

Alpha-diversity was assessed using the Shannon H diversity index [165] with *APOE* statistical significance determined by Kruskal-Wallis tests. Additional alpha-diversity tests included Margalef taxon richness, Pielou's evenness and the Simpson index with APOE statistical significance determined by Jonckheere–Terpstra nonparametric tests. Beta-diversity was assessed using Principal Coordinates Analysis (PCoA) of Bray-Curtis matrices with statistical significance determined by Permutational Multivariate Analysis of Variance (PERMANOVA) [166]. Taxonomic levels that associate with *APOE* status were determined using a classical univariate analysis with a Kruskal–Wallis test. A false discovery rate (FDR) approach was used to correct for multiple testing [161]. Heatmaps of family-level bacterial relative abundances were generated for male and female mice as a function of *APOE* status by using the Ward analysis of variance clustering algorithm that used Pearson Correlation Coefficient distance measures.

2.3 Results

To investigate the hypothesis that *APOE* is associated with gut microbial community structure, we began with LefSe analysis and visualized the results with cladograms (Figs. 1, 2, Figs. S1, S2). This robust approach provides a visual means to identify statistically significant and phylogenetically-related taxa associated with *APOE* status [164]. These LefSe results are also presented as LDA histograms to provide a quantitative representation of the LefSe analyses (Fig. S3). Results are presented with *APOE* status stratified as *APOE2* carriers, *APOE3* and *APOE4* carriers (pooled) (Figs. 1A, 2A) and with *APOE* status as separate genotypes (Figs. 1B, 2B). These two representations of the data provide insights into whether *APOE* allelic effects are dominant or co-dominant. Robust gut microbiome differences were observed in male mice compared to female mice at 3-months of age (Figs. 1, 2) with similar results found at 5- and 7-months of age (Figs. S1, S2). The microbiome of both male and female mice showed *APOE4*-associated increases in members of the Actinobacteria phylum (Figs. 1, 2). In contrast, only male mice showed *APOE4*-associated increases in the Erysipleotrichia and Gammaproteobacteria classes and *APOE2*-associated increases in the Cyanobacteria phylum. To gain further insights into these findings, we parsed the results into individual genotypes. This expanded our findings by showing that members of the Clostridia class were significantly associated with *APOE2/E2* and *APOE2/E3* (Fig. 1B). Overall, these results indicated that a subset of bacteria were consistently associated with *APOE* status, especially in males. The male population captured the majority of taxa significantly associated with *APOE* in the female population. In the following results, we present analyses of data from male mice at 3-months of age with analyses for all ages and sexes included within the Supplemental Files.

Microbiome alpha- (within sample) diversity was assessed by the Shannon H index and Simpson index, measures of taxon richness and evenness, as well the Margalef taxon richness index and evenness index [167]. A robust association between alpha-diversity and *APOE* was not detected in female mice (Shannon index p-values in Table 1, all measures of alpha-diversity in Supplemental Files). In contrast, male mice showed a stepwise trend towards higher alpha-diversity with *APOE2-APOE3-APOE4* at the genus level, and this trend became more defined and statistically significant at every higher level through phylum (phylum and family depicted in Fig. 3, all levels presented in Table 1 and Supplemental Files, additional ages, and female data in Table S1). When the results are parsed into separate genotypes, the alpha-diversity of the *APOE* heterozygous animals tended to be intermediate relative to the homozygous animals. Hence, *APOE* was associated with alpha-diversity in male but not female mice and *APOE* allelic affects appeared co-dominant.

Beta-diversity is a measure of between sample microbial communities based on their composition. Beta-diversity was visualized by using PCoA based on Bray-Curtis distance matrices [168-171], and analyzed using a PERMANOVA. We found that *APOE* status was significantly associated with microbiome beta-diversity in male mice (Fig. 3, Table 2, additional ages and female in Table S2). When the data were analyzed with each separate *APOE* genotype, beta-diversity was still significant at each of the same taxonomic levels. Overall, these results demonstrate that the microbiome is robustly associated with *APOE* genetics in male mice.

Variation in bacterial relative abundance per sample was visualized using heatmaps, as seen in Figure 4. While cladograms identify bacterial phylogenetic branches

that correlate with high abundance in association with a specific *APOE* status, heatmaps provide a per sample depth of information for each *APOE* status. Inspection of the heatmaps suggests that the data are relatively complex although some bacterial patterns of association with *APOE* status are discernible, e.g., *Ruminococcaceae* and *Erysipelotrichaceae* in male mice (Figure 4).

Although the use of LefSe analysis and cladograms provides insights into the microbiome and has been optimized for this purpose [164], another perspective is provided by taxon-by-taxon classical univariate analysis using a Kruskal-Wallis test for significance and an FDR correction for multiple testing. To highlight the taxa most robustly associated with *APOE*, we applied classical univariate analysis to identify results that were significant with both approaches. Classical univariate analysis of the 3-month old female mice found no ASVs, genera, families, orders, classes, or phyla that were significantly associated with *APOE* in either *APOE* model. However, this approach applied to 3-month old male mice in the dominant model found 12 *APOE*-associated ASVs, as well as 12 genera, six families, five orders, five classes, and one phylum (statistics for all taxa in each age group are listed in Supplementary Tables S1.1–S3.4). In the co-dominant model, this approach found eight ASVs, eight genera, three families, four orders, four classes, and no phyla that were significantly associated with *APOE*. A graphical representation of the findings in 3-month old male mice is depicted in Fig. 5. Several bacteria showed stepwise associations with *APOE* on multiple taxonomic levels and were overall increased with *APOE2*. For example, the Clostridia class, Clostridiales order and two major families within this phylogenic branch, *Ruminococcaceae* and *Lachnospiraceae* showed an increase in their relative abundance from *APOE4* to *APOE3*

to *APOE2* (Fig. 5). The most abundant genera within the *Ruminococcaceae* family significantly associated with *APOE* were *Ruminiclostridium* (Fig. 5A,E), *Ruminiclostridium_5* and *Ruminiclostridium_9,* which in aggregate represent approximately half of the *Ruminococcaceae* family. At 5- and 7-months of age, other genera within *Ruminococcaceae* were associated with *APOE* (Supplemental Tables S5.0–S7.5). Genera within the other major family, *Lachnospiraceae,* that increased with *APOE2* were *Acetifactor* and *Lachnoclostridium* (Fig. 5B,C,F,G).

In contrast, the relative abundance of other taxa was increased in APOE4 mice, most notably bacteria within the phylogenetic branch defined by the Erysipelotrichia class, confirming the findings from the LefSe cladograms (Figure 1). Bacteria within the Erysipelotrichia branch that were significantly associated with *APOE* included the order Erysipelotrichiales, its family *Erysipelotrichaceae* and its genera *Turicibacter* and *Dubosiella* (Figure 6, data for all ages shown in Supplementary Tables S1.1-S3.4). Consistent within this branch, bacterial relative abundance was near zero in the *APOE2* mice, moderate in *APOE3* and highly enriched in *APOE4* (Figure 6). Hence, both the LefSe and classical approaches identified members of the Clostridia class as enriched in *APOE2* mice while members of the Erysipelotrichia class were enriched in *APOE4* mice.

To discern whether the Clostridiales and Erysipelotrichiales phylogenetic branches associated with *APOE* in this murine *APOE*-TR model are also associated with *APOE* in humans, we turned to a recent GWAS that evaluated the relationship between the gut microbiome and human polymorphisms [172]. This meta-analysis included data from as many as 18,340 individuals [172]. The only genetic locus that reached genome wide statistical significance was rs182549, which is associated with lactose intolerance.

Interestingly, this SNP is modestly associated with the risk of AD (p=0.003, N=445,779) [24], consistent with the possibility that the gut microbiome may influence AD risk. Focusing on *APOE*, the alleles of *APOE2*, *APOE3* and *APOE4* are defined by two SNPs, rs7412 and rs429358. The minor allele of rs7412 defines *APOE2* while the minor allele of rs429358 determines *APOE4* status. The Clostridiales and Erysipelotrichiales phylogenetic branches were not significantly associated with rs7412 (*APOE2*) at any phylogenetic level. However, the class Erysipelotrichia, the order Erysipelotrichales and the family *Erysipelotrichaceae* were nominally associated with rs429358 (Table 3). For each of these taxa, the minor *APOE4* allele was associated with an increase in the relative abundance of these bacteria, reproducing the findings observed in the murine *APOE*-TR model.

2.4 Discussion

The primary finding reported here is that murine gut microbiome profiles are significantly associated with *APOE* status in a study wherein the *APOE*-TR mice were maintained in an optimized fashion for microbiome analyses. The microbiome association with *APOE* was observed in alpha- and beta-diversity, encompasses multiple bacterial lineages and was predominately in male mice. Both LefSe and classical univariate analyses identified specific taxa that were associated with *APOE*. This association occurred in a stepwise fashion in the mice with the progression from *APOE2-APOE3-APOE4*. The stepwise association between indices of the gut microbiome and *APOE2-APOE3-APOE4* reported here are reminiscent of *APOE* allelic association with other phenotypes ranging from LDL-cholesterol to AD risk [47,144]. Additionally, at least one of these associations, an increase in the Erysipelotrichia phylogenetic branch with

APOE4, is also observed in the human gut microbiome. Overall, these findings confirm and extend prior reports that *APOE* genetics are associated with the gut microbiome [94,115,116].

To identify the impact of *APOE* alleles on the microbiome, we used several approaches in this study. These approaches included alpha-diversity, beta-diversity, LefSe and classical univariate analyses. Alpha- and beta-diversity analyses aggregate multiple variables to provide an assessment of overall microbiome diversity and of microbiome profile similarity, respectively. In contrast, LefSe and classical univariate analyses provide an indication of differences in the relative abundance of specific taxa between experimental groups. In this discussion, we will highlight the primary significant findings from these various analyses.

APOE4 was associated with increased alpha-diversity as assessed by the Shannon H index. A stepwise progression was observed with lowest alpha-diversity in *APOE2* moderate in *APOE3* and highest in *APOE4.* Alpha-diversity is a measure of the number of distinct taxa and the evenness of these numbers across taxa. High alpha-diversity in the gut microbiome has been associated with improved gut health and microbiome homeostasis (reviewed in [173]). The *APOE4* association with increased alpha-diversity observed here is consistent with prior observations that *APOE4* is associated with better response to diarrheal infections in a third-world environment [107,108]. Indeed, the enrichment of *APOE4* in people indigenous to Amazonian basin has been proposed to be a result of evolutionary selection in this environment with insufficient sanitation [174].

A primary finding of this study was that both the LefSe and classical univariate analyses found that taxa within the Clostridia class were increased with *APOE2* status,

confirming results from our prior study [115] and that of Tran et al [116]. This phylogenetic branch included the Clostridiales order, *Ruminococcaceae* family and several genera within this family. The Clostridiales order was increased in *APOE2* mice compared to *APOE3* and *APOE4* mice. This was most robust in the three-month males with similar findings at five- and seven-months. The two major bacterial families within this order, *Ruminococcaceae* and *Lachnospiraceae*, were also both increased with *APOE2*. The stepwise fashion of the decline in *Ruminnococcaceae* relative abundance from *APOE2* to *APOE3* to APOE4 confirms the stepwise pattern seen previously [94,115] and extends it along the phylogenetic branch from the Clostridia class to associated genera, such as *Ruminiclostridium, Ruminiclostridium_5, Ruminiclostridium_9*. Interestingly, Tran et al. also reported an increase in relative abundance of the Clostridiales order and *Ruminococcaceae* family in *APOE2/E3* humans compared to *APOE3/E4* and *APOE4/E4* humans [116]. This suggests that this increase in Clostridiales and *Ruminococcaceae* with *APOE2* may extend to humans. Two additional genera in the Clostridia class, within the *Lachnospiraceae* family, i.e., *Acetifactor* and *Lachnoclostridium,* also increased with *APOE2* status in the current study. However, this finding was not replicated by Tran et al., who reported that *Lachnospiraceae* increased in *APOE4* mice compared to *APOE3* mice [116].

Our study strengthens the associations between *APOE* status and the Clostridiales order, *Ruminococcaceae* family and related genera, and the *Acetifactor* and *Lachnoclostridium* genera by demonstrating a stepwise pattern with *APOE* allelic status across the entire phylogenetic branch from the Clostridia class down to related genera. *Ruminococcaceae* and *Lachnospiraceae* are bacterial families that highly express genes

responsible for the metabolism of resistant starches in the large intestine, generating short chain fatty acids (SCFA)s. The presence of SCFAs in the gut affect human health in general (reviewed in [175,176]) and have been reported to promote microglial maturation and function in particular [123]. Treatment with SCFAs has been shown to reduce microglial pro-inflammatory signals and promote a homeostatic profile that is neuroprotective [177-180]. Considering these findings relative to disease pathology associated with the stepwise *APOE2-APOE3-APOE4* phenotype, we propose a tentative model wherein (i) *APOE2* is associated with an increase in the relative abundance of microbiome bacteria *Ruminococcaceae, Acetifactor* and *Lachnoclostridium*, relative to *APOE3* and *APOE4*, (ii) this shift in bacterial profile increases the production of SCFAs and (iii) this increase in SCFAs promotes microglial homeostasis and disease-ameliorating signaling, as suggested by robust genetic evidence [31,181-188], (reviewed in [189,190]). While speculative, this model serves as a framework for future studies.

Another primary finding detected by both the LefSe and classical univariate analyses was that the Erysipelotrichia phylogenetic branch was significantly associated with *APOE* status in a stepwise *APOE2-APOE3-APOE4* pattern. This finding appeared to extend to humans and replicates the increase of the *Erysipelotrichaceae* family in *APOE4* mice that we observed previously [115]. This parallels the association of the Erysipelotrichia class, Erysipelotrichales order and *Erysipelotrichaceae* family with the *APOE4* minor allele rs429358 in human GWAS data. Our current study also extends this finding from the Erysipelotrichia phylogenetic order to its major genera, i.e. *Turicibacter* and *Dubosiella*. However, Tran et al. reported *Erysipelotrichaceae* were significantly increased in *APOE3* compared to *APOE4* mice. *Erysipelotrichaceae* has been shown to

increase in animals fed a high-fat diet and to decrease in patients on a low-fat diet [191,192]. Hence, diet variation between the mice in our study and those of Tran et al. may account for the *Erysipelotrichaceae* difference, noting that our Teklad Global 18% (2018) chow has a fat content that accounts for 18% of total calories, whereas the RPM3, Special Diet Services chow used in the Tran et al study has a fat content that accounts for 12% of total calories [116]. Since *APOE* genetics have been associated with BMI and obesity [144], there may be a complex interplay between diet, *APOE* genotype and relative abundance of *Erysipelotrichaceae* in the gut.

2.5 Conclusions

In this study in which mice were maintained with optimized conditions for microbiome analysis, we report a significant association between *APOE* status and gut microbiome profiles in three-month male mice that reproduces at five and seven months of age. The Clostridia class, Clostridiales order, its related family *Ruminococcaceae*, as well as related genera *Ruminoclostridium,* and *Acetifactor* and *Lachnoclostridium* of the *Lachnopsiraceae* family increase with *APOE2,* which may reflect an increase in resistant starch metabolism with *APOE2*, and a possible impact on SCFA levels. The Erysipelotrichia class, Erysipelotrichiales order, *Erysipelotrichaceae* family, and *Turicibacter* and *Dubosiella* genera increase with *APOE4.* The findings with the Erysipelotrichia phylogenetic branch appear to extend to humans. Understanding the effects of *APOE* genetics on the gut microbiome may provide novel approaches to counter deleterious *APOE* genetic effects on human disease.

Alpha-diversity

	3 month p-values		5 month p-values		7 month p-values	
Taxonomic level	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
genus	$4.5x10^{-1}$	$5.8x10^{-1}$	$4.6x10^{-1}$	$7.9x10^{-1}$	$5.4x10^{-1}$	$7.7x10^{-1}$
family	**$5.8x10^{-4}$**	$3.2x10^{-1}$	**$2.0x10^{-2}$**	$4.1x10^{-1}$	$5.0x10^{-1}$	$7.4x10^{-1}$
order	**$7.7x10^{-5}$**	$7.7x10^{-1}$	$8.4x10^{-2}$	$6.1x10^{-1}$	$1.4x10^{-1}$	$2.8x10^{-1}$
class	**$7.7x10^{-5}$**	$7.3x10^{-1}$	$7.3x10^{-2}$	$6.3x10^{-1}$	$1.4x10^{-1}$	$2.4x10^{-1}$
phylum	**$1.1x10^{-5}$**	$6.5x10^{-2}$	$2.8x10^{-1}$	$9.5x10^{-2}$	$9.2x10^{-1}$	$2.5x10^{-1}$

Table 1. Alpha-diversity p-values for *APOE* mice.

Microbiome alpha-diversity was significantly associated with *APOE* status in three-month male but not female mice. P-values reflect nominal p-values and were determined using Kruskal-Wallis tests. P-values less than 0.5 are bolded.

Beta-diversity							
		3 month		5 month		7 month	
Taxonomic level		**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Genus	**p-value**	**0.001**	0.408	**0.044**	0.283	**0.005**	**0.038**
	R^2	0.146	0.058	0.0085	0.066	0.123	0.095
Family	**p-value**	**0.005**	0.496	0.080	0.060	**0.001**	0.201
	R^2	0.165	0.052	0.088	0.048	0.164	0.077
Order	**p-value**	**0.003**	0.688	0.089	0.789	**0.006**	0.165
	R^2	0.206	0.036	0.100	0.031	0.179	0.091
Class	**p-value**	**0.002**	0.656	0.082	0.830	**0.006**	0.133
	R^2	0.208	0.037	0.102	0.029	0.18	0.097
Phylum	**p-value**	**0.028**	0.263	0.846	0.059	0.360	0.105
	R^2	0.122	0.069	0.023	0.046	0.055	0.102

Table 2. Beta-diversity values for *APOE* mice.

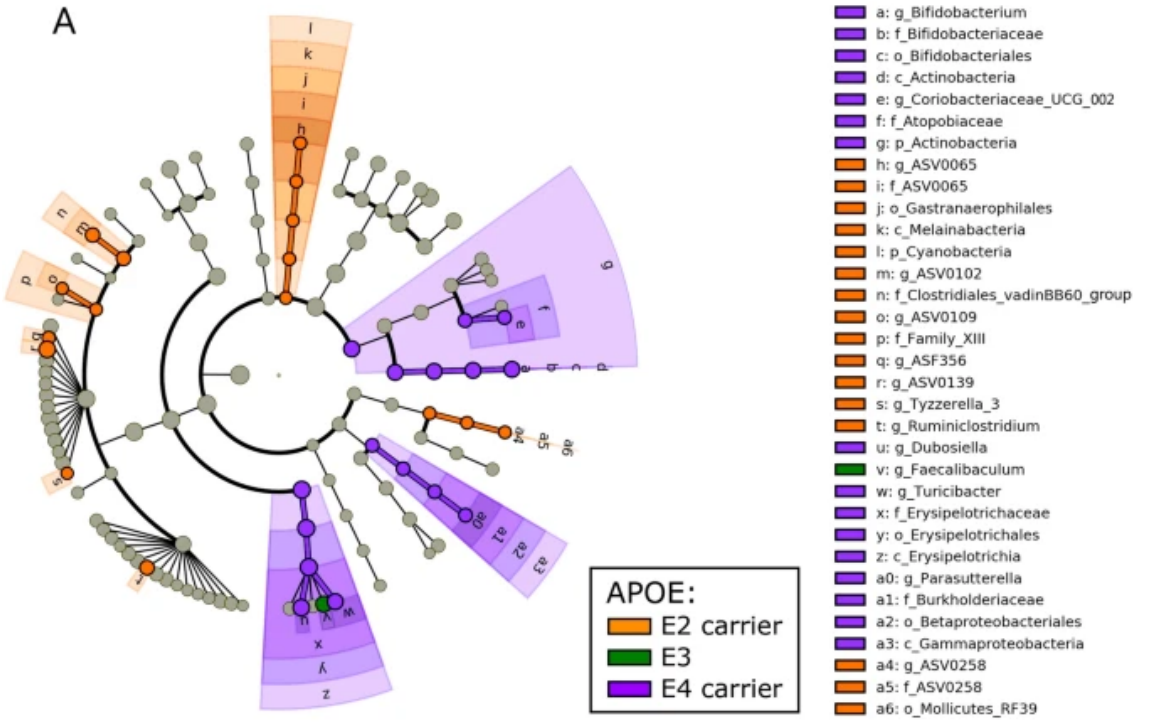
Microbiome beta-diversity significantly associated with *APOE* status in male, but not female, mice. The R2 values represent the proportion of the variance captured by *APOE* alleles. The PERMANOVA results were derived from 999 permutations.

Bacteria	SNP	Reference Allele	Effect Allele	Beta	SE	P-value	M
Class: Erysipelotrichia	rs429358	T	C	0.032	0.015	**0.035**	18097
Order: Erysipelotrichales	rs429358	T	C	0.032	0.015	**0.035**	18097
Family: Erysipleotrichaceae	rs429358	T	C	0.032	0.015	**0.035**	18097
Genus: Turicibacter	rs429358	T	C	0.011	0.021	0.87	8921

Table 3. Human GWAS for Erysipelotrichia taxa.

Bacteria in the Erysipelotrichia phylogenetic branch are nominally associated with rs429358 in humans. The positive beta values reflect that the bacterial taxa are increased with the minor *APOE4* allele of rs429358. These results combine data from men and women and are supplemental data from a large microbiome genetics study [172].

Three month males: dominant model



Three month males: co-dominant model

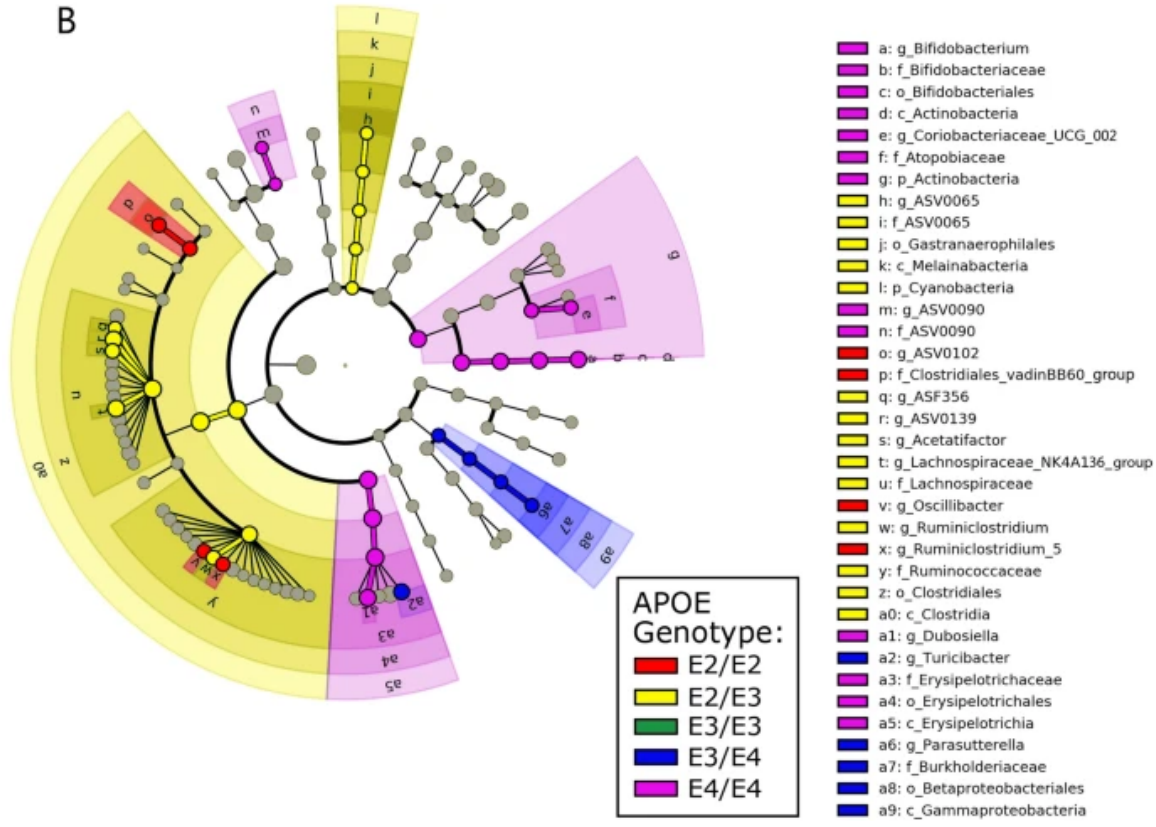
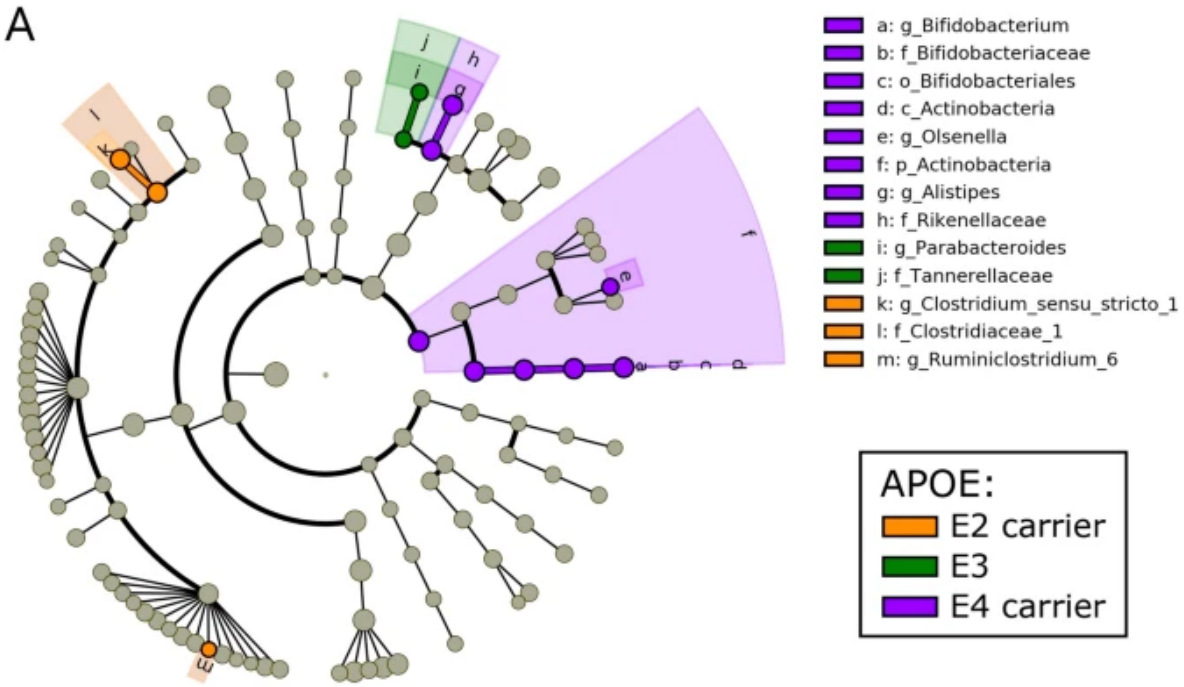


Figure 1. Cladograms of taxa differential for male *APOE* mice.

Cladograms reveal microbial phylogenetic branches associated with *APOE* status in males. Taxa are represented as nodes and are connected by lines based on the phylogenetic relatedness of all taxa present in each experimental cohort. For example, the end node, a. represents the genus *Bifodobacterium* which is connected to other nodes representing higher level taxa related to *Bifodobacterium* including; b. the family *Bifidobacteriaceae*, c. the order Bifidobacteriales, and d. the class Actinobacteria. Many taxa are associated with *APOE* genetics, with node colors indicating the *APOE* associated with highest levels of that taxa. Statistical significance reflects both $p < 0.05$ for Kruskal–Wallis tests and a logarithmic LDA score > 2.0.

Three month females: dominant model



Three month females: co-dominant model

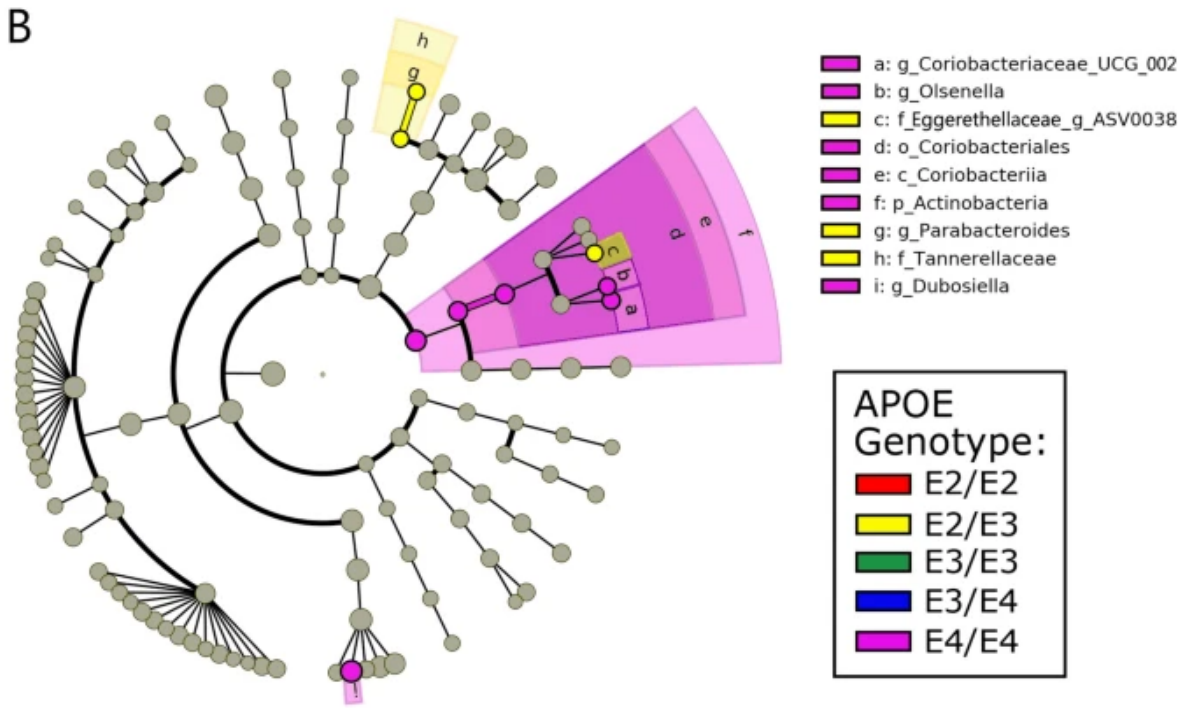


Figure 2. Cladograms of taxa differential for female *APOE* mice.

Cladograms reveal microbial phylogenetic branches associated with *APOE* status in females. Taxa significantly associated with *APOE* are highlighted ($p < 0.05$ for Kruskal–Wallis tests and a logarithmic LDA score > 2.0).

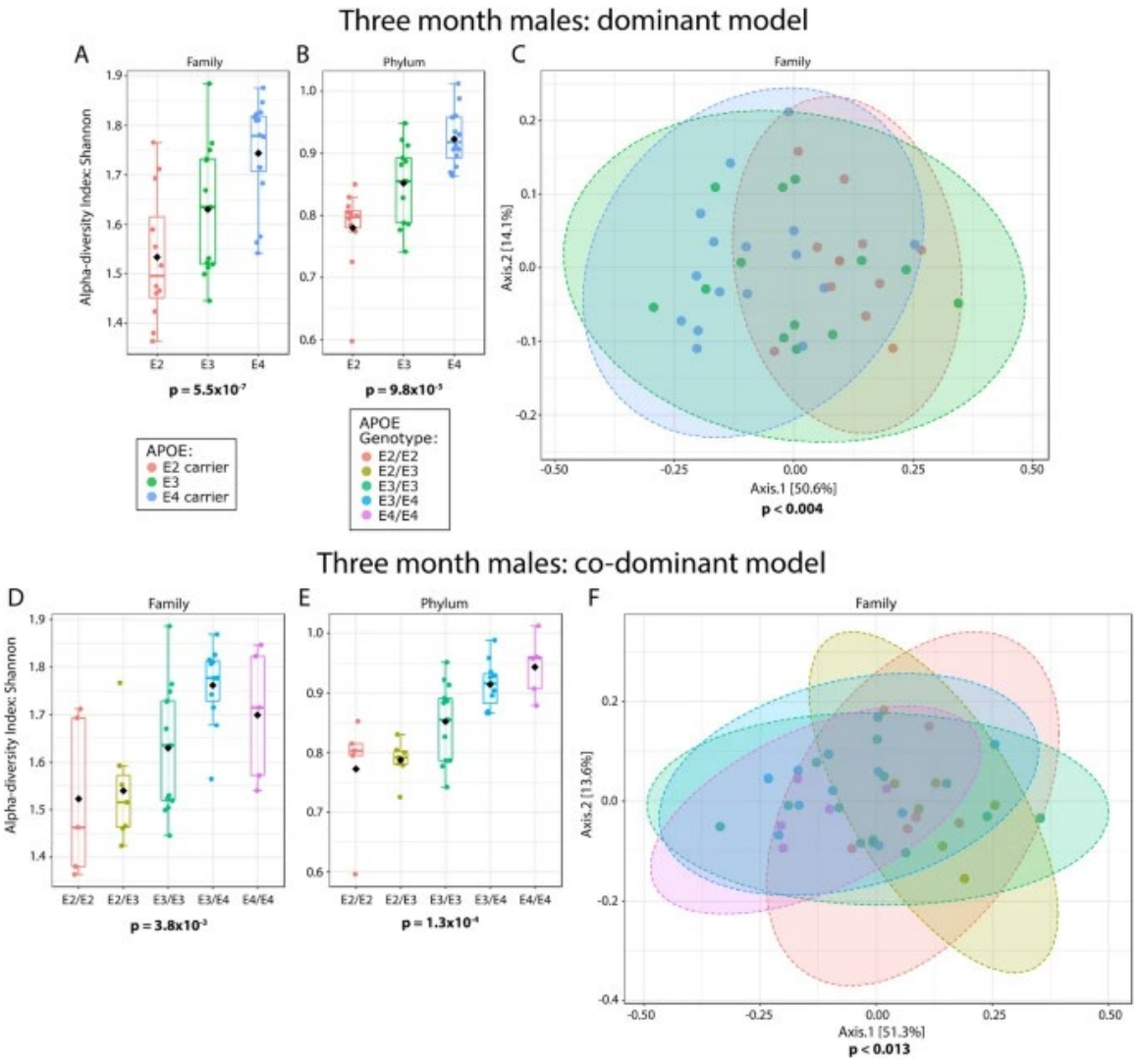


Figure 3. Alpha- and beta-diversity plots for *APOE* mice.

Microbiome alpha- and beta-diversity as a function of *APOE*. Alpha-diversity is depicted as boxplots (A,B,D,E) and beta-diversity as PCoA plots (C,F). These results are from male mice at 3-months of age. Statistical significance for the findings is indicated below each graph. Ellipses in C and F represent 95% confidence intervals. Dominant model (C) R2 = 0.171 and co-dominant model (F) R2 = 0.206. Beta-diversity was also analyzed using a PERMDISP, which had no significant p-values, indicating that variances were not significantly different as a function of *APOE*.

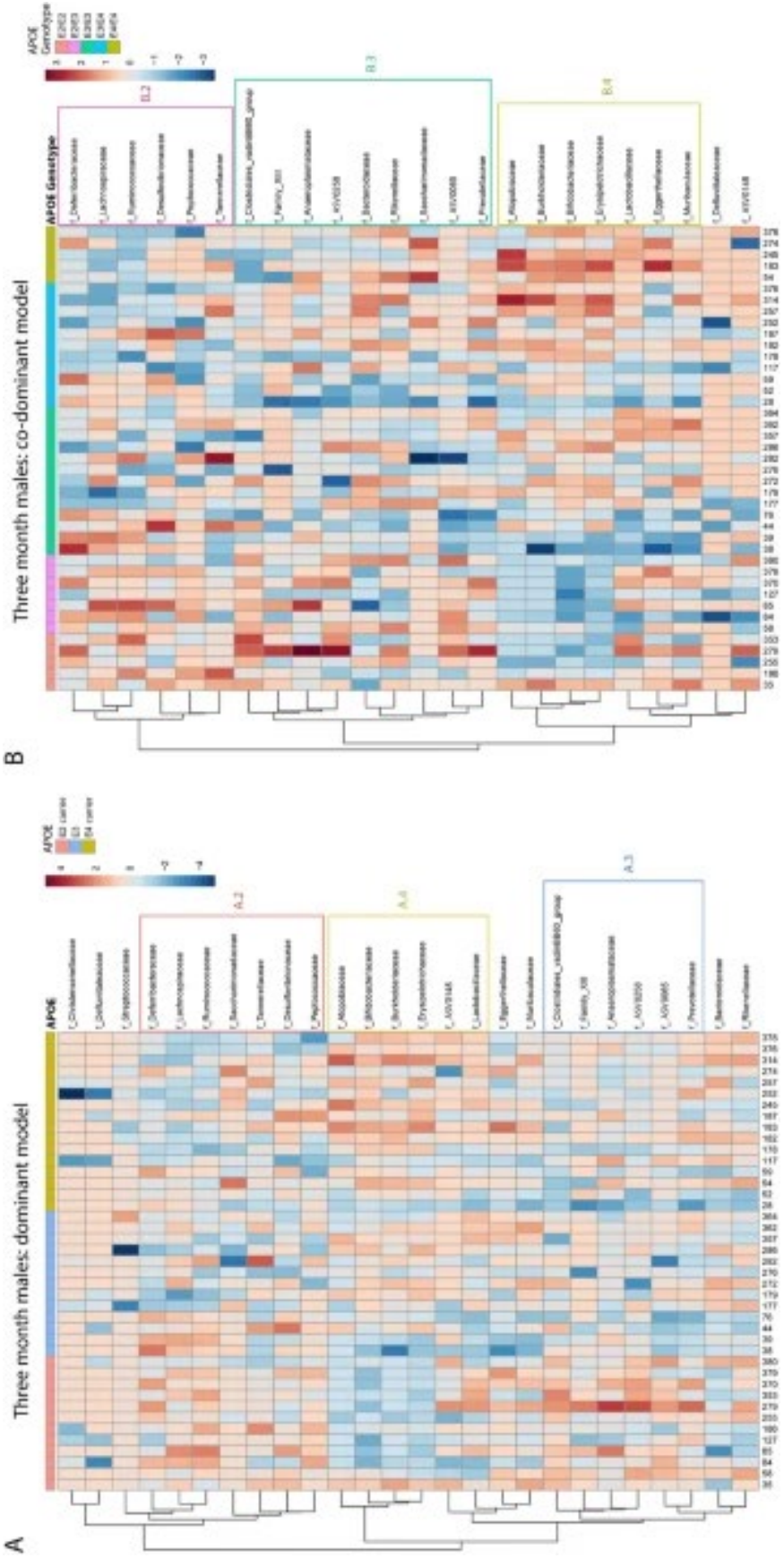

A
Three month males: dominant model
APOE
B
Three month males: co-dominant model
APOE Genotype

Figure 4. Heatmaps of bacterial taxa for all groups of mice.

Heatmaps depict overall microbiota profiles grouped by *APOE* status. These heatmaps depict per-sample relative abundance for family-level bacteria in female and male mice. Columns were grouped by *APOE* status, rows were grouped by the Ward clustering algorithm using Pearson Correlation Coefficient distance measures. Colored boxes highlight groups of taxa that follow either an *APOE2*- (A.2 and B.2), *APOE3*- (A.3 and B.3) or *APOE4*-associated pattern (A.4 and B.4).

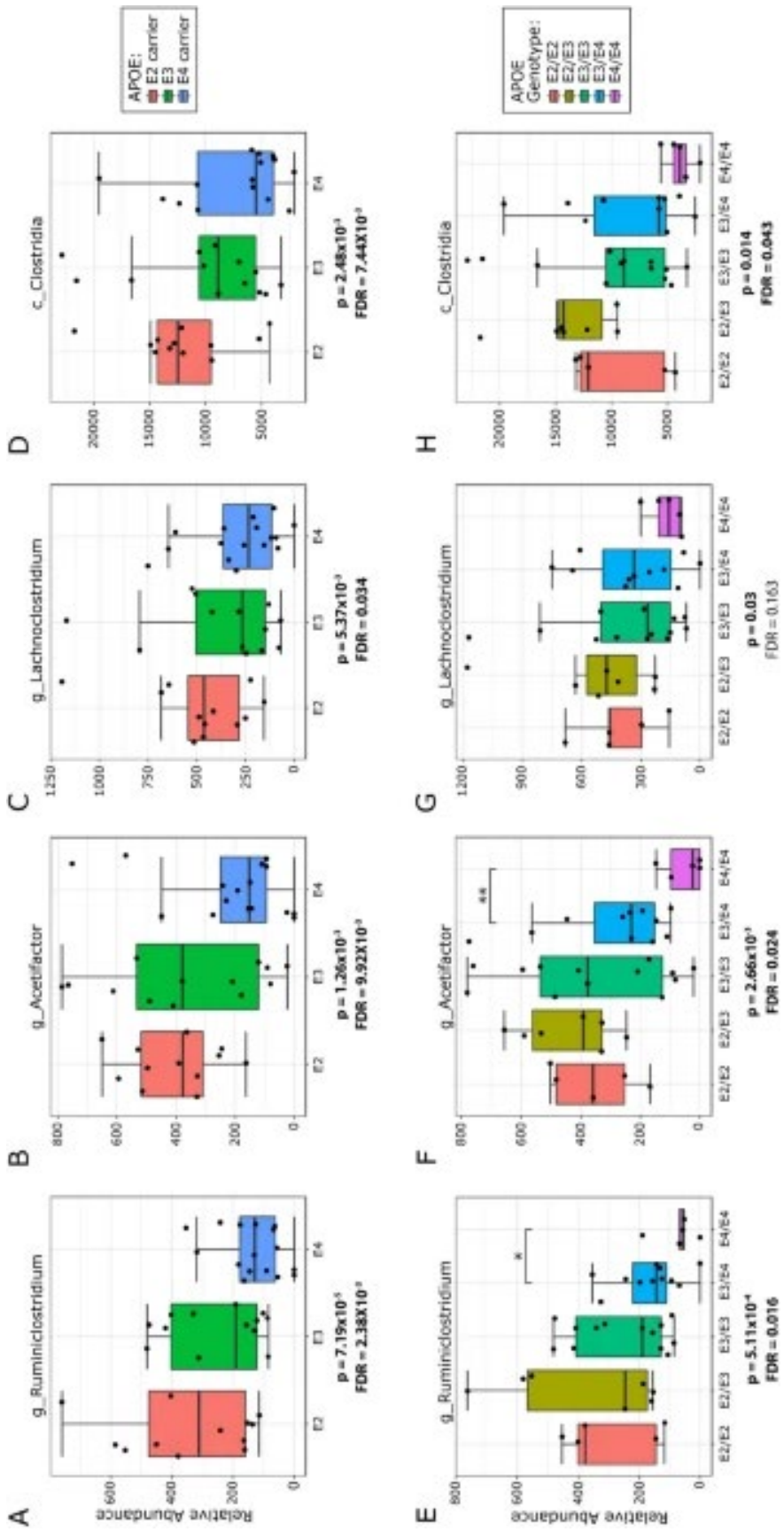

A
g_Ruminiclostridium
Relative Abundance
p = 7.19x10⁻⁵
FDR = 2.38X10⁻³
B
g_Acetitfactor
p = 1.26x10⁻³
FDR = 9.92X10⁻³
C
g_Lachnoclostridium
p = 5.37x10⁻³
FDR = 0.034
D
c_Clostridia
APOE:
E2 carrier
E3
E4 carrier
p = 2.48x10⁻³
FDR = 7.44X10⁻³
E
g_Ruminiclostridium
Relative Abundance
p = 5.11x10⁻⁴
FDR = 0.016
F
g_Acetitfactor
p = 2.66x10⁻³
FDR = 0.024
G
g_Lachnoclostridium
p = 0.03
FDR = 0.163
H
c_Clostridia
APOE Genotype:
E2/E2
E2/E3
E3/E3
E3/E4
E4/E4
p = 0.014
FDR = 0.043

Figure 5. The Clostridia branch in association with *APOE* in male mice.

The phylogenetic branch defined by Clostridia and its lower taxa shows a significant association with *APOE* in male mice. The relative abundance of each depicted bacteria was significantly associated with *APOE* status. The relative abundance of these bacteria decreased in a stepwise fashion from *APOE2* to *APOE3* to *APOE4*. P values have been corrected using an FDR approach. (A–D) Are the plots depicted using the dominant model representation, while (E–H) are the plots depicted using the co-dominant model representation. These data are derived from the 3-month male mice with data for all ages provided in Supplementary Tables S5.0–SS7.5.

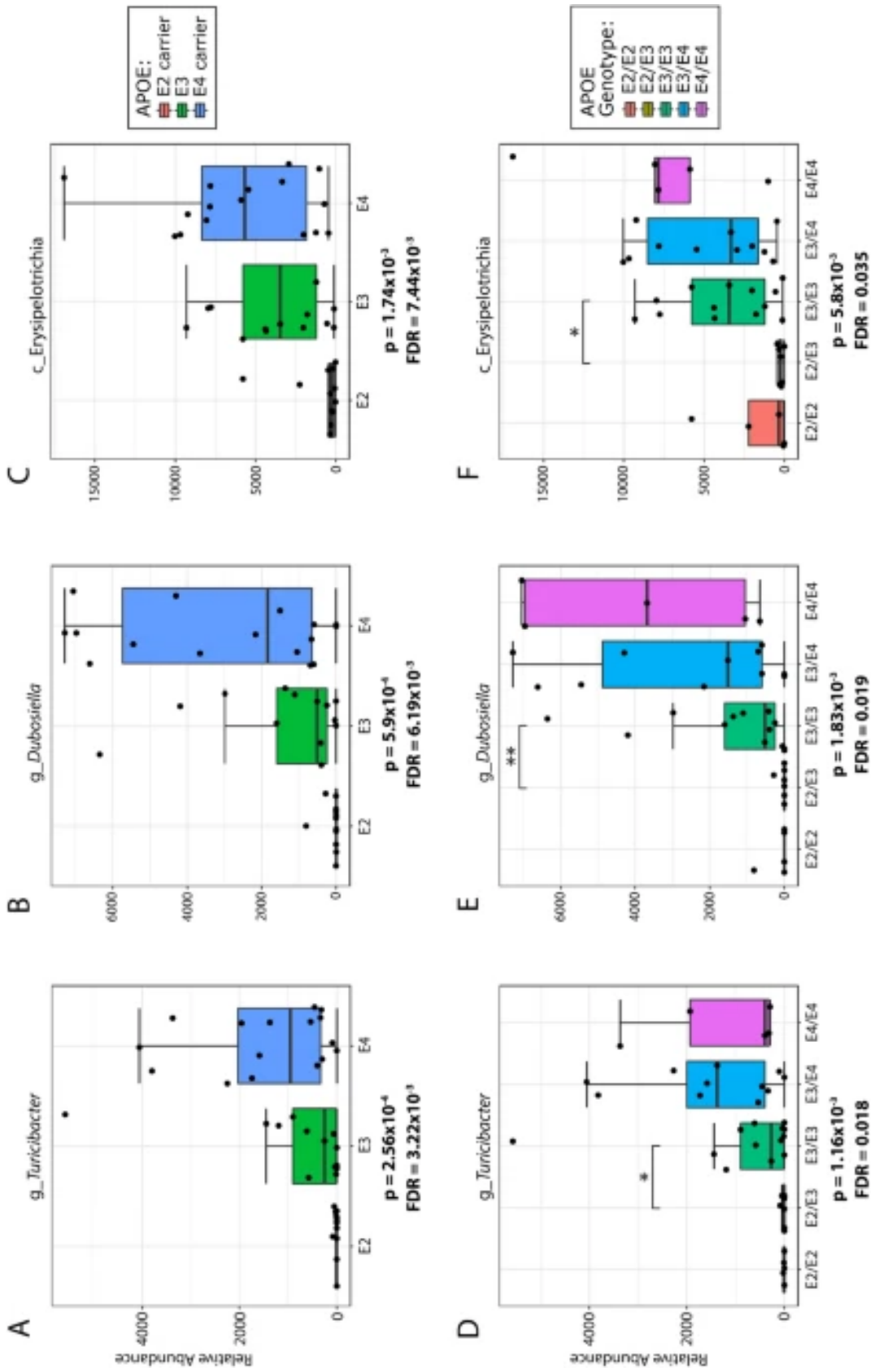

A
g_Turicibacter
Relative Abundance
p = 2.56x10⁻⁴
FDR = 3.22x10⁻³
B
g_Dubosiella
p = 5.9x10⁻⁴
FDR = 6.19x10⁻³
C
c_Erysipelotrichia
p = 1.74x10⁻³
FDR = 7.44x10⁻³
APOE:
E2 carrier
E3
E4 carrier
D
g_Turicibacter
Relative Abundance
p = 1.16x10⁻³
FDR = 0.018
E
g_Dubosiella
p = 1.83x10⁻³
FDR = 0.019
F
c_Erysipelotrichia
p = 5.8x10⁻³
FDR = 0.035
APOE Genotype:
E2/E2
E2/E3
E3/E3
E3/E4
E4/E4

Figure 6. The Erysipelotrichia branch in association with *APOE* in male mice.

The phylogenetic branch defined by Erysipelotrichia and its lower taxa shows a significant association with *APOE* in male mice. All depicted bacteria were significantly associated with *APOE* status. The relative abundance of these bacteria increased in a stepwise fashion from *APOE2* to *APOE3* to *APOE4*. P values have been corrected using an FDR approach. (A–C) Are the plots depicted using the dominant model representation, while (D–F) are the plots depicted using the co-dominant model representation. These results are derived from 3-month male mice.

CHAPTER 3. EXOGENOUS SHORT-CHAIN FATTY ACID EFFECTS IN APP/PS1 MICE

[This section contains material adapted from the published manuscript: Zajac DJ, Shaw BC, Braun DJ, Green SJ, Morganti JM, Estus S. Exogenous Short Chain Fatty Acid Effects in APP/PS1 Mice. Front Neurosci. 2022 Jul 4;16:873549. doi: 10.3389/fnins.2022.873549. PMID: 35860296; PMCID: PMC9289923.]

3.1 Introduction

The impact of the gut microbiome on Alzheimer's Disease (AD) is an area of intense current scrutiny (reviewed in [193,194]). Several studies have suggested differences in the gut microbiome between AD and non-AD individuals [83,84,88]. Whether the relationship between the gut microbiome and AD risk extends from correlation to causality is unclear, although several reports have found that Aβ burden in murine models is reduced in gnotobiotic mice or mice treated with antibiotics [97-102]. Hence, the gut microbiome may emerge as a modulator of AD risk.

SCFAs are a major microbiota metabolite that have been suggested to mediate gut microbiome effects in the brain (reviewed in [127,128]. The major source of SCFAs in the body is microbial digestion of resistant starch. Recently, SCFA treatment was reported to increase amyloid burden in specific-pathogen-free (SPF) APP/PS1 mice [101] while butyrate treatment was reported to decrease amyloid burden in SPF 5xFAD mice [44] and in 5xFAD mice maintained on a conventional microbiome [129]. We refer to a conventional microbiome as the microbiome of mice that were conventionally raised, as opposed to germ free or SPF mice, which have laboratory controlled/limited microbiomes. Here, we tested the effects of SCFAs on mice maintained under standard

laboratory conditions with a conventional microbiome. For this effort, five-month-old APP/PS1 mice were treated with SCFAs until ten months of age, and then evaluated for microbiome profile, spatial memory deficit, glial activation, and amyloid burden. We report that SCFA treatment impacted the gut microbiome but not memory impairment, glial activation, or amyloid burden in this paradigm.

3.2 Methods

3.2.1 Animals

APP/PS1 (APPswe,PSEN1dE9) are double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a human *PSEN1* gene lacking exon 9 (PS1-dE9) [195]. We chose this mouse model because the mice begin to develop Aβ deposits by six months of age, with abundant plaques in the hippocampus and cortex by nine months [195]. Plaques continue to increase up to around 12 months of age [196]. This is a less aggressive amyloid phenotype with a delayed onset compared to other mouse models, such as the 5xFAD [197], and we hypothesized that a mild agent, such as SCFA treatment, would be more likely to have an effect in the APP/PS1 model. Behavioral deficits have been reported across cognitive domains, although severity and timing depend on the specific behavioral tests [198]. Mice were bred by crossing *APP/PS1* carriers with wild-type C57Bl/6J mice. At weaning, mice were separated by sex and housed as mixed genotypes with 2-5 mice per cage. Non-APP/PS1 (WT) littermates served as control mice.

Mice were maintained on standard mouse chow (Teklad Global 18% Protein Rodent Diet) in individually ventilated cages. This diet consists of ground wheat, ground

corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, and brewers dried yeast, as well as vitamins and minerals [199]. Soluble starches such as inulin that contribute to SCFA production are present in these ingredients, although the specific amounts are not available. Another dietary component, brewers dried yeast, is also known to impact the gut microbiome [200]. Each of the mice in this study were maintained on the same chow for the duration of the study.

Beginning at five months of age, drinking water was supplemented with either SCFAs (67.5 mM sodium acetate, 25mM sodium propionate, 40 mM sodium butyrate, pH 6.8) or with sodium chloride (132.5 mM) [123]. This solution was administered via water bottles and was made fresh weekly for a total of five additional months. Equivalent amounts of water were consumed by each group. This approach has been previously used and shown to significantly increase plasma concentrations of acetate, propionate and butyrate in murine models, including an APPPS1 mouse model [101,201].

3.2.2 Microbiome Analysis

Fecal samples were collected on the day of euthanasia. The number of mice included 34 males (16 APP/PS1 which included eight on SCFA and eight on saline, and 18 WT which included 11 on SCFA and seven on saline), and 32 females (14 APP/PS1 which included five on SCFA and nine on saline, and 18 WT which included 10 on SCFA and eight on saline). DNA was isolated by using a QIAamp PowerFecal Pro DNA Kit (QIAGEN). Genomic DNA was polymerase chain reaction (PCR) amplified with primers CS1_515F and CS2_806R (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and

GGACTACNVGGGTWTCTAAT) targeting the V4 regions of microbial small subunit ribosomal RNA genes. Amplicons were generated using a two-stage PCR amplification protocol as described previously [156]. The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2). First stage PCR amplifications were performed in 10 microliter reactions in 96-well plates, using MyTaq HS 2X mastermix (Bioline). PCR conditions were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30", 55°C for 45" and 72°C for 60".

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-well plates. A mastermix for the entire plate was made using MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). Cycling conditions were: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". Samples were then pooled, purified, and sequenced on an Illumina MiniSeq platform employing paired-end 2x153 base reads. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. De-multiplexing of reads was performed on instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Genome Research Core (GRC) within the Research Resources Center (RRC).

Forward and reverse reads were merged using PEAR [157] and trimmed based on a quality threshold of p=0.01. Ambiguous nucleotides and primer sequences were removed and sequences shorter than 225 bp were discarded. Chimeric sequences were identified and removed using the USEARCH algorithm with a comparison to the Silva 132_16S reference database [158,159]. Amplicon sequence variants (ASVs) were identified using

DADA2 [160] and their taxonomic annotations determined using the UCLUST algorithm and Silva 132_16S reference with a minimum similarity threshold of 90% [158,159].

This sequencing effort yielded 4,443,016 reads. Raw sequence data files were submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) (BioProject #: PRJNA809693). One sample with fewer than 30,000 reads each was discarded. The average read count per sample was 67,318, where the minimum was 40,337 and the maximum was 134,816. Using MicrobiomeAnalyst [161] (updated version October 2021), samples were rarified to the minimum library size for each dataset. Low abundance ASVs were removed, *i.e.*, ASVs with < three counts in > 90% of the samples were removed, and low variance ASVs were also removed, *i.e.*, ASVs whose inter-quantile range was in the lowest 10% [161]. These corrections reduced the number of ASVs from 156 to 87 and 84 ASVs in the male and female mice datasets respectively. Count data were normalized with a centered log-ratio transformation.

Alpha-diversity was assessed using the Shannon H diversity index [165] with statistical significance determined by Kruskal-Wallis tests. Beta-diversity was assessed using Principal Coordinates Analysis (PCoA) of Bray-Curtis matrices with statistical significance determined by Permutational Multivariate Analysis of Variance (PERMANOVA) [202]. Taxa that associated with SCFA or *APP/PS1* status were determined using a classical univariate analysis with a Kruskal–Wallis test. A false discovery rate (FDR) approach was used to correct for multiple testing [161].

Bacteria associated with SCFA treatment or *APP/PS1* status were identified by a linear discriminant analysis effect size (LefSe) approach [164]. Significance thresholds were set to 0.05 for the alpha values for Kruskal-Wallis/Wilcoxon tests and 2.0 for the

logarithmic linear discriminant analysis (LDA) score, using a one-against-all multi-class analysis approach. These results were then plotted as a cladogram using the Huttenhower Galaxy resources to document the phylogenetic relatedness of SCFA associations with the bacteria at each taxonomic level [164].

3.2.3 Behavior Tests

Testing was performed by the Sanders-Brown Rodent Behavior Facility. Since robust behavioral deficits were previously identified with a Radial Arm Water Maze (RAWM) [203], we used this learning and memory task which takes advantage of the simple motivation provided by immersion into water. The radial arm water maze has been well characterized and used many times to detect a deficit in reference and working memory in the APP/PS1 mouse model [204-206]. The two-day RAWM test of spatial reference memory [207] was performed as previously described [208,209]. Mice were trained to find a hidden platform in one of eight arms using extramaze visual cues and were scored for number of errors made before finding the platform. The platform was kept in the same goal arm for each mouse, with the start arm sequence randomized such that all mice started from each of the other five arms (not including the goal nor the two arms directly adjacent) three times per day. Each trial lasted until the mouse found the platform or 60s had elapsed, whichever occurred first. Mice that failed to reach the platform in 60s were gently guided there and allowed to remain for 15s. Errors were counted as a mouse fully entering an incorrect (non-goal) arm or spending 15 consecutive seconds or longer in the same non-goal zone. On day one, mice were trained with 12 alternating hidden and visible platform trials followed by three hidden platform trials;

averaged across three consecutive trials into five blocks. On day two, mice again underwent 15 trials but with a hidden platform only. To ensure that any observed effects were not due to differences in vision or swimming ability, each mouse was tested in an open pool with no obstacles and the platform clearly identified (Figure S1). Each of the mice analyzed for microbiome were analyzed for behavior. Since females are known to have a larger amyloid burden in this model [210], results from males and females were analyzed separately by using a general linear model with treatment and transgene status and a treatment-transgene interaction term as main effects.

3.2.4 Gfap Expression and Amyloid β (Aβ) Accumulation

A random subset of mice were analyzed further for Gfap expression and Aβ quantitation. The number of mice included 22 males (nine APP/PS1 which included five on SCFA and four on saline, and 13 WT which included six on SCFA and seven on saline), and 16 females (eight APP/PS1 which included four on SCFA and four on saline, and eight WT which included three on SCFA and five on saline). Mice were deeply anesthetized with 5% isoflurane and then underwent transcardial perfusion with 50 ml ice-cold phosphate-buffered saline (PBS) at a flow rate of 10 ml/min before decapitation and brain removal and dissection. The right hemisphere was post-fixed in 4% paraformaldehyde for 24 h at 4 °C and cryo-protected in 30% sucrose for at least 48 h at 4 °C. Brains were then embedded in a solid matrix at 40 per block and sectioned coronally (MultiBrain processing by NeuroScience Associates, Knoxville, TN). For Gfap immunohistochemistry, free floating sections were treated with hydrogen peroxide, blocked and immunostained with Gfap (Dako, Catalog#: Z0334,1/1000), incubated overnight at room temperature, and labeled cells detected with a biotinylated secondary

antibody (Vector Lab), and diaminobenzidine tetrahydrochloride (DAB). To visualize amyloid deposits, sections were then subjected to a Campbell–Switzer silver stain. A detailed protocol for this stain can be found online at the NeuroScience Associates website: http://www.neuroscienceassociates.com/Documents/Publications/campbell-switzer_protocol.htm.

Gfap and amyloid staining was quantified in the dorsal hippocampus and overlying cortex by manually outlining these regions of interest in the HALO analysis suite (Indica Labs, version 2.3.2089.34) by an investigator blinded to experimental groups. The algorithm minimum intensity settings for all analyses were manually thresholded based upon negative control. Cortical and hippocampal analyses of Gfap and amyloid staining was quantified by using the area quantification algorithm (Area Quantification v.2.2.1) applied to the traced region across three-to-four sections per animal to give a single average count per square millimeter of tissue per region. Results from males and females were analyzed separately by using a general linear model with treatment and transgene status and a treatment-transgene interaction term as main effects.

3.2.5 MesoScale (MDS) Multiplex ELISA

Hippocampi and cortices were dissected from the left hemisphere to approximate the regions outlined for the amyloid staining analyses in the right hemisphere. Samples were snap frozen and kept at -80°C until Aβ quantitation. For this analysis, a random subset of APP/PS1 mice included nine males (which included four on SCFA and five on saline), and nine females (which included five on SCFA and four on saline). Soluble Aβ peptides were then quantified with an MSD approach as described previously [211]. Briefly, the PBS-soluble tissue fraction was prepared from each mouse by

homogenization with an Omni Bead Ruptor 24 (Omni International). Samples were homogenized in PBS lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma #P7626), 0.5 mM EDTA, and 0.2X Halt Protease Inhibitor Cocktail (Thermo Scientific #87786) and centrifuged at 12,000×*g* for 20 min at 4 °C. Supernatants were collected for $A\beta_{1–40}$ (Aβ40)/$A\beta_{1–42}$ (Aβ42) measurement using a human 6E10 Aβ kit (K15200E). All samples were run undiluted. Aβ peptide levels were normalized to the total mass of protein in the sample as determined by BCA Protein Assay (ThermoFisher #23225). Results were analyzed by using a general linear model with sex and treatment status as main effects.

3.3 Results

The purpose of this study was to test the effects of SCFA supplementation on APP/PS1 mice maintained in a standard laboratory environment. The SCFA and saline control treatments were well-tolerated by the mice. Mice maintained healthy coats and body weights were unaffected (Figure 1).

The effects of SCFA supplementation on the gut microbiome have not been reported previously. Therefore, we analyzed 16S rRNA gene amplicon sequencing results from fecal DNA samples. Since sex impacts the amyloid burden in the APP/PS1 model, males and females were analyzed separately [210]. We began with microbiome alpha-diversity (Shannon H index), which is a measure of within-sample diversity based on the richness and evenness of the taxa present. An alpha-diversity score was calculated using the Shannon H index for each sample. Both male and female mice showed a trend towards higher alpha-diversity with SCFA treatment, with male mice reaching statistical

significance at the taxonomic levels of order (p=0.0027), class (p=0.0027) and phylum (p=0.043) and female mice reaching significance at the genus (p=0.049) taxonomic level (Figures 2A and 2C, p-values for all taxonomic levels in Table 1). The presence of the transgene had no significant effect on alpha diversity at any taxonomic level in males or females ($p>0.05$). An increase in gut alpha-diversity, as associated with SCFA treatment here, is generally considered to be an indication of a healthier gut [212].

Beta-diversity is a measure of similarity of the microbial communities between samples. Beta-diversity scores were visualized using PCoA plots with Bray-Curtis distance measures. In both male and female mice, beta-diversity was generally not significantly affected by SCFA treatment (Table 2). However, in male mice, beta-diversity was significantly associated with SCFA treatment on the genus level ($R^2=0.073$, $p<0.049$), while in female mice, it was significantly associated with SCFA treatment on the species level ($R^2=0.086$, $p<0.009$) (Figures 2B and 2D, and scores for all taxonomic levels in Table 2). Although the p-values are significant, the R^2 values are low, a result of variability within group and overlap between groups, so these results may not have biological meaning.

Since alpha- and beta-diversity measures suggest some significant SCFA effects on the gut microbiome, additional analyses to identify specific taxa were performed. Taxa significantly associated with SCFA treatment were visualized with cladograms portraying phylogenetic relatedness between the taxa (Figures 3A and 4A). Significance was calculated using a linear discriminant analysis of effect size (LefSe). An additional classical univariate analysis with FDR-corrected p-values was used to generate box plots of individual taxa significantly associated with SCFA at all taxonomic levels (Figures 3B

and 4B). Both analyses identified similar taxonomic trends, with the classical univariate analyses identifying additional taxa.

In male mice, SCFA supplementation resulted in a significant increase in the relative abundance of the phylum Actinobacterium, which includes the order Bifidobacteriales of the class Actinobacterium and the order Coriobacteriales of the class Coriobacteria (Figure 3A). In contrast, SCFA treatment resulted in a significant decrease in the families *Prevotellaceae* and *Christensenellaceae* and the genus *Olsenella* (Figure 3, Supplemental files S1.1-1.6).

In female mice, SCFA supplementation significantly increased relative abundance of the genus *Anaeroplasma* of the phylum Tenericutes, and the genus *Lactobacillus* of the class Bacilli. In both sexes, the genus *Olsenella* was significantly increased in association with SCFA treatment, while the relative abundance of the family *Prevotellaceae* was decreased. Interestingly, SCFA treatment resulted in a significant increase of the species *Intestinale* from genus *Muribaculum* in female mice (Figure 4B), but a decrease in male mice (Figure 3B).

To determine whether SCFA supplementation improved cognition in the APP/PS1 mice, the animals were subjected to a RAWM test. The number of errors observed in male and female mice was determined on day one and day two (Figure 5). Since larger amyloid burden has been observed has been observed in female mice in this model [210], results from male and female mice were analyzed separately. These results were analyzed by a general linear model that included treatment status, transgene status, and treatment-transgene interaction (Figure 6).

The overall statistical model for male mice on day one was statistically significant ($F_{3,60}$=3.674, p=0.017), where transgene was significant (p=0.005), but treatment and treatment-transgene interaction were not significant ((p=0.789) and (p=0.264) respectively). Regarding transgene effects, the SCFA-treated APP/PS1 mice made more errors than the SCFA-treated WT mice (p=0.004, Figure 5). However, the number of errors committed by the saline-treated APP/PS1 mice was not significantly different from saline-treated WT mice (p=0.218). Overall, these findings indicate no treatment effect, and a transgene effect only in SCFA-treated mice.

The overall statistical model for female mice on day one was statistically significant ($F_{3,39}$=3.077, p=0.039), where transgene was significant (p=0.005), but treatment and treatment-transgene interaction were not significant ((p=0.454) and (p=0.351) respectively). Regarding transgene effects, SCFA-treated APP/PS1 mice made more errors than the SCFA-treated WT mice (p= 0.016, Figure 5). In contrast, saline-treated APP/PS1 mice were not significantly different from saline-treated WT mice (p=0.103). Overall, these findings again indicate no treatment effect in either APP/PS1 or WT mice and a transgene effect only in SCFA-treated mice.

The overall statistical model for males on day two was not statistically significant ($F_{3,58}$=1.068, p=0.370), where the overall transgene effect, treatment, and treatment-transgene interaction were not significant (p=0.334, p=0.816 and p=0.153, respectively, Figure 6). Overall, male mice did not display any significant difference in errors made on day two based on treatment, transgene, or a combined treatment-transgene interaction.

The overall statistical model for females on day two was statistically significant ($F_{3,39}$=3.746, p=0.019), where the overall transgene effect was significant (p=0.003),

while the treatment and treatment-transgene interaction were not significant (p=0.445 and p=0.606, respectively). On day two, the female saline-treated APP/PS1 mice made more errors than the saline-treated WT mice (p=0.008, Figure 6). The female SCFA-treated APP/PS1 mice showed a similar trend towards more errors than the SCFA-treated WT mice, but this trend did not reach significance (p=0.08, Figure 6). In summation, the female mice showed a robust transgene effect, especially in the saline-treated mice, while treatment and a treatment-transgene interaction were not significant.

To determine SCFA effects on glial activation in the mice, Gfap immunohistochemistry was performed (Figure 7). HALO was used to quantify the extent of robust Gfap staining in cortical and hippocampal slices. Results are presented as the percent of the region of the interest that was strongly Gfap positive (Figure 7). As detailed below, SCFA treatment did not significantly affect staining in the cortex or hippocampus in either male or female mice. The presence of the APP/PS1 transgenes significantly increased Gfap staining only in the cortex (Figure 7).

The overall statistical model for cortical Gfap staining in male mice was significant ($F_{3,17}$=3.554, p=0.037), where the transgene effect was significant (p=0.008), the treatment effect was not significant (p=0.333), and the transgene-treatment interaction was not significant (p=0.622, Figure 7). Specifically, the presence of the APP/PS1 transgenes significantly increased Gfap in the SCFA-treated mice (p=0.020), while the saline-treated mice showed a similar trend (p=0.106).

The overall statistical model for cortical Gfap staining in female mice was also significant ($F_{3,12}$=9.205, p=0.002), where the transgene effect was significant (p<0.001), and neither the treatment effect nor the transgene-treatment interaction were significant

(p=0.273 and p=0.637, respectively). Specifically, the presence of the APP/PS1 transgenes significantly increased Gfap in both the SCFA-treated mice (p=0.014) and the saline-treated mice (p=0.002).

The overall statistical models for hippocampal Gfap staining in male mice and female mice were not significant (($F_{3,17}$=0.443, p=0.0726) and ($F_{3,14}$=0.680, p=0.579), respectively). For each model, transgene, treatment, and transgene-treatment interaction were not significant.

To determine SCFA effects on amyloid accumulation in the APP/PS1 mice, amyloid burden was quantified in the cortex and in the hippocampus by both histochemistry and by MSD. For the histochemistry, HALO was used to quantify the percent area of the cortex and hippocampus ROI that was amyloid positive, and results analyzed by using a general linear model (Figure 8). The overall statistical models for cortical and hippocampal amyloid load were statistically significant (($F_{3,33}$=69.199, p<0.001) and ($F_{3,33}$=68.610, p<0.001) respectively). In the cortex, SCFA treatment again had no significant effect on amyloid ($F_{1,33}$=0.174, p=0.679). In the hippocampus, SCFA treatment had no significant effect ($F_{1,33}$=0.071, p=0.791) on amyloid burden.

Independent of treatment, female mice appeared to have robust amyloid deposition while male mice appeared to have a lighter amyloid burden (Figure 8A), as has been reported previously for this murine model [210]. Statistical analysis of the amyloid staining confirmed that female mice had a greater amyloid burden than male mice in the cortex ($F_{1,33}$=14.126, p<0.001) and in the hippocampus ($F_{1,33}$=14.484, p<0.001, Figure 8B).

To discern Aβ40 and Aβ42 independently, soluble amyloid peptide levels were quantified by MSD analyses. The results revealed robust levels of Aβ40 and Aβ42 in both the cortex and hippocampus (Figure 9). The overall models for cortical Aβ40 ($F_{2,15}$=5.360, p=0.018), hippocampal Aβ40 ($F_{2,15}$=9.146, p=0.003), cortical Aβ42 ($F_{2,15}$=21.492, p<0.001), and hippocampal Aβ42 ($F_{2,15}$=6.712, p=0.008), were statistically significant. SCFA treatment did not significantly impact cortical Aβ40 ($F_{1,15}$=0.375, p=0.549), hippocampal Aβ40 ($F_{1,15}$=1.069, p=0.318), cortical Aβ42 ($F_{1,15}$=3.549, p=0.079), or hippocampal Aβ42 ($F_{1,15}$=1.204, p=0.290).

Independent of treatment, female mice had significantly more cortical Aβ40 ($F_{1,15}$=10.705, p=0.005), hippocampal Aβ40 ($F_{1,15}$=16.077, p=0.001), cortical Aβ42 ($F_{1,15}$=42.798, p<0.001), and hippocampal Aβ42 ($F_{1,15}$=11.238, p=0.004) compared to the male mice (Figure 9). Overall, these findings that female mice had significantly more amyloid than male mice, but that SCFA treatment had no significant effect on amyloid, confirm the results of the histochemistry analyses.

3.4 Discussion

The goal of this study was to test the effects of SCFA supplementation in APP/PS1 mice maintained in a conventional laboratory animal environment. Our primary findings were that SCFA treatment significantly impacted the gut microbiome but had no effect on spatial memory deficits, glial activation nor amyloid burden in this model. In both male and female mice, SCFA supplementation was associated with increased alpha-diversity and increased relative abundance of several taxa associated with SCFA-production. Although a behavioral deficit was associated with the APP/PS1 transgenes, no effects

were detected following SCFA treatment. SCFA supplementation also had no significant effect on Gfap expression or amyloid burden. Overall, we interpret these findings as indicating that SCFA supplementation affects the gut microbiome, but not the hallmarks associated with this preclinical model of AD.

SCFA supplementation increased several bacteria in male and female mice. Based on LefSe analyses, the genera *Bifidobacterium, Olsenella* and *Odoribacter* increased in male mice, and the genera *Lactobacillus, Olsenella* and *Anaeroplasma* increased in female mice. Interestingly, of these taxa, the genera *Bifidobacterium* and *Lactobacillus* are known to produce SCFAs and SCFA precursors (reviewed in [213]). Overall, these findings suggest a possible feedforward effect of SCFA supplementation, in which SCFAs increase the relative abundance of bacteria that produce SCFAs. Further studies are needed to corroborate this finding.

Several studies report a role of SCFAs in the brain. For example, treatment of astrocyte or microglia *in vitro* with the SCFA acetate has been shown to reverse LPS-induced astrocytic activation and inhibit NFkB signaling [177,214]. *In vivo*, SCFA treatment impacts microglial morphology, transcriptome, and response to stimuli, such as LPS [123]. The mechanisms by which SCFAs act on cells within the brain is under intense scrutiny. SCFAs have been found to inhibit histone deacetylation, thereby affecting gene expression and inflammation (reviewed in [215]). For example, treatment of mice with sodium butyrate reduces histone deacetylase (HDAC) activity in the gut, associated immune cells and the central nervous system ([216]; reviewed in [215]). A second mechanism that may mediate SCFA activity in the brain is the binding of SCFAs to free fatty acid receptors FFAR2 and FFAR3 (reviewed in [176,215]). While these receptors are

mostly expressed in intestinal mucosal cells and immune cells, FFAR3 is also expressed by neurons in the periphery [217]. FFAR2 and FFAR3 expression in the brain has not been reported. SCFA actions in the brain depend upon their transport across the blood brain barrier. This is mediated by monocarboxylate transporters which are expressed at high levels in the endothelial cells of the blood brain barrier (reviewed in [215]).

Several factors may influence SCFA levels *in vivo*. First, diet has been shown to clearly modulate SCFA levels because components of soluble fiber such as inulin are metabolized to SCFAs by the gut microbiome (reviewed in [218,219]). In this study, the mice were maintained on the Teklad Global 18% Protein Rodent Diet which contains ingredients such as ground wheat, ground corn and wheat middlings that are a source of soluble fiber that is metabolized to SCFAs by the gut [199,218,219]. Second, the profile of bacteria within the gut impacts SCFA levels because certain bacteria are particularly proficient at generating SCFAs ([220], reviewed in [213]). Third, genetics may impact SCFA levels. For example, *APOE4*, which is associated with increased LDL-cholesterol and AD risk, relative to *APOE3*, is associated with a gut microbiome profile with reduced SCFA-producing bacteria, such as *Ruminococcaceae* [94,115-117].

Because the gut microbiome produces SCFAs and has been shown to modulate AD pathology, and SCFAs may act in the brain, several groups have investigated the effects of SCFA treatment on amyloid accumulation in the brain. Results have not been consistent (Table 3). Colombo et al. found that SCFA treatment increased amyloid burden in APPPS1 mice [101]. In contrast, Fernando et al. and Jiang et al. found that sodium butyrate treatment reduced amyloid burden in 5xFAD mice [44,129]. Here, we treated APP/PS1 mice with SCFAs and found no effect on amyloid burden. In the

following paragraphs, we will compare and contrast prior results with the results presented here.

Differences in the experimental designs of these studies are multiple (Table 3). One difference is the mode of SCFA administration and type of SCFA. Our study and that of Colombo et al. were similar in that SCFAs were administered in the drinking water at identical concentrations. However, they differ in that the control group in Colombo et al. received water while the control group in our study received saline (132.5mM) as their drinking water such that their sodium intake was equal to that of the SCFA treated mice. Interestingly, APP/PS1 mice that received about three-times more sodium chloride than our control group were reported to have reduced amyloid plaques [221,222]. Whether comparing SCFA treatment to saline treatment may have obscured a SCFA effect in our study is not clear. Jiang et al. used intraperitoneal injection of sodium butyrate while Fernando administered sodium butyrate via chow [44,129].

These SCFA studies also used different mouse models (Table 3). The Colombo et al. and Jiang et al. studies used mice maintained on a SPF microbiome background while mice in our study and Fernando et al. were maintained with a conventional microbiome. Since altering the microbiome with antibiotics reduces amyloid burden, differences in the microbiome may contribute to the differences observed in these studies [97,98,102]. The mouse models also differed in that Colombo et al. used an APPPS1 model wherein the *APPPS1* transgenes were driven by the Thy1 promoter, and the PS1 mutation was L166P [101]. This mouse model begins to deposit amyloid at six weeks of age and Columbo et al. started SCFA treatment at eight weeks of age for a duration of five weeks. Jiang et al. and Fernando et al. used the 5xFAD murine model wherein extracellular amyloid deposits

begin at eight weeks of age. Both studies began butyrate treatment at eight weeks. Jiang et al. treated for two weeks while Fernando et al. treated for 12 weeks. In our study, the *APP/PS1* transgene was driven by the mouse prion protein promoter, and the PS1 mutation was deletion of exon 9 [195]. This mouse model begins to deposit amyloid at four to six months of age. We began SCFA treatment at five months of age for a duration of five months. Hence, the studies are similar in that mice underwent SCFA treatment during the time that amyloid was accumulating. The studies are different in that (i) Colombo et al, Jiang et al. and Fernando et al. used mouse models with earlier amyloid deposition compared to the APP/PS1 model in our study; and (ii) Colombo et al. and Jiang et al. used SPF mice while Fernando et al. and our study used mice with a conventional microbiome.

Considering these variables and the mixed study results, we speculate the treatment with butyrate per se reduces amyloid burden because similar results were found with two different routes of administration and with SPF and conventional microbiomes (Table 3). In contrast, treatment with a mixture of acetate, propionate and butyrate produces results that appear model dependent. We propose that future studies investigating the effects of individual SCFAs may provide clarity to this field.

In conclusion, this is the first study to robustly evaluate SCFA supplementation effects on the gut microbiome itself, in addition to brain pathology and behavior, which have been reported on in previous studies. We found that SCFA treatment increased levels of SCFA-producing bacteria *Lactobacillus* and *Bifidobacterium* in a possible feedforward mechanism. Consistent with prior reports, female APPswe/PSEN1dE9 mice had a greater amyloid burden and memory deficit than male mice [210]. However,

inconsistent with prior reports, we did not detect an effect of SCFA supplementation on behavioral impairment or amyloid burden. We recognize that murine models of AD are pre-clinical, and so results are used to inform more physiologically relevant human studies. Given the conflicting results in these pre-clinical models, further studies are necessary to provide clarity to this emerging area.

Alpha-diversity p-values		
	Males	**Females**
Species	0.17	0.43
Genus	0.056	**0.049**
Family	0.19	0.37
Order	**0.0027**	0.15
Class	**0.0027**	0.18
Phylum	**0.043**	0.29

Table 4. Alpha-diversity for SCFA mice.

Microbiome alpha-diversity was significantly associated with SCFA supplementation in male mice. P-values reflect nominal p-values and were determined using Kruskal-Wallis tests. P-values less than 0.05 are presented in bold font.

Beta-diversity scores			
Taxonomic level		**Males**	**Females**
Species	**p-value**	0.300	**0.009**
	R^2	0.036	0.140
Genus	**p-value**	**0.049**	0.140
	R^2	0.073	0.051
Family	**p-value**	0.120	0.160
	R^2	0.052	0.051
Order	**p-value**	0.310	0.550
	R^2	0.035	0.023
Class	**p-value**	0.280	0.720
	R^2	0.038	0.015
Phylum	**p-value**	0.440	0.440
	R^2	0.026	0.028

Table 5. Beta-diversity for SCFA mice.

Microbiome beta-diversity did not significantly associate with SCFA treatment in both male and female mice. The R^2 values represent the proportion of the variance captured by SCFA versus saline treatment. The p-values were derived from analysis of 999 randomized permutations. P-values less than 0.05 are presented in bold font.

Study	Treatment	Mouse Model	Microbiome	Mouse Age during Treatment	Effect on Amyloid Burden
Fernando et al [129]	NaB was added to chow pellets at a concentration of either 40 mg/kg or 120 mg/kg where mice would receive either 5 mg/kg/day, or 15 mg/kg/day	5xFAD with APOE3	Conventional	From 8 weeks to 20 weeks	Reduced
Jiang et al [44]	0.2 g/kg daily intraperitoneal injection of NaB (0.1 ml/10 g) *	5xFAD	SPF	From 8 weeks to 10 weeks	Reduced
Colombo et al [101]	67.5 mM sodium acetate, 25mM sodium propionate, 40 mM sodium butyrate, pH 6.8 in drinking water	APPPS1	SPF	From 8 weeks to 13 weeks	Increased
Zajac et al [223]	67.5 mM sodium acetate, 25mM sodium propionate, 40 mM sodium butyrate, pH 6.8 in drinking water *	APP/PS1	Conventional	From 20 weeks to 40 weeks	No change

Table 6. Comparison of studies evaluating SCFA effects on murine amyloid models.

* SCFA treatment was compared to saline control.

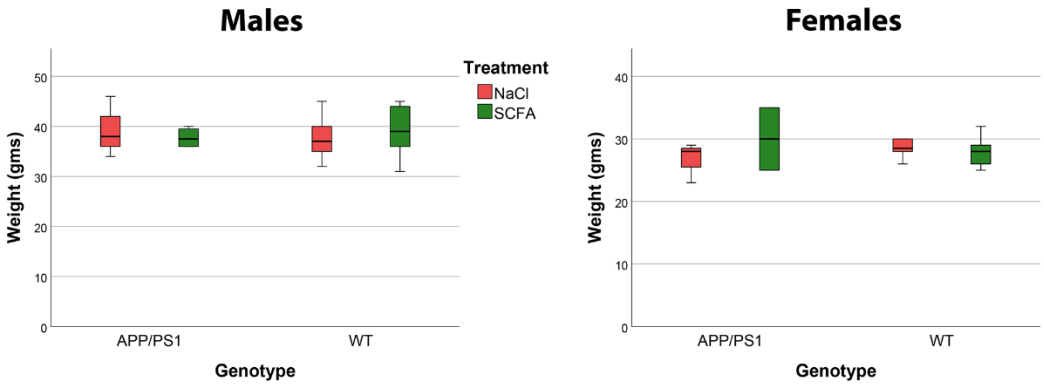
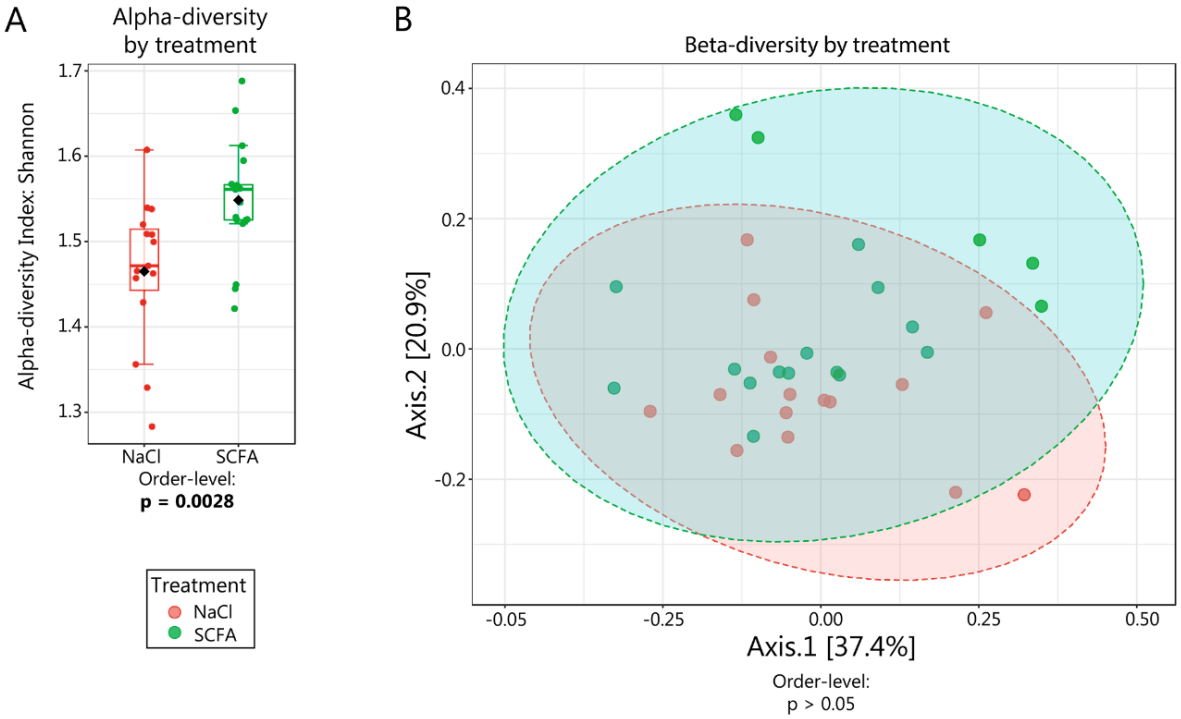


Figure 7. Mouse body weights in the SCFA study.

Mouse body weights were not affected by short chain fatty acid (SCFA) supplementation. Male mice weighed significantly more than female mice ($p < 0.0001$), but body weight was not influenced by SCFA-supplemented drinking water (A) or by the presence of the APP/PS1 transgenes (B) ($p > 0.05$). These data reflect weights on the day of euthanasia.

Males: Fecal Microbiome



Females: Fecal Microbiome

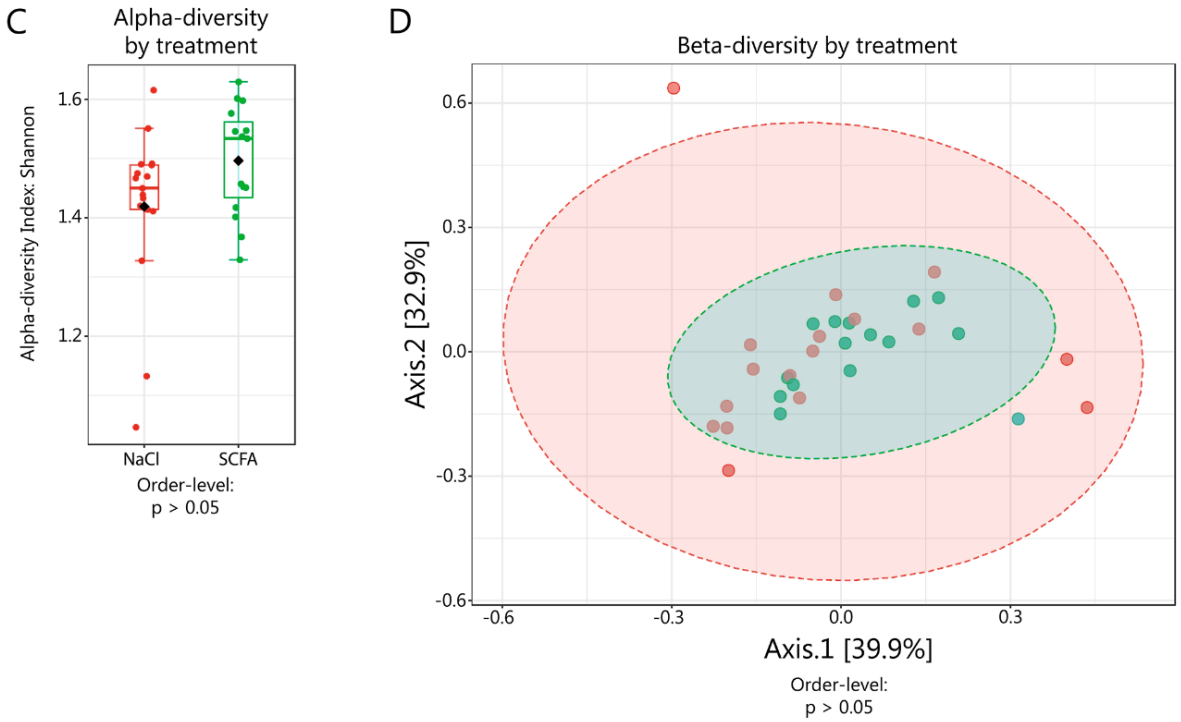


Figure 8. Alpha- and beta-diversity plots for SCFA mice

Microbiome alpha- and beta-diversity as a function of short chain fatty acid (SCFA) treatment. Alpha-diversity (Shannon H index) data are depicted in boxplots (A,C). Beta-diversity analyses are visualized in PCoA plots (B,D). Statistical significance for the findings is indicated below each graph. Ellipses in (B,D) represent 95% confidence intervals. For male mice (B) $R2 = 0.035$ and for female mice (D) $R2 = 0.023$. Beta-diversity was also analyzed using PERMDISP, which had no significant p-values except in females at the species level ($p = 0.027$), indicating that there was generally no difference in dispersion between SCFA vs saline treated groups.

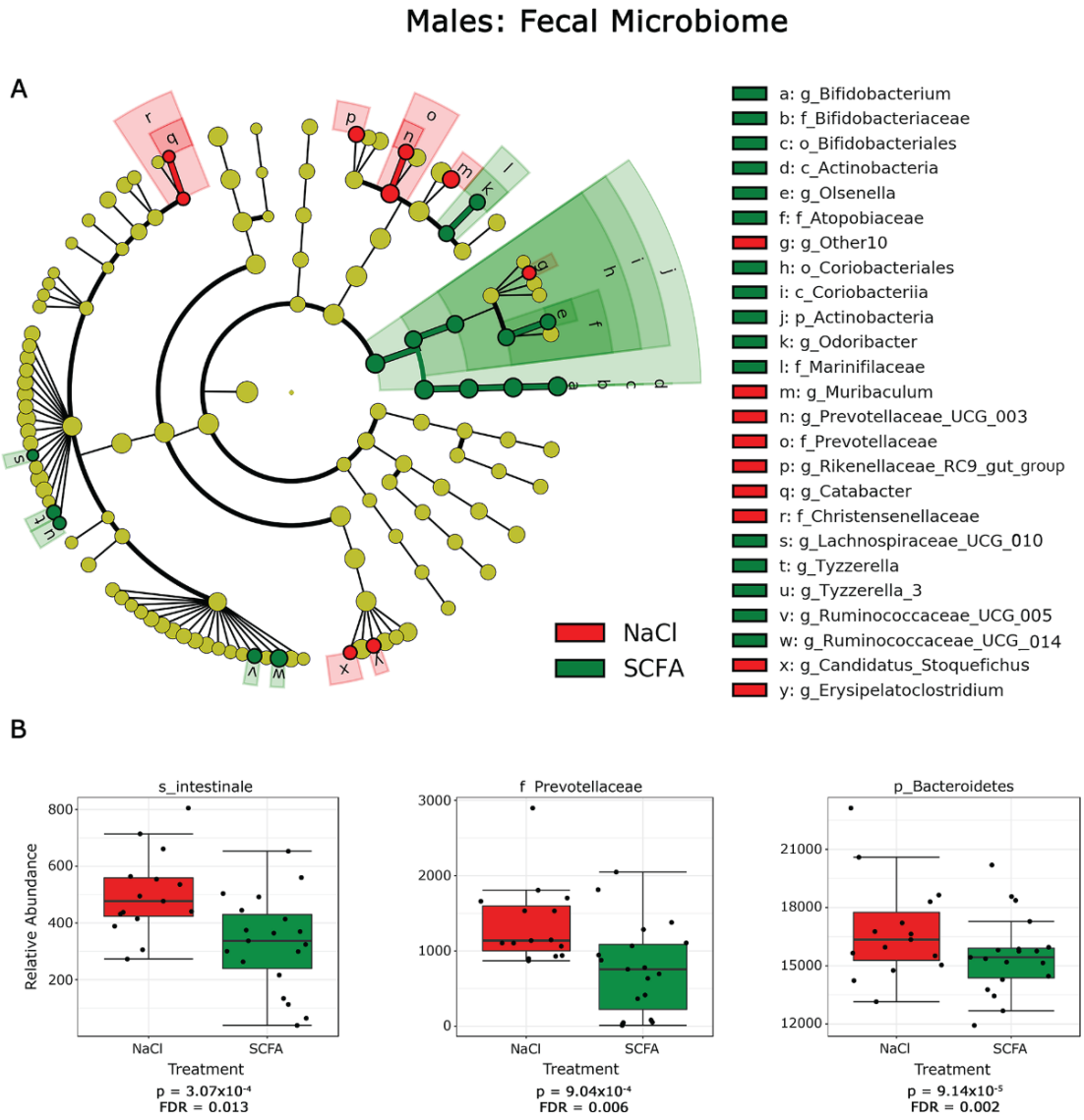


Figure 9. Cladograms and box plots of taxa for male mice.

Cladograms and box plots of individual taxa reveal microbial phylogenetic branches associated with short chain fatty acid (SCFA) supplementation in male mice. (A) Taxa are represented as nodes that are connected by lines based on the phylogenetic relatedness of all taxa present in each experimental cohort. For example, the end node, a, represents the genus *Bifodobacterium* which is connected to other nodes representing higher level taxa related to *Bifodobacterium* including b the family *Bifidobacteriaceae*, c the order Bifidobacteriales, and d the class Actinobacteria. Many taxa are associated with SCFA vs saline treatment, with node colors indicating treatment with highest levels of each taxa. Statistical significance reflects both $p < 0.05$ for Kruskal-Wallis tests and a logarithmic LDA score > 2.0. (B) Box plots present the relative abundance of individual taxa that are significantly associated with SCFA supplementation by classical univariate analysis. A full list of taxa with nominal p-values and FDR corrected p-values is provided in Supplementary Material 1.1–1.6.

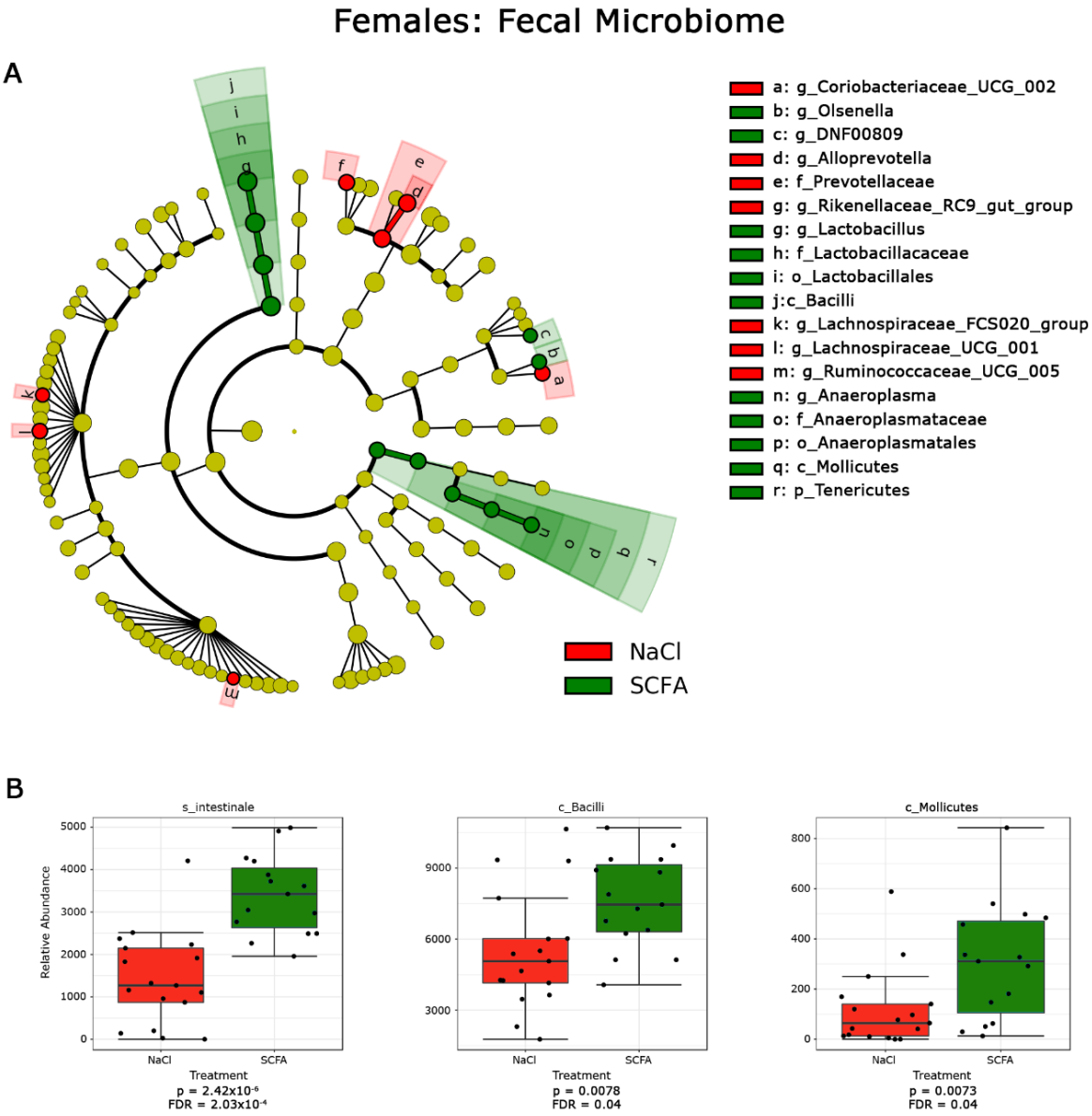


Figure 10. Cladograms and box plots of taxa for female mice.

Cladograms and box plots of individual taxa reveal microbial phylogenetic branches associated with short chain fatty acid (SCFA) supplementation in female mice. **(A)** Statistical significance reflects both $p < 0.05$ for Kruskal-Wallis tests and a logarithmic LDA score > 2.0. **(B)** Box plots present the relative abundance of individual taxa that are significantly associated with SCFA supplementation by classical univariate analysis. A full list of taxa with nominal p-values and FDR corrected *p*-values is provided in Supplementary Material 1.1–1.6.

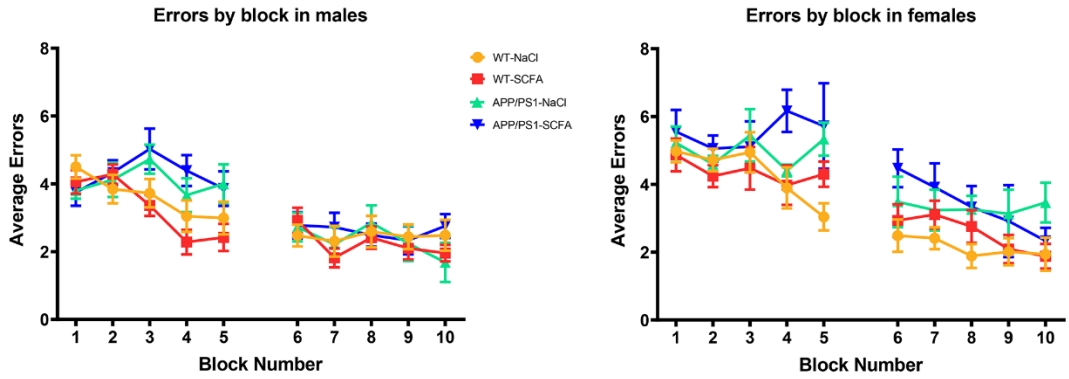


Figure 11. RAWM results for day one and day two trials.

RAWM results for day one and day two trials. Data are represented as means of three trials per block of all animals within the group, with five blocks per day. Day one was training day, while day two was testing day. Error bars are standard deviation of the mean.

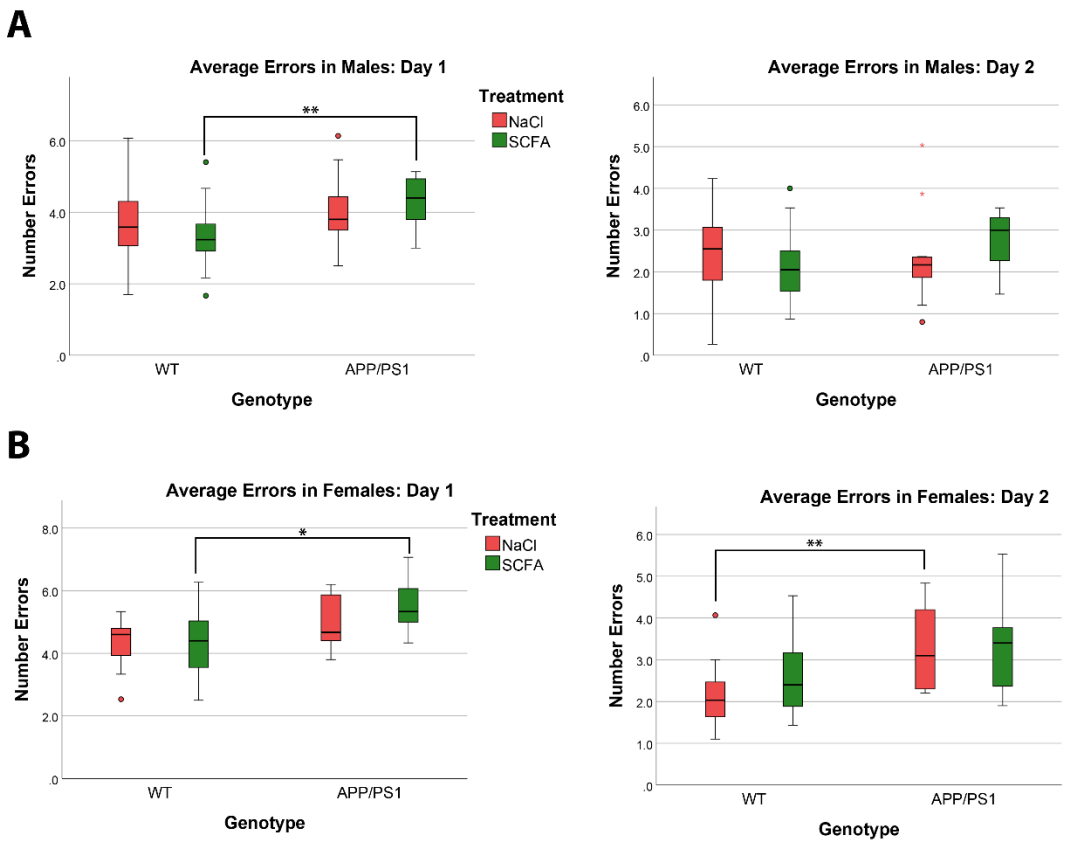


Figure 12. RAWM average errors in the mice.

Female but not male mice show cognitive deficits that are unaffected by short chain fatty acids (SCFA) treatment. The average number of errors on day one and day two are shown. On day one, significant differences were observed for both male and female mice between SCFA treated WT vs. APP/PS1 mice (A,B). On day two, significant differences were observed only for female saline-treated WT vs. APP/PS1 mice (B). Statistical significance was determined by using a general linear model (* = $p < 0.05$, ** = $p < 0.01$).

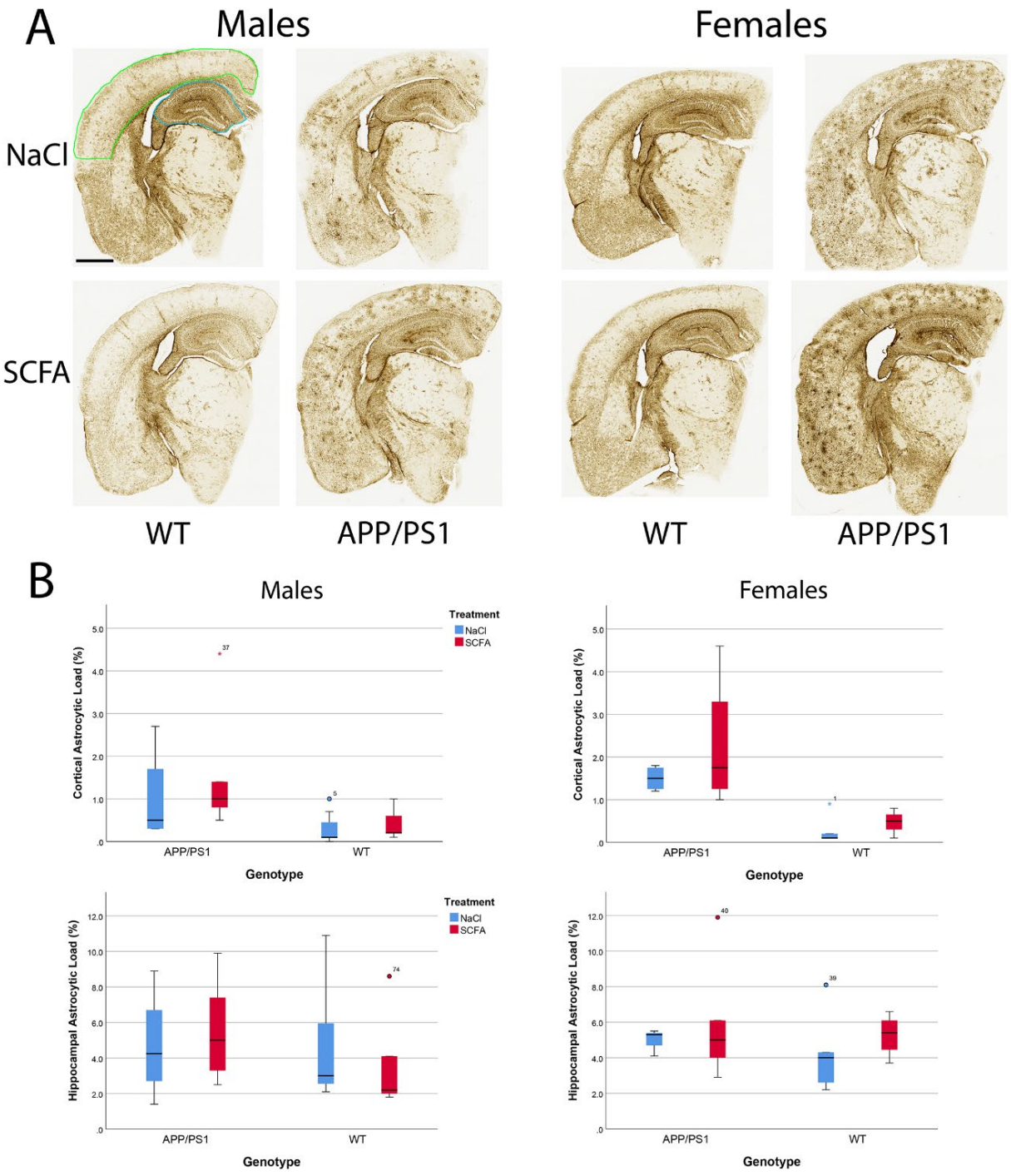


Figure 13. Gfap staining and quantification.

Gfap staining and quantification indicate short chain fatty acids (SCFA) has no effect although labeling is increased in cortices of APP/PS1 mice. Gfap expression was detected by Gfap immunohistochemistry (A). Scale bar represents 1 mm. Green outlining indicates the typical ROI for cortical staining while blue outlining indicates the ROI for hippocampal staining. Statistical analysis confirms a significant difference with APP/PS1 transgenes but not SCFA treatment in the cortex (B). Staining was quantified using HALO Area Quantification v.2.2.1. Statistical significance was determined by using a general linear model. Datapoints marked as circles represent outliers (3rd quartile + 1.5 × interquartile range) while asterisks mark extreme outliers (3rd quartile + 3 × interquartile range).

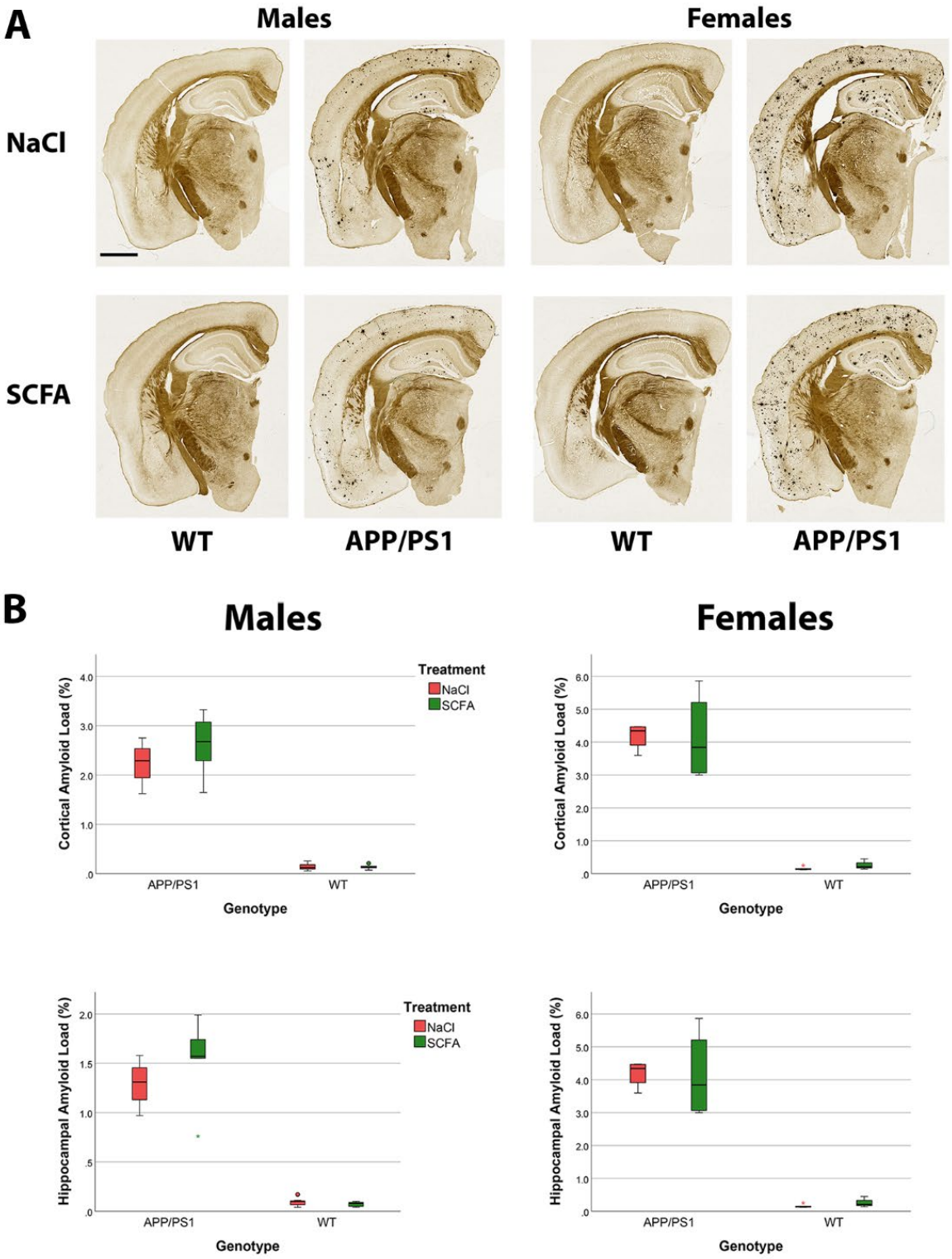


Figure 14. Amyloid staining and quantification.

Amyloid staining and quantification indicate no significant short chain fatty acids (SCFA) effect although an increased amyloid load is observed in female mice. The Campbell–Switzer silver stain labels both parenchymal amyloid and cerebral vascular amyloid (A). Scale bar represents 1 mm. Statistical analysis confirms a significant difference with sex and genotype but not SCFA treatment (B). Staining was quantified using HALO Area Quantification v.2.2.1. Statistical significance was determined by using a general linear model. Datapoints marked as circles represent outliers (3rd quartile +/− 1.5 × interquartile range) while asterisks mark extreme outliers (3rd quartile +/− 3 × interquartile range).

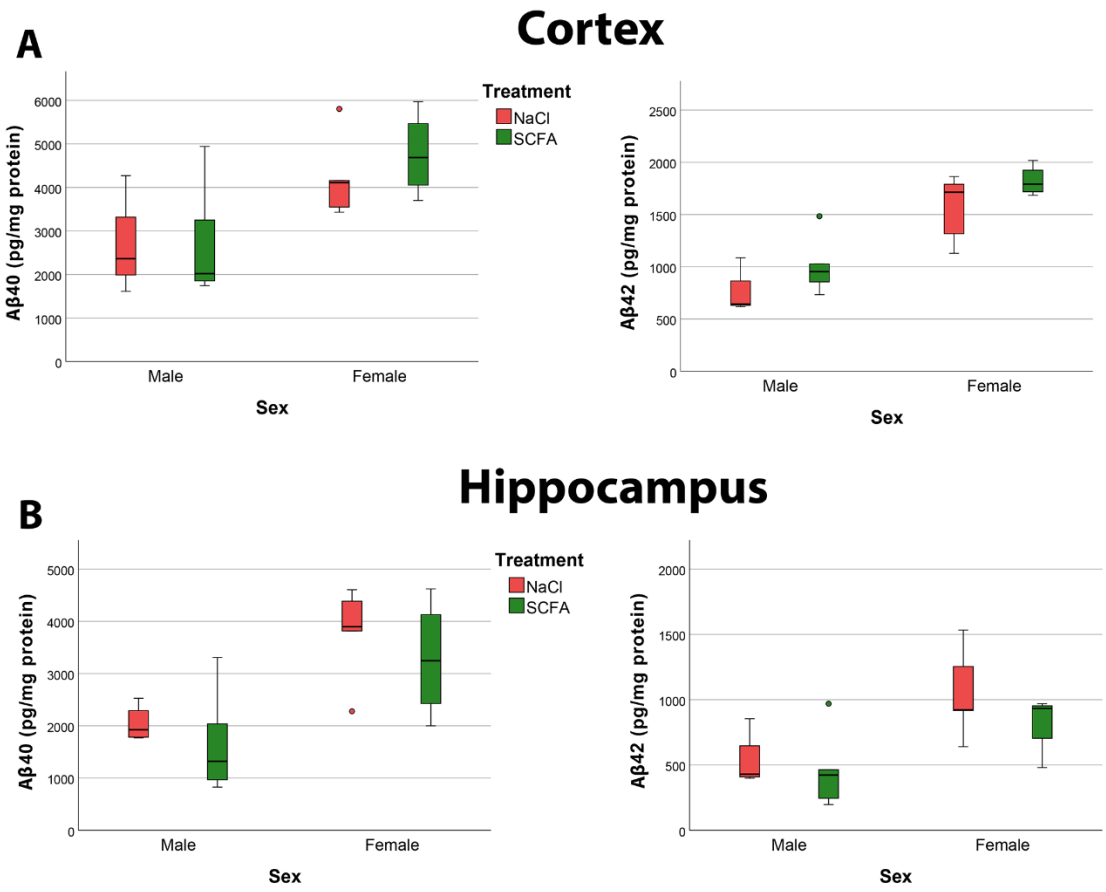


Figure 15. Amyloid MSD quantification of Aβ40 and Aβ42 .

Amyloid MSD indicates no significant effect of short chain fatty acids (SCFA) on Aβ40 and Aβ42 and confirm that female mice had significantly more Aβ. Quantification of Aβ40 and Aβ42 in the cortex (A) and hippocampus (B) reveals a significant difference with sex but not with SCFA treatment. Statistical significance was determined by using a general linear model.

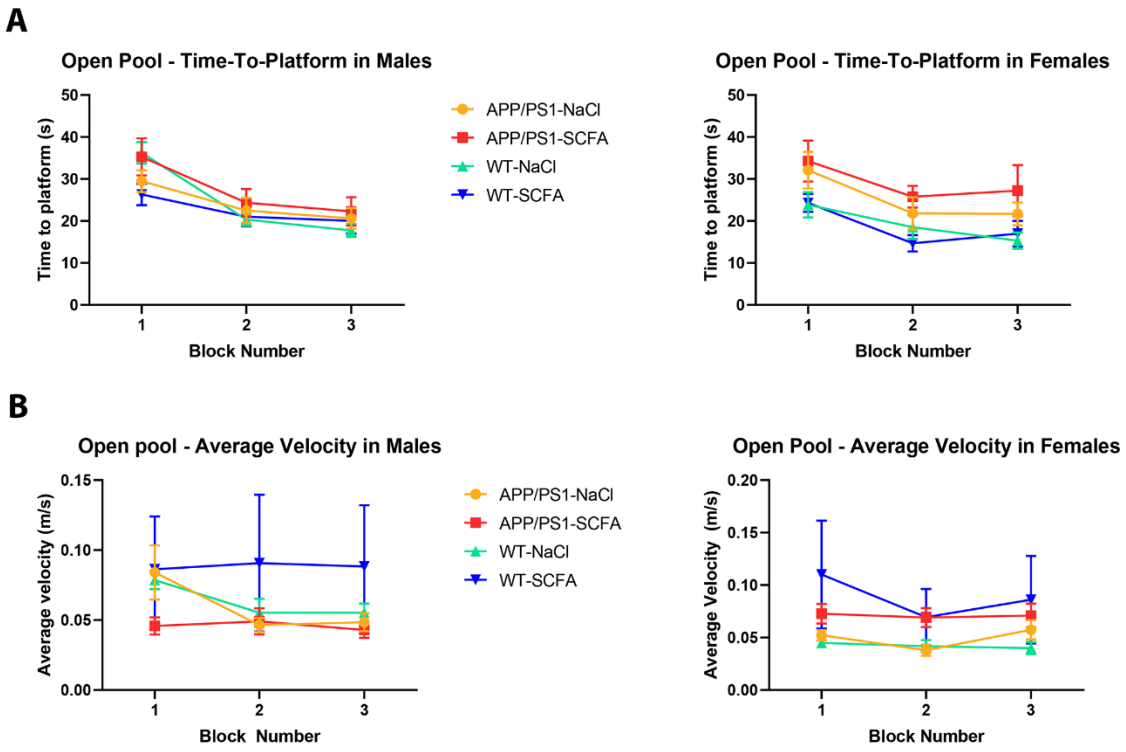


Figure 16. Supplemental Figure S1 shows Open Pool data.

Supplemental Figure S1 shows Open Pool data for all groups to validate the normal physical ability of the mice to swim and navigate in the water.

CHAPTER 4. DISCUSSION AND FUTURE DIRECTIONS

4.1 Primary findings and summary of dissertation

The primary findings of this dissertation are two-fold: The first is that *APOE* genetics are differential for murine gut microbiome profiles in a way that may potentially inform a gut microbiome influence on disease risk. The second is that SCFAs increase murine gut microbiome diversity, but alone may not be sufficient to cause significant changes in cognition or AD-like pathology.

First, *APOE4* status is associated with increased murine gut microbial diversity, and *APOE2* status is associated with an increase in bacteria known to produce SCFAs. The findings presented in Chapter 2 support previous research in that *APOE4* status can be GI protective as evidenced by increased alpha-diversity. This may be an evolutionary trait that is still beneficial in third-world environments, but also has detrimental effects later in life, increasing risk for AD. The finding that *APOE2* status is associated with increased SCFA-producing bacteria, specifically Ruminococcaceae and Lachnospiraceae, should be further explored, as other researchers have found that specific genera from these families increase in *APOE2* carriers, while other genera from these families increase in *APOE4* carriers. Functional pathway analysis for these bacterial taxa will shed light on the mechanisms involved, and whether the *APOE*-specific gut microbiome influences *APOE*-associated AD pathology or is simply a product of it.

Second, although SCFA treatment affects the murine gut microbiome, it is not necessarily sufficient to affect amyloid pathology in the brain. Specifically, in Chapter 3 I show that SCFA treatment of APP/PS1 mice has a sex-specific effect, where male mice show an increase in some SCFA-producing taxa, while female mice show a decrease in

the same taxa. Regarding the effects of SCFA treatment on cognition and pathology, there were no significant changes, although some trends could be observed that suggest this is an area that needs further study. Findings from Fernando et al., Jiang et al. and Colombo et al. only complicate the interpretation of the data, where they see opposing effects that seem dependent on the AD mouse model and the microbial environment of the mice. 5xFAD mice, both SPF and those raised maintained on a conventional microbiome, showed a reduction of amyloid pathology with SCFA treatment [44,101,129]. However, APPPS1 mice maintained in an SPF environment showed increased amyloid pathology with SCFA treatment [101], whereas APP/PS1 mice in our study maintained on a conventional microbiome showed no effect of SCFA treatment on amyloid pathology. A careful review of the literature leads me to suggest that the effects of SCFA treatment may only be discernable in mice with a depleted gut microbiome, such as SPF or GF. In addition, basal plasma and brain levels of SCFAs should be measured, as well as during and post-treatment, to ascertain what amount is entering the bloodstream and crossing the BBB. However, it is unknown if SCFAs must cross the BBB to modulate microglia activity and other functions in the brain. The mechanisms by which SCFAs produced in the gut may modulate CNS functions are still being investigated and future studies may inform us of the pathway involved in SCFA microglial modulation.

The major findings regarding the effects of *APOE* on the murine gut microbiome and in related studies suggest that *APOE2* status is associated with an increase in SCFA-producing bacteria and lower alpha-diversity, while *APOE4* status is associated with an increase in bacteria from the family Erysipelotrichaceae and higher alpha-diversity. The *APOE4* association with increased alpha-diversity supports the findings by Oria et al. that

APOE4 protects children from GI insult [107,108]. Although SCFA studies show varying results that may reflect different factors in the study design, such as mouse model, treatment method and the laboratory microbial environment (i.e. conventional vs SPF or GF mice), the results from my study suggest that male APP/PS1 mice have increased SCFA-producing bacteria (i.e. *Ruminococcaceae* and *Lachnospiraceae*) with SCFA-treatment, while female mice show a decrease in these bacteria with SCFA-treatment. Human studies show an association of AD with increased levels of fecal SCFA levels [81]. In our study, there is no correlation between SCFA-producing bacterial relative abundance and alpha-diversity, as there is no significant sex difference in alpha-diversity, so it cannot be assumed that SCFA-producing bacteria alone increase diversity in the gut microbiome. In addition, increased alpha-diversity is generally associated with a healthy gut barrier, decreased inflammation and reduced risk of chronic diseases (reviewed in [167,224]). The AD-protective allele *APOE2* is associated with reduced alpha-diversity in these mouse studies, but further evidence parsing AD vs control patients for dysbiosis, as a factor of *APOE* status is needed to understand this complex relationship. Although increased gut diversity may overall be protective from chronic diseases such as AD, current evidence does not clarify the relationship between SCFA's, gut diversity, *APOE* status and overall health in humans, nor in animal models.

Similar work in the field illustrates the complexity of the gut microbiome-AD relationship and is evidence that further work is needed to understand *APOE* effects on the gut microbiome and the viability of SCFA treatment for the reduction of AD pathology.

4.2 Future Directions: *APOE* effects on the gut microbiome

My work on *APOE* genetics leading to altered gut microbiome profiles, as well as the work of other researchers, provides evidence of an *APOE* bias on gut microbial diversity. Indeed, studies in both humans and animal models of *APOE* corroborate that *APOE4* carriers are resistant to GI insult and gut dysbiosis. There is also evidence suggesting that gut microbiome modulation influences AD pathology, and that gut microbiome profiles differ significantly in patients with MCI and AD compared to healthy controls. This suggests that *APOE* effects on the gut microbiome may be an additional pathway by which *APOE* modulates AD risk. Current studies of *APOE* effects on the gut microbiome provide us with insight into which, if any, bacterial taxa are most differential for *APOE*. Currently, evidence supports that taxa of the *Erysipelotrichaceae* family are increased in *APOE4* carriers, in both humans and murine models, while SCFA-producing taxa of the *Ruminococcaceae* and *Lachnospiraceae* families are increased in *APOE2* carriers. However, whether these taxa also modulate disease is unclear. Additionally, the strongest *APOE* effects are seen in male mice compared to female mice, where female mice may be more resistant to gut microbiome changes. I suggest future studies of the *APOE*-gut microbiome-AD relationship focus on investigating the therapeutic potential of these differential taxa. One possible model that is used is the addition of specific bacteria to GF mice, which can be done in AD models such as APP/PS1 or 5xFAD. So, a possible study could be to inoculate GF 5xFAD mice with the *APOE2*-associated *Ruminococcaceae* taxa versus the *APOE4*-associated *Erysipelotrichaceae* taxa, and measuring changes in the gut microbiome, amyloid pathology and cognition. The hypothesis would be that mice inoculated with Ruminococcaceae taxa would display a phenotype similar to an *APOE2*-crossed 5xFAD

mouse, and that mice inoculated with *Erysipelotrichaceae* taxa would display a phenotype similar to an *APO4*-crossed 5xFAD mouse. *APOE4*-5xFAD mice typically have increased levels of Aβ, Aβ deposition, and amyloid plaque pathology in the brain compared to *APOE3*-5xFAD mice, with the lowest levels in *APOE2*-5xFAD mice (reviewed in [225]). This would suggest that *APOE* effects on the gut microbiome are an additional risk factor for AD.

Fecal microbiome transplant (FMT) models are also widely used to investigate the effects of the gut microbiome on disease state [226-229]. I propose another study wherein *APOE*-crossed 5xFAD mice that develop AD pathology reflective of *APOE* status have their gut microbiomes antibiotically knocked down and are reinoculated with the gut microbiome of their opposite *APOE* counterparts. Specifically, *APOE4* mice will receive FMT from *APOE2* mice, and *APOE2* mice will receive FMT from *APOE4* mice, without any additional changes to diet or environment. The hypothesis would be that *APOE4* mice with *APOE2* gut microbiomes would show a reduction in amyloid pathology and rescue in cognition, and the opposite for *APOE2* mice with an *APOE4* gut microbiome.

4.3 Future Directions: SCFAs as a potential therapeutic agent for Alzheimer's Disease

Our current understanding of the health benefits of dietary fiber intake suggests that gut bacterial metabolites, such as SCFAs, are also beneficial to our health. Butyrate, one of the primary SCFAs, is the main source of energy for colonocytes and is essential for the healthy function of the colon, strengthening the integrity of the mucosal wall and preventing leaky gut syndrome, which is highly associated with many inflammatory

peripheral dysfunctions [230,231]. In Chapter 3, I investigate the effects of SCFA treatment on the gut microbiome, memory, astrocyte activation and amyloid pathology in APP/PS1 mice. My findings provide evidence that SCFA treatment can be used to increase gut microbial diversity, but that this treatment may not be sufficient on its own to ameliorate the disease phenotype. When comparing to similar studies of SCFA treatment on AD model mice, I observe that mouse model, treatment duration, and background microbial environment/diet may lead to contrasting effects of SCFA treatment. Furthermore, studies investigating the effects of SCFA treatment in murine models report mixed results regarding plasma concentrations of SCFAs post-treatment [101,223,232], whereas some studies simply do not report the plasma levels. In my study presented in Chapter 3, I find no significant changes to plasma SCFA levels post-treatment, unlike Colombo et al. who shows a significant increase in plasma SCFA levels post-treatment. I hypothesize this may be dependent on whether the murine model is GF or SPF versus maintained on a conventional microbiome. To gain a clearer understanding of the impact of SCFAs in relation to AD, I propose several avenues of investigation: (i) SCFA treatment may not be sufficient to alter SCFA plasma levels, so further clarity as to the most effective treatment method is needed. I propose future studies to include brain and plasma concentrations immediately post-treatment and hours after to distinguish the relationship between gut, blood, and brain levels of SCFAs post-treatment. (ii) since studies where mice are maintained on a limited microbiome environment (i.e. GF or SPF) seem to have a more profound impact on AD pathology, which may be due to increased microbial diversity rather than specifically SCFAs, future studies should compare recolonization of the gut with random bacteria/bacterial metabolites to SCFA-specific

treatment. (iii) our study found a sex difference where SCFA-treated male mice showed an increase in SCFA-producing bacteria but female mice showed a decrease in the same bacteria, so it is unclear how SCFAs as a probiotic affect the levels of SCFA-producing bacteria in the gut, and why this may be different between males and females. To elucidate if SCFAs act in a sex-dependent manner, I propose a SCFA study be done comparing gonadectomized AD mice to controls. Ours was the only SCFA AD-mouse study that included gut microbiome profiling between treatment groups, so future studies would benefit from gut microbiome data that will further clarify the effect of SCFA treatment on the gut microbiome between different mouse models, sexes and administration methods.

4.4 Future Directions: Gut microbiome as a proposed therapeutic target for Alzheimer's Disease

Overall, there is evidence that AD genetics may be differential for gut microbiome diversity and specific taxa, possibly associated with SCFA production. Current studies investigating the validity of the gut microbiome as a possible therapeutic for AD have focused on pro- and pre-biotic treatments, as well as plant-based diets high in dietary fibers, such as the Mediterranean diet, which improves gut health and increases SCFAs [233], as dietary intervention strategies [234] (reviewed in [235,236]). Indeed, the Mediterranean diet and Dietary Approaches to Stop Hypertension (DASH) diet are recommended interventions to delay neurodegeneration and decrease risk for AD [237,238]. These studies provide evidence that these dietary interventions can indeed lower the risk for cognitive decline and dementia [239,240]. In addition, when assessed with additional risk factors, such as APOE status, pro- and pre-biotic treatments show more

profound significant effects. A study in Italy of 848 aged participants found that an intervention strategy of increased dietary fiber intake (increase of 5g/d) in APOE4 participants was significantly associated with a 30% decreased risk for cognitive decline [241].

Dietary intervention is a potential strategy to decrease risk for AD, along with lifestyle changes such as increased aerobic exercise and novel tasks for mental training (reviewed in [242]). However, these strategies may not be sufficient to reduce amyloid and tau pathology, neurodegeneration, and cognitive decline in patients with MCI or AD. But, there is still hope, where the power of the gut microbiome can make a difference in a patient's life. More drastic gut microbiome alterations have the potential to reverse cognitive decline in patients with AD [134,135,228,243-247]. Specifically, there are cases studies where patients with progressive cognitive decline receive FMT for treatment of C. Difficile infection and show a rescue of cognition as measured by cognitive tests [134,247]. Patients treated by FMT for Familial Amyloid Polyneuropathy were reported to have reduced amyloid deposits and improved cognition following transplantation and for two years post-FMT [137]. Similar results have been seen in AD mouse models [226,229] and other models of cognitive dysfunction [227,245,246]. The safety and efficacy of FMT for treatment of patients is fairly well established, and future FMT treatments may be leaning towards more targeted microbiome therapies [248-250].

Finally, with the rise in big data analysis and sequencing technologies, more and more evidence connecting diet and gut metabolites to cognitive decline and AD genetic risk provides additional avenues for therapeutic insight [24,61,65,251,252]. Currently, genetic association studies have found genetic links to the gut microbiome [253-255], but

those targeted for specific diseases are lacking. In line with my background of statistical genomics and bioinformatics and values, I would like to propose a "call to action" for the collection and analysis of metabolic, dietary and gut microbiome patient data across all disease states. These types of analyses will allow us to better identify potential risk loci associated with dietary patterns and gut dysbiosis.

4.5 Closing Remarks

We cannot deny that AD is a multi-faceted disease, where diet and lifestyle have significant influence on disease risk, and therefore therapeutic strategies should also be a multi-faceted approach. Although genetics is a major contributor to AD risk, evidence from gut microbiome studies and from studies focused on other lifestyle factors illustrates the validity of lifestyle intervention for mitigation of AD risk. Additional lifestyle factors to consider are mental health and mindfulness [256-261] , physical exercise [262,263], sleep [264-266], stress [267-269], and spiritual wellness [270-272]. In conjunction with AD treatments targeting the neuropathology, such as amyloid antibodies that reduce amyloid burden in the brain of high-risk patients, holistic approaches to therapy may improve lifestyle and increase the likelihood of cognitive recovery in patients with MCI and AD [273,274]. At the very least, therapeutic approaches that improve digestion and quality of life of patients are important for essential care. In this dissertation, I provide evidence as to how gut microbiome interventions have the potential to rescue and ameliorate aspects of AD, slowing the decline and improving quality of life for patients. It is of my expert opinion that targeting the gut microbiome—as a potential lifestyle risk factor for AD—should be used to complement current existing pharmacological and cognitive therapies of AD. It is of my humble option, based on peer-reviewed literature and personal

experiences, that targeting the overall physical, mental, emotional, and spiritual well-being of individuals will lead to the greatest health outcomes in our population as a whole.

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  B.A. Biochemistry
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Relevant Research Experience

- *PbD Dissertation Project* 2018—2023
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  University of Colorado
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  Protein-Protein Interactome Analysis for Mycobacterium abscessus and Mycobacterium avium
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  DePaul University, Chicago
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  Thresholded Hierarchical Itemset Clustering for Expert Explorations

Professional Development

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Awards, Honors, and Scholarships

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National Presentations

- **Zajac D.J.**, Simpson, J.F., Morganti, J.M. and Estus, S. *Expression of the Microglial INPP5D Isoforms as a Function of Alzheimer's Disease Status and Genetics*. Alzheimer's Association International Conference, Poster Presentation, August 2021, Virtual

- **Zajac D.J.**,, Simpson, J.F., Morganti, J.M. and Estus, S. *Microglial INPP5D Isoform Expression as a Function of Alzheimer's Disease Status and Genetics.* Society for Neuroscience, Poster Presentation, November 2021, Virtual
- **Zajac D.J.**,, Simpson, J.F., Morganti, J.M. and Estus, S. *Genetics of Microglial INPP5D Isoform Expression in Alzheimer's Disease.* Society for Neuroscience, Poster Presentation, November 2022, San Diego, CA

Publications and Preprints

- Parikh, I.J., Estus J.L., **Zajac D.J.**, Malik M., Weng J.M., Tai L.M., Chlipala G.E., LaDu M.J., Green S.J., and Estus S.. "Murine Gut Microbiome Association With APOE Alleles." *Frontiers in Immunology* 11 (2020).
- **Zajac D.J.**, Green S., Johnson L., Estus S.. "APOE Genetics Influence Murine Gut Microbiome." *Sci. Rep* 12, 1906 (2022).
- **Zajac D.J.**, Shaw B.C., Braun D.J., Green S.J., Morganti J.M., Estus S.. "Exogenous Short Chain Fatty Acid Effects in APP/PS1 Mice." *Front. Neurosci. – Neurodegeneration* (2022).
- Shaw B.C., Snider H.C., Turner A.K., **Zajac D.J.**, Simpson J.F., Estus S.. "An Alternatively Spliced TREM2 Isoform Lacking the Ligand Binding Domain is Expressed in Human Brain." *J Alzheimer's Dis* (2022)
- **Zajac D.J.**, Simpson J.F., Zhang, E., Parikh, I.J., Estus S.. "Expression of INPP5D isoforms is increased in Alzheimer's Disease brains and suggests an allele-specific SNP effect." *Genes* (2023)

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