Investigating the Microbe-Specific Effects in Multiple Sclerosis and CNS Autoimm

Investigating the Microbe-Specific Effects in Multiple Sclerosis and CNS Autoimmunity

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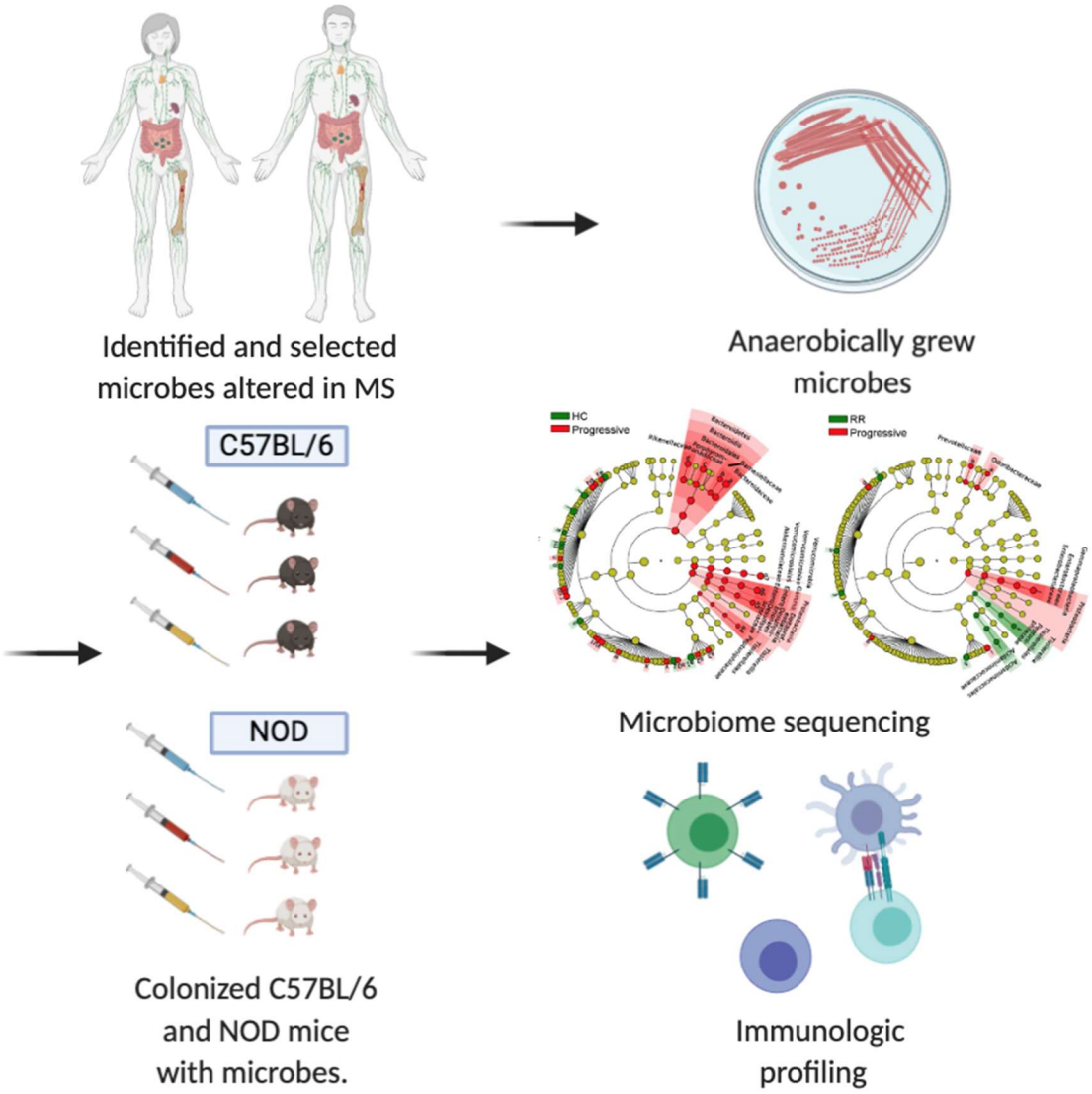
Abstract

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) resulting in the demyelination of neurons. The gut microbiome consists of commensal and opportunistic pathogenic bacteria, viruses and fungi and is emerging as a potent modulator of the immune system contributing either to inflammatory or regulatory responses. Patients with MS have significant differences within their gut microbiome as compared to healthy controls; however more research is needed to fully understand specific microbe contributions to CNS autoimmunity. Antibiotics are commonly used to deplete the microbiome of pathogens but more often than not, cause a complete wipeout of commensal organisms, leaving the body vulnerable to pathogenic infection. Commensal microbes are essential to promote proper gut health and homeostasis, but may be altered in MS. I hypothesize that certain microbes that are altered in MS may contribute to the disease by regulation of CNS autoimmunity. I tested my hypothesis by colonizing mice with bacteria that we have identified as altered MS, then induced experimental autoimmune encephalomyelitis (EAE) to model disease onset and progression in both the C57BL/6 and NOD mouse strains, which model the acute and progressive forms of the disease, respectively. In AIM I, I investigated whether the course of experimental autoimmune encephalomyelitis differs between animal vendors in the acute C57BL/6 (abbrev. C57) and progressive NOD/ShiLtJ (abbrev. NOD) animal models, because previous studies have shown differences in pro-inflammatory Th17 cells in mice from Jackson laboratories and Taconic Farms. I found that while mice from both animal vendors showed EAE symptoms, mice from Jackson Laboratories had worse disease in the C57 model and mice from Taconic Farms had worse disease in the NOD EAE model. To test whether microbes

associated with clinical disease in MS patients contribute to CNS autoimmunity, in AIM II, I colonized C57 and NOD mice with *Anaerotruncus colihominis* and *Clostridium bolteae,* associated with worse disease, and *Faecalibacterium prausnitzii* and a combination of butyrate producing bacteria, associated with improved clinical parameters, then induced EAE. I found that *A. colihominis* had a protective effect blunting the severity of EAE in C57 and NOD models of disease. *F. prausnitzii* had a protective effect in the NOD animal model of disease. Finally, to identify potential mechanisms by which these bacteria may affect MS, in AIM III, I characterized peripheral and central immune responses. I found that treatment with *Anaerotruncus colihominis* decreased the secretion of pro-inflammatory cytokines and increased the secretion of anti-inflammatory cytokines, which may contribute to lower disease burden. Treatment with *Faecalibacterium prausnitzii* produced limited effects on immune responses however, we found some increases in the levels of regulatory immune cells which may limit host immune responses and produce better disease outcomes. These studies are important to biologically validate correlations that we identified to be associated with differential disease outcomes in our MS patient population. This new information can allow for more targeted treatment opportunities in patients with the disease.

Frontispiece



Dedication

I would like to dedicate my thesis to my grandmother Linda Gayle Collins whose optimism in the face of challenges continues to inspire me.

Acknowledgments

I would like to begin by thanking my gracious thesis director Dr. Howard Weiner for allowing me to study and perform experimental research in his laboratory at the Ann Romney Center for Neurologic Diseases at Brigham and Women's hospital. Words cannot express the gratitude I have for the opportunity to contribute research to a disease that my own personal family has been affected by.

I would also like to thank my thesis supervisor Dr. Laurie Cox for agreeing to mentor me this past year. I have learned so much from her both scientifically and professionally. Her guidance has led me to produce research that I am truly proud of and am confident makes a great difference in the future of disease treatment.

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Chapter I.

Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) which results in autoreactive T and B-cells infiltrating from the blood to the spinal cord and brain. This inflammatory response results in demyelination and eventual neurodegeneration. Symptoms of MS can appear in the form of weakness or numbness in extremities as well as dizziness and visual dysfunction. The average age of onset of MS is typically between 20-50 years with approximately 1 million individuals living with MS in the United States alone. This number has nearly doubled since the 1970's (National MS society, n.d.), suggesting that there is something in the environment that could be increasing the prevalence of MS. Furthermore, this increase has been more profound in women. 20 years ago, only twice as many women than men were affected by MS, whereas today, three times more women than men are affect by MS (Algrehn et al., 2011). We hypothesize that the changes in the gut microbiota may be an environmental factor that is contributing to the increase in MS prevalence. Because women are more affected by the disease, our studies will focus on female mice. Further studies are warranted to explore environmental factors driving the increased vulnerability to MS in women vs. men.

RRMS is the most common form of MS, with about 85% of clinical diagnoses occurring as this type (Goldenberg, 2012). The chronic progressive subtypes of MS consisting of primary progressive (PPMS) and secondary progressive (SPMS) comprises

approximately 10% of diagnoses. Many individuals initially diagnosed with RRMS will eventually progress to this subtype. Chronic progressive disease in MS consists of consistent inflammatory attacks with no remission.

1.2 The Gut Microbiome

The microbiome is home to over 100 trillion various bacteria, fungi and viruses, which contribute to a wide variety of bodily processes including educating the immune system (Peterson et al., 2009). The gut microbiome can influence onset and progression of many different types of diseases including metabolic diseases (e.g. diabetes and obesity), cancer, and neurologic diseases (e.g. anxiety, depression, autism, Parkinson's disease, and MS). Microbes are an integral part of diseases concerned with inflammation. One example is segmented filamentous bacteria (SFB), which attach to intestinal epithelial cells and induce Th17 cell differentiation (Ivanov, Cell, 2009, Atarashi et al., 2015). Th17 cells can have protective function in excluding intestinal pathogens, however, proinflammatory Th17 cells contribute to autoimmune diseases, including rheumatoid arthritis and multiple sclerosis.

Initial studies using animal models of experimental autoimmune encephalomyelitis (EAE) showed that depleting the gut microbiota with antibiotics ameliorated disease. This was some of the first evidence that the gut microbiota could contribute to CNS autoimmunity (Ochoa-Reparez et al., 2009). It was also shown that germ-free mice do not develop EAE, but mono-colonizing mice with SFB, which induced Th17 cells, restored disease (Lee, 2010).

Antibiotics are useful tools to treat pathogens; however, in many ways there can be a major immunologic disadvantage when depleting the gut microbiome of commensal

bacteria. As antibiotics target growing microbes, use of antibiotics can deplete the gut of protective commensals which provide colonization resistance against pathogenic organisms as explained by the theory of disappearing microbiota (Blaser, 2017). The colonization resistance that the microbiome provides is extremely important in avoiding pathogenic infections including one of the most common healthcare associated pathogens, *C. difficile* (Sassone-Corsi and Raffatellu et al., 2015).

Many researchers have attempted to study the risk and or possible benefits that antibiotics may have on patients with MS (Abdollahpour et al., 2018 & Nørgaard et al., 2011). It is presumed that patients who receive antibiotics for bacteria that could worsen the disease and enhance progression in MS should see favorable outcomes. One Canadian study showed that use of the common oral antibiotic Minocycline prevented individuals with CIS (clinically isolated syndrome, which is one of the first inflammatory neurologic attacks that patients with MS commonly suffer preceding their disease diagnosis) from fully developing the disease (Metz et al., 2017). It is not always the case to see better disease outcomes in MS following antibiotic exposure. A study conducted by Nørgaard et al, concluded that the use of penicillin and other antibiotics contributed to increased risk of MS development. It is then also assumed in this case and by other scientists that antibiotic use that removes beneficial bacteria from the gut may aggravate the disease and result in worse prognoses.

1.3 Gut Microbiota Alterations in MS

A study performed by Jangi et al. 2016, analyzed the microbiome in MS patients versus healthy controls showed that there are significant differences in the abundance of certain microbes within each subset. They found that the commensal microbe

Akkermansia muciniphila was increased in MS patients (Jangi et al., 2016). While this *Akkermansia* was originally thought to be detrimental in MS, further work has shown that *Akkermansia* may help with the resolution of disease as demonstrated in animal models (Liu et al., 2019).

It is found that induction of EAE in mice will also change the gut microbiome profile assumingly to accommodate disease. Previous studies have found that specific microbes may protect the host from CNS autoimmunity. EAE studies show that *Prevotella histicola, Bacteriodes fragilis*, and Clostridia clusters XIVa and IV influence secretion of anti-inflammatory cytokines and lower disease score when transferred to mice with induced disease. These microbes are reportedly decreased in patients with MS. This loss of beneficial microbes may contribute negatively to disease. Other bacterial species found to be decreased in MS include *Butyricimonas, Lachnospiraceae and Ruminococccceae* (Chu et al., 2018).

1.4 Potential for the Microbiome as a Treatment for MS

Many of the currently approved therapeutics available to treat multiple sclerosis involve immunoregulation since MS is an autoimmune disease. For example, Rituximab is an anti-CD20 monoclonal antibody, which binds to the surface of B cells and triggers their death, and decreases inflammatory responses that damage the myelin sheath. Microbiome based-treatments could provide an alternative or additive therapeutic approach to immune-suppressing treatments currently available to patients with MS.

The microbiome can be modulated by a number of various methods. Fecal microbiome transplant (FMT) is becoming more widely used to treat chronic diseases. FMT from healthy to disease burdened individuals has been linked to better health

outcomes. Interestingly in the case of MS, microbes transferred from mice at peak EAE greatly reduced disease in EAE induced mice (Liu et al, 2019). Prebiotics used to amplify growth of certain beneficial microbes, or probiotics containing live microorganisms are also potential ways to modulate the microbiome.

Previous studies including the use of probiotics have identified changes in the microbiome and immunologic profile of patients with MS. Patients were given a cocktail of presumed beneficial bacteria including various strains of *Bifidobacterium*, *Lactobacillus,* and *Streptococcus* for two months and then a washout phase was performed for five months. Scientists found that the microbes did not stably colonize post washout, however immune changes were lasting (Tankou et al., 2018).

1.5 Potential for Butyrate Producing Bacteria as a Novel MS Probiotic

While many of the over-the-counter probiotics use lactic acid bacteria (e.g. *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*), these do not stably colonize the adult G.I. tract. Furthermore, the use of probiotics with these lactic acid bacteria is widespread, yet MS persists, indicating that disease-specific microbiota interventions are needed. Resident gut microbiota that produce butyrate may have increased efficacy since they stably colonize and have beneficial effects on G.I. function. Butyrate is a short chain fatty acid with many roles tied to gut health and immunity. Recent studies demonstrate that butyrate may be among the most important short chain fatty acid that facilitates communication between the gut and the brain (Fachi et al., 2019).

Previous experiments conducted in the Weiner Lab analyzed the microbiome in patients with different forms of multiple sclerosis by sequencing the V4 region of the 16S rRNA gene. They found that *Faecalibacterium prausnitzii* was decreased in RRMS and

progressive MS patients and was negatively correlated with disease severity scores (Fig. 1&2). *Faecalibacterium prausnitzii* is a Gram-positive anaerobic bacteria belonging to the class bacillus. F. prausnitzii is one of the highest butyrate-producing microbes within the large intestine and has long been considered a hallmark of gut health (Filippis et al., 2020). Other studies have echoed the result of this preliminary data by reporting decreased levels of *Faecalibacterium* in MS patients vs healthy controls. A common treatment option for MS includes the use of immunomodulating drugs, however they may change the composition of the microbiome by altering levels of beneficial microbes as well. *Faecalibacterium* was also decreased in patients on the immune-modulating drug glatiramer acetate reportedly increased after untreated MS patients were supplemented with vitamin D, a co-factor commonly decreased in individuals in MS (Cantarel et al., 2016). Butyrate production from this microbe helps homeostatic gut function by providing the major energy source for colonocytes and by regulating immune responses which can help ameliorate many gastrointestinal disorders and diseases (Lopez-Siles et al., 2017). Butyrate can also induce T regulatory cells, which may help decrease CNS inflammation in MS.

Roseburia intestinalis is a prominent butyrate-producing bacteria. Specifically, this microbe can metabolize plant polysaccharides including ®-mannans by upregulating hydrolases responsible for the transportation and esterification of this protein. ®-mannans is a plant polysaccharide, widely found throughout the human diet and acts as fiber. Fiber in the human diet is essential for gut health and it has been reported that low levels of this bacteria positively correlate with disease (La Rosa et al., 2019).

Agathobacter rectalis is a Gram-positive anaerobic bacteria of the phylum Firmicutes. This microbe, previously named *Eubacterium rectale,* is a major butyrate producer in the gut. This microbe is still not well understood and little research has been done to characterize its effects on disease and immunity. However, as *A. rectalis* is a prominent member of the *Firmicutes* phylum, it's by products, namely butyrate are, quite beneficial to the host and it is also therefore presumed to be a beneficial microbe (Karcher et al., 2020).

1.6 Potential for Mucosa Adhering Bacteria to Contribute to MS

Th-17 cells are a subset of T-cells that secrete the pro-inflammatory cytokine IL-17. Epithelial cell adhesion is an important signal for Th-17 cell induction (Atarashi et al., 2015). In the case of segmented filamentous bacteria (SFB), this bacteria adheres to the intestinal wall and as a result induces differentiation of Th-17 cells. Researchers attempted to identify members of the human gut microbiota that could exert immunological effects equivalent to those of SFB. They identified 20 strains of bacteria that were correlated with a positive increase in Th-17 cells from human microbiota collected from patients with ulcerative colitis. Among these 20 strains were *Clostridia* cluster IV containing *A. colihominis* and *Clostridia* cluster XIV containing *C. bolteae* other species of *Bifidobacterium* were included as well. The bacteria are considered prominent butyrate producers. As previously suggested, butyrate producing bacteria is associated with better disease outcomes and lower disability. This finding produces evidence in the case that not all butyrate producing bacteria may be good in association with disease (Atarashi et al., 2015).

Anaerotruncus colihominis is a rod-shaped species of bacteria first discovered in 2004 when isolated from children with late-onset autism. *A.colihominis* is a Gram-positive anaerobe, and can only grown in the strict absence of oxygen. While related to other members of the Clostridia class, this microbe is different from other bacterial clusters as it is not a spore-former (Lawson et al., 2004). According to previous data from the Weiner lab, *A. colihominis*, was upregulated in both RRMS and Progressive MS and associated with worse disease outcomes in both disease subtypes (Figures 1&2). This patient data provided an avenue for further exploration as to the immunological effects this specific microbe may have on the CNS.

Clostridium bolteae is a Gram-positive obligate anaerobic bacterium belonging to the *Lachnospiraceae* family. This bacteria was first isolated in 2003 from a juvenile autistic patient (Song, 2003). Further studies also found that that *C. bolteae* was increased in the gastrointestinal tracts of autistic patients. (Pequegnat et al., 2013). According to previous patient data from the Weiner lab, *C.bolteae*, was found to be increased in both RRMS and Progressive MS and associated with worse disease outcomes (Figures 1&2).

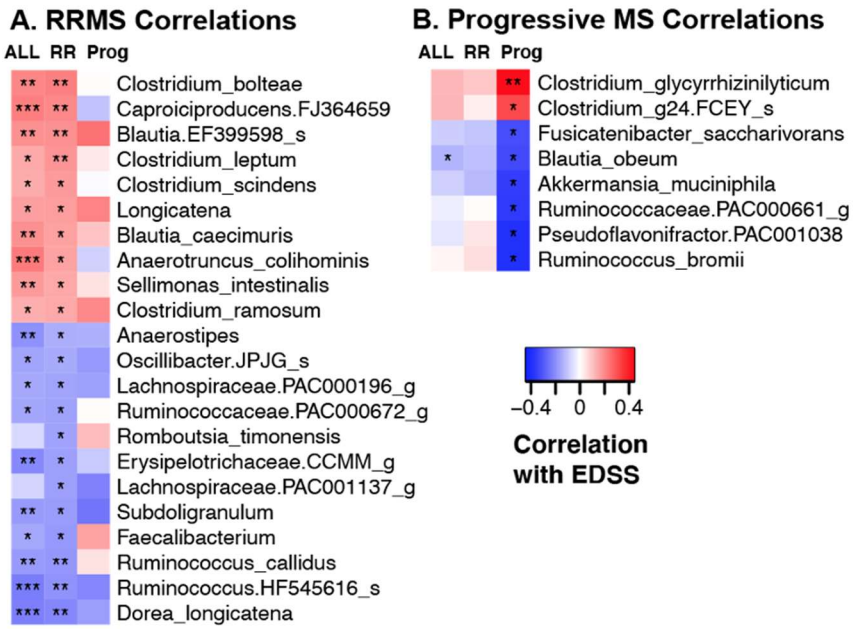

Figure 1: Microbiota correlations with EDSS scores show unique relationships in RRMS (A) and progressive MS (B), *Spearman correlations, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

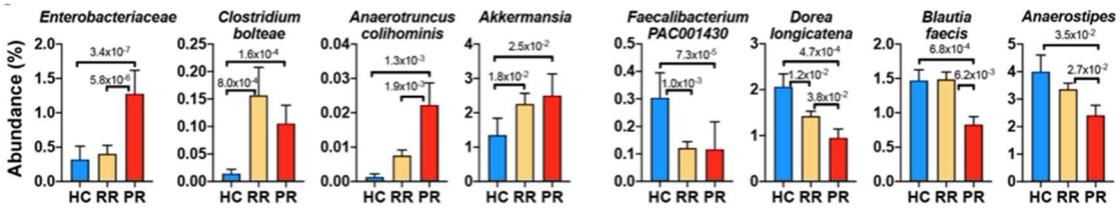

Figure 2: Compositional differences in the microbiota of progressive and relapsing MS. *The relative abundance of selected microbiota altered in RRMS (n = 202) and progressive MS (n = 42) vs healthy controls (n = 41), determined by linear discriminant analysis effect size (LEfSe).*

1.7 The Microbiome in Animal Models of Multiple Sclerosis

Various murine strains have been utilized to model the disease course in MS. Mice are immunized with proteins in the myelin sheath that wraps the neuros (e.g. myelin oligodendrocyte glycoprotein, MOG, and proteolipoprotein, PLP) in combination with complete Freund's adjuvant to illicit an autoimmune attack on neurons. This process induces experimental autoimmune encephalomyelitis (EAE), which is models the CNS autoimmune processes in multiple sclerosis. Of these models, the C57BL/6 is one of the most widely used EAE models within research (McCarthy et al., 2012). Common disease course within the C57BL6 model presents as a monophasic peak from 19-21 days of disease induction. This model demonstrates immunologic consistencies with the relapsing remitting form of MS, including the infiltration of Th1 and Th17 cells into the CNS and has been successfully used to discover new disease modifying therapies that are now used to treat MS.

Figure 3: The C57BL/6 mouse model demonstrating paralysis and a (score of 3.5 out of 4) by induction of EAE. This paralysis is consistent with paralysis seen in human patients with MS.

Another murine strain more recently utilized to represent disease in MS is the NOD (non-obese diabetic) mouse model immunized with MOG/CFA. This mouse strain was originally developed to model type I diabetes mellitus (Dang et al., 2015). The EAE induced disease course of this mouse model presents differently from the C57BL6 model as it consists of a biphasic relapsing remitting phase from days 22-71 after disease induction. This relapsing remitting phase then develops into a chronic progressive phase (Levy et al., 2010). It is believed that the NOD model disease transition captures a very integral facet of human disease which is the progression of RRMS to SPMS (Colpitts et al., 2017). The motor symptoms observed in the NOD mouse model during active EAE are similar to the C57 model. Weight loss is of the first symptoms to be experienced, following decreased motor function and paralysis (Dang et al., 2015).

In validation testing of the NOD model, it was observed that mice induced with EAE and a control group without disease induction had significantly different microbiome composition. There is considerable variation in symptoms in NOD EAE mice, and studies have found that NOD mice with worse disease had alterations in their microbiome compared to NOD mice that did not develop severe disease following immunization (Colpitts et al., 2017).

Figure 4: The NOD mouse model demonstrating paralysis and a (score of 3 out of 4) by induction of EAE. *This paralysis is consistent with paralysis seen in human patients with MS.*

1.8 Animal Vendor Specific Effects

Several labs, including our own, have variable results in EAE induction, in which the same protocol, same operator, and same animal vendor produce robust disease in one experiment and fail to induce disease in other experiments. In a recent study in a model of amyotrophic lateral sclerosis, C9orf72 mice showed motor dysfunction in the Harvard Institutes of Medicine animal facility but failed to show symptoms across the river at the Broad Institute. This affect was linked to the microbiota and swapping microbiota between facilities swapped the disease susceptibility (Burberry et al., 2020).

Due to some previous complications inducing EAE, I conducted a pilot study to test the efficacy of the MOG-CFA protein induction of EAE, but also investigated vendor specific effects. A study done by Ivanov et al., demonstrated that different vendors can produce different phonotypic characteristics within their mice. Jackson Laboratories and Taconic Farms are two of the most common vendors from which labs from across the country order animals utilized for research purposes. Results from this study found that mice from Taconic farms were colonized with SFB, which as previously mentioned is a

potent Th17 cell inducer. Therefore, mice from this vendor tend to experience worse disease outcomes than mice from Jackson Laboratories (Ivanov et al., 2009).

The Weiner lab had several experiments in which EAE had not been robustly induced, and it was imperative to this experiment to determine if successful EAE induction would be possible in these animal models and also which vendor would be best to move forward with to create the most representative MS animal model possible.

1.9 Hypothesis and Specific Aims

Hypothesis 1: The resistance to experimental autoimmune encephalomyelitis observed in the Weiner lab may be due to differences in the microbiota from different animal vendors.

AIM I: Investigate whether the course of experimental autoimmune encephalomyelitis differs between animal vendors in the acute C57BL6 and progressive NOD animal models.

Hypothesis 2: Specific microbes associated with clinical outcomes in multiple sclerosis regulate CNS autoimmunity

AIM II: Test whether microbes associated with worse clinical disease in MS patients, including *A. colihominis* and *C. bolteae,* contribute to CNS autoimmunity. Test whether microbes associated with a lower disease burden in MS patients, including *F. prausnitzii* and a combination of butyrate producing bacteria, protect from CNS autoimmunity.

AIM III: Identify potential mechanisms by which MS-associated microbes may affect CNS autoimmunity by characterizing peripheral and central immune responses.

Chapter II.

Materials and Methods

Safety Statement: Animals were kept in a specific pathogen-free facility (pilot experiment) or a biosafety level-2 facility (experiments I/II) on a 12 hour light/dark cycle at the Harvard center for Comparative Medicine. Mice were 8–10 weeks of age and cohoused, five mice from same experimental condition per cage. Mice were fed an ad-libitum diet of Picolab Rodent Diet 5053 and distilled water without added preservatives (provided by BWH animal facility). All animal experiments described in this paper were approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School and carried out in accordance with those approved animal experiment guidelines.

2.1: AIM I: Investigation of Vendor Specific Effects

Animals: In a pilot experiment to distinguish vendor specific effects on regulation of CNS autoimmunity, we ordered 20 mice from Taconic farms and 20 from Jackson Laboratories. Both C57BL6 and NOD mice (n = 10 per vendor) were used to represent different models of MS.

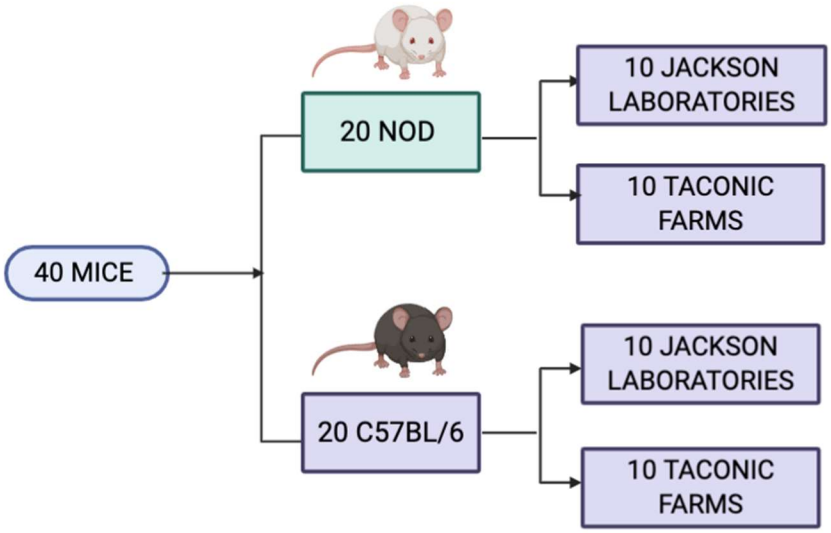

Figure 5: Study design for pilot experiment.
Consisting of n=40 mice total, n=20 C57BL/6 and n=20 NOD, n=10 mice per vendor.

Inducing EAE: A total of 150 ⌈g MOG was injected per mouse, mixed 1:1 MOG-CFA, injection volume is 200 ⌈L, 100 ⌈L per flank. A total of 200 ng PT in a volume of 200 uL (1 ng/⌈L) was injected per mouse on the day of MOG induction and 48 hours later (Hooke Labs, n.d.). Disease progression was evaluated using a 5-point scoring system, detailed below. Mice were humanely euthanized if they scored a four for two consecutive days or appeared moribund.

Scoring Criteria: (1) Limp tail, (2) limp tail and weakness of hind legs, (3) limp tail and complete paralysis of hind legs, (4) limp tail, complete hind leg and partial front leg paralysis, (5) moribund.

Microbiome Characterization: DNA was extracted with the Qiagen PowerLyzer kit. We constructed 16S rRNA sequencing libraries using our 960 barcoded-fusion primers and sequence the libraries on the MiSeq (Jangi et al., 2016, Tankou et al., 2018, Liu et al., 2019).

Data Analysis Plan. Quantitative insights for microbial ecology 2 (QIIME2) was used to demultiplex and quality filter sequences, assign taxonomy, calculate ⟨-diversity metrics such as phylogenetic diversity, richness. Shannon's index was used for evenness and to calculate ®-diversity between samples to examine the magnitude of group wise differences based on UniFrac distance (Lozupone et al., 2010), calculate relative abundance, and visualize the data (Caporaso et al., 2010). Because *C. bolteae* is absent from the commonly used taxonomic databases Green Genes and Silva (McDonald et al., 2011, Yilmaz et al., 2013), it is possible that this species was missed in other MS studies. Thus, we used the bacterial database from EZBioCloud to assign bacterial taxonomy as we have done for our preliminary data (Yoon et al., 2016).

Statistical Analysis: Microbiome sequencing statistical significances were determined by LEfSe for taxonomic differences (Morgan, Metagenomic biomarker discovery), PERMANOVA for beta diversity and the t-test for differences in alpha-diversity.

2.2 Aim II: Investigation of MS Associated Bacteria, Experiment I, *A. colihominis* and *F. prausnitzii*

Animals: This experiment included 60 mice in total. Of the 60 mice, 30 C57BL6 mice were obtained from Jackson Laboratories and 30 NOD mice were obtained from Taconic Farms based on the previous data from the pilot study.

Study Design: Based on our preliminary data, we tested whether *A. colihominis* worsened EAE in the C57BL/6 and NOD model, and whether *F. prausnitzii* ameliorates disease. C57 mice from Jackson Laboratories and NOD mice from Taconic mice were initially colonized with anaerobically cultured microbes 3x by oral gavage prior to immunization for the first week and then once weekly for 3 weeks. The mice were scored every day to record motor function and phenotypic effects. Mice were also weighed 3x weekly to monitor weight loss/gain.

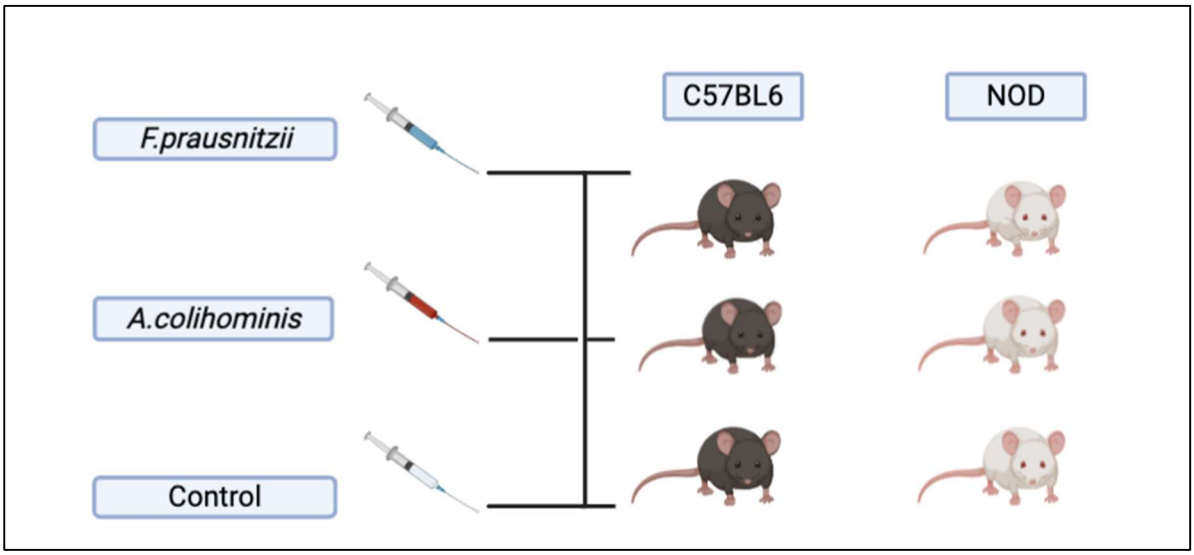

Figure 6: Study design model for Experiment I.
This experiment consisted of n=30 C57BL/6 mice and n=30 NOD mice. Both model groups were treated with either n=15 F. prausnitzii, n=15 A. colihominis, or n=15 PBS.

EAE Induction and Scoring: Disease was induced by immunizing with MOG35-55/CFA plus pertussis toxin and disease progression was monitored as previously described in AIM I.

Scoring Criteria: Animals were scored daily by the criteria indicated in AIMS I.

Bacterial Culture: Bacterial strains were grown in pure culture under anaerobic conditions on BHI for *A. colihominis* and YCFA agar for *F. prausnitzii*.

Microbial Administration: Mice were colonized with selected bacteria via oral gavage. The microbes were given 3x per week for the first week and once weekly for 3 weeks following.

Preparation of microbial inoculum: Microbial suspensions were made to deliver a dose of 10^8 bacteria/mL. Bacterial growth was collected after 3-5 days and diluted in 8mL PBS. Statistical Analysis: Differences in disease severity were determined by two tailed Students t-tests.

2.3 AIM II: Investigation of MS Associated Bacteria, Experiment II, *C.bolteae* and a combination of *R. intestinalis* and *A. rectale*

Animals: This experiment included 45 C57BL6 mice. All 45 were obtained from Jackson Laboratories.

Study Design: Based on our preliminary data, we tested whether *C.bolteae* (n= 15 per group) worsened EAE in the C57BL/6 and NOD model, and whether a combination of *R. intestinalis* and *A. rectale* (n=15 per group) ameliorates disease. C57 mice from Jackson Laboratories For this experiment mice were initially colonized with anaerobically cultured microbes 3x by oral gavage prior to immunization for the first week and then once weekly for 3 weeks. The mice were scored every day to record motor function and phenotypic effects. Mice were also weighed 3x weekly to monitor weight loss/gain.

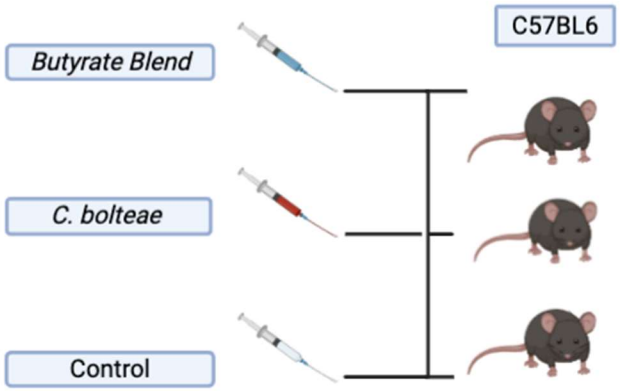

Figure 7: Study design model for Experiment II.

This experiment consisted of n=45 C57BL/6 mice from Jackson Laboratories. Mice were treated with either n=15 Butyrate blend (R. intestinalis & A. rectale), n=15 C. bolteae, or n=15 PBS.

EAE Induction: EAE was induced as described in AIMS I

Bacterial Culture: Bacterial strains will be grown in pure culture under anaerobic conditions on BHI for *C. bolteae* or YCFA agar for *A. rectale* and *R. intestinalis*.

Microbial suspensions were made to deliver a dose of 10^8 bacteria/mL.

Preparation of microbial inoculum: Microbial suspensions were made to deliver a dose of 10^8 bacteria/mL. Bacterial growth was collected after 3-5 days and diluted in 8mL PBS.

Microbial Administration: Microbes were administered as described in AIMS II EXPT I.

Statistical Analysis: Differences in disease severity were determined by two tailed Students t-tests.

2.4: AIM III: Investigation of the Effect of MS-Associated Bacteria on Immune Responses

Animals: This experiment included mice listed in AIMS II, Experiment I.

In vivo: We cultured *Anaerotruncus* associated with worsening disease and *Faecalibaculum* associated with lower disability burden. In order to assess how these MS-associated bacteria affect immune responses, we collected mesenteric lymph nodes, spleen and spinal cord tissue at end point from mice colonized with MS-associated strains as described in Aims II. As a control, in one naïve cohort was gavaged with a saline solution. Samples were collected 26 days after immunization.

Immunologic characterization of peripheral immunity: T cells from the spleen, mesenteric lymph nodes, and CNS were recovered in a single cell suspension, and stained for T cell subsets, monocytes, and activation makers. Cells were stimulated with PMA ionomycin, and stained for intracellular cytokine production with antibodies against interferon-gamma, IL-17, GM-CSF, and IL-10.

Chapter III.

Results

AIM I: Investigate Whether the Course of Experimental Autoimmune Encephalomyelitis Differs Between Animal Vendors

Because severity and development of MS can depend on environmental factors (O'Gorman et al., 2012), and because the microbiome can differ between animal vendors and affect immune responses (Ivanov, 2009), it is important to determine if there is a significant difference in EAE induction in mice obtained from different vendors. This experiment was performed to ensure successful EAE induction and to create the most representative MS mouse model possible. This was also an important control experiment because the Weiner lab had several previous experiments in which their standard EAE immunization protocol failed to effectively induce EAE.

3.1 EAE Severity in C57 and NOD Mice from Jackson Labs and Taconic Farms

The initial pilot EAE experiment induced EAE using MOG/CFA in 2 different mouse strains from 2 separate vendors to investigate the effect of genotype and environment on. The C57 mice model an acute phase of EAE, whereas the NOD mice present initially with a relapsing remitting disease, that eventually transitions to a progressive disease. We selected two animal vendors, Jackson Labs, which were previously reported to have lower Th17 cells because of an absence of SFB, and Taconic

Farms, which were previously reported to have higher Th17 cells, linked to the presence of SFB.

We found that the C57BL6 model began to display symptoms of EAE approximately 10 days post induction and showed a rapid increase in disability from days 10-19, regardless of vendor (Figure 8.A). Jackson mice showed a consistent disease course and continued to gradually worsen throughout the experiment, whereas Taconic mice briefly started to recover from days 19-23, followed by a relapse of worsening disease. By day 29, the Taconic mice had caught up to the Jackson EAE severity. Total cumulative disease over the 42-day observation period was not significantly different between vendors (Figure 8.B). Based on these data, we were able to successfully validate our EAE induction protocol and were concluded that vendor differences in the C57 model were minimal between Jackson and Taconic mice.

As previously described, the NOD mouse model initially presents with a relapsing remitting disease course, where there can be almost complete recovery, and then severe symptoms can be seen much later in disease course. We found that the NOD mice immunized with MOG/CFA began to display symptoms of EAE severity approximately 7-10 days post induction (Figure 8.C), which was similar to disease onset in the C57 mice. Consistent with the literature, we observed a relapsing remitting disease course until approximately day 50 in NOD mice from both animal vendors, with slightly earlier periods of paralysis in the Taconic mice. After day 50, animals from both vendors show sustained disability, however mice from Taconic Farms had worse disability from day 60 until the end of observation (Figure 8.C**)** and also had increased cumulative disease severity (Figure 8.D). Of note, we observed some behavioral differences in the NOD

mice between the vendors. The mice from Jackson Laboratories demonstrated heightened anxiety, including jumping and running away quickly. It is hypothesized that increased anxiety may have a masking effect on the overall phenotype of these mice. The significance in the increase of EAE phenotype in Taconic Farms obtained NOD mice is consistent with the previous research from the Ivanov et al. study demonstrating that mice from Taconic Farms have a more severe paralysis in response to MOG based on differences in the gut microbiota, which affects overall disease outcomes and progression (Ivanonv et al., 2009). Based on these data, the Taconic mice showed worse EAE severity as compared to the Jackson mice, especially in the progressive phase of the disease. In addition, we were able to validate our EAE induction protocol for the NOD mouse model.

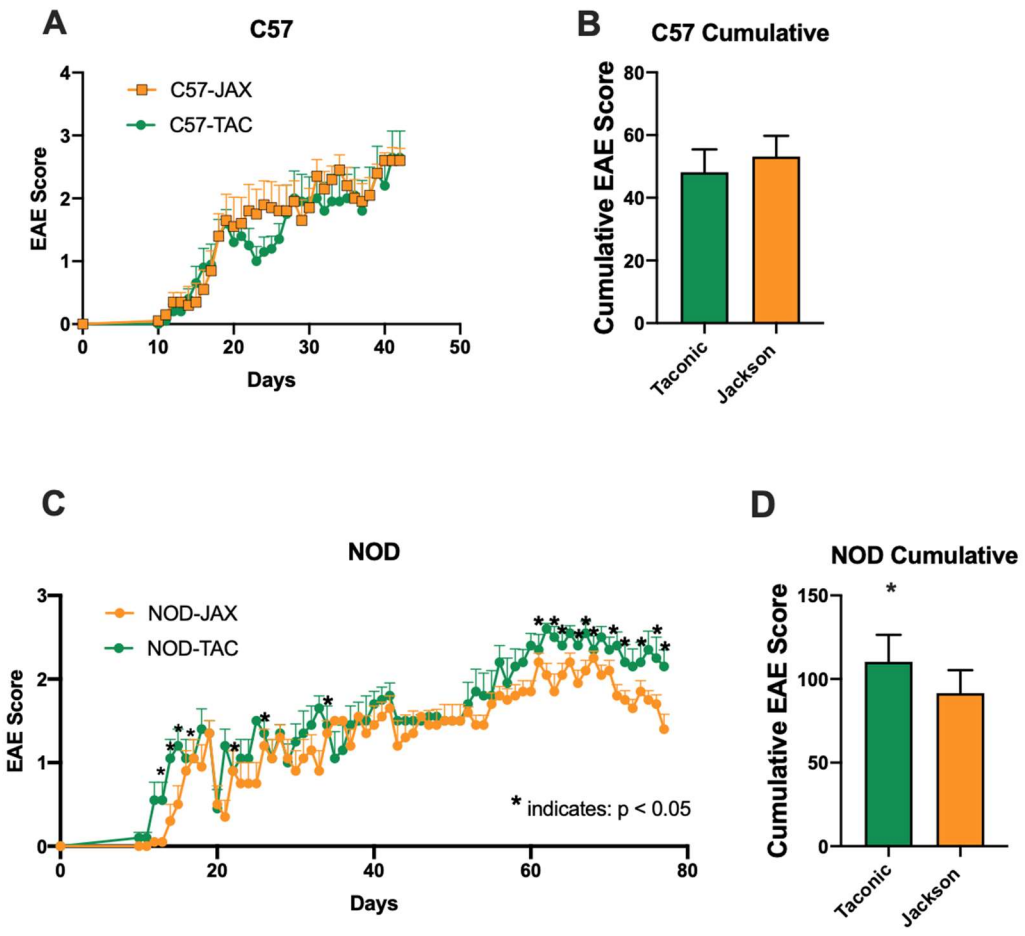

Figure 8: Vendor specific differences in EAE phenotype.

*(A) Difference in disease course of C57 mice from either Jackson Laboratories or Taconic Farms. (B) The cumulative disease score for C57 mice from both vendors. (C) Difference in disease course of NOD mice from either Jackson Laboratories or Taconic Farms. (D) The cumulative disease score for NOC mice from both vendors, *indicated p-value<0.05.*

3.2 Microbiota Differences in EAE Between Animal Vendors in NOD Mice

Because we found a difference between disease severity in the NOD Jackson and Taconic mice, we next looked at changes in their gut microbiome. We extracted DNA from ileal and colonic samples from NOD mice from Jackson Laboratories and from

Taconic Farms (n = 10 each), and then sequenced the microbiome targeting the V4 region of the16S rRNA gene on the Illumina MiSeq platform. (Caporaso et al., 2012). We use the 16S rRNA gene because it is highly conserved, and thus is a good representation of phylogeny within the gut microbiome. Primers have been designed to anneal to conserved regions to capture most microbes, and then amplify unique sequences in the variable regions.

We first evaluated the quality and our depth of coverage in our sequencing run (Figure 9.A). Based on our quality scores, we decided to trim our read at 230 base pairs. At this length, approximately 50% of the reads had a Phred score of Q20, which corresponds to an error rate of 1%. Trimming at Q20 has been shown to be optimal in other microbiome studies (Bokulich et al., 2013). We next looked at the distribution of the depth of coverage, also known as the number of microbial sequences, captured per sample (Figure 9.B). In our dataset, we successfully sequenced 37 samples, with a median depth of coverage of 5,320 sequences (minimum:1,773, maximum: 11,620).

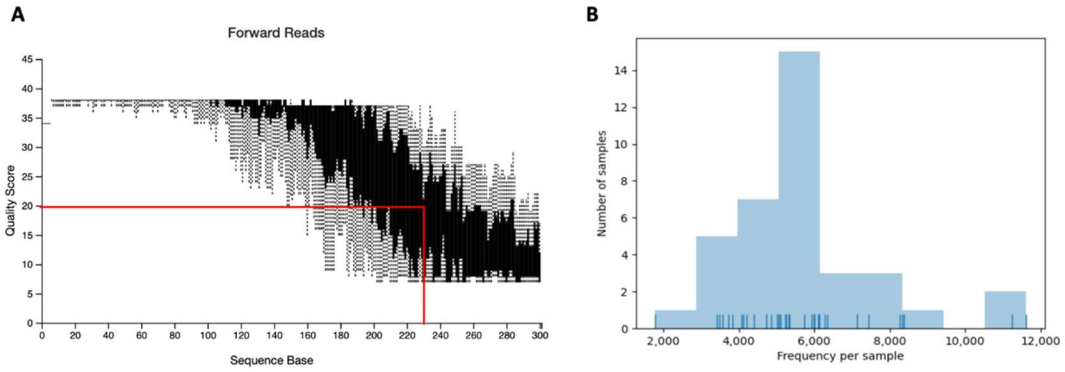

Figure 9: Sequence quality and depth of coverage.

We sequenced the microbial V4 region of the 16srRNA gene on the illumine MiSeq platform and used QIIME to evaluate read quality (A) and reads per sample (B).

We next analyzed the alpha diversity of the gut microbiota from mice from Taconic and Jackson labs. Alpha diversity is defined as the total ecological diversity within a sample. Low alpha diversity has been reported in liver disease, inflammatory bowel disease and *Clostridium difficile* infections (Kriss et al., 2018). However, the Weiner lab and other laboratories have not found any differences in alpha diversity in MS patients (Jangi et al., 2016). The alpha-diversity of the gut microbiome can be measured several different ways. Richness, or number of amplicon sequence variants (ASVs), reports how many different types of bacteria are detected, without considering phylogenetic relationships. For example, the same weight is given if E. coli + *Lactabacillus johnsonii* are detected as compared to *Lactobacillus reuteri* + *Lactabacillus johnsonii* are detected. Faith's phylogenetic diversity measures branch lengths along a phylogenetic tree, thus a microbiome composed of mostly closely related bacteria (e.g. *L. reuteri* + *L. johnsonii*) would have low phylogenetic diversity, whereas a microbiome composed of unrelated organisms would have a high phylogenetic diversity (*E. coli* + *Lactabacillus johnsonii*). Finally, the Shannon Diversity metric accounts for both richness in the microbiome and evenness (e.g. does each microbe have the same relative abundance? Or are there predominant vs. minor populations). We found that colonic samples had higher alpha-diversity in comparison to ileum samples by all three metrics, including observed ASVs, Faith phylogenetic diversity and Shannon diversity (Figure 10.B,C, Table 1), but there was no effect between animal vendor.

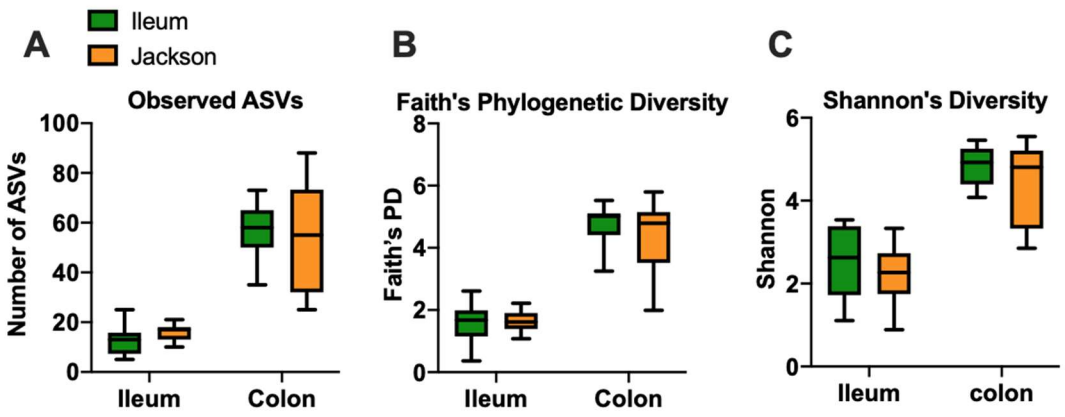

Figure 10. The effect of animal vendor on microbiome alpha diversity. Faith's phylogenetic diversity (B) Shannon's phylogenetic diversity (C) Observed OTU's group significance.

		Faith's PD	Shannon	Observed OTUs
Vendor Comparison	Jax Ileum vs Tac Ileum	p= 0.85	p= 0.56	p= 0.10
	Jax Colon vs Tac Colon	p= 0.60	p= 0.65	p= 0.68
Anatomical comparison	Jax Ileum vs Jax Colon	p= 0.00033	p= 0.00045	p= 0.00023
	Tac Ileum vs Tac Colon	p= 0.00038	p= 0.00038	p= 0.00036

Table 1: PERMANOVA p-values for differences in alpha diversity.

We next looked at differences between the overall microbiota composition, known as beta-diversity, or the differences in composition between ecosystems. Principal coordinate analysis plots allow us to visualize similarities and dissimilarities within obtained samples, in which the more similar the communities, the closer the dots in the

PCoA plot. In the unweighted UniFrac PCoA plot (Figure 11) we can see a clear clustering of the colon samples from Jackson and Taconic, which is significant by the PERMANOVA test (Table 1). We also see significant clustering of the ileum samples from both of these vendors (Figure 11, Table 1). This clustering pattern is expected as these are two different body sites and should share a lot of similarities. However, we also see differential clustering between the organ samples from each vendor one oriented to the left of the plot and the other oriented on the right. The different clustering patterns demonstrate clear differences both by anatomical region and by animal vendor.

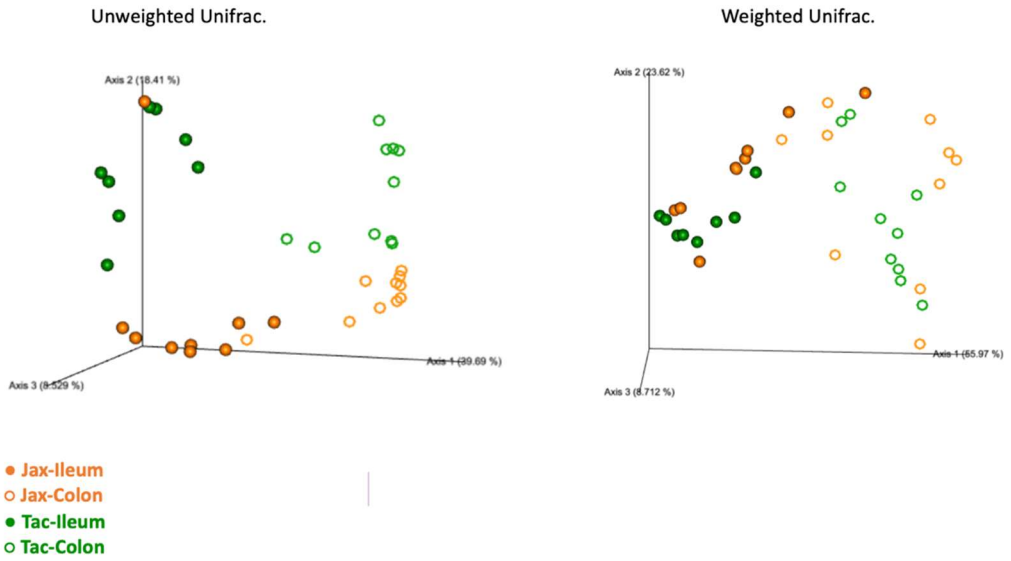

Figure 11: Weighted and Unweighted UniFrac by anatomical sample site by vendor.

		Unweighted UniFrac	Weighted UniFrac
Vendor Comparison	Jax Ileum vs Tac Ileum	p= 0.01	p= 0.055
	Jax Colon vs Tac Colon	p= 0.001	p= 0.248
Anatomical comparison	Jax Ileum vs Jax Colon	p= 0.001	p= 0.001
	Tac Ileum vs Tac Colon	p= 0.001	p= 0.001

Table 2: PERMANOVA p-values for differences in beta-diversity. *Beta diversity metrics include Unweighted unique fractions and Weighted unique fractions.*

We next examined specific changes in the bacterial taxa of Jackson and Taconic mice by using linear discriminant analysis effect size (LEfSe) (Segata et al., 2011). LDA scores represent the overall enrichment (or effect size) of bacteria that are significantly different (determined by the Mann-Whitney U non-parametric test). Bacteria are reported as significant if the p value for the Mann-Whitney U is < 0.05 and if the effect size is greater than 2. We found several differences, with a greater number of changes in the colon than in the ileum (Figure 12.A,B).

We found that several members of the family of *Erysipelotrichaceae*, including *Faecalibaculum rodentium*, *Dubosiella newyorkenesis*, and an unclassified species of *Turicibacter* were increased in Taconic mice, and also found unclassified species of *Clostridium* and *Bifidobacterium* to be increased in Taconic mice (Figure 12.C). The bacterial family.

Erysipelotrichaceae has been determined to be implicated in various metabolic and inflammatory diseases (Cox et al., 2017). Isolated strains of *Erysipelotrichaceae* can also produce immunologic effects similar to MOG, an adjuvant used to induce EAE in mice. This adjuvant increases Th-17 responses in mice to produce inflammation and drive disease pathogenesis. Mice co-colonized with two strains of bacteria, *Lactobacillus reuteri* and an unclassified member of the *Erysipelotrichaceae* OTU0002, produced synergistic effects that drove disease pathogenesis (Miyauchi et al., 2020).

Bifidobacterium is a bacterium commonly found to be depleted in the gut of MS patients and can be administered orally in a probiotic as treatment. Treatment with *Bifidobacterium* has been shown to decrease EDSS (Expanded disability disease score) and improve outcomes (Toghi et al., 2019). The increased levels of Bifidobacterium in Taconic mice as compared to Jackson mice can seem contradictory to the previous phenotype data, as previous studies have shown that Bifidobacterium can lessen inflammation, and the Taconic mice developed a much stronger EAE phenotype than Jackson mice. As previous data has demonstrated its beneficial effects, it is not expected to be responsible for the worse disease in Taconic mice but may be either a bystander or a compensatory change in the gut microbiota to help recover from the disease. One example of a compensatory change in EAE is the elevated levels of *Akkermansia*. Studies have found that *A. muciniphila* is upregulated in MS patients and in animals at peak EAE, and until recently it was thought to be contributing to pathogenesis due to its increase. Instead, transferring microbiota at peak EAE with high *Akkermansia*, or directly administering *Akkermansia*, can ameliorate disease. A similar phenomenon may occur with *Bifidobacterium* but remains to be tested. An alternate explanation is that

Bifidobacterium may be higher in mice coming from Taconic, but not related to the disease process at all.

The involvement of *Clostridia* in either protection or promotion of the disease would depend on the exact species. *Clostridia* species appear to be elevated in patients with MS as indicated by many studies (Ventura et al., 2019). The effects of *Clostridium* on disease and immunity highly species specific. Some species of *Clostridium* can activate intestinal epithelial cells to further induce the release of TGF-® among other Treg inducing factors. Tregs are incredibly important to protect the host from overt immune stimulation and to balance immune responses by producing anti-inflammatory cytokines such as IL-10. Other species of *Clostridium* including *C. difficile* one of the most common hospital acquired infections can induce the secretion of Th-17 cells producing increased inflammation. Other studies have shown that species of Clostridium can produce effects similarly to SFB by adhering tightly to epithelial cells and (Atarashi et al., 2015).

We also identified bacteria to be increased in Jackson mice, including an unclassified species of *Lachnospira* and *Alistipes finegoldii*. *Lachnospira* is a butyrate producer has been implicated in a host of metabolic and inflammatory diseases. *Alistipes finegoldii* it is described as bile-resistant and to live symbiotically in the intestines of humans. In summary, we were able to identify specific bacteria in Taconic mice, which had a worse EAE course in the progressive model, and these bacteria may contribute to worsening of disease.

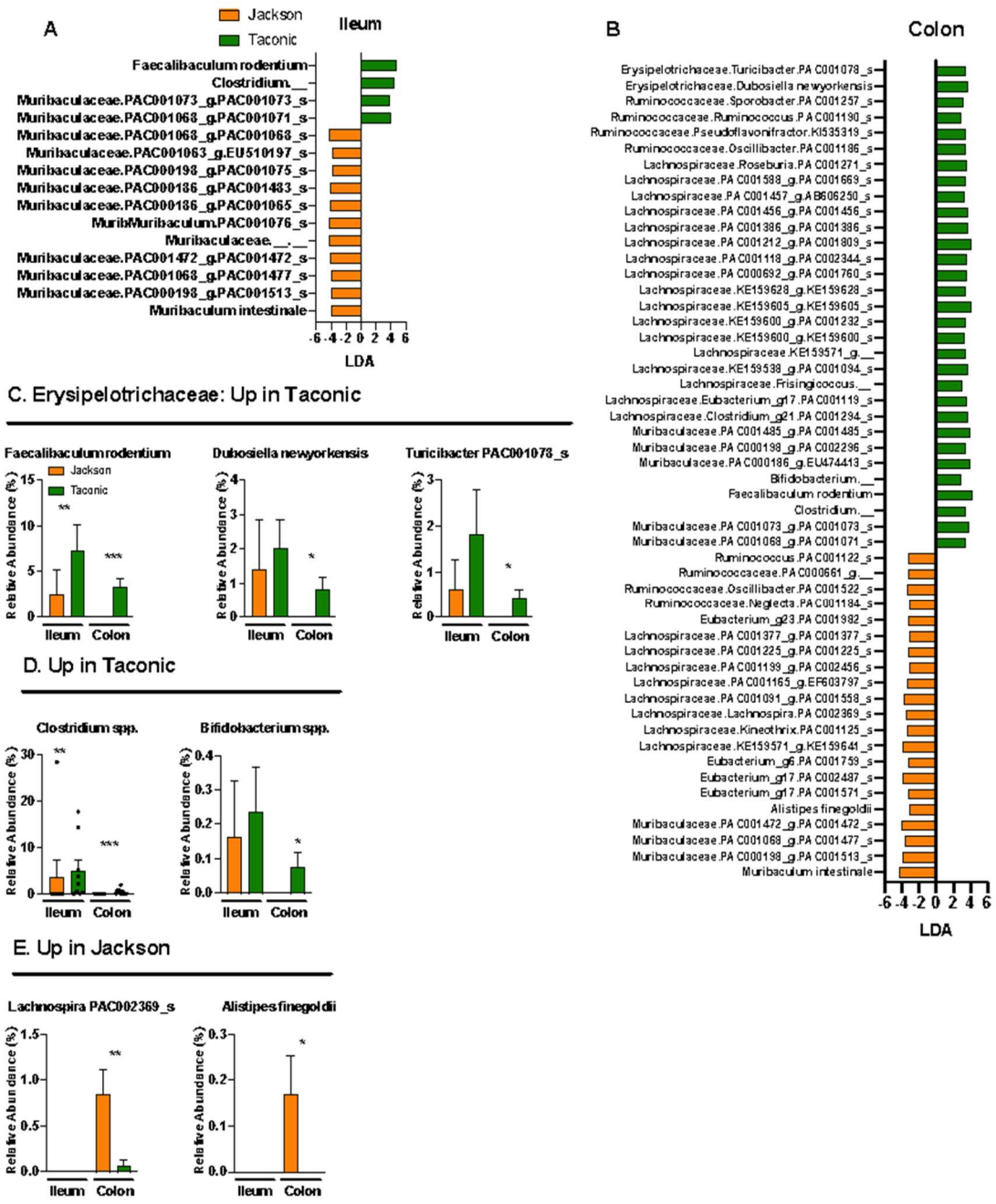

Figure 12. Vendor specific microbe differences *(A) Ileal bacteria shared between Jackson and Taconic mice. (B) Colonic bacteria shared between Jackson and Taconic mice. (C) Erysipelotrichaceae elevated in Taconic mice. (D) Unclassified species of Clostridia and Bifidobacterium elevated in Taconic mice. (E) Unclassified species of Lachnospira and Alistipes finegoldii elevated in Jackson mice.*

3.3 Aim II: Experiment I, Investigate the Effect of *A. colihominis* and *F.prausnitzii* on EAE in C57 and NOD Mice

As we have identified microbes associated with worse disease outcomes in patients with MS, we will test the effects of these microbes in mice induced with EAE and characterize their effects on immunity. This experiment will produce a deeper look into the mechanisms underlying immune responses to certain bacteria and how this may promote or prevent disease.

We previously found *Anaerotruncus colihominis* elevated in progressive MS and associated with worse disease disability in MS and found decreased *Faecalibacterium* in both RR and progressive MS and an association with better clinical parameters. In order to test whether *A. colihominis or Faecalibaculum* affected CNS autoimmunity, we colonized C57 and NOD mice with these bacteria and induced EAE by immunizing with MOG/CFA. Mice were treated with bacteria 3 times for the first week then once weekly for three weeks. Surprisingly, we found that *Anaerotruncus* significantly lowered EAE severity in C57 mice and had an overall protective effect when compared to the control (Figure 13.A, B). Mice treated with *A. colihominis* also had a lower cumulative EAE score when compared to the *F.prausnitzii* treated and control mice (Figure 13.C). When analyzing the phenotypic data in the NOD mice we found that *A. colihominis* initially had a protective effect in EAE from days 20-50. Around day 50 the disease scores began to converge with the control group and *F. prausnitzii* treated groups. We also found that *F. prausnitzii* initially had a protective effect as well from days 22-53, though not as substantial as those effects produced from *A. colihominis*. Though there is an apparent convergence of these EAE scores near the endpoint, it is possible that booster doses of *A.*

colihominis and *F. prausnitzii* may be needed to produce longer lasting effects and stable colonization. Though these two microbes initially produced a protective effect in EAE, *A. colihominis* significantly delayed disease course which was also a positive and rather unexpected outcome from treatment of this bacteria.

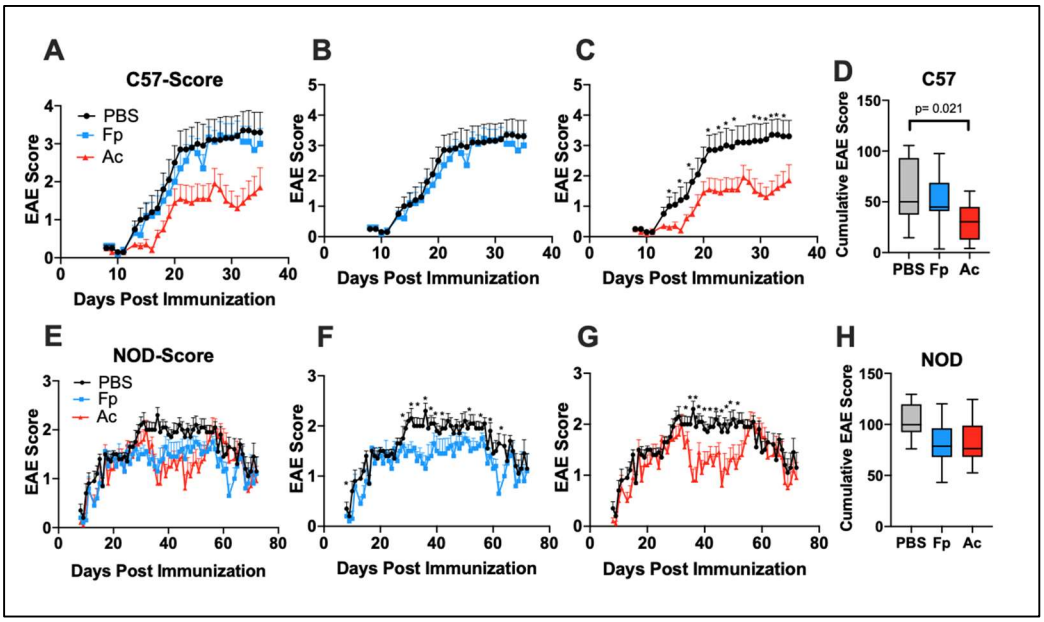

Figure 13 Disease phenotype by treatment of A. colihominis, F.prausnitzii or PBS in C57 and NOD mice *(A) Disease score phenotype of all treatment groups in C57 mice. (B) Disease phenotype of mice colonized with F.prausnitzii versus control saline treated C57 mice. (C) Disease phenotype of mice colonized with A.colihominis versus control saline treated C57 mice. (D) Cumulative EAE score for all treatment groups in C57 mice. (E) Disease score phenotype of all treatment groups in NOD mice. (F) Disease phenotype of mice colonized with A.colihominis versus control saline treated NOD mice. (H) Cumulative EAE score for all treatment groups in NOD mice. *Indicates p-value<0.05.*

While the data from this experiment contradicts our preconceived hypothesis that *Anaerotruncus* may contribute to disease severity, it may be a compensatory change that

helps the disease. Previous studies have found that elevated *Akkermansia* in MS, which in fact plays a protective role. *Anaerotruncus* is a butyrate producer, which may in fact have a beneficial effect in increases regulatory T cells.

3.4 Aim II-Experiment II: Investigate the Effect of *C. bolteae* and a Combination of *A. rectale* and *R. intestinalis* On EAE in C57 Mice.

We next decided to colonize mice with another bacteria determined to be associated with worse disease in MS, C. bolteae, and a combination of two bacteria A. rectale and R. intestinalis, potent butyrate producers that we hypothesized would decrease disease severity in MS. We verified the efficacy of our MOG protein and our protocol for inducing EAE in our pilot experiment. In experiment I we were also able to successfully induce EAE and produce the expected phenotype in mice. For this experiment, we induced EAE with the exact same protocol and materials shown in our prior experiments and began to assess mice at days 7 post immunization. Unexpectedly, the mice did not develop symptoms consistent with the expected EAE phenotype. We followed these mice from days 7-24, however the EAE phenotype never developed (Figure 14.A,B,C). We decided to end the experiment at day 24 because the control mice did not develop disease, thus we could not determine whether the bacteria could make EAE better or worse. One factor that may have failure to induce disease is the breakdown of the immunization components, however, none had expired, and all had been stored properly. A second possibility is that the new mice shipped from Jackson labs may have a different microbiota that is protective for EAE. To evaluate whether or not this is a possibility, an experiment consisting of microbiota transfer from symptomatic mice from the control group in the previous experiment (Figure 13.A) vs microbiota from the PBS group from

this experiment (Figure14.A) could help determine whether microbiota differences are the root cause of this issue.

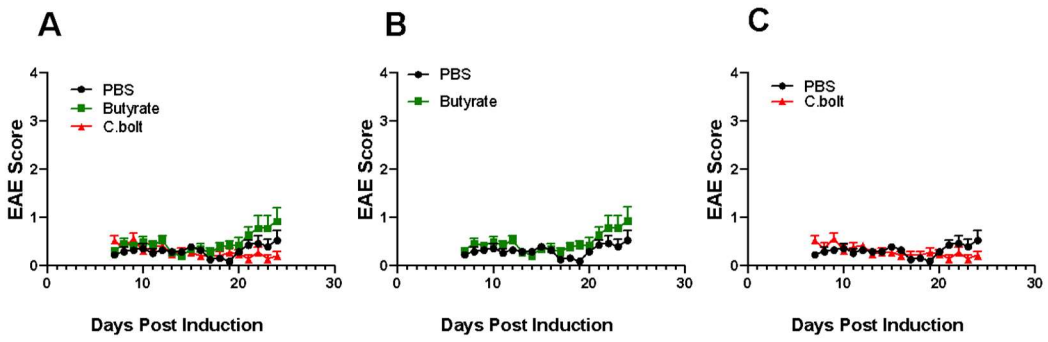

Figure 14: Disease phenotype by treatment of C.bolteae, Butyrate blend consisting of R. intestinalis & A.rectale) or PBS in C57 and NOD mice. Disease score phenotype of all treatment groups in C57 mice. (A) Disease phenotype of mice colonized with the Butyrate producing combination (A.rectale and R.intestinalis) versus control saline treated C57 mice. (C) Disease phenotype of mice colonized with C.bolteae versus control saline treated C57 mice.

3.5 : Investigating the Effect of *A. colihominis* and *F. prausnitzii* on Peripheral and Central Immune Responses in C57 Mice, Experiment I.

We previously found that *Anaerotruncus* ameliorated EAE in C57 mice, whereas there was no effect of *Faecalibacterium*. In order to characterize the microbe specific changes in CNS autoimmunity, fluorescence activated cell sorting (FACS) analysis was performed on a series of cytokines and t-cell panels on 3 organs, the spleen, spinal cord, and mesenteric lymph node. For these FACS analysis panels, we chose cytokine markers based on their specificity to CNS autoimmunity. MS is commonly associated with large increases in pro-inflammatory cytokines secreted by T cells, including IFN-©, IL-17 and GM-CSF. Decreases of anti-inflammatory cytokines such as IL-10 are also common in MS.

In health, it is uncommon to have T cells infiltrate the spine, so even small population differences effected by these microbes may be important. *A. colihominis* treatment reduced the number of spinal cord IFN-© CD4 T cells from approximately 10 cells to 1, reduced the number of GM-CSF CD4 cells from ~19 to 4, and reduced the number of IL-17+ GM-CSF+ ©™ T cells from 10 to 1 in the spine when compared to control mice (Figure 15). They also increased the number of CD8 IL-10+ secreting cells from 2 to 7, and the number of CD8 IL-17 cells from 1 to 3. While CD8 IL-17 cells may have a pathogenic role, an average of 3 cells is much smaller than other changes we observed and may be less biologically relevant.

We also examined how *A. colihominis* and *F. prausnitzii* altered the proportions of cytokine secreting T cells in the spine by calculating the relative abundance of each population. We found that the proportion of CD4 T-cells were decreased in *A. colihominis* and the *F. prausnitzii* treated mice. Consistent with our count data, we also found that *A. colihominis* decreased the proportion of CD4 IFN-©+ cells, decreased ©™ IL-17+GM-CSF+, and decreased CD-8 IL-10 and IL-17 secreting cells compared to control mice (Figure 16).

We next looked at activation markers and inhibitory molecules on CD4, CD8, and ©™ T cells. We found an increase in CD4/CD8 TIM-3, CD4: FOXP3+ LAP+, γδ:LAG-3, TIM-3, CXCR3, and effector memory cells in the *A. colihominis* treated mice when compared to the control. We also found that *F. prausnitzii* treated mice had an increase in CD4: LAG-3 and TIM-3, γδ:LAG-3, TIM-3, CXCR3, and effector memory cells (Figure 17).

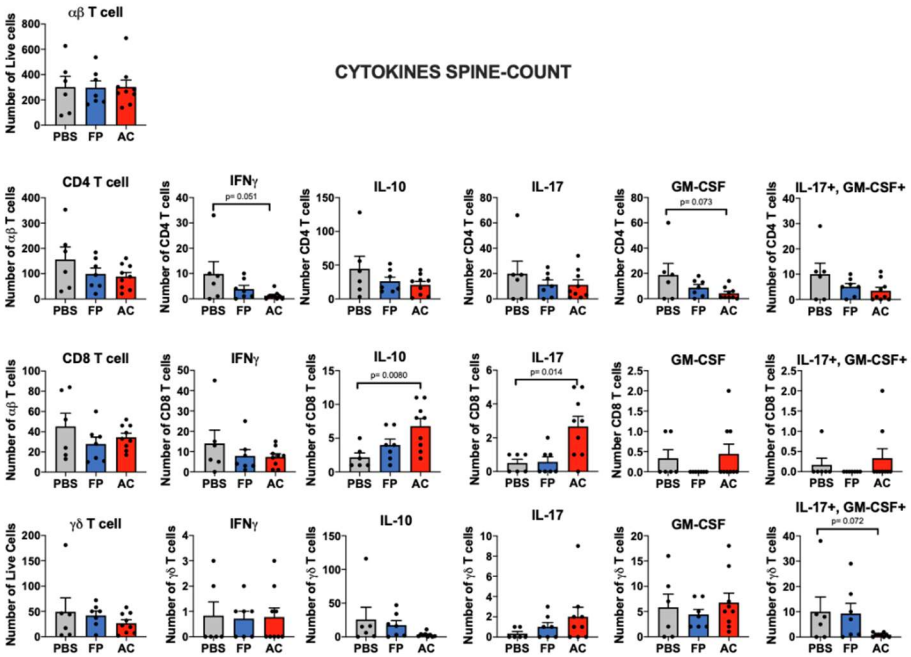

Figure 15: The population effects of Faecalibacterium (FP) and Anaerotruncus (AC) on cytokine responses in the spine-count. *Cells were stimulated with PMA Ionomycin and cytokines were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM. N=6 PBS, 7FP, 9AC.*

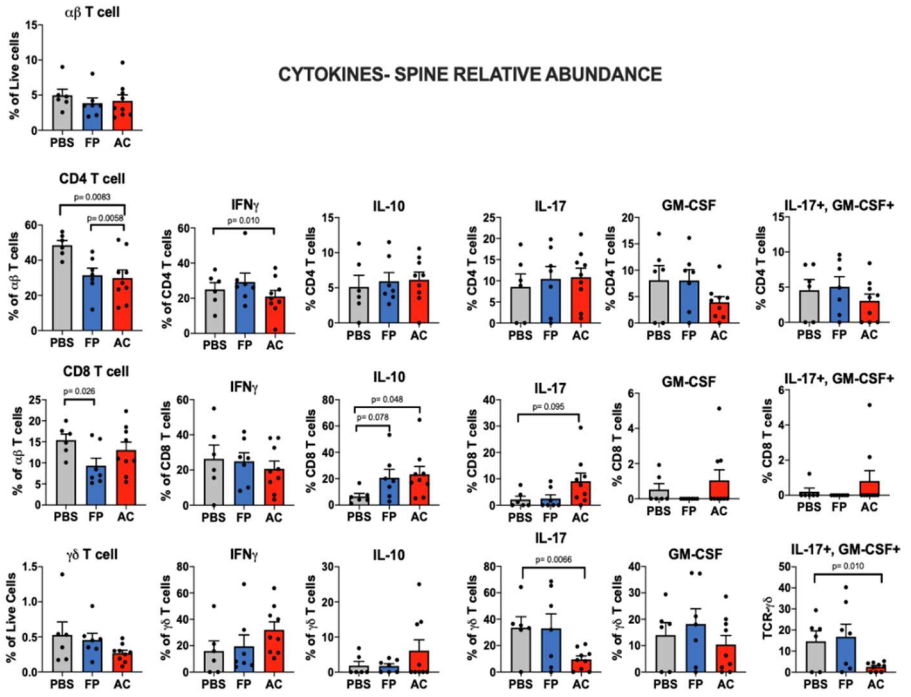

Figure 16: The effect of Faecalibacterium (FP) and Anaerotruncus (AC) on cytokine responses in the spine-relative abundance. *Cells were stimulated with PMA Ionomycin and cytokines were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM.n=6 PBS, 7 FP, 9 AC.*

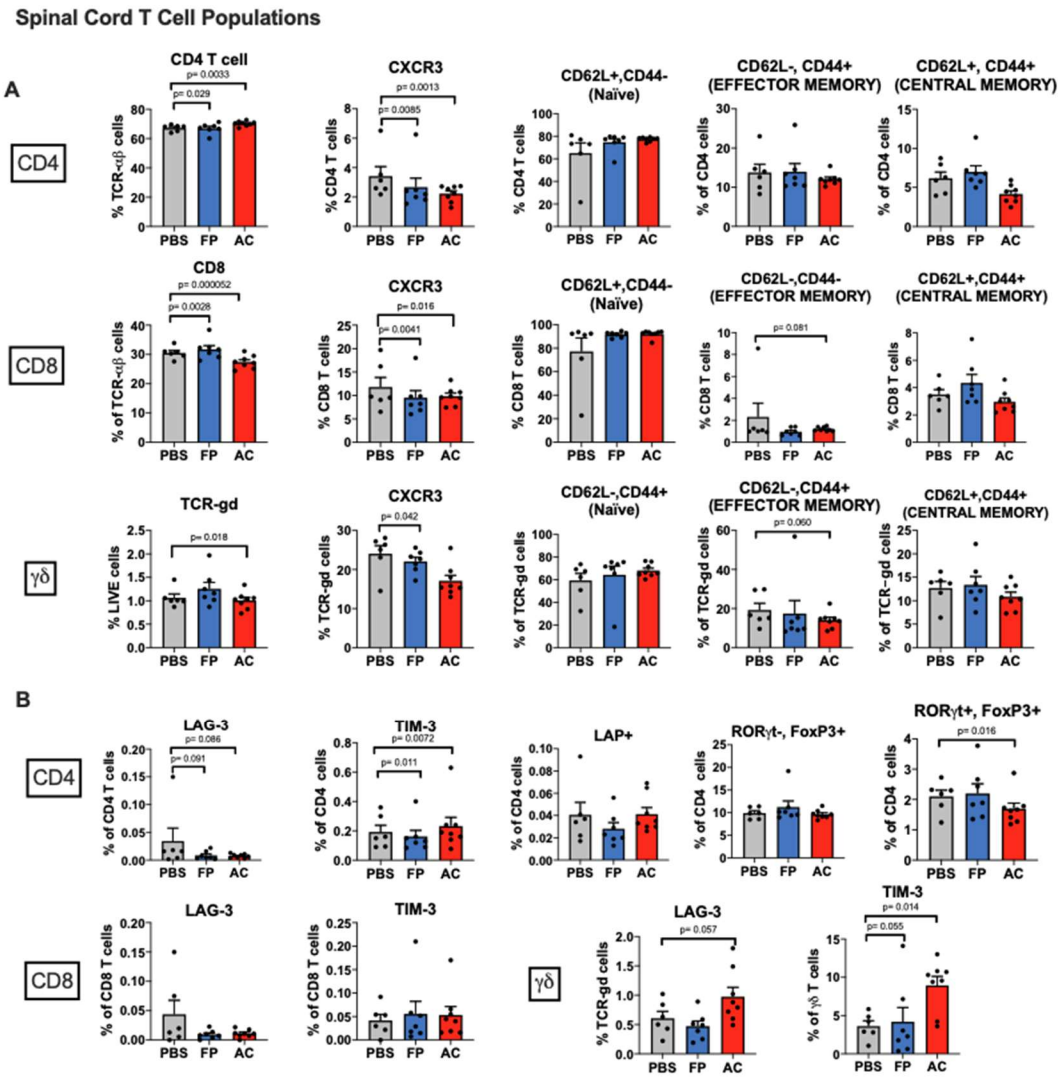

Figure 17: The effect of Faecalibacterium (FP) and Anaerotruncus (AC) on t-cell responses in the spine-population count. *Cells were stimulated with PMA Ionomycin and t-cells were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM.n=6 PBS, 7 FP, 9 AC.*

The spleen is a pivotal component of the immune system as it allows filtration of the blood to contain pathogens as well as regulating T and B cells responses (Lewis et al., 2019), thus we analyzed the relative abundance of cytokines from the spleen. We found

a decrease in production of IL-17 and GM-CSF from CD4 T cells and decreased production of IFN©, IL-17, and GM-CSF by ©™ T-cells in A. colihominis treated mice vs. control mice. There were fewer changes in the F. prausnitzii treated mice, including a trend of a decrease in CD8 IFN© and IL-10 production (Figure 18). These immunologic changes are consistent with the protective effect of Anaerotruncus in the C57 model and the minimal change in EAE course in mice given Faecalibacterium. This suggests that Anaerotruncus may help prevent EAE by decreasing the secretion of inflammatory cytokines in the periphery. A. colihominis treated mice had increases in CD4/©™: TIM-3 and CD8: T Effector cells when compared to control mice. F. prausnitzii treated mice had increases in CD4: LAG-3 when compared to the control (Figure 19).

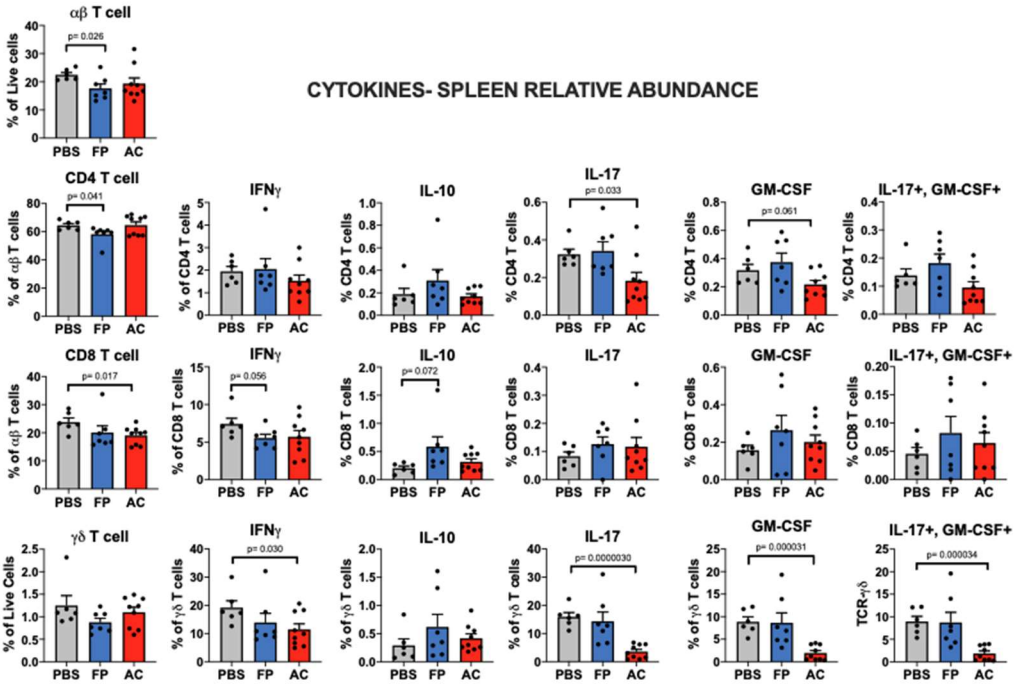

Figure 18. The effect of Faecalibacterium (FP) and Anaerotruncus (AC) on cytokine responses in the spleen-relative abundance. *Cells were stimulated with PMA Ionomycin and t-cells were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM.n=6 PBS, 7 FP, 9 AC.*

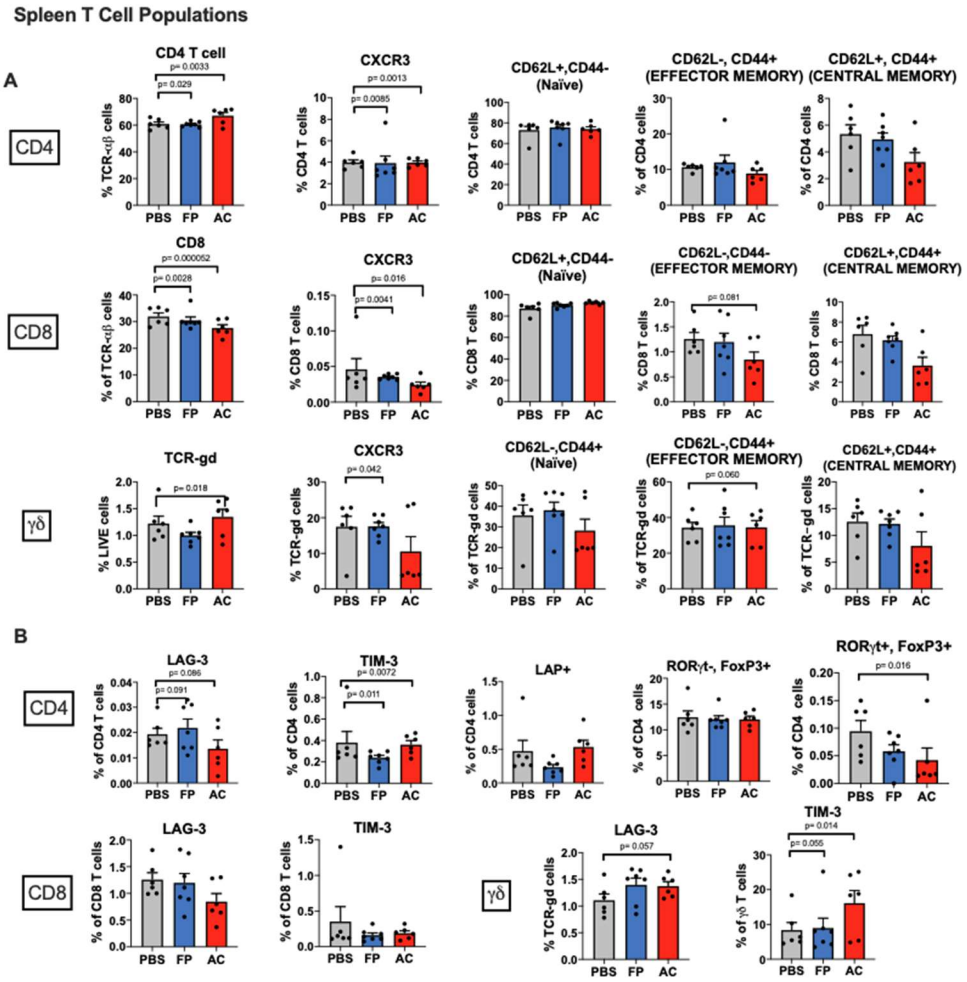

Figure 19: The effect of Faecalibacterium (FP) and Anaerotruncus (AC) on t-cell responses in the spleen-population count. *Cells were stimulated with PMA Ionomycin and t-cells were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM.n=6 PBS, 7 FP, 9 AC.*

The mesenteric lymph nodes (MLNs) were the final organ to be chosen to analyze because of its key regulatory roles in the immune system. The MLN acts as the primary site for antigens from the gut microbiota to pass into the lymphatic circulation. (Macpherson et al., 2006). We found a decrease in the relative abundance of CD8 cells in the *A. colihominis* treated group vs the control mice, as well a decrease in the secretion of

IFN-© in the *A. colihominis* treated group when compared to the control (Figure 20). There are not any significances in the cytokine panel besides a decrease in the amount of ⟨® T cells when comparing the *F.prausnitzii* treated group to the control group in the MLN. These minimal changes are consistent with the lack of an effect on EAE course. However, this data further emphasizes the protective effects of *A. colihominis.*

In our T-cell panel we found an increase in the amount of CD4: ⟨®, TIM-3 and ©™: LAG-3 in the *A. colihominis* treated group. We found decreases in CD4: CXCR3, LAG-3 and CD8: ⟨®, CXCR3, ©™ CXCR3, TIM-3 in the *F. prausnitzii* treated group versus the control (Figure 21). An increase in ©™ LAG-3 from treatment with *A. colihominis* can further exemplify this bacteria's potential therapeutic effects. LAG-3 acts an immune checkpoint inhibitor and is an important component to maintain homeostasis within the immune system. TIM-3 can act as both an inhibitory or co-stimulatory t-cell within the immune system depending on the context in which it is activated (Banerjee et al., 2018). Decreases in CD8 t-cells are a common early hallmark and predictor of disease prognosis in MS (Pender et al., 201

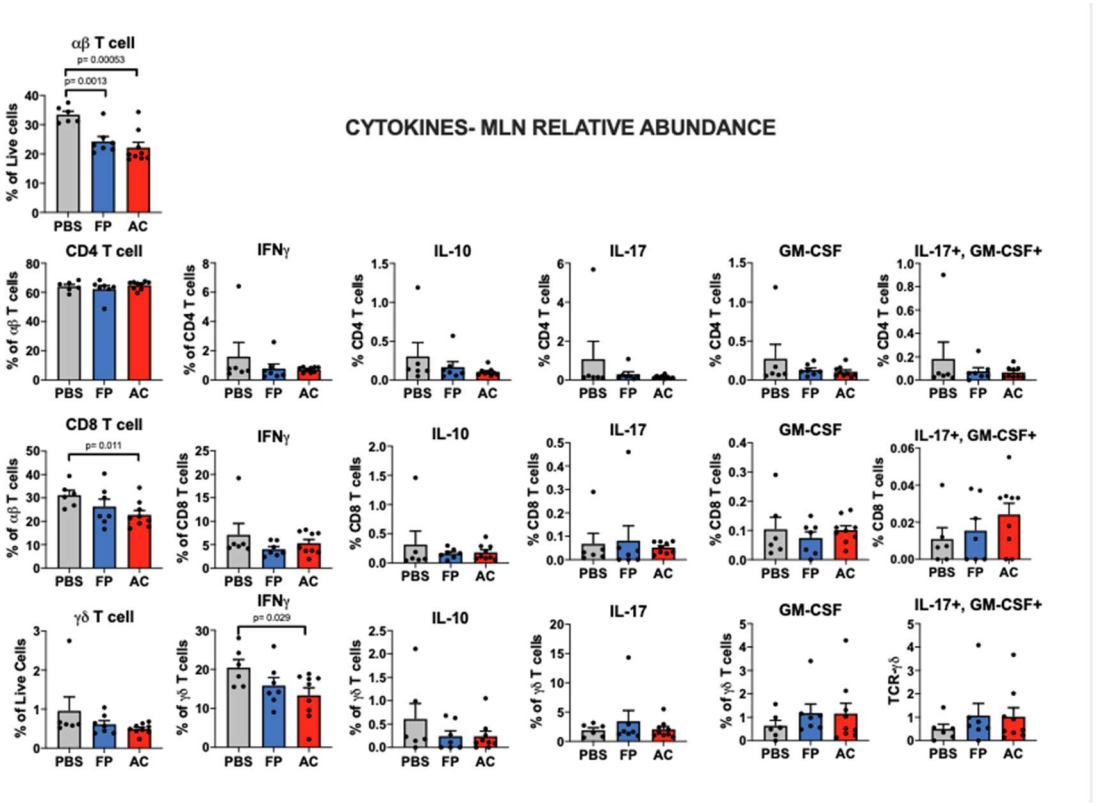

Figure 20: The effect of Faecalibacterium (FP) and Anaerotruncus (AC) on cytokine responses in the mesenteric lymph nodes-relative abundance. *Cells were stimulated with PMA Ionomycin and t-cells were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM.n=6 PBS, 7 FP, 9 AC.*

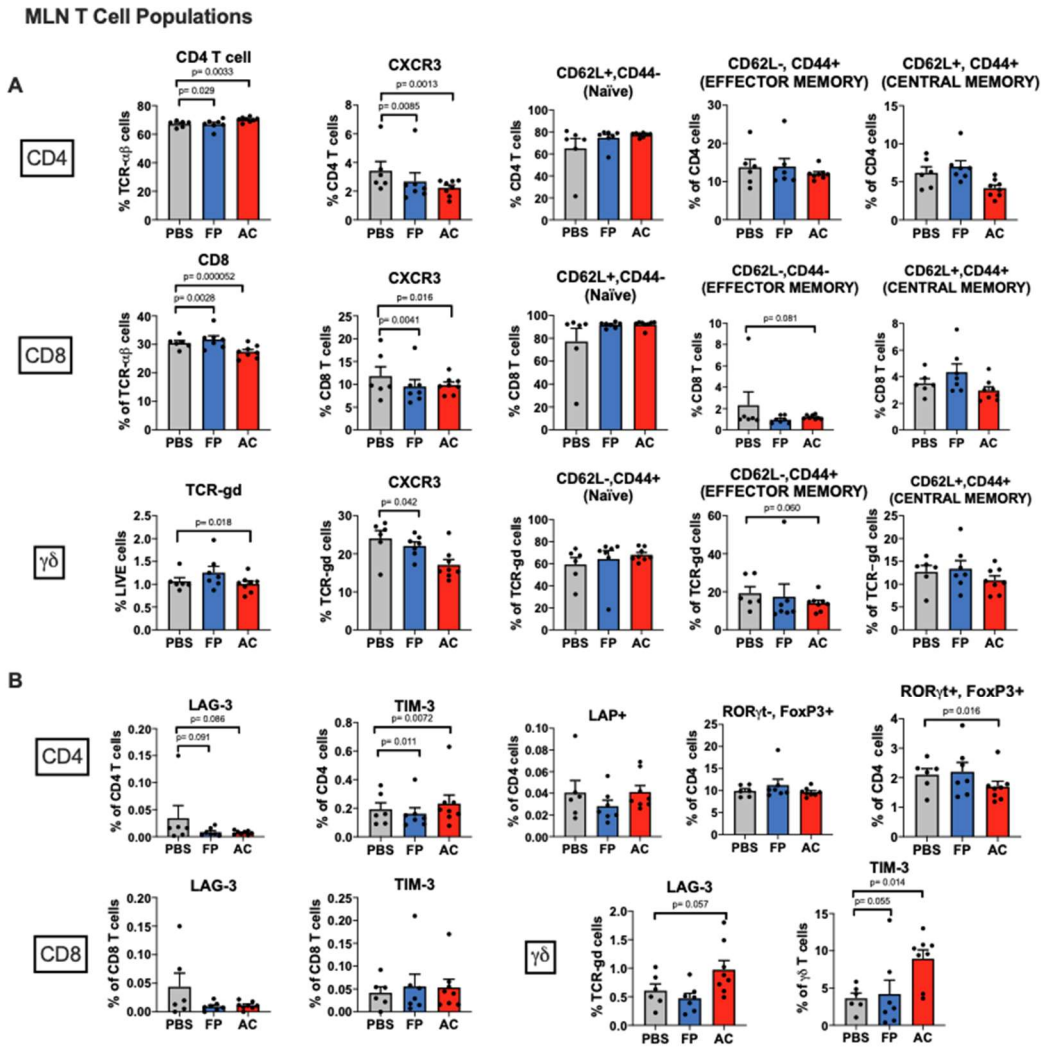

Figure 21: The effect of Faecalibacterium (FP) and Anaerotruncus (AC) on t-cell responses in the mesenteric lymph nodes-cell population. Cells were stimulated with PMA Ionomycin and t-cells were measured by flow cytometry in CD4, CD8 and gd-T cells. Bars show mean +/- SEM.n=6 PBS, 7 FP, 9 AC.

Chapter IV.

Discussion

Our major goal was to experimentally investigate whether bacteria associated with multiple sclerosis could worsen CNS autoimmunity and identify relevant immune populations that were affected. Because of experimental variability in the EAE MOG model, it was first important to establish a working protocol for EAE induction (AIM I). We were able to successfully induce EAE in our pilot experiment and determine vendor specific phenotypes between mice from different vendors. This gives us insight into the important role that the environment can play in determining microbiota composition and disease susceptibility.

While many bacteria are altered in MS, we selected specific bacteria associated with improved or worse disease from our MS patient population. While we found these bacteria associated with worse or better disease, it was important to move past correlation to address causation. We found that *A. colihominis* had a protective effect in the C57 model while there were no significances in the NOD model of disease. We also determined that treatment with *F. prausnitzii* initially had a protective effect though not as substantial as those effects produced from *A. colihominis.*

The spine is one of the organs most affected in MS. Spinal lesions produce long lasting disability and correlate with disease severity. Though spinal infiltration of Cytokines and T cells is less common, it is interesting to see immune cell variance in this organ which may contribute to disease. The studies conducted provide further evidence that microbes can significantly impact immunity in a specific way.

The studies conducted provide further evidence that microbes can significantly impact immunity in a specific way. *Anaerotruncus colihominis* has shown to have impactful protective effects by decreasing secretion of pro-inflammatory cytokines including IFN-©, IL-17 and GMCSF, all potent immune system defenses that can be destructive in MS. In some areas, *A. colihominis* also increased levels of anti-inflammatory IL-10 secretion, which can limit host immune responses. This bacteria had a more prominent effect early in treatment which appeared to taper off near the endpoint. It is speculated that a change in the animal facility may have occurred that could be responsible for its diminishing protective effects, however delaying disease course is also welcomed in treatment for progressive MS.

A. colihominis also increased IL-17 producing CD4 populations in the spine, however the effect was low in magnitude, increasing the average number of cells from 1 to 3. Though this result may seem to contradict our other findings including less secretion of these pro-inflammatory cytokines in other organs and other T cell subset of mice treated with *A. colihominis*, there is a subset of Th-17 cells that are capable of secreting the anti-inflammatory IL-10. A study performed on patients with Acute Myeloid Leukemia (AML) found an increase in the levels of Th-17 secreting IL-10 cells which was associated with immunosuppression (Musuraca et al., 2015). In the case of autoimmune diseases, immunosuppressive drugs are commonly used to prevent further damage from the over-active immune system.

Contrary to previous evidence that *F. prauznitzii* can ameliorate inflammatory diseases, treatment with *F.prausnitzii* had limited protective effects on EAE severity and

on immune responses. However, we found increases of both TIM-3 and LAG-3 which can have regulatory and inhibitory effects on the immune system depending on context.

The microbiome provides a new innovative approach to individually tailored treatment for patients with MS. More studies need to be conducted on the various microbes isolated in patients with MS that correlate with either positive or negative disease outcomes to further determine the unique implications that microbes may have on the disease. However, this study provides an avenue for further scientific exploration.

4.1 Pitfalls and Alternatives

The mice in Experiment 2 did not display the expected EAE phenotype despite immunization, including in our PBS control. Though the batch of MOG-CFA was pre-tested in the pilot experiment for efficacy in inducing EAE in mice, there may be a few reasons for the mice to be resistant to immunization of this protein. The first of these reasons is that this group of mice may have come from a different breeder parent in the Jackson Laboratories which may have affected their microbiome composition. As previously discussed, mice that are deficient in SFB a potent Th17 cell inducing microbe, are resistant to EAE immunization.

Another possible explanation for resistance in the Experiment 2 mice, could be indirect handling of the mice from animal technicians within the facility breaking specific sterility and exposing the mice to a breadth of diverse microbes which can affect CNS autoimmunity. The third explanation for the phenotypic absence could be the colonization of the determined beneficial microbe for EAE protection, A. colihominis. After scoring each of the experimental groups the fume hood is thoroughly cleaned with an anti-bacterial, however A. colihominis is a spore former and may have aerosolized in

the hood, unknowingly exposing the mice in other experiment groups and exerting its protective effects against EAE.

As previously discussed, there are various mouse strains that may be used to model disease and induce EAE. Another murine strain that has been used in EAE experiments is the Biozzi ABH mouse model. This mouse model produces both a relapsing-remitting and secondary progressive disease phenotype. This mouse model however is age specific, and mice develop variance in disease phenotype depending on age of EAE induction (Peferoen et al., 2016).

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