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The bi-directional relationship between gut microbiota and autoimmunity

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The Bi-directional Relationship Between Gut Microbiota and Autoimmunity

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ABSTRACT

The Bi-directional Relationship Between Gut Microbiota and Autoimmunity

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Humans serve as a major reservoir for a vast number of microbiota. These microbes have evolved symbiotic relationships with humans due to their close proximity with their host. As a result, the immune system adapts to the microbiota thus modulating immunological function. Autoimmunity is a state in which there are aberrant immune responses produced against host tissue. Intestinal bacteria are directly impacted by instances of inflammation brought on by autoimmunity. The complicated nature between autoimmunity and bacterial modulation demonstrates a bi-directional relationship. Here, we utilize experimental autoimmune encephalomyelitis, a model for multiple sclerosis, to explore the bi-directional relationship that disease and microbiota share. We demonstrate that disease severity, as well as time point post disease induction, shapes the structure and function of the gut microbiome. Additionally, antibiotic intervention modulates the symptoms of disease by upregulating the expression of T regulatory cells. Further, we show that a genetically engineered *Lactococcus lactis* expressing the colonization factor antigen I from *Escherichia coli* acting as an immunomodulatory probiotic reduced the severity of disease.

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Overview

Bacteria are prokaryotic microorganisms with a vast array of functions and characteristics. Diverse bacterial metabolism allows them to occupy almost every niche possible on Earth. Their range spans everywhere from extreme conditions such as deep sea hydrothermal vents to more mild conditions such as on plants growing in temperate climates. As a consequence, every facet of human biology is exposed to bacteria. Whether it be from dust in the air to the food consumed, humans are constantly exposed to bacteria.

Generally, there is a misconception that bacteria are harmful to humans and pose a threat to human health. There are more bacteria that establish symbiotic relationships with us, commensal or mutualistic, than bacteria that are pathogenic. The symbiotic bacteria participate in with their human host are analogous to symbioses present in traditional ecological settings. Commensal bacteria will reside in or on the human host and receive nutrients from their host's diet and or metabolites produced from other bacteria. These bacteria do not help their host but they also do not cause disease for their human host. Mutualistic bacteria will behave much like commensal bacteria but their presence will provide a direct benefit to their host. As science continues to investigate the bacteria associated with humans, their apparent function and benefits become more apparent.

The Gut Microbiome

Bacteria must compete and interact with other microbes such as other bacteria, viruses, fungi, archaea, and single celled eukaryotes as well as interact with the environment around them. These interactions form intricate microbiomes; a microbiome is best defined as the summation of all these microbes as well as the combination of all the genetic material from them in a defined space and time. The sum of all microbes within a given niche is defined as microbiota. Several types of microbiomes exist and are generally defined by the physical space in which they are located. Although scientists study all types of microbiomes, particular interest has been focused on human associated microbiomes due to their relevance to human health. Because of this, and in association with its highly complex structure and broad array of effects, the best studied microbiome is the human gut microbiome (and by proxy the murine gut microbiome).

The best studied microbiome is the human gut microbiome (and by proxy the murine gut microbiome) that spans the entire digestive tract including the stomach, small intestine, caecum, large intestine, and rectum. Across the geographic location, the gut environment varies dramatically in both its physical and chemical nature. The physical and chemical variation dictate the structure of the microbiota present. For example, the approximate pH of the duodenum is 6 whereas the pH of the terminal ileum ranges from 6-7.4.[1] Mucus levels tend to differ between the large and small intestines as well which directly regulates the bacterial communities present.[2] Although the physical properties of the gastrointestinal tract shape bacterial populations, microbes persist and thrive. Therefore, the gastrointestinal tract physiology evolved to interact and respond to the microbiota present.

Bacterial metabolism also plays a role in which taxa are present at differing geographic locations. For instance, fatty acids and simple carbohydrates derived from food are absorbed in the small intestines and therefore bacteria that can ferment complex carbohydrates are the ones that can survive.[3] As a consequence, the *Bacteroides* are the most studied taxa that can perform this metabolic task.[4] Additionally, human diet plays a critical role at shaping the gut microbiome. Studies in which humans switch their diets from being primarily plant based versus primarily animal based experience a profound effect on the composition of the gut microbiota.[5] The effect of diet on the composition of the gut microbiome generally is centered on the fact that it dictates what nutrients are available for the microbiota. Therefore, nutrient availability as well as retention is critical.

Antimicrobials play an additional role at shaping the structure of the gut microbiome. Specialized epithelial immune cells known as Paneth cells secrete antimicrobial compounds that alters the growth of bacteria near the mucosal surface.[6] These compounds are cationic peptides that interacted with charged membranes of bacteria. Some bacteria, however, have evolved to respond to these charged peptides; some gram negative bacteria have modifications in the lipid A component of the outer membrane which renders them resistant to these peptides.[7] Interestingly, however, the concentration of antimicrobials are higher towards the proximal end of the small intestine which results in higher abundance and diversity in the distal ends.[3]

Secreted Immunoglobulin A (sIgA) and other immune system mediated responses dictates what bacteria persist where. Intestinal mucosa contains large quantities of sIgA to monitor the gut microbiota.[3] The non-pathogenic bacteria becomes coated in sIgA to

maintain tolerance from the host. Coats of sIgA on bacteria reduces inflammatory signaling and reduces changes to bacterial gene expression.[8] This process allows homeostasis between the host and the microbiota to be maintained. However, this process can be sometimes utilized by pathogenic bacteria as well. In the case of some species of *Helicobacter*, these pathogens are coated in sIgA as well resulting in an inappropriate tolerance response by the host.[9] Therefore the complexity of homeostasis between non-pathogenic bacteria and the host is more complicated than simply just sIgA coating. Some believe that the bacteria themselves have to promote their own tolerance to the host for their own survival. This is shown the case of *Bacteroides fragilis*. The polysaccharide A component of the bacterial capsule on *B. fragilis* has been shown to exert anti-inflammatory properties by stimulating the secretion of IL-10 by regulatory T (Tregs) cells.[10] This process is seen in other bacteria as well suggesting that the need for self-promoting tolerance is necessary.

The Anatomy of the Gut Epithelium

The gastrointestinal tract is the site of interaction between the body's largest concentration of immune cells and the gut microbiota.[11] The gut epithelium acts as a major barrier between the external environment and the host's internal environment. The human digestive tract essentially is a long tube; starting from the mouth and extending down the esophagus, past the stomach and through the small intestines and large intestine, finally ending past the colon at the anus; everything humans eat needs to be separated from the inside of the body.

Goblet cells in the intestinal epithelium produces mucus to form a matrix between the external environment and the intestinal epithelial surface.[12] Besides the mucus layer,

only a single layer of epithelia separates the intestinal lumen contents from the underlying connective tissue and interior milieu.[13] Due to this, the intestinal epithelium developed specialized cells types to deal with the exposure. As previously discussed, Paneth cells play a critical role in maintaining the security of the gut epithelium by secreting antimicrobial peptides. Additionally, cells expressing CD24 resides in the colonic crypts which elicit similar responses to the Paneth cells.[14]

The thin layer of epithelium generates a barrier that can prevent material from the intestinal lumen from entering the interstitial space of the body. Tight junction protein complexes regulate the paracellular permeability of the intestinal epithelium.[13] The permeability of the epithelium varies across the intestines geography and is largely determined by the amount of tight junction protein complex expression. The protein complex consists of transmembrane proteins such as occludin, claudin, junctional adhesion molecules, tricellulin, and intracellular scaffold proteins like zonula occludens.[13] The pathogenesis of several diseases such as inflammatory bowel disease, celiac disease, and even food allergies have been attributed to the hyperpermeability of the intestinal barrier.[15] Interestingly, alterations in normal gut microbiota has been noted to cause changes in intestinal permeability.[16] In the case of Gulf War Illness, chemical injury results in the alteration in intestinal microbiota resulted in a down regulation of occludin expression and an increase in intestinal permeability. The leachates then cause endotoxemia leading to the upregulation of toll like receptor-4 activation in the intestine as well as the brain.[16]

Because of the high intake of microbes via digestion and the critical role intestinal epithelia plays, the immune system and intestinal physiology evolved to monitor the

traffic. The lymphatic system is a network of vasculature that allows leukocytes to travel the body and monitor antigens. For the gut, there is a specialized set of lymphatic tissue known as the gut associated lymphoid tissue (GALT). The GALT is comprised of the Peyer's patches, mesenteric lymph nodes, lymphatic vasculature, as well as other lymphoid aggregates. These structures work in tandem to protect the gut from pathogenic microbes while monitoring the commensal or mutualistic bacteria.

The Gut/Immune System Nexus

As bacteria and their metabolites persist in the gut, the GALT-associated immune cells must monitor every aspect of the intestinal lumen to catch and eliminate pathogens. M cells, a specialized epithelial cell of mucosa-associated lymphoid tissue, transports antigens from the intestinal lumen to immune cell populations. M cells allow dendritic cells to sample antigens from the lumen and endogenously prepare the antigen for presentation to T effector cells via the major histocompatibility complex (MHC) class II molecule. This process then, in turn, can activate the T cells and differentiate them into specialized effectors to perform functions necessary for dealing with the microbe expressing the activating antigen. Whether it is to mount an immunological response or to disregard the antigen, the interaction between the host immune system and the microbe will occur. This process is mediated by the subsets of T cells as well as anergy.

In the case of pathogenic antigens, the need to activate pro-inflammatory T cells may be necessary. T helper (Th) cells such as Th1, Th2, and Th17 can help mobilize and recruit innate immune cells to the site of the pathogen. This process is mediated by the secretion of cytokines and chemokines. Cytokines are molecules such as interferons, interleukins, growth factors, and other compounds that have some effect on other immune

cells. CD4 T cells express surface receptors known as T cell receptors that can identify antigens presented by MHC molecules. Upon this union, the naïve T cell will differentiate into one of the various T helper cell subsets depending on the additional signaling molecules the naïve T cell encounters during co-stimulation. As an example, Th1 cells can be activated after co-stimulation and go on to secrete interferon-gamma (IFN-γ) to activate other Th1 cells in the immediate area.[17] The non-cognate stimulation of Th1 cells allows the immune system to clear pathogenic intracellular bacteria. These cytokines and chemokines recruit innate immune cells such as neutrophils to the site of the infection; the immune system then can eliminate the threat of the pathogen via phagocytosis, neutralizing antibodies, or antibody mediated engulfment.

In the case of non-pathogenic antigens, there is a need to activate anti-inflammatory T cells to prevent immune cell mediated clearance of the pathogen. As previously stated, not all of bacteria are pathogenic but rather commensal or mutualistic. Therefore it is critical for the immune system to have a way to virtually ignore the antigens of these non-harmful bacteria. Previously discussed, *B. fragilis* had the ability to stimulate its own immunotolerance by stimulating the production of Tregs which would secrete IL-10.[10]

Additionally, anergy could also be used to prevent an immunological response against non-pathogenic bacteria. Anergy is defined as being the absence of a normal immunological response to an antigen; achieving anergy against non-pathogens could be another way to ignore non-pathogenic antigens. Toll like receptor-7 can be engaged resulting in an intracellular calcium flux with the activation of a NFATc2-dependent anergic gene expression program that ultimately results in T cell non-responsiveness.[18]

The processes that maintain the balance between pro- and anti-inflammatory processes are complicated and in some cases depend on the bacteria or viruses present.[10,18] Additionally, the gut microbiome is shaped by various factors including host genetics, geographical location, diet, lifestyle choices, prescribed pharmaceuticals, mode of delivery during birth, antibiotic exposure, and possibly even disease states themselves. When these factors tip the gut microbiome out of balance, there can be an induced imbalance between these pro- and anti-inflammatory responses that can result in disease. This proposed disease model is known as dysbiosis: gut microbial imbalances that result in, or are a result of, disease states. Generally, labs are investigating whether these alterations in gut microbiota result in disease. However, it is possible that alterations in the balance between pro- and anti-inflammation can alter the structure and function of the gut microbiota as a function of diseased states.

Multiple Sclerosis and Autoimmunity

Autoimmunity is best described as being a process in which the host's immune system fails to distinguish self from non-self and begins to elicit immunological responses against host tissue. Mechanisms that drive autoimmunity remain to be elucidated; however, it generally is understood that exacerbated pro-inflammatory responses can exacerbate the tissue damage that characterizes the process of autoimmunity. Therefore, understanding the extent of inflammation induced by dysbiosis becomes critical to investigate. The importance of gut microbiota in context to immune function can be seen when comparing traditional mice to gnotobiotc mice. Gnotobiotic mice exhibit reduced immune function with physiological abnormalities such as increased intestinal permeability. Dysbiosis can be induced by the same environmental

factors that have been described to potentially illicit autoimmunity. Moreover, dysbiosis has been noted in several experimental models of autoimmunity as well as patients suffering from autoimmunity.[19-22] These phenomena can provide evidence that intestinal dysbiosis is critical in the development of autoimmune disorders.

Our lab primarily focuses on Multiple Sclerosis (MS), a clinically common autoimmune disease. MS affects millions of individuals worldwide; many of these individuals reside in northern geographic locations such as the Pacific Northwest. For these individuals the quality of life slowly diminishes as the disease progresses, as the host immune system attacks the insulating structure known as the myelin sheath that surrounds the axon shaft of neurons in the spinal cord.[23] When the sheath is degraded, the ability for electrical signals to be sent through the axon is reduced thus resulting in paralysis and other symptoms. The most prevalent form of MS is relapsing-remitting MS (RRMS) that affects 85% of the total patient population; RRMS is initially diagnosed as a syndrome of neuronal dysfunction with a repeating series of relapses and remissions that follow over time.[24] Approximately 70% of RRMS patients develop secondary-progressive MS (SPMS), which causes a steady and progressive neurological impairment.[24,25] The precise reason as to why immune cells destroy myelin remains unclear.[24,25] In MS, the myelin sheath that surrounds the axons of neuronal cells is degraded by host immune cell populations. Although the exact etiology is debated, there are hallmark events that undeniably occur.

Generally, MS arises in genetically susceptible individuals. Genetic variation is attributed to about one third of the disease risk.[26] Environmental conditions as well as lifestyle choices play an additional factor in disease risk. Lacking a predominant

exogenous risk factor, there is ambiguity as to whether MS starts in the periphery or in the central nervous system (CNS).[27] In peripheral models of MS, pathogenic T cells are activated and subsequently released to the draining lymph nodes.[27] From the draining lymph nodes, these pathogenic T cells can enter circulation and gain access to the central nervous system by trafficking with activated B cells and monocytes.[27] Intrinsic models propose that the rise of autoreactive lymphocytes is secondary to intrinsic CNS damage.[27] Additionally, autoreactive B cells can be found in the meninges, parenchyma, and cerebrospinal fluid.[27] These autoreactive B cells can secrete antibodies which tend to increase with age in MS patients.[28] There is a lack of known autoantigens which makes the mechanisms behind autoreactive B cell pathology speculative;[27] however, next-generation sequencing has provided evidence that antigen-experienced B cells potentially go through maturation prior to entering the CNS.[29] Inflammation is a primary result from autoreactive lymphocytes. These responses cause axonal damage and could potentially trigger a self-sustaining chronic neurodegenerative process.[27] As a result, resident CNS cells such as the microglia and astrocytes additionally secrete inflammatory molecules further exacerbating the neurodegeneration.[30] The extent to which all these cell populations work in tandem to cause disease highlights the need to prevent the initial onset of inflammation and neurodegeneration.

Compounding the magnitude of autoreactive immune cell populations, defective Treg cells have also been noted in MS.[31] Defective Tregs could contribute to the production of autoreactive lymphocytes and additionally exacerbate the effects of preexisting autoreactive lymphocytes. Studies show that Tregs are lower in numbers and

have reduced functionality in patients with MS.[32] Ultimately, it is the occurrence of defective Treg cells that can help explain why autoreactive immune cells arise.

The degradation of the myelin sheath by host immune cells is generally mediated by the T helper 17 (Th17) cells.[33] The imbalance between effector T cell and Tregs leads to pro-inflammatory states which characterizes MS. The increased levels of Th17 cells secrete pro-inflammatory cytokines and chemokines that recruit immune cells for the degradation events. Reduced and dysfunctional Tregs will fail to keep the exacerbated Th17 in check resulting in myelin degradation.

Gut microbial imbalances tend to shift towards a pro-inflammatory state that have profound effects on the intestinal physiology of the individual. Additionally, dysbiosis has been associated with intestinal barrier disruptions. When the integrity of these tight junction protein complexes diminishes there is an increase in intestinal permeability; the bacterial antigens can pass out of the intestinal lumen and travel to other locations in the body. As a result, levels of antigens, like the endotoxin lipopolysaccharide, can increase in the blood circulation which could have systemic inflammatory effects.[34] Systemic translocation of bacterial antigens can have a profound effect on CNS immunity and impact the integrity of the blood-brain barrier.[35] This process can result in the ultimate passage of autoreactive lymphocytes into the CNS and have direct access to the myelin sheath.

The Bi-directionality of the Gut Microbiome

As previously discussed, autoimmunity has been shown to be impacted by the gut microbiome. However, it has also been theorized that disease itself can shape the

structure and function of the gut microbiome. What this implies is that there is a bi-directional relationship between diseased states and the structure and function of the gut microbiome. This then raises more questions; what comes first: the disease or the aberrant gut microbiome?

In instances of some autoimmunity, the aberrant gut microbiome precedes the onset of disease. This phenomenon can be seen directly with Type 1 Diabetes (T1D). Intestinal dysbiosis has been noted in both humans and animal models of T1D. In a study conducted by Costa *et al*., the murine model for T1D was protected against the development of insulitis when treated with antibiotics.[36] The model utilized in this study was a streptozotocin-induced T1D models in which the onset of insulitis is brought on upon by the bacterial translocation event. The utilization of antibiotics prevented bacterial translocation and thus prevented the onset of disease. Moreover in humans, bacterial translocation has been theorized as being a major factor that leads to insulitis. Bacterial translocation through the duodenum as a result of intestinal dysbiosis can cause the bacterial antigens in the pancreatic ducts to recruit immune cell populations resulting in the destruction of β-cells.

On the other hand, there is an increasing interest in determining how disease itself shapes the gut microbiome. Risk factors that have been associated with autoimmunity also impact the gut microbiome.[37] Additionally, autoimmunity can directly impact how the immune system responds to the gut microbiota. In the case of inflammatory bowel diseases, the immune system targets resident microbiota thus altering the overall structure of the gut microbiome.[38] Targeting non-pathobionts and clearing them from the intestines could have profound impacts on the immune system of the host. If a

populations of bacteria that promotes anti-inflammatory responses are eliminated it is possible that unchecked systemic inflammation could occur thus further exacerbating the initial autoimmune disease.

In the case of MS, dysbiosis is present.[39-41] Therefore, there is ample data to support the hypothesis that the gut microbiome can play a role in the development of MS. However, very little is known about how MS affects the composition of the gut microbiome. Understanding how MS pathology affects gut microbiota can give insight to novel therapeutic approaches to impact disease progression. Similarly, dysbiosis drives disease progression in the inflammatory bowel disease model, therefore it is possible that dysbiosis also promotes inflammation in the MS model. It is hypothesized that there is a bi-directional relationship between the gut microbiota and MS. The structure and function of the gut microbiome shapes MS pathology at the same time MS disease progression also shapes the structure and function of the gut microbiome.

The structure of the dysbiotic MS gut is so influential to disease progression that fecal transplantation of the gut microbiome can influence the progression of experimental autoimmune encephalomyelitis (EAE), a model for MS in mice. When fecal material from discordant monozygotic twins was transplanted into mice, there was a profound impact on spontaneous EAE disease incidence.[42] The MS gut microbiome had the ability to increase the likelihood of spontaneous EAE induction in mice as opposed to the healthy twin. Moreover, the stool from MS patients also increased the severity of EAE in mice.[43] Therefore, it is clear that targeting the gut microbiome might have profound impacts on MS pathology.

Targeting the Gut Microbiome with Therapeutic Options

As summarized earlier, recent experimental evidence and clinical data suggest that the gut microbiome might be a major factor regulating autoimmunity. Targeting the gut microbiome with therapeutics could have profound effects in disease progression as well as managing symptoms of disease. The extent and approach towards modulating the gut microbiome can follow many directions; it is crucial to balance the positive and negative impacts of each therapeutic option. Gut microbiome-based therapeutic approaches could have beneficial effects in terms of disease management but also have unintended adverse side effects. Furthermore, some therapeutic options can work for some autoimmune diseases but not others. Therefore, again, assessing proposed therapeutics are crucial and developing therapeutics with less negative consequences is essential.

Antibiotic Therapy

Utilizing antibiotics to therapeutically target the gut microbiome has been proposed for models of Type 1 Diabetes as well as Fulminant Ulcerative Colitis.[44,45] Additionally, a study conducted by Nakamura *et al*. set out to test the efficacy of an antibiotic cocktail on an experimental autoimmune uveitis (EAU) model. Autoimmune uveitis has both a genetic and environmental culmination that impacts disease susceptibility, and is characterized by a distinct increase in Th17 cell populations with a decrease in Tregs cell populations.[46] Utilizing an antibiotic cocktail consisting of ampicillin, vancomycin, neomycin, and metronidazole, the researchers noted that clinical scores of EAU were significantly reduced compared to the control group when the antibiotics were administered orally. The bacterial phyla *Firmicutes* and *Bacteroidetes* as

well as the class of *Alphaproteobacteria* were reduced while there was an increase in the class of *Gammaproteobacteria*. More importantly, the utilization of antibiotics significantly increased the expression of CD4+Foxp3+ Treg cell populations with a reduction in IL-17 producing Th17 cells.[46] Although the reduction in Th17 cells was not significantly reduced, it was still reduced substantially. Taking this all into consideration, the treatment of broad spectrum antibiotics conferred protection against the EAU pathology.

Experimental Autoimmune Encephalomyelitis (EAE), a model disease for MS, has also been explored in the context of antibiotic intervention. A study by Ochoa-Repáraz *et al*. demonstrated that broad-spectrum antibiotic interventions affected the balance between inflammation and inflammatory regulation in EAE by modulating intestinal microbiota and, as a consequence, Treg cell populations.[47] Additionally, Yokote *et al*. reported similar findings but noted that the alterations in intestinal microbiota impacted the Natural Killer T cell populations.[48] These experiments analyzed the impacts antibiotic intervention could have on modulating EAE in SJL as well as C57 mice. In the case of germ-free mice, mice with no microbiota, it was noted that colonization with segmented filamentous bacteria induced intestinal Th17 cell production.[49] Additionally, the colonization of segmented filamentous bacteria was correlated with the increased expression of inflammatory genes.[49] In the case of EAE, when germ-free mice were monocolonized with segmented filamentous bacteria they experienced exacerbated symptoms of EAE which generally are not present in the germ-free model of EAE.[50]

CNS diseases also have studied in terms of how antibiotics impact disease pathology. Parkinson's disease (PD) patients report gastrointestinal distress as well as exhibit intestinal inflammation well before symptoms of motor deficits.[51-53] A study conducted by Sampson *et al*. hypothesized that alterations in the gut microbiota by antibiotic interventions could alleviate PD pathology due to the association of intestinal complications and the tight interactions between the gut and the CNS.[54] The treatment of broad-spectrum antibiotics did, in fact, confer protection against motor dysfunction in a murine model. These findings support the notion that therapeutic targeting of the gut microbiome with antibiotics may be efficacious.

In the cases above, antibiotic interventions have conferred protection in these models of disease by altering the gut microbiome. However, the usage of broad spectrum antibiotics can also have adverse effects by the same mechanism. *Clostridium difficile*, an opportunistic pathogen, can infect the host after antibiotic treatments with symptoms ranging from diarrhea to pseudomembranous colitis and can be in some cases life threatening.[55] The exposure to antibiotics cause structural changes in the gut microbiome leaving the host susceptible to opportunistic infection from *C. difficile* as well as other enteric pathogens.[56] Children exposed to antibiotics within the first three years of life exhibit lower diversity in their gut microbiome. Moreover, these microbiome structures are less stable in exposed children than in non-exposed children.[57]

Phage Therapy

In response to the negative impacts of broad spectrum antibiotic usage the notion of using bacteriophage (phage) as a therapeutic has been discussed. Phage are bacteria-specific viruses that can be altered to target bacterial populations of interest while leaving

others untouched. The oral delivery of phage has been considered to be safe and have the capability to bypass intestinal epithelia and penetrate the GALT as well as the blood stream.[58,59] This ability to penetrate the GALT as well as the blood stream makes phage therapy extremely attractive to deal with bacterial translocation that can lead to diseased states such as those seen in T1D.

Phage play a critical role in shaping the gut microbiome naturally and constitutes the bulk of the intestinal viriome.[60,61] However, phage can also be pathogenic by contributing to intestinal dysbiosis; their uncontrolled destruction of beneficial bacteria can have consequences to the overall structure and function of the gut microbiome.[62] Additionally, phage also can have the unintended impact of horizontally transferring antibiotic resistance to their bacterial hosts.[63] Lysogenic phage have had antibiotic resistance genes associated within their genomes.[64] To some, this has rendered the notion of phage therapy obsolete. However, researchers have been designing "smart" phage cocktails that bypass horizontal gene and appear promising.[65] Ultimately, there is still a lot of unanswered questions and concerns in regards to the safety and efficacy of phage therapies in terms of unintentional microbiome impacts.

Fecal Transplantation

The approach of antibiotic interventions and phage therapeutics is to target bacterial populations and remove or reduce them from the gut microbiome. Fecal transplantations act as whole gut microbiome replacements in hopes of correcting aberrant gut microbiome structures and functions. The efficacy of fecal transplantations is also quite high; thus, fecal transplantations is a common therapeutic to treat patients with *C. difficile*.[66,67] Conceptually, by eliminating the host's aberrant gut microbiome

and replacing it with a healthy gut microbiome, the diseased state will be rectified. This largely is the case in patients suffering from *C. difficile* infections and has revealed infection clearance with a single treatment.[66,67] The efficacy of fecal transplantations has promise in other diseases, including autoimmunity and neurological disorders, as well.

In the instance of autoimmunity, again, fecal transplantations has been proposed as a therapeutic strategy to treat irritable bowel syndrome as well as inflammatory bowel disease.[68] In inflammatory bowel disease, there is instances of dysbiosis with an increase in pro-inflammatory bacterial species.[69] A meta-analysis from Colman *et al.* showed that fecal transplantation therapy put 45% of patients into clinical remission and reduced the need for other forms of anti-inflammatory therapeutics.[70] Symptom rescue was attributed to microbiota manipulation; the gut microbiome changes in the patients in response to fecal transplant therapy.[71]

Satisfactory results from fecal transplantation are apparent, however there are still concerns regarding safety. For example, some of these concerns regard the route of administration, frequency of applications, screening the microbiota from the donor, the preparation protocol of the stool sample from the donor, what antibiotics should be administered and their frequency prior to fecal transplantation, among others.[69] Some of these concerns are very valid and have been partially explored. For example, in the study by Friedman-Korn *et al.*, instances of aspiration occurred in two patients when the fecal transplantation was administered via gastroscopy.[67] It was proposed that that colonoscopy would be a safer route for the therapy. This claim was then assessed later in the study by Bamba *et al.*[66] Still, several concerns remain. The impact donor stool can have an immense effect on an individual. Data supports the notion that fecal

transplantations can play a role in the development of obesity.[72] Ultimately, the risks and rewards of fecal transplantation therapy still needs extensive exploration.

Dietary Supplementation

Diet, itself, plays a major role in shaping the structure of the gut microbiome. By manipulating compounds and nutrients the gut is exposed to, it is possible to alter the structure and function of the gut microbiome. Western diets generally consist of high amounts of saturated fats and carbohydrates which can lead to chronic inflammatory states.[73] Recognizing the impacts of the Western diet and identifying routes to supplement the diet to reduce the negative impacts and chronic inflammatory states could be a therapeutic avenue to assess.

Naturally occurring compounds have been noted to have profound impacts on metabolic health. Chlorogenic acid is a phenolic acid commonly found in beverages commonly ingested by humans worldwide, such as coffee and tea.[74] It has been shown to inhibit glucose-6-phosphate translocase as well as hepatic glucose-6-phosphatase.[75] These properties would limit the amount of carbohydrate absorption by the small intestine as well as the extent in which glucose is released.[75] Chlorogenic acid has also been noted to have antimicrobial activities against some bacterial species that constitute parts of the gut microbiome.[76] Glycyrrhizin, a compound found in the roots of the *Glycyrrhiza glabra* plant, has also been identified for its anti-inflammatory properties.[77] When glycyrrhizin was administered into high-fat induced obese rats, the glycyrrhizin caused the rat to have reduced insulin resistance, lose weight from the diet, and reduced obesity-induced oxidative stress.[78] The impact glycyrrhizin has on bacteria has not been noted nor has it been explored in context to the gut microbiome. The overall impacts

these plant based compounds have on the gut microbiome is vastly unknown and warrants further investigation.

Utilizing dietary supplements as a therapeutic avenue for MS is still largely unexplored. The usage of probiotics however, has been gaining attention. A probiotic is generally described as bacteria that is administered orally to have some beneficial impact on the health of the individual. Moreover, probiotics are a non-toxic immunomodulatory agent that can orally be used in conjunction with current therapeutics for MS.[79] Studies have shown that oral administration of probiotics have positive immunomodulatory effects that could work in tandem with current pharmaceutical therapeutics. Probiotics will further be discussed later.

Limitations of Current Therapeutics

Ultimately, all these therapeutic options have not been explored fully been for MS. Moreover, the therapeutics available for MS are quite limited. There is no cure for MS and most of the therapeutics available only treat symptoms of the disease. Current pharmaceuticals exist that act as immunosuppressive or immunomodulatory agents; however, many of them have limited efficacy and have in some cases severe side effects.[80] Moreover, there is yet no commercial therapeutic for secondary progressive MS. Because of the current limits of the pharmaceuticals for MS, there is a need to explore novel therapeutic avenues.

Testing a potential therapeutic option for MS

The effect of the gut microbiome on MS pathology is a promising avenue of investigation. Therefore, characterizing the microbial profile of the MS gut as compared

to healthy gut microbiomes, as well as other autoimmune diseases, could be insightful. By investigating the structure of these microbiomes it is possible to understand what microbes are contributing to the pro-inflammatory state and lack of anti-inflammation. From this data, it is possible to then develop probiotic mixtures containing critical bacterial populations, which can increase Treg numbers, but are diminished in the MS gut microbiome.

Here, we propose to explore the bi-directionality between the etiology and pathology of MS and the gut microbiome of the host. To achieve this goal, we will analyze the structure and function of the gut microbiome when CNS inflammatory demyelination is induced in EAE mice, and in healthy control mice. From there, we will confirm the role the gut microbiome plays on symptoms of MS by subjecting EAE mice to broad-spectrum antibiotics and assessing the clinical scores of these mice during disease progression (as previously done in other models of EAE).[47,48] From the data obtained from the structural analysis of the EAE gut microbiome, we will test the efficacy of a probiotic on the symptoms of EAE in mice.

We hypothesize that the gut microbiome of EAE differs between individuals based on the severity of disease as well as the time point after disease induction. The broad-spectrum antibiotic cocktail will reduce disease severity when mice are subjected to the antibiotics during the early, more pro-inflammatory phase of EAE as opposed to later stages of disease. Moreover, we also propose that the oral treatment of EAE mice with a genetically-engineered probiotic strain of *Lactococcus lactis* designed to promote anti-inflammatory responses will confer protection in the severity of EAE.

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Chapter 2: Analyzing the Effect of CNS inflammatory demyelination on the Composition of the Gut Microbiome using a murine model of Multiple Sclerosis

Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) MS in which the host's immune cells degrade the myelin sheath of neurons. This degradation is brought on by a breakdown in self-tolerance which allows the production, mobility, and function of these auto-destructive immune cells.[1] The exact mechanism of why this phenomenon occurs is yet to be defined. However, it is believed that this could be a result of a deficit in T regulatory cell (Treg) populations.[1] Moreover, when therapies targeting enhancement of defective Treg populations are applied in the murine model of experimental autoimmune encephalomyelitis (EAE), symptoms of disease progression are ameliorated.[1] Seeing that the dysfunctional Tregs may play a critical role in MS pathology, it is critical to explore what is causing the defect in Tregs.

Recently, researchers have begun to make the association between the defunct Treg populations in MS and the changes in the gut microbiome. A recent study by Cekanaviciute *et al*. showed that certain microbial taxa were diminished in MS patients as compared to healthy individuals.[2] More so, some of these reduced taxa are associated with the expression of IL-10^{+}FoxP3^{+} Tregs.[2] Moreover, the gut microbiome of MS patients can exacerbate the severity of EAE in murine models.[2] For instance, MS patient's stool can cause spontaneous autoimmune encephalomyelitis in murine models.[3] These results show the clear connection between the gut microbiome and its role in the etiology of MS. Further understanding the potential mechanisms of this relationship is required.

The gut microbiome has been attributed several inflammatory conditions as well as CNS disorders including MS.[2-6] An investigation by Ochoa-Repáraz *et al*. showed that the gut microbiome plays a role in disease pathology in the EAE model as well.[7] Moreover, the gut microbiota regulates the function of regulatory immune cells and could provide protection from EAE.[7] Therefore, understanding the composition of the gut microbiome in the EAE model could provide insight into avenues for novel therapeutics. It is important first to characterize the gut microbiome at various points in EAE progression as well as to compare the differences between gut microbiome compositions in differing severities of disease.

The non-obese diabetic (NOD) mouse model of EAE is characterized by an early stage of low grade disease severity followed by a more prolonged and exacerbated phase. By being a longer more drawn out model of disease, it allowed us to analyze the gut microbiome in a controlled fashion; it permitted for the composition of the gut microbiome at various time points of the disease as well as the severity of the disease to be analyzed. This makes the NOD model a more attractive model organism as opposed to C57 or SJL strains. Further, in EAE studies, mice that suffer from milder forms of disease are not evaluated exclusively; we aim to show structural and functional differences between the mild form of EAE, the severe form of EAE, and mice no subjected to EAE.

In this study, we hypothesize that the gut microbiome composition will differ between the various stages of EAE progression in a murine model of disease already established in the NOD mouse.[8-10] Further, we also hypothesize that the gut microbiota will differ depending on the severity of the disease. These alterations in gut microbiota

will lead to altered metagenomic functions and potentially explain differing severities of EAE. Our experimental aim is to provide evidence that the gut microbiota differs as a function of disease status.

Materials and Methods

Mice and Treatments

Ten-week old female NOD ShiLt (NOD/ShiLt) mice obtained from the Jackson Laboratories were utilized for the experiment. All aspects of animal use and care were conducted in accordance with the institutional policies for animal health and well-being under Eastern Washington University.

EAE induction

EAE was induced using Hooke Kit™ for EAE induction (Hooke Laboratories, EK-2110) containing 200 µg MOG_{35-55} emulsified in 200 µl of complete Freund's adjuvant (CFA). Each mouse was initially challenged with a single subcutaneous challenge. On days 0 and 1 post-challenge, each mouse received 400 ng of *Bordetella pertussis* toxin intraperitoneally (List Biological Laboratories, Campbell, CA; provided with the Hooke Kit™ for EAE induction). Mice were monitored and the disease progression was scored daily by blinded observers as previously described: 0, clinically normal; 0.5, limp tip of the tail (when picked up by the base of the tail, the tail still has tension except for the tip); 1, limp tail (no tail tension observed); 1.5, limp tail and inhibition of the hind legs with a slight wobble when the individual walks; 2, limp tail and hind leg weakness, the wobble in each step is more pronounced, mouse exhibits poor balance;2.5, limp tail, and the hind legs drag; mouse exhibits poor balance; 3, limp tail,

hind legs exhibit complete paralysis, or limp tail with one front leg and one hind leg exhibiting complete paralysis; 3.5, limp tail, hind legs exhibit complete paralysis, and mouse can move, but if placed on its side it cannot right itself back up; 4, limp tail, hind legs exhibit complete paralysis, the front legs are starting to show signs of paralysis, and the mouse is moving minimally but still appears to be alert and eating; 5, limp tail, hind legs exhibit complete paralysis, mouse exhibits minimal movement in front legs, and the mouse expresses minimal or no reaction to contact.[11] In accordance to IACUC policies, mice exhibiting a score of 3.5 or higher were sacrificed with primary chemical euthanasia via carbon dioxide asphyxiation followed by secondary physical euthanasia via cervical dislocation. The first two days of concurrent scores of 0.5 or higher were considered and documented as the onset of disease per individual. Type 1 diabetes will spontaneously develop in NOD mice at week 14-20 of age unless CFA is administered.[12-14] For breeding purposes, we administered CFA to the breeding pairs to prevent the onset of insulitis. Diabetic mice were not treated with CFA or MOG_{35-55}.

Fecal pellet isolation and 16S rRNA sequencing

Aseptically, fecal pellets were collected on days 0, 14, 30, and 58 and were stored at -80 ^{o}C. Samples were sent to AKESOgen (Norcross, GA) for 16S rRNA analysis of the gut microbiome. Qiagen DNA stool extraction kits were used to isolate DNA. 1 ng/ml of DNA aliquots were analyzed using PCR with primers specific to the variable region 4 (V4) of the prokaryotic 16S rRNA gene. Library preparation and sequencing for V4 amplicon sequencing was performed on the Illumina MiSeq V2 (2 x 250bp) chemistry. AKESOgen used a protocol that combined the 2-steps in 1-step of amplification with forward primer (515F) and indexed reverse primer (806R). Once the

sequencing was performed, we used a cloud-based web application that facilitates the analysis of microbiome data from the Office of Cyber Infrastructure and Computational Biology (OCICB), National Institute of Allergy and Infectious Disease (NIAID). Utilizing Nephele from the Nation Institute of Health (NIH) (http://nephele.niaid.nih.gov/) in 2016 the data was analyzed using QIIME and R was used for the statistical analysis.

A total of 60 samples were compared using the QIIME FASTQ paired end protocol. Samples were pre-processed with a Phred quality score with a mean of 19 and a 99% base pair accuracy was observed. Quality filters were applied to obtain a median sequence length of 253.0 per sample. 23435 total observations were found. The maximum and minimum number of counts obtained per sample were 573,715.0 and 174,345.0 respectively, with a mean of 287,059.183 +/- 75,305.628 (+/- the standard deviation). Reads that were demultiplexed were clustered into OTUs open reference approach by comparison with the Greengenes database allowing sequences clustered at 97% similarity. Analysis included the identification of chimeras with 6,119 chimeras found and removed using UCHIME. The OTU table was rarified to 174,344 sequences per sample. From the OTU table, the data was normalized for chromosomal redundancy in QIIME and chromosomal data was collected from the Greengenes database per OTU read. This generalized data was then assigned putative metagenomics functions based on each OTU read. This data was set aside from the OTU table for further statistical analysis in R.

The abundance of each taxa was analyzed using the phyloseq package in R.[15] We visualized the compositional heterogeneity of the microbial community of each sample at

every time point of collection and at each taxonomical level using non-metric multidimensional scaling (NMDS),[16] the ordinate function in the phyloseq package and the metaMDS function in the vegan package, and the Bray-Curtis dissimilarity Index. We used 6 dimensions as it produced the lowest amount of stress, defined as the difference between the original dissimilarity and the dissimilarity based on a simplified combination of the original taxa.

Public access to raw sequences can be found at Sequence Read Archive (SRA) at NCBI (BioProject ID: PRJNA383155).

Statistical Analysis

Area under the curve analysis followed by one-way ANOVA and multiple comparisons tests were applied to show differences in EAE scores of no EAE, mild EAE, and severe EAE mice. Weights were compared by two-way ANOVA followed by multiple comparisons test. To compare severity index as well as the onset of disease, Mann-Whitney tests were used. p-values < 0.05, <0.01, <0.001, and <0.0001 were indicated. The effect of disease on the overall microbiota structure was visualized by plotting the results by NMDS in 2 dimensions. Plots were obtained using the R vegan package. After multiple iterations, the one showing the lowest stress was used to generate visuals and for statistical permutational Multivariate Analysis of Variance Using Distance Matrices, ADONIS,[18] a permutational form of multivariate analysis of variance (MANOVA). Since it is a permutational method, it produces slightly different p-values each time. To obtain reliable p-values, we repeated the ADONIS method 10,000 times and reported the average of those p-values for each taxonomical level, each time-point, and each comparison (Table 2.2). Genus-level counts were compared using the non-

parametric Wilcoxon Rank-Sum test (when comparing 2 sample or matched samples) (Fig. 2.4) Differences were considered significant with a p-value < 0.05. Putative metagenomic functions assigned to the intestinal microbiota at each time point of fecal collection was analyzed by two-way ANOVA followed by multiple comparisons test (Table 2.3).

Results

We induced active EAE in 10 week-old female NOD mice as described previously.[8-10] EAE in NOD mice shows an incidence rate of disease of approximately 75%. In our study 31 out of the total 45 mice experimentally induced with EAE (68.9%) showed symptoms of disease, which is similar to what has been previously described (Fig. 2.1A, Table 2.1). The mice initially develop a short phase of disease with lower clinical scores that progressed into more severe symptoms of EAE. These animals were considered to be part of the severe EAE group. The other 14 mice (31.1% of the mice experimentally induced with EAE) showed a milder form of the disease which can be characterized by the initial mild disease severity that did not progress later into more severe scores as seen in the severe EAE group. These mice exhibited average scores much less than the severe EAE group and therefore considered to be the mild EAE group (Fig. 2.1A, Table 2.1). One animal in the mild EAE group did exhibit a clinical score of 2 on day 34; however, it did not progress into a more severe form of EAE and therefore was still considered in part of the mild EAE group.

Scores for both the severe EAE group as well as the mild EAE group exhibited an initial phase of disease ranging from day 9 to day 35 with scores averaging between 0.5 and 1. At day 35, the clinical scores the severe EAE group progressed into the more

severe clinical score range and did not exhibit any signs of remission. The mild EAE group, however, did exhibit signs of a remission post day 35 (Fig. 2.1A). The area under the curve was greater for the severe EAE group as compared to both the mild EAE group as well as the no EAE group (Fig. 2.1B). The no EAE group were mice that were not challenged with EAE and therefore exhibited no symptoms of EAE (Fig. 2.1A). There also was no significant difference between the area under the curve for the no EAE group and the mild EAE group (Fig. 2.1B). Even though the severity of the disease differed between the severe EAE group and the mild EAE group, there was no difference in the onset of EAE between these groups (Fig. 2.1C).

The body weights of every animal were monitored and recorded weekly. There was no significant difference in body weight between all of the groups (data not shown). However, when the percentage of the initial weight was compared based on the form of disease that developed (mild vs. severe), we observed a significant decrease in weight gain in the severe EAE group versus the no EAE control group (Fig 2.1D).

To assess whether or not disease severity affected the composition of the gut microbiota, we longitudinally evaluated the mice at each time point of fecal pellet collection (days 0, 14, 30, and 58 post EAE induction) and correlated this with the severity of EAE symptoms for each mouse. These groups consisted, again, of the control no EAE group, the mild EAE group, and the severe EAE group. The clinical scores at the times of the collection are shown in Figure 2.2A. Of all the stool samples collected, we analyzed 15 of the mice. We chose mice that were never caged with mice belonging to mice of other experimental groups. To generate relative abundances on each taxonomical level, 16S rRNA gene amplicon sequencing was used. To assess whether disease

severity affected stool microbial composition, we analyzed the similarity between each individual animal analyzed. To establish a genetic background baseline to compare deviations in the stool microbial composition, we compared stool collected prior to EAE induction on day 0. The analysis was conducted at the genus level by ordination analysis using nonmetric dimensional scaling (NMDS) in 2 dimensions (Fig. 2.2B). We used the NMDS combination that yielded the lowest stress. Stress is defined as deviations between the original dissimilarity of samples generated after the simplified combination of the original taxa levels. After the NMDS analysis, we used the ADONIS multivariate non-parametric ANOVA and ran the ADONIS analysis with 10,000 permutations to obtain a distribution of p-values per comparison. No significant differences were observed between the groups at this time point of day 0 ($p = 0.895$) (Fig. 2.2 and Table 2.2). Further, when the groups were compared individually (no EAE vs. mild EAE, no EAE vs. severe EAE, and mild EAE vs. severe EAE) there were no significant differences observed (Table 2.2).

The Shannon diversity index, a measure of taxa richness and evenness, of the stool samples isolated from mild EAE group on day 14 was higher when compared to the no EAE group (Fig. 2.2C; $p = 0.0267$). It also showed a trend towards elevation ($p =$ n.s.) in the severe EAE mice when compared to the no EAE group (Fig. 2.2C). When comparing the amount of observed taxa at the species level between severe EAE mice and no EAE mice, there was a significant reduction at day 58 indicating that a lower level of species was identified in EAE mice at the end of the experiment (Fig. 2.2C; $p = 0.0234$).

We next compared the overall composition of the gut microbiome of the three different groups on days 14, 30, and 58 at the genus level as well by 2-dimesional NMDS (Fig. 2.3A and Table 2.2). We started by comparing day 0 for the control no EAE group with days 14, 30, and 58. We noted no significant difference between the gut microbiome composition at these various time points (day 0 vs. day 14: $p = 0.0961$; day 0 vs. day 30: $p = 0.0960$; day 0 vs day 58: $p = 0.0962$). This was also true when comparing day 14 with day 30 and day 58 both with p-values of 0.0962 (graph not shown).

When comparing the taxa at the genus level on day 14, there were significant differences between the overall compositions of the gut microbiome between the control no EAE group, the mild EAE group, and the severe EAE group ($p = 0.0034$) (Fig. 2.3A and Table 2.2). This was also the case between the compositions on day 30 ($p = 0.0096$) (Fig. 2.3A and Table 2.2). However, there were no significant differences between the groups on day 58 ($p = 0.1915$) (Fig. 2.3A and Table 2.2). When analyzing the gut microbiome between groups at day 14, there were still significant differences; no EAE group vs. mild EAE group ($p = 0.0406$) and no EAE group vs. severe EAE group ($p = 0.0167$) (Table 2.2). Further, on day 14 there also was a significant difference between the structure of the gut microbiome of the mild EAE group vs. the severe EAE group ($p = 0.0327$) (Table 2.2).

On day 30, there also was significant differences between the gut microbial composition of the control no EAE group vs. the severe EAE group ($p = 0.0168$) and between the mild EAE group vs the severe EAE group ($p = 0.0407$) but not between the control no EAE group vs. the mild EAE group ($p = 0.3183$) (Table 2.2). At day 58 post-EAE induction there were no overall differences between the three groups ($p = 0.1915$)

(Fig. 2.3A and Table 2.2). Similar NMDS analyses and statistical comparison were run at every taxonomical level but no significant differences were seen between the gut microbial compositions on day 58 (not shown). From these results we do believe that EAE does in fact alter the composition of the gut microbiome in NOD mice. Further, the extent of disease itself seems to alter the composition as well; this is clearly seen when examining the severe EAE group vs. the mild EAE group and then further the control no EAE group vs. the mild EAE group.

We next compared the effects of disease progression and severity on the relative abundance of genera among the major phyla. The relative abundances at the genus level are shown in Figure 2.3B (average relative abundances for the n = 5 per group and time point). We observed changes in genus-level sequence counts of gut microbiota primarily in the early stages of disease. An undetermined genus of the family *Ruminococcaceae* and the genus *Akkermansia* from the family *Verucomicrobiaceae* were increased in the severe EAE group vs. the control no EAE group. The undetermined genus of the family *Ruminococcaceae* differed between the control no EAE group vs. the severe EAE group at day 14 (raw p = 0.008, adj. p = 0.17) (Fig. 2.4). The genus *Akkermansia* differed between the control no EAE group vs. the severe EAE at day 14 as well (raw p = 0.031, adj. p = 0.57) (Fig. 2.4). The opposite was seen with an undetermined genus of the family *Christensenellaceae* the genus *Lactobacillus* of the family *Lactobacillaceae*, in which there was a decrease in sequence counts for these taxa between the severe EAE group vs. the control no EAE group. The undetermined genus of the family *Christensenellaceae* differed between the control no EAE group vs. the severe EAE group at day 14 (raw p = 0.008, adj. p = 0.22) (Fig. 2.4). The genus *Lactobacillus*

differed between the control no EAE group vs. the severe EAE at day 14 as well (raw p = 0.008, adj. p = 0.17) (Fig. 2.4).

When taken together, our results for the retrospective analysis of stool from mice suffering from various severities of EAE show that the extent of disease as well as time point during disease progression alters the structure of the gut microbiome. Moreover, these alterations can have a direct impact on specific bacterial taxa. These changes, however, are short lived and occur primarily in early more inflammatory stages of disease. Due to the limited number of samples compared, it is possible that our data would preclude to put statistical significance on these changes, although interesting trends that require further study were observed.

Using putative metagenomics reads based on using the PICRUSt algorithm showed differing functionality in the gut microbiome based on what taxa were altered (Fig. 2.5). The heat-map generated shows that metagenomics functions differed in the severe EAE group, but did not in the mild EAE group or the no EAE group. Metagenomic function irrelevant to bacterial function were not included (Fig. 2.5). Further, metagenomic functions between the various time points of disease were analyzed using two-way ANOVA followed by multiple comparisons test (Table 2.3). Significance under a p-value of 0.05 were noted in red. Taking data from disease severity and stage of disease, amino acid metabolism and carbohydrate metabolism were further analyzed based on disease severity from stool collected on day 14 (Fig. 2.6).

Discussion

The understanding of how important the gut microbiome is in context to disease has increased dramatically in recent scientific history. Our work as well as others highlights just how important the immunomodulatory nature of the gut microbiome, particularly the bacteria, is in the case of progression for experimental animal models of CNS autoimmunity.

In this experiment, we show and characterize just how disease itself (specifically EAE) alters the gut microbiome. Our results indicated that gut microbiota altered early in mice that developed EAE as opposed to those that did not. Statistical analyses were unable to detect specific deviations in specific bacterial genera associated with disease-induced dysbiosis. The trends show preliminary data that specific taxa that exhibit immunomodulatory effects as well as regulate disease progression are affected. The genus *Lactobacillus* was reduced (raw $p = 0.008$, adjusted: n.s.). *Lactobacillus* is known for promoting immunomodulation and a mixture of *Lactobacillus spp.* has been shown to promote protection against EAE in mice.[19] This reduction in *Lactobacillus* could have a profound impact on disease severity as well as progression. Another genus that was reduced in the severe EAE mice was *Christensenellaceae.* Very little is known about the functionality of *Christensenellaceae* and its role in the gut microbiome. This, in turn, makes it quite interesting that it was reduced in the EAE murine model. On the other hand, the genus *Akkermansia* as well as an unspecified genus of the family *Ruminococcaceae* were increased in the severe EAE mice. Interestingly enough, a study reported that the fecal transplantation of stool from MS patients into mice caused the spontaneous development of EAE.[3] The stool obtained from those MS patients had

elevated levels of *Akkermansia* as well.[3] These findings are interesting especially when considered in the context to our study.

Additionally, we explored how alterations in gut microbiota would impact how the gut microbiota functions. To do this, we utilized PICRUSt as part of the pipeline analysis through Nephele by the NIH. Part of the analysis generated metagenomics reads that could be quantified between the experimental groups. The severe EAE group had a different function when compared to the mild EAE group and no EAE group (Fig. 2.5). Additionally, the difference between microbiome function was noted specifically during day 14 post EAE induction (Table 2.3). We then further were interested in amino acid metabolism as well as carbohydrate metabolism due to their immunogenic importance. We noted that there was an increase in gene expression for amino acid metabolism and carbohydrate metabolism in the severe EAE group when compared to the mild EAE group and the control group.

The data generated from PICRUSt is putative, but it provides interesting insight to how disease severity and disease progression alters the gut microbiome. The composition of the gut microbiome appears to be heavily influenced by both disease severity as well as the time point post disease induction. These structural changes have a profound impact on the way the gut microbiome can then function; by reducing bacterial populations such as *Lactobacillus*, but increasing *Akkermansia*, there appears to be changes in how the community behaves. These perturbations in the gut microbiota must have some immunomodulatory impact that might be increasing the extent of inflammation in the CNS. It is unfortunate, however, that the data generated by PICRUSt is speculative, but the data can be a used to design further studies. In this experiment, we

noted an increase in amino acid metabolism. This could hold some functional importance due to the fact that gamma-aminobutyric acid (GABA) is a derivative of amino acids, and its production is dependent on the gut microbiota.[20] Moreover, GABA levels has also been noted to be reduced in MS patients. Additionally, our lab is currently evaluating the effects of GABA on EAE and determining its efficacy in reducing disease severity; although speculative, perhaps the increase in amino acid metabolism could be reducing GABA levels in the EAE mice.

In conclusion, this study aimed at exploring the extent in which EAE altered the composition of the gut microbiome in NOD mice. This experiment further strengthens the notion that the gut microbiome plays a critical role in disease pathology.

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Figures and Tables

	Total/disease mice	% Disease	Day of onset (mean +/− SD)
EAE (45 mice)			11.8 +/− 0.6
Mild	14	31.1	12.1 +/− 0.9
Severe	31	68.9	11.5 +/− 0.8
No EAE (25 mice)	0	0.0	NA

Table 2.1. Number of mice used, number and percentage of mice that developed mild and severe EAE, and day of disease onset.

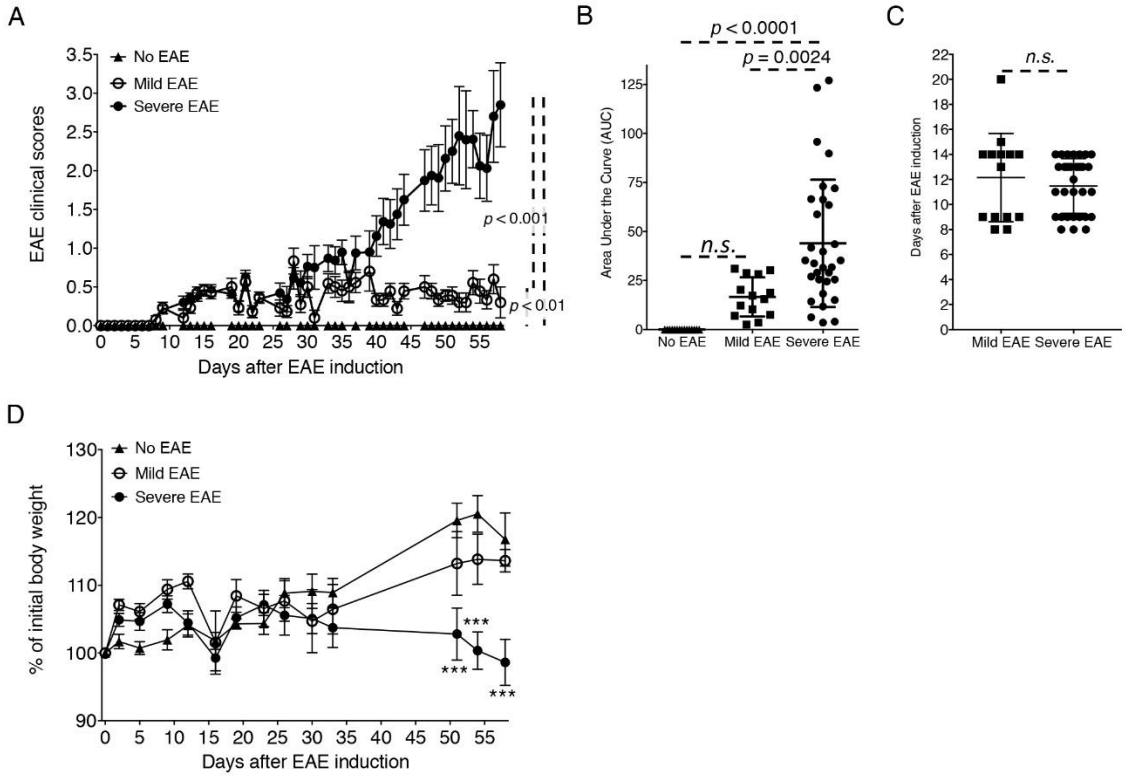

Figure 2.1. Active EAE induces a progressive CNS disease in mice. EAE was induced in NOD mice with a subcutaneous injection of MOG_{35-55} emulsified in CFA and 2 doses of pertussis toxin (day 0 and 1) given intraperitoneally. Disease was induced in a total of 45 mice in 3 independent experiments. 25 NOD mice were used as controls (no EAE). A) Graph depicts EAE clinical scores using a 0-5 scale from one of 3 experiments performed (no EAE n = 5; EAE-induced, n = 15). Scores were compared by one-way ANOVA analysis followed by multiple comparisons test (no EAE vs. mild EAE, mild EAE vs. severe EAE, and no EAE vs. severe EAE: $p < 0.001$). B) Graphs indicate the area under the curve pf the clinical scores of all mice from the 3 experimental groups: no EAE n = 25, mild EAE n = 14, and severe EAE n = 31 mice. The area under the curves were compared by one-way ANOVA followed by multiple comparisons test. C) Day of EAE onset for all mice from the 3 experimental groups, mild EAE n = 14 and severe EAE n = 31 mice, compared by student's t-test. D) Body weights of no EAE, mild EAE, and severe EAE mice from one of the three experiments performed (no EAE n = 5, mild EAE n = 5, severe EAE n =

10). Percentages of body weights were compared by two-way ANOVA followed by multiple comparison test (no EAE vs severe EAE: ***, p < 0.001).

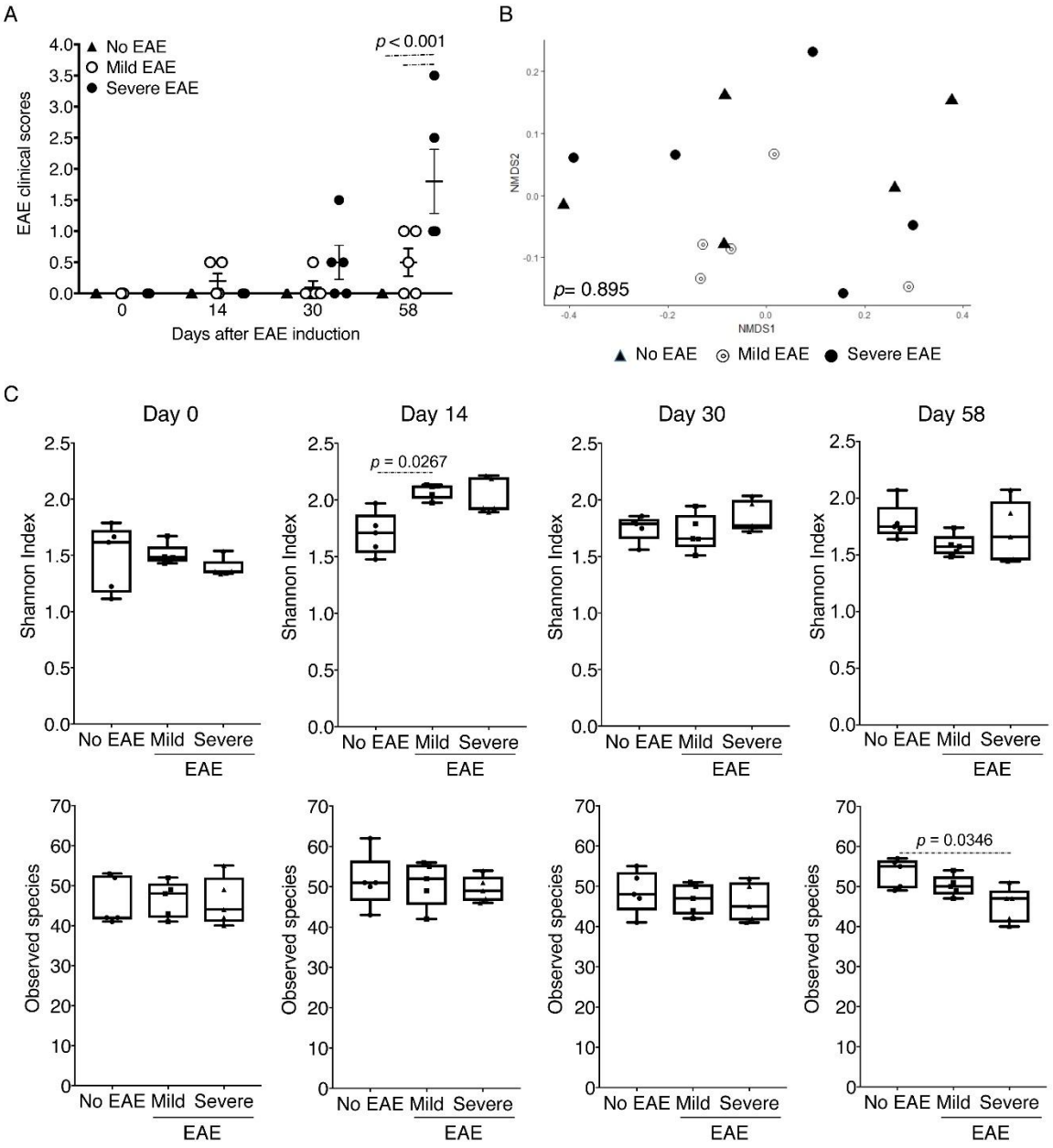

Figure 2.2. Composition of the microbiome at day 0, α diversity and species richness during disease. Stool samples analyzed from no EAE control mice (n = 5), mice that developed mild disease (n = 5), and mice that developed severe EAE (n = 5). A) Samples were collected from all NOD mice. Clinical scores (mean +/- SEM) of mice at each time point of fecal collection (day 0, 14, 30, and 58 post-EAE induction) were compared by two-way ANOVA. B) To examine the compositional heterogeneity of the microbial communities found in the three groups, we used non-metric dimensional scaling (NMDS) in R to ordinate the microbial communities of samples

based on the Bray-Curtis dissimilarity index using QIIME after the 16S rRNA gene sequencing analysis of stool samples, using the phyloseq package. Shown are the NMDA graph for the genus taxonomical level of OTUS identified in the analysis at day 0. The statistical comparison was performed using the permutational Multivariate Analysis of Variance Using Distance Matrices, ADONIS, in the vegan package. C) Shannon index of α diversity and observed species measure in the 3 experimental groups compared by one-way ANOVA.

	Day 0	*Day 14*	*Day 30*	*Day 58*
Overall	0.8956	0.0034*	0.0096*	0.1915
No EAE vs. Mild	0.5000	0.0406*	0.0168*	0.1516
No EAE vs. Severe	0.9602	0.0167*	0.3183	0.36548
Mild vs. Severe	0.8094	0.0327*	0.0407*	0.2230

Note. *, p <0.05 after ADONIS analysis repeated 10,000 times for each time point and comparison.

Table 2.2. Averages of the p-values obtained after 10,000 different ADONIS analysis of NMDS graphs for the genus level shown in Fig. 2 and 3A.

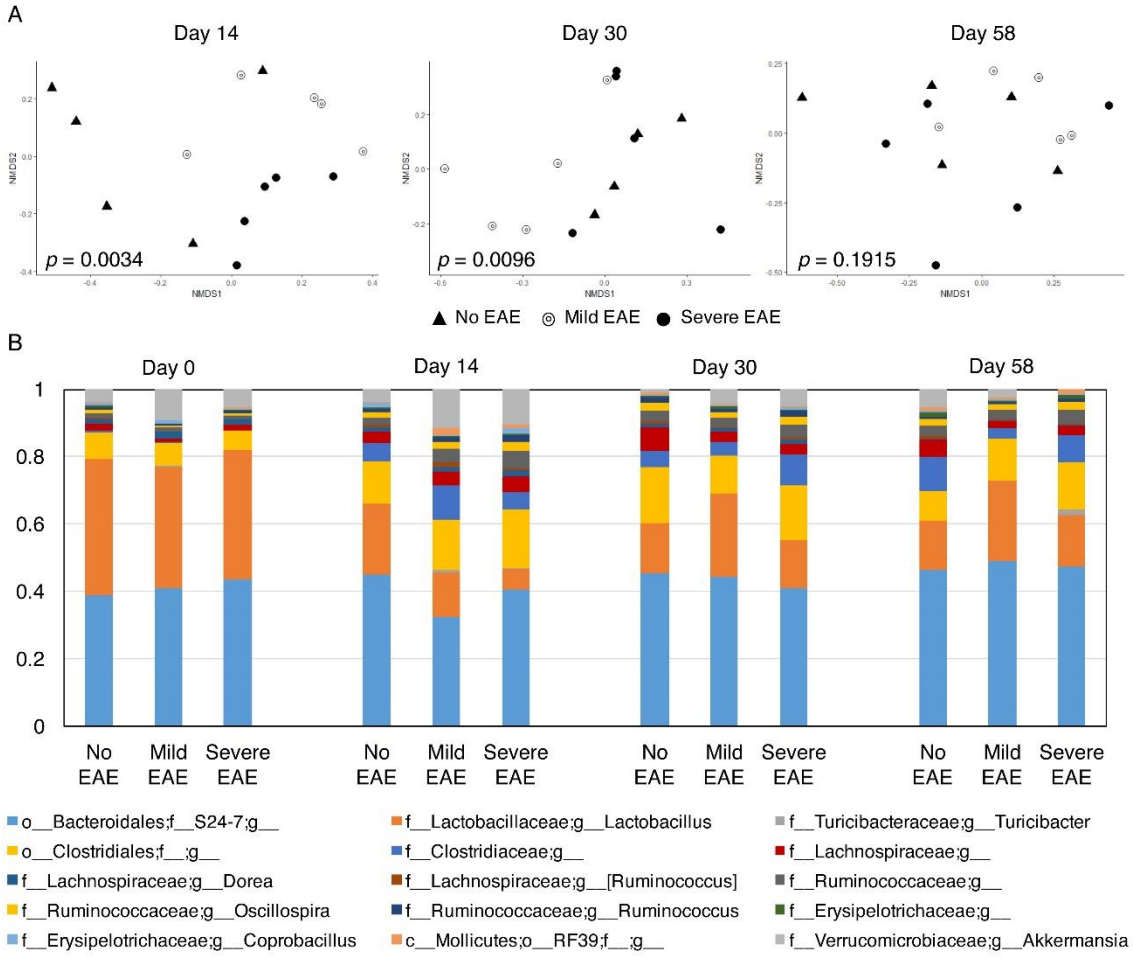

Figure 2.3. Early stages of EAE significantly affect the overall composition of the gut microbiome. A) NMDS analysis for the genus taxonomical level of OTUs identified in the analysis at day 14, 30, and 58. B) Relative abundances observed in analysis of the gut microbiota

at the genus level. The bar graph represents the average of relative frequencies per group (n = 5) per time point for abundances equal or above 1%.

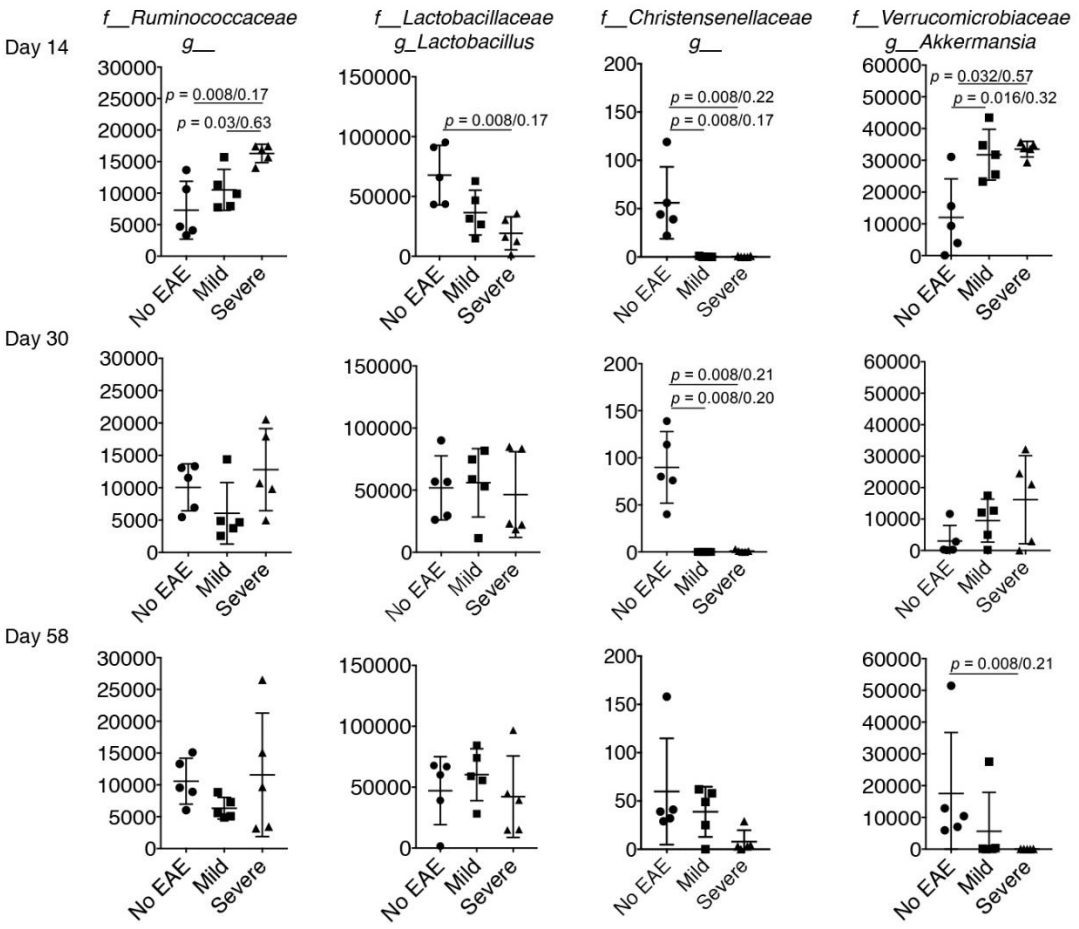

Figure 2.4. Taxonomical differences between the no EAE, mild EAE, and severe EAE groups. OTUs observed were compared at the genus level using the non-parametric Wilcoxon Rank-Sum test was used.

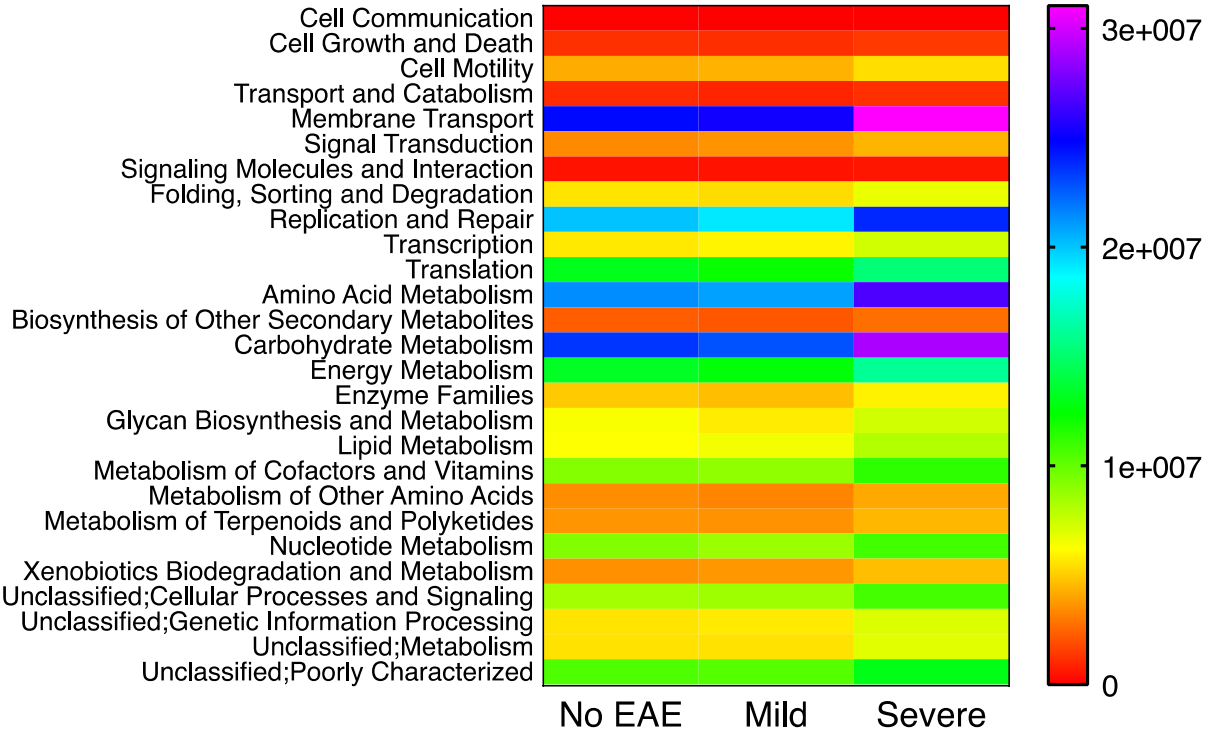

Figure 2.5. A heat-map was generated based on the magnitude of metagenomics reads based on disease severity from pellets obtained on day 14 post EAE induction. From the fecal pellets, 16S rDNA amplicon sequencing was performed and OTUs were generated using Nephele through the NIH. Additionally, PICRUSt annotations of putative metagenomics function were applied to the OTUs after being normalized.

	Day 0 vs. 14	Day 0 vs. 30	Day 0 vs. 58	Day 14 vs. 30	Day 14 vs. 58	Day 30 vs.58
Cell Communication	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
Cell Growth and Death	0.9754	>0.9999	0.9999	0.9699	0.9613	>0.9999
Cell Motility	0.0663	0.9138	0.9454	0.2691	0.224	0.9996
Transport and Catabolism	0.9885	>0.9999	0.9999	0.9846	0.9797	>0.9999
Membrane Transport	<0.0001	0.8803	0.8373	<0.0001	<0.0001	0.3922
Signal Transduction	0.3414	0.9989	0.9999	0.4231	0.306	0.9967
Signaling Molecules and Interaction	0.9987	>0.9999	>0.9999	0.9979	0.9974	>0.9999
Folding, Sorting and Degradation	0.1624	0.9986	0.9834	0.1153	0.0708	0.9969
Replication and Repair	<0.0001	0.9935	0.8066	<0.0001	<0.0001	0.9219
Transcription	0.032	0.9931	>0.9999	0.0657	0.0268	0.9873
Translation	<0.0001	0.9848	0.9037	<0.0001	<0.0001	0.9877
Amino Acid Metabolism	<0.0001	0.9963	0.9268	<0.0001	<0.0001	0.8372
Biosynthesis of Other Secondary Metabolites	0.8323	>0.9999	0.9992	0.8116	0.7633	0.9997
Carbohydrate Metabolism	<0.0001	0.9958	0.5042	<0.0001	<0.0001	0.65
Energy Metabolism	<0.0001	0.9964	0.8998	<0.0001	<0.0001	0.9646
Enzyme Families	0.2334	>0.9999	0.9966	0.2228	0.1534	0.9977
Glycan Biosynthesis and Metabolism	0.1597	0.9894	0.9377	0.0789	0.0394	0.9932
Lipid Metabolism	0.029	>0.9999	0.9815	0.0282	0.0091	0.9828
Metabolism of Cofactors and Vitamins	0.0009	>0.9999	0.9882	0.001	0.0003	0.9865
Metabolism of Other Amino Acids	0.5803	0.9999	0.9978	0.536	0.4648	0.9995
Metabolism of Terpenoids and Polyketides	0.4944	0.9997	0.9957	0.4362	0.3578	0.9991
Nucleotide Metabolism	0.006	0.9982	0.9661	0.0033	0.0011	0.991
Xenobiotics Biodegradation and Metabolism	0.3715	>0.9999	0.9967	0.3653	0.2629	0.9972
Unclassified; Cellular Processes and Signaling	0.0012	>0.9999	0.9783	0.0014	0.0002	0.9732
Unclassified; Genetic Information Processing	0.0747	>0.9999	0.9949	0.0784	0.0397	0.9936
Unclassified; Metabolism	0.0936	>0.9999	0.9937	0.0949	0.0486	0.9933
Unclassified; Poorly Characterized	0.0002	0.9995	0.9347	0.0001	<0.0001	0.9629

Table 2.3. Metagenomic functions differed on day 14 post EAE induction. Utilizing PICRUSt, metagenomic annotations obtained from mice on day 0, day 14, day 30, and day 58 were generated. Using two-way ANOVA followed by multiple comparisons test, each metagenomics function was analyzed. Significance was considered for p-values below 0.05 and are denoted in red.

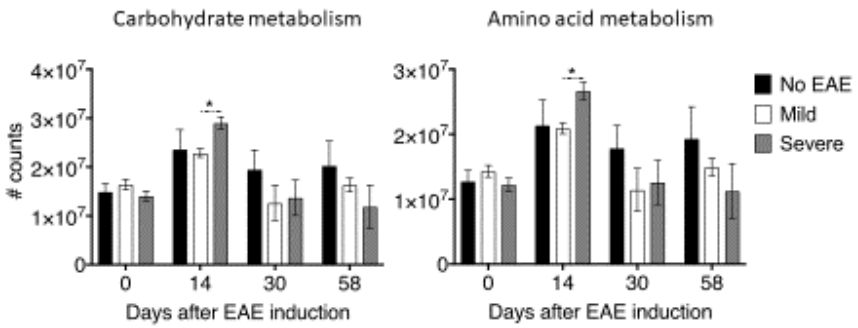

Figure 2.6. Carbohydrate and amino acid metabolism differed between the severe EAE group on day 14. Using two-way ANOVA followed by multiple comparisons test, significance was noted ($p < 0.05$).

Chapter 3: Exploring Outcomes of Altering the Gut Microbiome in a murine model of Multiple Sclerosis

Introduction

The use of antibiotics to target the gut microbiome for therapeutic merit has been explored in the case Type 1 Diabetes, Fulminant Ulcerative Colitis, and experimental autoimmune uveitis.[1-3] Utilizing antibiotic cocktails to modulate intestinal microbiota caused changes in disease severity. In the model of experimental autoimmune uveitis, the expression of CD4+Foxp3+ Treg cell populations was increased while a reduction in IL-17 producing Th17 cells was observed.[3] These results, especially in the case of experimental autoimmune uveitis, suggests insight that antibiotic interventions may confer protection against symptoms of EAE, a model disease for MS, in NOD mice. Increased levels of Th17 cells and reduced levels of Tregs have also been noted in MS.[4,5]

EAE has previously been explored in the context of antibiotic intervention. A study by Ochoa-Repáraz *et al*. demonstrated that broad-spectrum antibiotic interventions disrupted the balance between pro- and anti-inflammatory responses by modulating Treg cell populations.[6] Additionally, Yokote *et al*. reported similar findings but in Natural Killer T cell populations.[7] These experiments analyzed the impact of antibiotic intervention on modulation of EAE in SJL as well as C57 mice. However, to the best of our knowledge, the impact of antibiotics on symptoms of EAE in NOD mice remains unknown.

NOD mice demonstrate a more chronic, long-term version of EAE whereas SJL and C57 mice exhibit a short, yet severe, course of EAE. By utilizing NOD mice as

opposed to SJL or C57 mice, it is possible to elucidate the impact intestinal microbiota plays at various points in EAE progression. Additionally, utilizing NOD mice could also provide novel insight into a potential therapeutic window for disease modulation; by demonstrating when antibiotics have the most prominent effect on disease progression, it is possible to infer the ideal time point in which therapeutics could have the most beneficial effect.

We propose to explore the role antibiotic intervention plays on EAE modulation at various time points of disease. Because of the heightened inflammation occurring in the early stages of EAE, we hypothesize that early antibiotic intervention would have the highest beneficial impact on disease severity. The later stages of EAE are defined as being more neurodegenerative and therefore antibiotic interventions during the middle and late stages of EAE are predicted to have lower impacts on modulating EAE severity. Further, it is also hypothesized that antibiotic interventions would increase Treg cell populations based on evidence obtained from Ochoa-Repáraz *et al*. in 2009.[6]

Materials and Methods

Mice and Treatments

Ten-week old female NOD ShiLt (NOD/ShiLt) mice obtained from the Jackson Laboratories were utilized for the experiment. All aspects of animal use and care were conducted in accordance with the institutional policies for animal health and well-being under Eastern Washington University.

Mice subjected to oral antibiotic treatment were given drinking water with neomycin (1 g/L) (Fischer Bioreagents, BP2669-25), vancomycin (0.5 g/L) (Fischer

Bioreagents, BP2958-1), metronidazole (1g/L) (Acros Organics, 210340050), and ampicillin (1 g/L) (Fischer Bioreagents, BP1760-25) for 2 weeks. Control mice received standard drinking water, and three different treatment windows were used depending on the experiment: 1) day 0 of EAE induction to day 14. 2) day 30 to day 44, or 3) day 69 to day 83. Body weights were measured on day 0 of EAE induction (at the initiation of treatment with antibiotics), every 3 days during antibiotic treatment, and weekly following the termination of antibiotic treatment.

EAE induction

EAE was induced using the Hooke Kit™ for EAE induction (Hooke Laboratories, EK-2110) containing 200 µg MOG_{35-55} emulsified in 200 µl of complete Freund's adjuvant (CFA). Each mouse was initially challenged with a single subcutaneous challenge. On days 0 and 1 post-challenge, each mouse received 400 ng of *Bordetella pertussis* toxin intraperitoneally (List Biological Laboratories, Campbell, CA; provided with the Hooke Kit™ for EAE induction). Mice we monitored and the disease progression was scored daily as previously described: 0, clinically normal; 0.5, limp tip of the tail (when picked up by the base of the tail, the tail still has tension except for the tip); 1, limp tail (no tail tension observed); 1.5, limp tail and inhibition of the hind legs with a slight wobble when the individual walks; 2, limp tail and hind leg weakness, the wobble in each step is more pronounced, mouse exhibits poor balance;2.5, limp tail, and the hind legs drag; mouse exhibits poor balance; 3, limp tail, hind legs exhibit complete paralysis, or limp tail with one front leg and one hind leg exhibiting complete paralysis; 3.5, limp tail, hind legs exhibit complete paralysis, and mouse can move, but if placed on its side it cannot right itself back up; 4, limp tail, hind legs exhibit complete paralysis, the

front legs are starting to show signs of paralysis, and the mouse is moving minimally but still appears to be alert and eating; 5, limp tail, hind legs exhibit complete paralysis, mouse exhibits minimal movement in front legs, and the mouse expresses minimal or no reaction to contact.[8] In accordance with IACUC policies, mice exhibiting a score of 3.5 or higher were sacrificed with primary chemical euthanasia via carbon dioxide asphyxiation followed by secondary physical euthanasia via cervical dislocation. The first two days of concurrent scores of 0.5 or higher were considered and documented as the onset of disease per individual. Type 1 diabetes will spontaneously develop in NOD mice at week 14-20 of age unless CFA is administered.[9-11] For breeding purposes, we administered CFA to the breeding pairs to prevent the onset of insulitis. Diabetic mice were not treated with CFA or MOG_{35-55}.

Cell preparation and flow cytometry

Single cell lymphocyte preparations from Peyer's patches were stained using conventional methods. A live/dead fixable fluorescence-labeled viability dye (BD Biosciences, 564407) was used in all staining protocols, and samples were gated on viable cells for subsequent analysis. Cell subsets were analyzed using fluorochrome-conjugated mAbs against T cell antigens CD3 (BD PharMingen, 553062), CD4 (BD PharMingen 553052), and CD39 BioLegend, 143804). Intracellular staining for Foxp3 was performed using fluorochrome labeled anti-Foxp3 mAb (clone FJK-16s; eBioscience, 175773-82). Flow cytometric results were acquired using BD Accuri C6 (BD Biosciences, San Jose, CA). Data was analyzed using FloJo software (FloJo LLC., Ashland, OR).

Statistical analysis

Parametric and non-parametric t-tests and one-way ANOVA followed by the Kruskal-Wallis comparisons of multiple groups was applied to show differences in flow cytometric analysis. Two-way ANOVA followed by multiple comparisons tests were used to compare the scores of EAE and EAE-ABX mice. In addition, area under the curve analysis followed by Mann-Whitney tests were used to compare scores of EAE and EAE-ABX mice. Weights were compared by two-way ANOVA followed by multiple comparisons test. To compare severity index, the Mann-Whitney test was used (p-values < 0.05, <0.01, <0.001, and <0.0001 were indicated).

Results

Previous work has documented the effects of the gut microbiota in regard to regulating the progression of acute EAE when SJL and B6 mice were treated orally with broad spectrum antibiotics before the induction of EAE.[6,7] Ochoa-Repáraz et al. showed that treatment with broad-spectrum antibiotics altered the balance of regulatory and pro-inflammatory responses in mice with EAE and that regulatory T cells (Tregs) were responsible for the protection achieved via microbiota modulation.[6] Nakamura *et al.* showed similar results in the case of the autoimmune uveitis.[3] Additionally, Yokote *et al.* reported similar findings but suggested that a different cell population was responsible.[7] Our experiment wanted to evaluate whether broad-spectrum antibiotics would affect the progression of the secondary, more severe, stages of EAE in NOD mice. The average clinical scores of the antibiotic treated mice were significantly reduced at multiple time points after day 40 (Fig. 3.1A). Analyzing the area under the curves showed a significant reduction in clinical scores of mice treated with the antibiotic cocktail when compared to

control mice (Fig. 3.1B; $p = 0.0162$). Additionally, the treatment with antibiotics also delayed the onset of disease (Fig. 3.1C; $p = 0.0005$) and reduced the overall severity index when compared to control mice (Fig. 3.1D; $p = 0.008$). Because of the unintended impacts to animal health from broad-spectrum antibiotic exposure, the weight of each animal was monitored weekly. At day 6 of treatment, we observed a significant reduction in body weight of antibiotic treated mice when compared to control mice (Fig. 3.1E). At day 10, the weights recovered and no overall significant differences were observed when comparing the area under the curve (not shown; $p = 0.7295$). Flow cytometric analysis showed that the frequency of $Foxp3^+$ Tregs increased in the Peyer's patches during EAE (Fig. 3.1F). We also noticed similar increases in $CD39^+$ T cells as well as $Foxp3^+CD39^-$ Tregs.

We then compared the ability of orally administered broad-spectrum antibiotics to reduce the severity of EAE when administered after the onset of disease. Using mice that survive the initial wave of CNS inflammation, we treated mice with antibiotics from day 30 to day 44 or day 69 to day 83 post EAE challenge. Again, control mice received normal drinking water. As a whole, the mice that received antibiotics during later stages of disease showed a mild improvement in clinical scores, but the difference was not statistically significant (Fig. 3.2). This indicates that early intervention of gut microbiota manipulation can affect the progression of EAE in NOD mice.

Discussion

Our work, as well as others, highlights just how important the immunomodulatory nature of the gut microbiome, particularly the bacteria, is in the case of disease progression for experimental animal models of autoimmunity. In this experiment, we evaluated the gut microbiome and how it modulates the severity of disease (in this case, EAE).

Our results indicate that disease progression is significantly altered when mice received broad-spectrum antibiotics orally. Two weeks of treatment also significantly delayed the onset of EAE as well (Fig. 3.1). Moreover, despite the long nature of EAE in NOD mice, the short therapeutic window of the early stages of disease can reduce the severity of the second phase of disease when intervention is undertaken early in disease development. This is logical in the context of the composition of the gut microbiome, as well as more inflammatory nature of the first phase of EAE.[12] The second phase of EAE in NOD mice is more neurodegenerative and, therefore, later therapeutic intervention with antibiotics would be predicted to have a diminished impact on disease modulation.[12]

We observed that antibiotic intervention at later time points of the disease (day 30-44 as well as day 70-84) did not have a significant impact on disease severity (Fig. 3.2). This is largely attributable to the fact that alterations in the gut microbiome are not as large or important at this stage of disease. In one study, on day 58 post EAE induction in NOD mice there was no significant differences in the gut microbiota structure between severely ill EAE mice, mildly ill EAE mice, and mice not subjected to EAE.[12] This may reflect the observation that, at this point, the inflammatory phase of disease has passed, and thus is logical that altering the gut microbiome would have no major impact on

disease severity; at this point, the onset of neurodegeneration is underway. Additionally, we demonstrated that the functional differences in the gut microbiome were noted on day 14 post EAE induction as opposed to day 30 or day 58. This further supports the notion that the antibiotics were unlikely to have a profound effect on disease modulation during late stages of disease.

In conclusion, this study was geared to explore the extent in which the gut microbiome modulates disease in NOD mice. This experiment further strengthens the notion that the gut microbiome plays a critical role in disease pathology.

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Figures and Tables

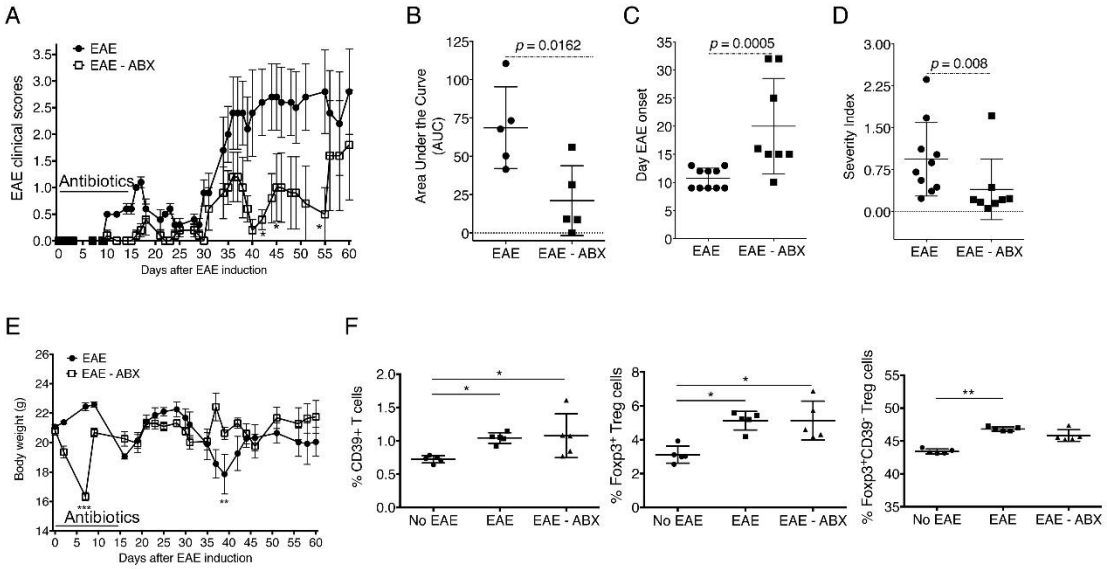

Figure 3.1. Early treatment with antibiotics reduces the severity of EAE in NOD mice. EAE was induced in NOD mice that were treated with antibiotics administered in the drinking water from day 0 to 14 or given normal drinking water as a control. A) EAE clinical scores are shown from one or two independent experiments (n = 5), for a total of n = 8 per group. Comparisons were made using a two-way ANOVA followed by multiple comparisons test (*, $p < 0.05$). Graphs indicate the area under the curve as calculated by Prism (B; n = 5 for the experiment depicted in panel A), the day of EAE onset (C), and the severity index (D). Panels C and D depict values obtained for all mice combined between the 2 experiments (n = 8). Significance was measured using Mann-Whitney test. E) Body weights shown from one or two independent experiments for a total of n = 8 per group. Two-way ANOVA followed by multiple comparisons test (**, $p < 0.01$; ***, $p < 0.001$). F) Graphs indicate frequencies of Tregs using flow cytometric analysis of live $CD4^+$ T cells in the Peyer's patches based on expression of Foxp3 and CD39 (n = 5 per group). Comparisons were made using a two-way ANOVA followed by multiple comparisons test (*, $p < 0.05$; **, $p < 0.001$).

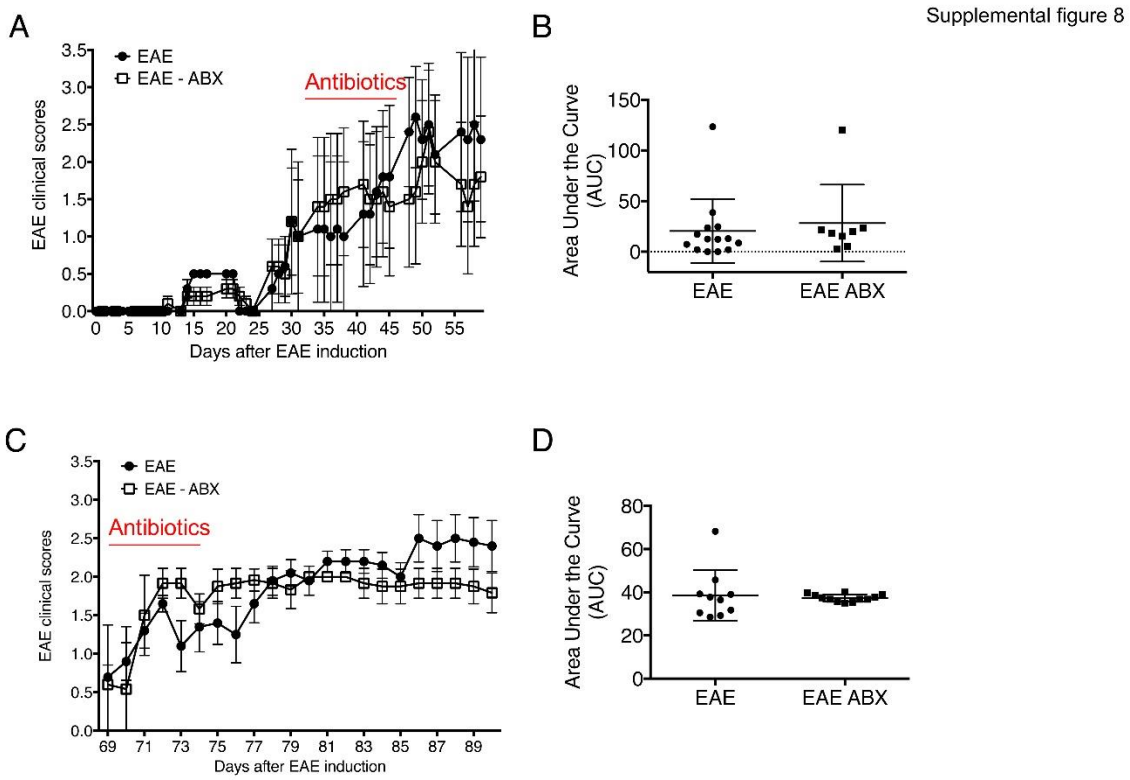

Figure 3.2. Late treatments with antibiotics does not reduce the severity of EAE in NOD mice. EAE was induced in NOD mice that were treated with antibiotics administered in the drinking water from day 30 to day 44 as well as day 70 to day 84. A) EAE clinical scores are shown from one or two independent experiments (n = 5), for a total of n = 8 per group. Two-way ANOVA followed by multiple comparisons test. Graphs indicate the area under the curve as calculated by Prism (B; n = 5 for the experiment depicted in panel A). Significance was measured using Mann-Whitney test. C) EAE clinical scores are shown from one or two independent experiments (n = 5), for a total of n = 8 per group. Two-way ANOVA followed by multiple comparisons test. Graphs indicate the area under the curve as calculated by Prism (C; n = 5 for the experiment depicted in panel D). Significance was measured using the Mann-Whitney test.

Chapter 4: Assessing the protective effects of an anti-inflammatory, genetically modified, Probiotic strain of Lactococcus lactis in a murine model of Multiple Sclerosis

Introduction

Tolerance is the control that our immune system has in the balance between overactive or underactive immune cells. The immune system also must balance inflammatory responses and anti-inflammatory responses, in conjunction with tolerance, to ensure that the body only attacks foreign or pathogenic material but not the residential microbes or self-components. If these mechanisms fail, the result is autoimmunity. Autoimmune diseases are diseases in which an exaggerated immune response eventually leads to immune system failure and an inability to distinguish self from non-self. The microbiome has a clear relationship with the immune system by influencing the balance of immune responses that control autoimmune diseases[1]; the immune system and autoimmune diseases share a bidirectional relationship with the gastrointestinal bacteria in an individual.[2] The disease state shapes the biodiversity and composition of gastrointestinal bacteria, and these bacteria help regulate and modulate the host's immune system.[1]

Using a MS model known as Experimental Autoimmune Encephalomyelitis (EAE) in Non-Obese Diabetic (NOD) mice, we previously showed that disease progression is associated with reduction in certain microbial taxa.[2] When antibiotics were orally utilized after 14 days post disease induction, the disease severity was attenuated.[2] When the gut microbial composition was analyzed, there was a severe reduction in *Lactobacillales* found in the guts of non-treated micel This suggests a

connection between the gut microbiome and autoimmune diseases as a bi-directional relationship.

In terms of treatment options for relapse-remitting MS (RRMS) patients, there are commercial drugs already available. However, therapeutics are not efficacious enough and come with a wide range of mild to severe side effects. Furthermore, there currently are no options available for secondary progressive MS (SPMS) treatment.[3] Thus, new and safer treatment options are needed. Our preliminary data suggests a focus on the order *Lactobacillales* would be prudent, since it is reduced significantly during an early therapeutic window.[4,5]

Previous experiments have been performed to induce oral tolerance, or immunomodulation, via the oral introduction of antigens.[4-6] Kasarello *et al*. used *Lactococcus lactis* to deliver self-antigens by inserting the genes into the *L. lactis* bacterium and found evidence that this methodology is useful for promoting oral tolerance.[5] What is problematic, however, is that this experiment also used self-antigens from myelin tissue.[5] In a clinical setting there is a reason of concern with the notion of treating MS patients with a self-antigen which could induce harm, and likely would not gain IRB-approval. By engineering the expression of CFA/I fimbria, we eliminate the use of self-antigens for oral tolerance and provide a novel and safe approach to achieving oral tolerance.

Because of the potential connection between the reduction in disease severity and the presence of *Lactobacillus*, MS becomes a target disease to investigate using *Lactobacillus*. The Pascual laboratory at the University of Florida, has developed a genetically modified form of *Lactococcus lactis* to express a Colonizing Factor Antigen I

(CFA/I) fimbriae which has been shown to confer protection from other autoimmune diseases such as rheumatoid arthritis.[7] MS has not been explored with this approach. CFA/I fimbriae modulates the immune system by inducing T regulatory cells (Tregs) in an ectoenzyme CD39 dependent fashion.[8] This immunomodulatory induction of Tregs by the CFA/I fimbriae is a central focus of our current design. Choosing the CFA/I fimbriae as a target antigen is ideal since the antigen is foreign and non-myelin in nature. We intend to utilize the CFA/I genetically modified *L. Lactis* bacterium to induce oral tolerance by performing oral gavage on EAE mice during the therapeutic window in which the presence of *Lactobacillus* is known to be reduced. We propose to explore the immunomodulation of SPMS in the C57 murine model via utilization of a predesigned probiotic *Lactococcus lactis*–CFA/I (*L. lactis*-CFA/I). We hypothesize that the oral treatment of *L. lactis*–CFA/I will reduce the severity of disease in EAE mice (reduced EAE scores).

Materials and Methods

Mice and Treatments

Ten-week old female NOD C57BL/6 mice obtained from the Jackson Laboratories were utilized for the experiment. All aspects of animal use and care were conducted in accordance with the institutional policies for animal health and well-being under Eastern Washington University.

EAE induction

EAE was induced using Hooke Kit™ for EAE induction (Hooke Laboratories, EK-2110) containing 200 µg MOG_{35-55} emulsified in 200 µl of complete Freund's adjuvant (CFA). Each mouse was initially challenged with a single subcutaneous challenge. One days 0 and 1 post-challenge, each mouse received 400 ng of *Bordetella pertussis* toxin intraperitoneally (List Biological Laboratories, Campbell, CA; provided with the Hooke Kit™ for EAE induction). Mice we monitored and the disease progression was scored daily as previously described: 0, clinically normal; 0.5, limp tip of the tail (when picked up by the base of the tail, the tail still has tension except for the tip); 1, limp tail (no tail tension observed); 1.5, limp tail and inhibition of the hind legs with a slight wobble when the individual walks; 2, limp tail and hind leg weakness, the wobble in each step is more pronounced, mouse exhibits poor balance;2.5, limp tail, and the hind legs drag; mouse exhibits poor balance; 3, limp tail, hind legs exhibit complete paralysis, or limp tail with one front leg and one hind leg exhibiting complete paralysis; 3.5, limp tail, hind legs exhibit complete paralysis, and mouse can move, but if placed on its side it cannot right itself back up; 4, limp tail, hind legs exhibit complete paralysis, the

front legs are starting to show signs of paralysis, and the mouse is moving minimally but still appears to be alert and eating; 5, limp tail, hind legs exhibit complete paralysis, mouse exhibits minimal movement in front legs, and the mouse expresses minimal or no reaction to contact.[9] In accordance to IACUC policies, mice exhibiting a score of 3.5 or higher were sacrificed with primary chemical euthanasia via carbon dioxide asphyxiation followed by secondary physical euthanasia via cervical dislocation. The first two days of concurrent scores of 0.5 or higher were considered and documented as the onset of disease per individual.

Preparation of *Lactococcus lactis* and Oral Gavages

For the experiment, two genetically modified strains of *Lactococcus lactis* were obtained from the Pascual laboratory at the University of Florida. The pBzMM153 strain (*Lactococcus lactis* expressing the CFA/I fimbriae) (*L. lactis* - CFA/I) and pMSP3535H3 strain (*Lactococcus lactis* not expressing CFA/I fimbriae but containing the same plasmid vector) (*L. lactis* empty) were grown at 30ºC in DIFCO M17 broth supplemented with 0.5% glucose with 10µg/mL of erythromycin in a 25 mL flask. The strains were frozen in 100 µL aliquots for subsequent gavages.

For the gavage treatments, a pre-inoculum was made of 6 mL of M17 DIFCO broth supplemented with 0.5% glucose and 1.2µL of erythromycin at a concentration of 50mg/mL. The pre-inoculum was inoculated with one 0.1mL aliquot previously frozen. The pre-inoculum was incubated overnight at 30ºC overnight without shaking. The next morning 3 mL of the pre-inoculum was introduced to 50 mL of pre-warmed fresh M17 DIFCO broth supplemented with 0.5% glucose and 1.2µL of erythromycin at a concentration of 50mg/mL. The solution was allowed to incubate at 30ºC until the

optical density was approximately 0.2 to 0.23. Subsequently, 100µL of nisin was added and the bacteria was allowed to incubate for 4 additional hours at 30°C. The approximate CFU level for both *L. lactis* strains was approximately 30-60 $x10^7$ per mL.

Prior to receiving treatment, each mouse was orally gavaged with sodium bicarbonate to neutralize the stomach pH and ensure the *L. lactis* would survive past the stomach and enter the intestines. Subsequently, mice received 5 x 10^8 CFU of either *L. lactis* - CFA/I, *L. lactis* empty, or sham (no *L. lactis*) on day 0, 3, and 7 post EAE induction, respectively.

Statistical Analysis

Two-way ANOVA analysis followed by multiple comparisons test was used to evaluate clinical scores on a daily basis. We compared *L. lactis* - CFA/I vs. control (sham), *L. lactis* - CFA/I vs. *L. lactis* unmodified vector, and *L. lactis* unmodified vector vs. control (sham) daily. To compare severity index, a one-way ANOVA analysis followed by multiple comparisons test was conducted (p-values < 0.05, <0.01, <0.001, and <0.0001 were indicated).

Results

Lactococcus lactis Expressing the CFA/I antigen confers long-term protection against EAE in C57BL/6 Mice

To assess the efficacy of each treatment in comparison to control (mice received PBS solution), we collected clinical scores of disease severity for each mouse every day during the progression of disease. Both the *L. lactis* - CFA/I as well as the *L. lactis* empty reduced the severity of EAE in mice (Fig. 4.1A). There were significant

differences between the control mice and the *L. lactis* - CFA/I mice starting on day 10 ($p < 0.05$) (Fig. 4.1A). Although statistical significance between disease severity in the control mice and the *L. lactis* - CFA/I mice was lost at day 18, statistical significance was observed at the termination of the experiment ($p < 0.001$) (Fig. 4.1A). From this data, we infer that *L. lactis* - CFA/I conferred long-term protection against EAE that remained throughout the experiment even when treatment ended on day 7 post EAE induction.

Additionally, there was statistically significant differences between the control mice and the *L. lactis* empty mice starting at day 11 ($p < 0.05$) (Fig. 4.1A). Statistical significance was lost between the control mice and the *L. lactis* empty mice on day 14, but returned for a few days on day 24 ($p < 0.005$) (Fig. 4.1A). Ultimately, there was no significant difference between the control mice and the *L. lactis* empty mice at the end of the experiment (Fig. 4.1A). This suggests that there was a degree of protection conferred by the *L. lactis* with an unmodified plasmid but the protection was short-term, especially when compared to the *L. lactis* - *CFA* group. On day 28, there was a statistically significant difference between the clinical scores of the *L. lactis* empty mice when compared to the *L. lactos* - CFA/I mice ($p < 0.05$) (Fig. 4.1A).

The extent of disease severity in each treatment group was evaluated with pie charts (Fig. 4.1B). Inside each treatment group, mice we grouped by clinical scores ranging from 0-1, 1.5-2, 2.5-3, and 3.5-5. Visually, there are differences between the distributions of disease severity in the three treatment groups. The *L. lactis* - CFA group had scores in the 0-1 range throughout the entire experiment whereas the *L. lactis* empty group no longer had mice with scores of 0-1 on day 28 (Fig. 4.1B). Interestingly, the control group lost individuals with a clinical score of 0-1 on day 14, had individuals with

those scores reappear on day 21, and lost them again on day 28 (Fig. 4.1B). This likely is due to natural fluctuations in EAE. No statistics were conducted on this data set.

We also compared the severity index for each group. The severity index is best described as being the cumulative scores divided by the number of days with disease symptoms. There was a statistically significant difference between the control mice and the *L. lactis* - CFA mice ($p = 0.022$) (Fig. 4.1C). There was no significant difference between the severity index of the *L. lactis* - CFA/I mice and the *L. lactis* empty mice (Fig. 4.1C).

Discussion

Lactococcus lactis has been shown to confer protection against EAE in rats previously.[5] These studies employed self-peptides to induce oral tolerance. By introducing self-antigens, it is possible the probiotic could induce unintended complications, especially since autoimmunity has a strong genetic component; being genetically susceptible towards autoimmunity may cause alternate autoimmunological responses to self-antigens. The work done by Kasarello *et al*. was done in rats.[5] When considering the simplicity of rodent models of autoimmunity in comparison to the complications of autoimmunity in humans, using a self-antigen for oral tolerance is not feasible.

Salmonella vectors containing CFA/I antigens have also been used to induce tolerance.[11] Moreover, the probiotic conferred production by inducing Treg cell populations that, once adoptively transferred into EAE mice, significantly reduced the disease severity.[11,12] The protection induced was effective and suggests that the induction

of oral tolerance is a viable therapeutic option.[11] However, the usage of *Salmonella* as a vehicle to deliver antigens is also infeasible in human studies. Generally, *Salmonella* is associated with pathogenicity. Therefore, utilizing *Lactococcus* as a vehicle to deliver CFA/I antigens is more attractive. *Lactococcus*, and more specifically *L. lactis*, is generally recognized as being safe and edible by the federal Food and Drug Administration. *Lactococcus* is commonly found in everyday foods like yogurt.

In our experiment, we observed a significant difference between the clinical severity of EAE in the control mice when compared to the mice receiving *L. lactis* - CFA/I. The protection conferred by *L. lactis* - CFA/I was seen starting around day 10 of the disease. The protection conferred was maintained throughout the duration of the experiment. A similar outcome was noted between the control mice and the *L. lactis* empty mice. The extent of protection, however, diminished near the end of the experiment around day 26 post-EAE induction. Interestingly enough, *L. lactis* has been shown to produce gamma-aminobutyric acid (GABA) naturally.[13] GABA has been shown to be reduced in MS patients; GABA has also been associated with protection in MS. Further, the genus *Lactobacillus* is also reduced in the gut of NOD mice suffering from EAE.[2] Therefore, it is expected that *L. lactis* not expressing CFA/I would, alone, confer protection against EAE. We are now evaluating the relevance of the production of GABA by *L. lactis* on EAE protection.

When examining the weights between the groups, there were significant differences between the percentage weight from before EAE induction and at the end of the experiment (data not shown). As the animals develop EAE they generally lose weight. As a result, examining differences in weight can help provide insight into

therapeutic efficacy. When comparing the weights as a percentage of each animal's initial weight prior to disease induction, it was clear that the *L. lactis* - CFA/I mice maintained their weight and even continued to grow naturally, whereas weight loss occured for both the control group and the *L. lactis* empty group at the conclusion of the experiment. This suggests that the probiotic alone is insufficient; the expression of the irrelevant antigen helps promote oral tolerance that confers protection from the disease.

Lastly, it is important to note that the severity of disease in the mice receiving the *L. lactis* empty increased at the end of the experiment. EAE in C57 mice is extremely acute and the mice exhibit quick and strong symptoms of EAE. While the animals were receiving weekly treatments, the *L. lactis* empty recipients maintained lower clinical scores than the control group. This phenomenon, however, ended when treatments ceased and therefore there was no lasting effect of the treatment. This was not observed in the group receiving *L. lactis* - CFA/I. Even when the weekly administrations ended, the *L. lactis* - CFA/I recipients maintained lower clinical scores than the control group and maintained a healthy weight gain expected of healthy mice. Although the data is preliminary, we speculate that there is enhanced long term protection conferred by the probiotic expressing the antigen as opposed to the probiotic alone.

Because we did not evaluate the immune cell populations in the animals, we cannot conclude that the mechanism of action of *L. lactis* – CFA/I is equivalent in the context of EAE disease. However, we can, at the least preliminarily, say that the usage of *L. lactis* expressing an irrelevant antigen can induce oral tolerance in the C57 mouse model of EAE. Other experiments suggest that the protection brought on by *L. lactis* -

CFA/I is in part due to the activation and maturation of Treg populations, which are important. Further experimentation is needed to expand upon our initial findings.

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Figures and Tables

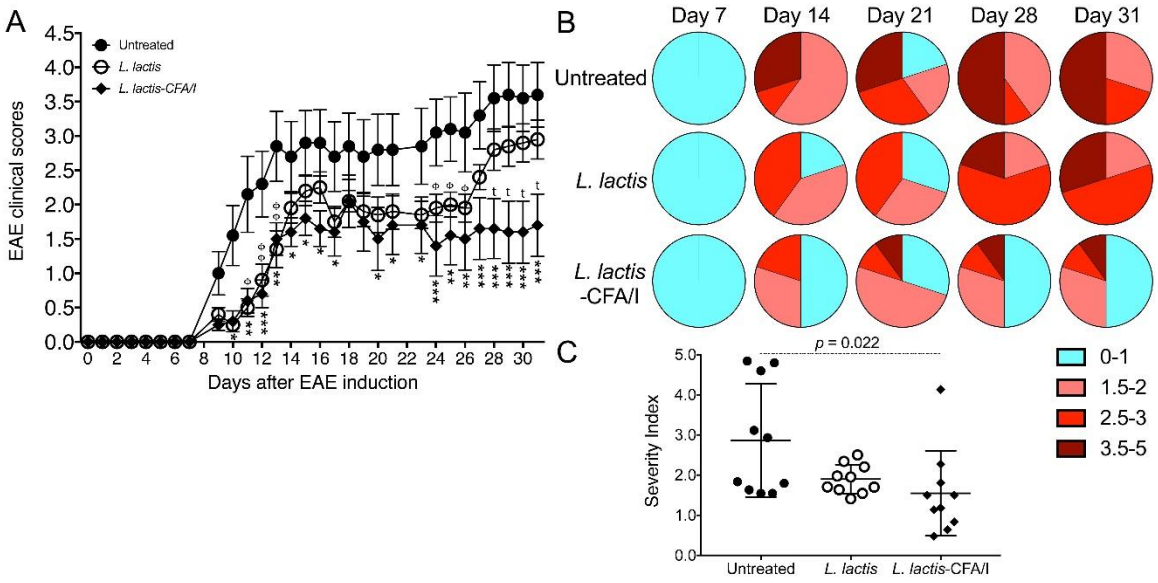

Figure 4.1. EAE was induced in 30 mice (n = 10/group) treated with sham, 5 x 10^8 CFU of *L. lactis* vector, or *L. lactis*-CFA/I on day 0, 3, and7 post-EAE induction. A) Daily clinical scores. Two-way ANOVA followed by multiple comparisons test: Untreated. Vs. *L.lacts*: φ, $p < 0.05$; φp < 0.01; Untrireated Vs. *L. lactis*-CFA/I: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$; *L. lactis* vs. *L. lactis*-CFA/I: ^{t}, $p < 0.05$. B) Distribution of EAE scores. C) Severity Index, analyzed by one-way ANOVA followed by multiple comparisons test.

Chapter 5: Digesting the Bi-directionality Between the Gut Microbiome and Autoimmunity via Directional Analysis of the Relationship and Interconnectedness Evaluated using a Therapeutic Probiotic

The importance of the gut microbiome in human health continues to garner interest. We realize, however, that the relationship between the gut microbiome and host health is complex. This complexity associates with many factors including the intestinal bacterial populations as well as the resulting health of the individual. There is overlap between these factors; for example, exercising is generally beneficial to human health, but studies show that exercise also is beneficial to shaping a health gut microbiome.[1] In the latter study, rats were subjected to no exercise following a bone fracture and surgery for repair. After a period of time, the rats were then able to exercise which resulted in mitigating metabolic distress as well as normalization of the gut microbiome to a healthy state. But what comes first: the metabolic recovery or the gut microbiome recovery? They likely are not mutually exclusive events but rather simultaneous and synergistic effects. This paradigm is just one example of the complicated bi-directionality between the gut microbiome and host health.

In the case of autoimmunity, a similar paradigm occurs. Factors that shape the development of autoimmune disorders also influences the gut microbial composition. Genetics, diet, lifestyle, geographic location, and disease history all shape the gut microbiome as well as predispose individuals to autoimmunity. The vast overlap between risk factors for autoimmunity as well as factors that shape the composition of the gut microbiome makes discerning the role each plays in health challenging. This

observation represents the platform of the current thesis, which is to highlight just how difficult it is to separate intestinal dysbiosis from autoimmunity.

Our first goal was to analyze the role autoimmunity has on shaping the gut microbiome. To achieve this, we examined how EAE shaped the composition of gut microbiota based on the time point during disease, as well as disease severity. Using 10-week old female NOD mice, we induced EAE; using stool collected just before EAE induction as a baseline, we analyzed the similarity in microbial composition at various times post EAE challenge as well as symptom severity. As expected, and in light of the interconnected relationship of autoimmunity with the gut microbiome, we saw that disease itself played a key role in shaping the structure of the gut microbiome. When compared to the control mice in which EAE was not induced, severely ill EAE mice had a vastly different gut microbiome composition. This phenomenon was also observed when comparing the mildly ill EAE mice to the control healthy mice; however, the extent in dissimilarity between the gut microbiome compositions was less dramatic than that seen when comparing severely ill EAE mice and healthy mice. What this means is that the extent of disease plays a role in shaping the overall composition of the gut microbiome.

To demonstrate the other side of the bi-directionality between the gut microbiome and autoimmunity, we sought to demonstrate that the gut microbiome plays a role in shaping disease severity in EAE. Again, taking 10-week old NOD mice, we induced EAE. Then, at various time points in the disease, we subjected the mice to broad-spectrum antibiotics. The theory behind the use of antibiotics was to eliminate the vast majority of the bacterial population in the gut in order to have an unbiased measure of the

extent in which the gut microbiome shapes disease. When the mice were subjected to antibiotics during day 0 to day 14 post EAE induction the mice exhibited significantly reduced symptoms of EAE. This suggests that the presence of the gut microbiome modulates the severity of disease. The gut microbiome has been recognized as playing a key role in educating host immune systems. In the case of autism spectrum disorder, children with dysbiotic guts have an unbalanced immune system.[2] Taking this further, new physiological pathways are being identified showing that the host's immune system communicates with the gut microbiome regularly and routinely.[3] Therefore, by wiping out the presence of the bacteria in the intestines, one can modulate the disease severity. That is exactly what we saw during the first phase of EAE in the NOD mice. Interestingly enough, we observed the similar effects when antibiotics were used at later periods of EAE. This is explained by the observation that the first phase of EAE is highly inflammatory whereas the second phase is more neurodegenerative.

We further sought to examine the cellular mechanism by which the antibiotics reduced EAE severity. Using previous work, we predicted there would be an effect on Treg populations when the gut microbiome was eradicated. We therefore performed flow cytometric analyses on the immune tissue from the gut associated lymphoid tissue. Here, we found that when mice were treated with broad-spectrum antibiotics there was an increase in $Foxp3^{+}CD39^{+}$ populations as well as $Foxp3^{+}CD39^{-}$ cell populations. This was expected based on previous literature, and the fact that we also noted genus level deviations between the EAE induced mice that was dependent on disease severity.

The genus *Lactobacillus* has been appreciated for its immunomodulatory properties,[4] and accordingly has been used for probiotic therapies.[5] We noted that there

was a reduction on *Lactobacillus* as well as an unspecified genus of the family *Christensenellaceae* between severely ill EAE mice and healthy controls. While these taxa were reduced between those mice, we noted that there was an increase in *Akkermansia*. Interestingly, *Akkermansia* has been attributed to being a key player in MS.[6] Therefore it was not surprising that we saw an increase in the relative abundance of *Akkermansia* in the severely ill EAE mice. Further, it follows that when we eradicated the gut microbiota, a reduction in EAE severity, as well as an increase in Treg populations, was observed. This suggests that an aberrant immunological response (as seen in cases of autoimmunity) associate with an aberrant gut microbiome. Our data clearly demonstrated that the gut microbiome plays a role in autoimmunity.

Since the gut microbiome is an excellent target for probiotic intervention, we next sought to use a genetically modified *Lactococcus lactis* expressing the CFA/I antigen as a therapy for EAE. It was clear that probiotic intervention had a beneficial impact on reducing disease severity of EAE. Ours represents the first to evaluate the efficacy of this genetically modified probiotic in EAE. The results of the experiment have obvious pharmacotherapeutic ramifications, but most importantly, it was clear that targeting the gut microbiome had an impact on autoimmunity.

This dissertation was targeted to an exploration of the bi-directionality between the gut microbiome and autoimmunity. Supplementing the gut microbiome with a probiotic clearly altered the extent of disease. We speculate that if a 16S rRNA amplicon analysis performed on the stool obtained from the animals treated with *Lactococcus latics* would reveal that the overall structure of the gut microbiome would only be minimally altered since symptoms of the disease were modulated. Therefore, future experiments

should simultaneously examine the gut microbiome and autoimmunity by manipulating both components simultaneously. Additionally, these future studies could be expanded to other forms of autoimmunity and those with other versions of intestinal dysbiosis. In the case of *Lactococcus lactis* expressing the CFA/I antigen, we sought to induce oral tolerance with an irrelevant antigen. We speculate that the modulation of oral tolerance associates with GABA increases which confer protection against EAE. We believe that manipulation of the gut microbiome has broad relevance to human health with expansive new pharmaceutical potential.

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Doctors of Philosophy (Pharmaceutical Science) 2018 - present

Washington State University – Spokane

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Thesis Dissertation: *The Bi-directional Relationship Between Gut Microbiota and Autoimmunity*

Bachelor of Science (Biology; Minor: Chemistry) 2014-2016

Eastern Washington University

Senior Thesis: *Exploring the effects of dietary change on the oral microbiome diversity in the brown rat, Rattus norvegicus.*

HONORS, AWARDS, FELLOWSHIPS, AND GRANTS

Department of Biology Mini Research Grant Recipient 2017

Effects of a Christensenella probiotic on a Type-1 Diabetes progression in the NOD murine model

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Ochoa-Repáraz, J., **Kirby, T.O**., Kasper, L.H. (2018). The Gut Microbiome and Multiple Sclerosis. *Cold Spring Harb Perspect Med*. pii: a029017.

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IN PROGRESS

Kirby, T.O., & Ochoa-Repáraz, J. (2018). Multiple Sclerosis and the Gut Microbiome: A Review.

POSTER SUBMISSIONS

Kirby, T.O., Keever, A., Seagrave, E., Turkistani, A., Lopez, S., Garcia, D., Johnson, C., McClendon, P., Ramelow, C., Harding, C., Tigranyan, A., Rogers, J., Gonzales, M., Martinez, C., Ralls, S., Magori, K., Ochoa-Repáraz, J. (2017, October). Exploring the differences in intestinal dysbiosis induced by CNS inflammatory demyelination and diabetes in non-obese diabetic mice. Washington State University, Pullman, WA.

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Kirby, T.O., Keever, A., Seagrave, E., Turkistani, A., Lopez, S., Garcia, D., Johnson, C., McClendon, P., Ramelow, C., Harding, C., Tigranyan, A., Rogers, J., Gonzales, M., Martinez, C., Ralls, S., Magori, K., Ochoa-Repáraz, J. (2017, October). Exploring the differences in intestinal dysbiosis induced by CNS inflammatory demyelination and diabetes in non-obese diabetic mice. Washington State University, Pullman, WA.

Kirby, T.O., Keever, A., Seagrave, E., Turkistani, A., Lopez, S., Garcia, D., Johnson, C., McClendon, P., Ramelow, C., Harding, C., Tigranyan, A., Rogers, J., Gonzales, M., Magori, K., Ochoa-Repáraz, J. (2017, May). Exploring the differences in intestinal dysbiosis induced by CNS inflammatory demyelination and diabetes in non-obese diabetic mice. American Association of Immunologists Annual Meeting 2017. Washington D.C.

RESEARCH EXPERIENCE

Research Tech. II, Washington State University 2018

Laboratory of Dr. Gibson.

Graduate Student Researcher, Eastern Washington University 2016-2018

Studied the gut microbiome of non-obese diabetic mice induced with experimental autoimmune encephalomyelitis. Compared the gut microbiome between the Multiple Sclerosis model and the Diabetic model in both structure and function. Collected fecal pellets for 16 S rRNA analysis, obtained gut associated lymphoid tissue for immune cell phenotyping, performed western blots on the intestinal epithelium to assess intestinal permeability, assessed clinical scores for the mouse model, performed statistical analysis and helped with the animal housing and care. Laboratory of Dr. Ochoa-Repáraz.

Undergraduate Student Researcher, Eastern Washington University 2015-2016

Studied the gut microbiome of non-obese diabetic mice induced with experimental autoimmune encephalomyelitis. Collected fecal pellets for 16S rDNA analysis, obtained gut associated lymphoid tissue for immune cell phenotyping, assessed clinical scores for the mouse model, and helped with the animal housing and care. Laboratory of Dr. Ochoa-Repáraz.

Undergraduate Student Researcher, Elmhurst College 2013-2014

Performed RNA extractions and dissections on brain tissue obtained from *Drosophila melanogaster*. The overall goal of the experiment was to observe the impacts of ethanol rearing on the JAK-STAT pathway. Laboratory of Dr. McClure

Assistant Laboratory Technician, Loyola University 2013

Helped doctoral students as well as post-docs by creating media for cloning genetically modified *E. coli* with the human TRIM-5α gene. Performed protein purification and extraction of the TRIM-5α protein. Laboratory of Dr. Campbell.

TEACHING EXPERIENCE

Hematology Graduate Teaching Assistant 2018

Eastern Washington University

Lecture and laboratory practice addressing the basics of Hematology. Taught students about erythrocytes, leukocytes, hematocrits, blood typing, identifying types of anemia and leukemia from blood smears, and how to prepare blood smears.

Microbiology Graduate Teaching Assistant 2016-2018

Eastern Washington University

Lecture and laboratory practice addressing the basics of microbiology. Taught students basic and differential staining procedures, bacterial plating and culture, bacterial streaking, aseptic technique, and common techniques of microbiology.

Departmental Microbiome Capstone Graduate Teaching Assistant 2017-2018

Eastern Washington University

Led students in study of current literature and development of individual research projects concerning the gut microbiome in murine models.

Elementary Medical Microbiology Graduate Teaching Assistant 2016-2017

Eastern Washington University

Lecture and general microbiology laboratory practice. Taught students basic and differential staining procedures, bacterial plating and culture, bacterial streaking, aseptic technique, and common techniques used in hospital laboratory settings.

COMMUNITY OUTREACH

Inland Northwest Concerned Scientists 2017-2018

Member; non-partisan organization that brings together scientists, professionals and concerned citizens to encourage the appreciation of science, science-based policy making, and to support current and future scientists in upholding these values, with special emphasis on local and regional issues.

Washington State Science Olympiad - Microbe Mission 2017-2018

Helped design, proctored, and graded exams for both middle school and high school students.

ADDITIONAL EXPERIENCE

Graduate Student Liaison 2017-2018

Attended faculty meetings and provided a line of communication between faculty and graduate students. Informed both populations of current events. Helped program lunch-ins for graduate students and prospective faculty members as well as provided campus tours for prospective faculty members. Coordinated social events for faculty and students to enrich the educational process.

Roving Naturalist 2012-2013

Roving Naturalist for the Brookfield Zoo under the Conservation, Education, and Training department
Performed inquiry based projects with the general public as well as my department. Most inquiries related to *Pteropus rodricensus* and *Desmodus rotundus.* The inquiries were designed to explore the social behavior between bat types and to investigate if human disturbances altered social behavior. Also worked with the locals to establish native species based gardens to benefit migrating *Danaus plexippus* populations. Educated general public at the Coast, the Garden, the Australasia House, and the Sting-ray Bay exhibits.

TECHNICAL EXPERTISE

Fluent in gut microbiome analysis and interpretation by 16S rDNA analysis and PICRUSt; Nephele through the NIH; Dissection and collection of immunological tissue; Immune cell phenotyping via flow cytometry; Sample analysis via SDS-PAGE electrophoresis and Western blotting; Bacterial culturing and analysis; buffer preparation; IACUC procedures; Murine husbandry; Murine exsanguination.

Experienced with writing IACUC protocols; Statistical analysis with R; DNA/RNA extractions; Administration of oral gavage; Prepping tissues for immunohistology assays; Protein extractions and purification from bacterial samples.