Spring 2021

Investigating the protective effects of intestinal GABA[subscript A] receptor activation on an animal model of Multiple Sclerosis

Hannah M. Kohl

INVESTIGATING THE PROTECTIVE EFFECTS OF INTESTINAL $GABA_A$ RECEPTOR ACTIVATION ON AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

A Thesis

Presented To

Eastern Washington University

Cheney, Washington

In Partial Fulfillment of the Requirements

for the Degree

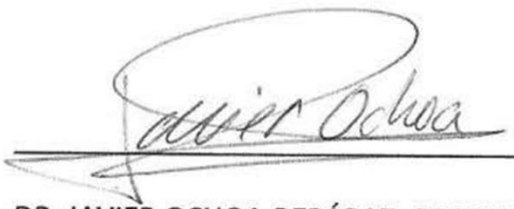
Master of Science in Biology

By

Hannah M. Kohl

Spring 2021

THESIS OF HANNAH M. KOHL APPROVED BY

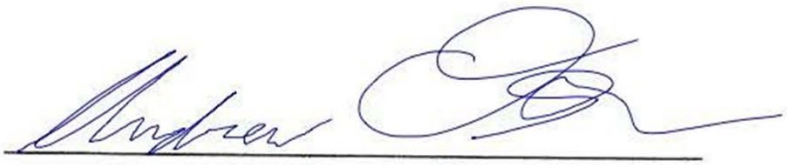


DATE 6/7/2021

DR. JAVIER OCHOA-REPÁRAZ, GRADUATE STUDY COMMITTEE

DATE 6.8.21

DR. ANDREA CASTILLO, GRADUATE STUDY COMMITTEE



DATE 6/7/2021

DR. ANDREW OSTER, GRADUATE STUDY COMMITTEE

ABSTRACT

INVESTIGATING THE PROTECTIVE EFFECTS OF INTESTINAL $GABA_A$ RECEPTOR ACTIVATION ON AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

By Hannah M. Kohl

Spring 2021

Gamma aminobutyric acid (GABA) is an inhibitory neurotransmitter, produced by neurons in the central nervous system (CNS) and by some species of intestinal bacteria. $GABA_A$ receptors are found not only in the CNS, but also in T cells and within the intestines. Past studies have indicated that GABA can have an anti-inflammatory effect and production of GABA by gut bacteria decreases in patients with multiple sclerosis (MS). We examined whether administration of a positive allosteric $GABA_A$ receptor modulator, farnesol, ameliorates the progression of experimental autoimmune encephalomyelitis (EAE), a murine model of MS and whether those effects correlated with a decrease in immune cell infiltration of the central nervous system. We observed a significant treatment effect on clinical scores, as well as immune cell infiltration. Seeking to determine if these effects were due to $GABA_A$ receptor activation or other anti-inflammatory effects of farnesol, we investigated whether modifying mice microbiota with GABA-producing bacteria influences EAE severity. We used a *Lactococcus lactis* strain (P8 GAD-*L. lactis*) genetically modified with an extra copy of glutamic acid decarboxylase (GAD) and glutamic acid/glutamate antiporter to produce high levels of GABA (Castillo, unpublished data). P8 GAD-*L.lactis* produced significantly more GABA

than the *L. lactis* control strain (pAC-*L. lactis*). EAE-induced C57BL/6 mice treated with P8 GAD-*L. lactis* (n=10) had significantly lower clinical scores than the sham control group (autoclaved media; n=10) and the *L. lactis* control group (pAC-*L.lactis;* n=10). Despite trying, we were unable to repeat this experiment due to difficulties inducing disease. We farther tested different C57BL/6 mouse providers (Envigo and Jackson Laboratory) to determine if the decrease in disease severity resulted from the provider (Envigo). The Envigo group (n=10) had fewer animals die from severe EAE compared to the Jackson group (n=10), but it wasn't significant. More experiments are needed to determine what may be causing this difference in disease severity and if oral treatment with a GABA producing probiotic protects against mouse EAE.

ACKNOWLEDGEMENTS:

I would like to thank my advisor Dr. Javier Ochoa-Reparaz for all his tremendous support and mentorship through this journey. I am also grateful to Dr. Andrea Castillo's assistance and mentorship, and for her work to generate P8 *L. lactis*. I would also like to thank my committee member Dr. Andrew Oster.

I am grateful for the help and advice of Dr. Jean-Baptiste Roullet at Washington State University, and his assistant Xutong Shi who taught me how to do ELISAs, as well as Dr. K. Michael Gibson.

I thank Rick Barido, the EWU vivarium manager, for sharing his considerable expertise in experimental animal care and handling, as well as his and his staff's excellent care of the animals in our experiments. Thanks to David French and John Shields for ordering required reagents needed for my research.

I thank my graduate colleagues who helped on this project; Kristina Hoffman, Lacey Sell, Christina Ramelow, and Tyrel Long. As well as the many undergraduate assistants who contributed; Kendall Staben, Killian Campbell, Marcos Monteiro, Jasleen Bains, William (Jake) Doyle, Alivia Sargent, Rachel Linton, Katelyn Dowling, and Iraia Repáraz

Finally, I would like to thank the National Institutes of Health (NIH) for their financial support through their grant (1R15NS107743), as well as the EWU Biology Department research funding mini-grant.

Contents:

INTRODUCTION

Multiple Sclerosis (MS)

MS is a major health concern affecting millions of people. It is the most common reason for non-traumatic disability in adults from developed nations. Nearly a million people in the United States have MS, with the northern states having the greatest prevalence (Wallin et al., 2019). Multiple sclerosis (MS) is a neurodegenerative disease characterized by inflammatory lesions of the central nervous system (CNS) that lead to demyelination of neurons. As the myelin sheath, the insulating layer around nerves, is damaged sclerosed plaques develop. These sclerosed plaques are where the disease gets its name (Dobson and Giovannoni, 2019). As the damage increases, neurons are unable to efficiently send electrical signals. Disruption of neuronal signaling leads to a wide variety of neurological symptoms such as vision problems, depression, fatigue, dizziness, and paralysis (Trapp et al., 1998; Thompson et al., 2018) and can ultimately lead to death (Scalfari et al., 2013). Symptoms can vary drastically between individuals.

MS is categorized by the progression of neurological decline in patients. Relapsing/remitting MS (RRMS) is the most common form. RRMS is categorized by periods of remission followed by relapses where neurological symptoms worsen. About 85% of newly diagnosed MS patients are diagnosed with RRMS. Ten to fifteen years following diagnosis, 50% of those patients will develop secondary progressive MS (SPMS), where neurological symptoms gradually worsen with few if any remissions. Around 15% of newly diagnosed patients are diagnosed with primary progressive MS (PPMS) where neurological symptoms gradually worsen with few remissions (De Angelis

et al., 2018). MS can be difficult to diagnose due to varying symptoms that often resemble other diseases. An MRI scan can identify brain damage, but MS lesions can look like damage caused by other diseases and trauma. To diagnose, doctors look for brain damage occurring in multiple locations at differing times, where other diagnoses have been ruled out (Miki, 2019). Multiple MRI scans are often required.

The inflammatory lesions of MS are caused by immune cells infiltrating the CNS and attacking the myelin sheath. Both innate and adaptive immune systems and their associated cells contribute to MS disease pathology. The innate immune system is the body's first line of defense against pathogens and injury; it is non-specific and deploys rapidly. Cells of the innate immune system include natural killer cells (NK cells), macrophages, neutrophils, dendritic cells, mast cells, basophils, and eosinophils. It is also responsible for activating the adaptive immune system. The adaptive immune system is the body's second line of defense. It requires activation, is antigen specific, and retains memory through memory T and B lymphocytes. The cells of the adaptive immune system are T and B lymphocytes.

Immune cells and signaling molecules that play a role in MS

Monocytes are phagocytes that ingest pathogens and dead or damaged cells. They are derived from granulocyte -macrophage progenitor cells and can in turn differentiate into antigen presenting cells (APCs) such as macrophages and dendritic cells

(DCs). These cells can be farther differentiated based on their location, function, and cell origin. Microglia are the resident macrophages of the CNS and reside in the parenchyma.

Activation and infiltration of the CNS by microglia and other macrophages contribute to the pathology of MS. Microglia and infiltrating macrophages are the main immune cells found near damaged axons (Trapp et al., 1998) and within CNS lesions in MS (Hemmer et al., 2015). Activated microglia and macrophages can secrete pro-inflammatory molecules such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) (Cavaillon, 1994; Hanisch, 2002) increasing neuronal inflammation and the process of demyelination. Macrophages remove dead or degenerate myelin and may be one of the main contributors to myelin damage in early MS lesions (Henderson et al., 2009).

T and B lymphocytes (T cells and B cells, respectively) also play a critical role in MS pathology. As part of the adaptive immune system, T cells are activated by antigen-presenting cells (APCs) presenting small peptides derived from processed antigens to them. T cells mature in the thymus and B cells mature in the bone marrow. B cells are able to be activated by unprocessed antigens and with help provided by T cells differentiate into plasma cells, which are responsible for producing antibodies. B cells are often found in early MS lesions. Some studies find antibodies against myelin in cerebral spinal fluid of some MS patients, but this result is inconsistent among all studies conducted (Karni et al., 1999; Reindl et al., 1999; Kennel De March et al., 2003; Khalil et al., 2006; Wekerle, 2017). In childhood onset MS, it has been theorized that certain antibodies found in patients, such as anti-myelin oligodendrocyte glycoprotein (MOG),

are one of the reasons for a different disease phenotype in children (Bar-Or et al., 2016). In addition, drugs targeting B cells, such as rituximab, have been used to successfully treat MS. This is consistent with a role for B cells in the pathology of MS.

T cells are one of the main drivers of MS pathology (Bhat and Steinman, 2009). T cells can be divided into two main groups CD4+ cells (Helper T cells and Regulatory T cells) and CD8+ cells (Cytotoxic T cells) both of which are involved in MS. The CD4+ and CD8+ refer to the expression of coreceptors (CD4 or CD8) on their cell surfaces, serving as essential markers for the phenotypic analysis of the cells by immunological methods, such as flow cytometry. Activated CD8+ T cells that function to directly destroy infected or damaged host cells, they are often found in MS lesions and have been shown to be capable of killing neuronal cells (Huseby et al., 2012). Activated CD4+ T cells differentiate into T helper cell (Th cells) subsets that depending on their nature release specific cytokines, small messenger proteins, that help coordinate immune responses. One specific subset of CD4+ Th cells are the T regulatory cells (Tregs), that help develop self-tolerance by controlling the proliferation of inflammatory cell subsets. Dysfunction in Tregs have been linked to increased MS disease severity (Danikowski et al., 2017).

Differentiated Th cells are classified depending on the type of cytokines they produce. Some produce inflammatory cytokines (such as IL-17A, GM-CSF, IFN-γ, TFN-α) and others anti-inflammatory cytokines (such as IL-10, IFN-1α, IFN-1β). Some categories of Th cells are associated with MS more than others. Specifically, Th17 CD4+ cells that produce interleukin 17A (IL-17A) which is a critical recruiter of inflammatory cells such as neutrophils, macrophages, and cytotoxic cells. Many of them also produce a cytokine

called granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF exacerbates the differentiation of CD4+ T cells into Th17 CD4+ T cells. The two cytokines are involved in a positive-feedback loop that increases inflammation and subsequent tissue damage causing demyelination and ultimately paralysis. Increased levels of these cytokines are associated with the disease pathology of MS, as observed in patients (Milosevic et al., 2015). Experimental evidence also suggests their role in pathogenesis. Mice who were genetically engineered to have no production of GM-CSF cytokines are resistant to induction of Experimental Autoimmune Encephalomyelitis (EAE), an animal model that resembles human MS (Ponomarev et al., 2007). The lack of IL-17A also significantly ameliorates the EAE (Komiyama et al., 2006).

Dysregulation of calcium has also been linked to a number of neurodegenerative disorders, including MS (Kattimani and Veerappa, 2018; Verma et al., 2018). Calcium is a major intracellular signaling molecule responsible for regulating numerous cellular processes such as muscle contraction and neurotransmitter release (Enders et al., 2020). Calcium is also responsible for oligodendrocyte (the cells that form the myelin sheath) maturation and myelin sheath formation (Cheli et al., 2015; Enders et al., 2020). Extracellular calcium also directs microglia migration to brain lesions (Tvrdik and Kalani, 2017), such as found in MS. Dysregulation of calcium can lead to excitotoxicity, where an overload of intracellular calcium causes apoptosis (cell death) (Rajda et al., 2017; Verma et al., 2018). Some inflammatory cytokines such as tumor necrosis factor α (TNF-α) increase calcium levels and activated T cells can also increase intracellular calcium levels (Enders et al., 2020).

Etiology of MS

The cause of MS is still uncertain. From 1990 to 2015, there was a 59% increase in global MS rates (Wilkins, 2019). There does seem to be a genetic component to the disease, as some people have a higher susceptibility to it. The first discovered and strongest genetic factor linked to MS are variants of the human leukocyte antigen region (HLA) locus, that codes for the highly polymorphic cell-surface glycoproteins which are key components of the immune system. Other genetic variants associated with MS include genes implicated in T-cell activation and expansion, cellular recruitment, escape from regulatory immune surveillance, and various other immune functions. Epigenetic changes may also play a role as some of the genes, differentially expressed in MS patients compared to controls, have been shown to be partially controlled by epigenetic factors; such as promoter methylation (Muñoz-Culla et al., 2013).

Genetics are not the only risk factor involved in MS. There is some evidence that it is associated with Epstein–Barr virus (EBV) infection. However, the association is indirect and might not correspond with a direct link, as 90% of Americans have been infected with the virus by the age of 20 (Guan et al., 2019). An infection with EBV seems to increase the risk of developing MS, as almost all MS patients have had the virus. Having an active infection increases the risk substantially (Ascherio and Munger, 2007) and increased quantities of EBV specific antibodies are associated with a heightened risk of developing MS (Lucas et al., 2011).

Other factors that increase the risk of MS are living at higher latitudes specifically during the formative childhood years. This may be due to a lack of vitamin D (Sintzel et al., 2017). There is also a significant gender difference in MS. Females are three times more likely to get MS than men. Yet, this has not always been the case. The incident rate of females getting MS has been increasing over the years (Voskuhl, 2020), which may imply lifestyle changes due to culture. Though males are less likely to get MS, those who do often have a worse prognosis and faster progression than females (Voskuhl, 2020). MS is more common in developed nations, particularly those in Northern Europe and North America (Browne et al., 2014), this may partially be due to an increase in the availability of medical care and reporting. Other variables that seem to be involved include diet, exercise, infectious disease, host microbiota, stress, and age (Joscelyn and Kasper, 2014).

Current MS treatments

There is currently no cure for MS. Of the treatments currently available, many are expensive, and they are not always effective and are associated with a wide array of side effects. Many of the current treatments focus on weakening the immune system to reduce inflammation. Often the treatment's effectiveness wanes over time requiring replacement with a more powerful drug as the disease worsens. In addition, some treatments have been associated with increased risk of infections. PML (Progressive Multifocal Leukoencephalopathy) is a dangerous disease of the brain caused by the John Cunningham virus. This disease is associated with some of the more aggressive MS treatment with anti-VLA-4 due to their weaking of the immune responses in the CNS.

An alternative treatment is the use of anti-inflammatory cytokines to decrease inflammation in the CNS. Type-I interferon beta-1α and 1β (INF-1α and INF-1β) are used as anti-inflammatory treatment in MS patients. This option has fewer side effects but is not effective for all patients. Other drugs directly target immune cells for destruction, reducing the ability of the immune system to exacerbate disease. The MS drugs Alemtuzumab, Ocrelizumab, and Natalizumab are all monoclonal antibodies that target immune cells. Hematopoietic stem cell transplantation (HSCT), where a patient's immune cells are all destroyed by chemotherapy and then replaced with bone marrow hematopoietic stem cells, is another possible treatment. These more aggressive treatments are often more effective than cytokine-based approaches, but also have more serious side effects (table 1). Side effects like PML (progressive multifocal leukonencephalopathy), a dangerous disease of the brain caused by the John Cunningham virus. Because of the lack of a completely effective and safe treatment, the search for a new therapeutic alternative must continue as it is an unmet clinical need.

Intestinal Microbiota

The microbiota refers to the sum of all microorganisms within a defined environment (Marchesi and Ravel, 2015). Increasingly studies have shown a link between intestinal microbes and MS. The human intestinal microbiota contains 10-100 trillion microorganisms (Gill et al., 2006). It is needed for the appropriate development of the immune system (Kiyono et al., 1982; Wannemuehler et al., 1982; Sudo et al., 1997), and altered compositions, named dysbiosis, has been associated with several inflammatory disorders, including MS (Ochoa-Repáraz et al., 2018; Kohl et al., 2020).

Germ free mice (born and raised without microbiota) show an altered immune cell balance between pro- and anti-inflammatory cells when compared to those raised with a normal intestinal microbiota (Olszak et al., 2012) that makes them less susceptible to different inflammatory-mediated diseases. The intestinal microbiome is markedly malleable, changing in response to, for example, diet, age, weight, and environment. This makes it a great target for therapeutic change. Many of the factors that seem to be involved in increased risk of MS are known to affect the gut microbiota as well (Ochoa-Repáraz and Kasper, 2014).

Mice orally treated with broad-spectrum antibiotics to reduce their gut microbiota had significantly lower EAE disease severity, compared to untreated controls, or mice that were treated with an intraperitoneal antibiotic that didn't affect the gut microbiota (Ochoa-Repáraz et al., 2009). The treatment with oral antibiotics is protective only when administered at early stages of the disease, when the inflammatory process is most prominent (Colpitts et al., 2017). Germ free mice develop EAE at a severely attenuated rate, have significantly reduced symptoms, and the number of pro-inflammatory cytokines associated with EAE pathology is reduced in comparison to mice colonized with a complete microbiota (Lee et al., 2011).

MS patients have a different microbiota population compared to healthy individuals (Cekanaviciute et al., 2017). Microbiota from MS patients transplanted into a transgenic mouse model of spontaneous brain autoimmunity, leads to a significantly higher incidence of autoimmunity than that which occurs from their healthy twin's

microbiota being transplanted (Berer et al., 2017). Fecal transplants from healthy individuals given to MS patients seem to decrease some of the neurological symptoms of their disease (Evrensel and Ceylan, 2016). Studies continue to come out showing a link between EAE or MS severity and gut microbiota composition.

Changes in the intestinal microbiota can lead to increased inflammation, in a process defined as dysbiosis. Intestinal inflammation has been associated with increased intestinal permeability, while increased intestinal permeability can lead to an increase in microbial translocation and entry of microbial components such as Gram-negative lipopolysaccharide (LPS, or endotoxin) to the lamina propria, resulting in an increase in systemic inflammation (Sánchez de Medina et al., 2014). Ten months after injection with LPS mice still had elevated brain levels of tumor necrosis factor-alpha (TNF-a) (Qin et al., 2007). TNF-a is a pro-inflammatory cytokine that is also found in elevated levels in MS brains and circulation. Patients with MS have increased intestinal permeability (Yacyshyn et al., 1996). Increased intestinal permeability, sometimes called leaky gut, has been reported at the onset of EAE in mice (Nouri et al., 2014). Furthermore, EAE severity is associated with corresponding increases in intestinal permeability (Secher et al., 2017). Certain types of bacteria, such as *Bacteroides fragilis,* help to ameliorate the effects of increased intestinal permeability (Hsiao et al., 2013), while other species exasperate it (Wilson et al., 2018). Germ free mice have been found to have fewer tight junction proteins, occludin and claudin-5 (Braniste et al., 2014), this results in more leaky epithelia, as tight junctions are what holds the epithelia cells tightly together preventing leakage. Tight junctions are involved in both increased permeability in the gut and in the

blood brain barrier (Braniste et al., 2014). One of the earliest symptoms of MS is increased blood brain barrier permeability and infiltration of lymphocytes and immune cells (Ortiz et al., 2014).

γ-Aminobutyric acid (GABA)

GABA is the principal inhibitory neurotransmitter in the CNS (Wong et al., 2003). Low GABA levels are associated with numerous neurological diseases, such as schizophrenia (Geffen et al., 2009), Huntington's disease (Garret et al., 2018), and epilepsy (Treiman, 2001), and MS(Cawley et al., 2015; Yalçınkaya et al., 2016). In addition to being produced by neurons in the CNS, certain intestinal bacteria such as *Lactococcus lactis* (Nomura et al., 1999) and *Lactobacillus* produce GABA by decarboxylating glutamate, through the enzymatic action of glutamic acid decarboxylase (Dhakal et al., 2012). It is also found in many fermented foods because lactic acid bacteria produce it (Dhakal et al., 2012).

There are 2 known types of GABA receptors which activate when bound to GABA; $GABA_A$ receptor and $GABA_B$ receptor. Originally, there were thought to be 3 types of GABA receptors with $GABA_C$ receptors being the third type. Additional research revealed that $GABA_c$ receptors are really a subtype of $GABA_A$ receptors (Martin et al., 2020). $GABA_A$ receptors were the first to be characterized and are well researched, due to their ability to bind with numerous pharmacological agents (Olsen and DeLorey, 1999). They are large pentameric transmembrane, chloride gated ion channel receptors (Chebib and

Johnston, 1999). $GABA_B$ receptors in contrast are metabotropic G protein binding receptors. Both types of receptors are widespread in the CNS (Martin et al., 2020).

In addition to their expression in the cells of the neuronal system, $GABA_AR$ are also expressed in immune cells. More importantly, targeting $GABA_AR$ promote significant functional changes in immune cells. T cell's express $GABA_AR$ (Mendu et al., 2012) and activation of those receptors inhibited antigen specific T cell proliferation (Tian et al., 1999). GABA has been shown to inhibit T cell activation cascades and decrease pro-inflammatory T cell responses without causing apoptosis and thereby immune deficiencies (Tian et al., 2004). As T cells are a major disease-driving mechanism in MS and murine EAE (Bhat and Steinman, 2009), this may be a mechanism for GABA effecting MS and EAE severity. Oral administration of homotaurine, which activates $GABA_A$ receptors, ameliorates EAE symptoms in mice (Tian et al., 2018). In the same study, researchers looked at the effect of the activation of $GABA_AR$ on cytokine production and found that it decreases the amount of proinflammatory cytokines (IFN-y and IL-17A) and increases the quantities of anti-inflammatory cytokines (IL-10) in EAE mice.

In addition, the oral treatment with Lactic acid producing bacteria (LABs) which produce intestinal GABA appear to be helpful in decreasing inflammation (Lavasani et al., 2010). Mice who received an oral dose of *Lactobacillus* had an increase in GABA levels in their brains (Janik et al., 2016), showing that increased intestinal GABA can impact brain GABA levels.

Activation of $GABA_AR$ in the gut could also affect the vagus nerve function (Auteri et al., 2015), as a mechanism linking the gut and the brain. The vagus nerve is the main nerve of the parasympathetic branch of the automatic nervous system (ANS), and regulates homeostasis, metabolism, and immune responses. Direct stimulation of the peripheral vagus nerve *in vivo* in rats induced with endotoxemia; inhibited TNF synthesis in liver, attenuated peak serum TNF amounts, and prevented the development of shock (Borovikova et al., 2000). Stimulation of the vagus nerve can also and increase production of GABA in the brain (Van Leusden et al., 2015). Chronic oral treatment of *L. rhamnosus,* a GABA producer, results in increased GABA production in the brain of mice in a vagus nerve-dependent mechanism. This is evidenced by the cessation of that GABA production when the vagus nerve was severed via subdiaphragmatic vagotomy (Bravo et al., 2011).

Recent evidence suggest that intestinal GABA plays a role maintaining the integrity of the intestinal barrier, possibly disrupted in the context of inflammatory diseases, as indicated above. In a study by Sokovic et al. (2019), supernatants containing GABA (and other supernatants without GABA as controls) were administered *in vitro* to intestinal epithelial cells previously exposed to IL-1b, a proinflammatory cytokine. The supernatants that contained GABA had a greater protective effect on intestinal integrity compared to ones without GABA, increasing the expression levels of zonulin, occludin and claudin 4, proteins involved in epithelial tight junctions (Sokovic Bajic et al., 2019).

It has been found that in EAE mice the amount of intestinal microbe GABA producers decreases in the early stages of the disease. In a study, by our lab (Colpitts et

al., 2017), we looked at the relative abundances of Lactobacilli after EAE was induced. There was a clear decrease in Lactobacilli numbers in the early stage of the disease, days 9-14. Previous data from our lab showed a significant decrease in clinical scores for EAE mice that were given an unmodified *Lactococcus lactis,* which produces GABA, as a treatment in comparison to controls.

Farnesol

Farnesol is a positive allosteric modulator of $GABA_A$ receptors (Jeevan et al., 2019). Farnesol (FOL) is a 15-carbon acyclic sesquiterpene primary alcohol. It is commonly found as essential oil in plants (Jung et al., 2018). Oral administration of FOL has been shown to have powerful anti-oxidant and anti-inflammatory effects in mice (Jahangir et al., 2005; Ku and Lin, 2015). FOL has been shown to protect against an LPS-induced murine model of neurodegeneration (Santhanasabapathy and Sudhandiran, 2015), as well as an acrylamide-induced model of neurotoxicity (Santhanasabapathy et al., 2015). Farnesol is known to block Ca^{2+} channels (Luft et al., 1999; Beedle and Zamponi, 2000), which may help protect against intraneuronal Ca^{2+} overload in MS.

Farnesol impacts the microbiota. It is an important quorum sensing molecule. Microbes use quorum sensing molecules to communicate between cells to detect and respond to cell density. Biofilms are often formed when a certain cell population density is detected. Biofilms can reduce the effectiveness of immune cells, such as neutrophils and macrophages, and some bacteria begin to produce inflammatory molecules after forming a biofilm (Watters et al., 2016). FOL has been shown to inhibit the formation of biofilm in certain species of inflammatory pathogenic gut flora such as *Candida albicans*

(Ramage et al., 2002) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Jabra-Rizk et al., 2006). While *Lactobacillus subtilis* biofilm formation, which has been shown to promote stress resistance, life extension, and protect against pathogenic infection in *C. elegans* (Smolentseva et al., 2017), is promoted by farnesol (Feng et al., 2014). Like GABA, FOL also strengthens epithelial integrity by increasing epithelial cell occludin expression. It also increases transepithelial electrical resistance and reduces paracellular flux (Fang et al., 2019).

Experimental Autoimmune Encephalomyelitis (EAE) Animal Model of MS

One of the most common animal models for MS is experimental autoimmune encephalomyelitis (EAE). This model has been used to study MS for over 100 years, its origins are linked to the encephalomyelitis originally observed in Louis Pasteur's rabies vaccine in the 19th century (Farooqi, Gran, & Constantinescu, 2010). When animals were injected with a spinal cord emulsion containing a weakened rabies virus to protect against rabies some of them developed CNS inflammation and paralysis symptoms despite not having rabies. Since then, EAE has been developed in primates, mice, and other mammals (Burrows et al., 2019). Due to its extensive use and ease, it works well for comparing treatments.

EAE induction can be either active or passive. In passive EAE induction, encephalitogenic T cells are isolated from the lymphoid tissues of animals already immunized with myelin antigen. These T cells are specific to myelin antigen and when transferred to naïve recipients attack the myelin sheath and start the disease. In active EAE induction, T-cell mediated immunity is induced by injection of myelin antigens (such

as, myelin oligodendrocyte glycoprotein peptides 35-55, or MOG_{35-55})(Burrows et al., 2019). In either case myelin specific CD4+ T cells cross the blood brain barrier into the CNS parenchyma. APCs activate them by presenting myelin peptides. Activation of T cells lead to a pro-inflammatory cascade mediated by cytokines. Continuous inflammation results in myelin sheath damage and eventual paralysis.

There are a few major differences between MS and EAE. While MS develops spontaneously, EAE requires an active sensitization of the immune system through exposure to CNS antigens. The exception being spontaneous EAE, which still requires special transgenic animals (Gold et al., 2006; Burrows et al., 2019). EAE also requires a strong immune adjuvant to boost the immune response, which is unlikely to occur in natural MS development even with infections. These differences may result in differences in how the T cells and other immune cells activate and attack, resulting in differing outcomes between various therapeutics in EAE model animals and real MS patients. Currently, not enough is known about the induction of MS to enable an exact repeat in an animal model (Farooqi et al., 2010). Active EAE induced in C57BL/6 mice (B6 mice) is the animal model selected for all experiments conducted in this project (see methods section).

In this study, we determined that oral administration of farnesol results in a decrease of mouse EAE severity, with the decreased disease severity associated with decreases of macrophages, monocytes, dendritic cells, and CD4+ cells in farnesol treated mouse spinal cords. Due to farnesol being a positive allosteric $GABA_A$ receptor

modulator, we looked at the possible protective effects of increased intestinal GABA production by treatment with a modified probiotic. Though early results were promising, we were unable to repeat the experiment due to difficulties inducing EAE.

RESEARCH HYPOTHESES

Hypothesis 1#

Oral administration of Farnesol, a positive allosteric $GABA_A$ receptor modulator, will result in decreased disease severity in a EAE murine model of MS, as measured by clinical scores. It will also result in a decrease in pro-inflammatory immune cell infiltration of the spinal cord.

Hypothesis 2#

Increasing the levels of intestinal neurotransmitter γ-Aminobutyric acid (GABA) by treatment with a GABA-producing probiotic will reduce disease severity as measured by clinical scores in a EAE murine model of MS.

METHODS

Animal strain and care

Female C57BL/6 mice were obtained from Envigo (Envigo RMS, Inc., Indianapolis, IN, USA), except in one experiment where another provider was also used (The Jackson Laboratory, Inc., Bar Harbor, ME, USA). The mice arrived at Eastern Washington University when they were 8 weeks old and were given time to acclimate before immunization at 10 weeks. At the time of disease induction, they weighed approximately 20 g. They were housed in Eastern Washington University's vivarium in wire-top cages (46cm x 25cm x 20cm) with bedding. Animals were placed in cages randomly with 5 animals per cage. The room environment was kept at 22 ± 1°C and 23-33% humidity with a 12-hour light/dark cycle. All animals had free access to food and water. When mice reached an EAE clinical score of 2.5 or higher crushed food soaked in water was placed in a shallow dish at the bottom of the cage to help facilitate access. Mice were fed pellet food (Teklad 2018) containing plenty of glutamate for bacterial synthesis of GABA. All animal care and procedures followed Eastern Washington University's Institutional Animal Care and Use Committee (IACUC) policies and approved protocols.

EAE Induction and Clinical Scoring

Mice were induced with EAE using the Hook Kit™ for EAE induction (Hooke Laboratories, EK-2110). To induce each mouse was given a subcutaneous injection of 250 µg myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) emulsified in complete Freund's adjuvant on the flanks at 2 sites on day 0. The same day and 48 hours later the

mice will receive an intraperitoneal injection of 400ng of *Bordetella pertussis* toxin (200 µL). In this model, the onset of the disease (where symptoms are first observed) is typically around days 9-14 and the peak of the disease occurs 3-4 days later.

All mice were monitored for the duration of the experiment, up to 28 days, and scored daily using the EAE clinical score scale for disease severity. The clinical scores are based on the degree of paralysis the animal exhibits. 0 is a healthy animal with no disease; 0.5, a distal limp tail; 1, completely limp tail or isolated weakness of gait without a limp tail; 1.5, a limp tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb or partial hind and front limb paralysis; 3.5, complete bilateral hind limb paralysis and partial front limb paralysis. 5, moribund or dead animal. In accordance with IACUC policies to minimize undue suffering, mice displaying a score of 3.5 or higher (unable to right themselves when placed on their sides) for more than two consecutive days were euthanized. Thereafter the mouse was listed as a 5 (the clinical score for a dead mouse). Mice were euthanized via carbon dioxide asphyxiation followed by cervical dislocation. Body weights were measured weekly and expressed as % body weight at time of EAE induction.

Flow Cytometry

Spinal cord and splenic cells were isolated from mice on day 19 (peak disease) after EAE induction, to determine the impact of the positive allosteric $GABA_a$ receptor modulator, farnesol, on CNS infiltration. Spinal cords were extracted from the vertebral cavity by flushing with 1 mL cold sterile phosphate buffer saline (PBS) using a 3-mL

syringe and 18-gauge needles. The spinal cords were then homogenized and passed through a 70-µm filter in 1 % bovine serum albumin (BSA) in sterile PBS. They were washed in PBS and centrifuged at 350xg for 5 minutes, excess liquid was removed, and the cell pellets were then resuspended in 30mL sterile PBS. They were passed through a 40-µm filter and centrifuged again at 350 x g for 5 mins. Excess liquid was removed, and the cell pellet was resuspended in 1 mL PBS/1% BSA.

To obtain splenocytes, spleens were homogenized and passed through a 70-µm filter in sterile PBS. The samples were centrifuged at 350 x g for 5 minutes. Excess liquid was removed, and the cell pellets were resuspended in 9 ml PBS and 1 mL red blood cell lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany, cat. # 11 814 389 001). They were incubated for 10 minutes at room temperature. 50 mL of PBS was added to each sample. They were passed through a 40-µm filter and centrifuged again at 350 x g for 5 mins. Excess liquid was removed and the cell pellet was resuspended in 1 mL PBS-1 % BSA.

The spinal cord cell and splenocyte samples were treated with an anti-mouse CD16/CD32 Fc block this blocks non-specific antibody binding via their Fc regions and decreases background staining. The samples were then stained with hamster anti-CD11b FITC (clone M1/70; BD Biosciences, San Jose, CA, cat. #. 553310), hamster anti-CD11c (clone HL3; BD Biosciences, cat. # 553802), and rat anti-mouse F4/80 APC (clone T45-2342; BD Biosciences, cat. # 566787) to differentiate monocyte/macrophages and CD11c-positive dendritic cells.

Samples were stained to detect Tregs using the mouse regulatory T cell staining kit #1 from eBiosciences (cat. # 88-8111-40; Thermo Fisher Scientific, Waltham, MA). Which contained the following antibodies: anti-CD4 FITC (RM4-5), anti-CD25 APC (PC61.5), and anti-mouse/rat Foxp3 PE (FJK-16s). We used rat anti-mouse IgG2a PE as an isotype control. Samples were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences) at Washington State University. Data processing was performed with the FloJo software (FloJo LLC, Ashland, OR).

Lactococcus lactis strains

Strains of *Lactococcus lactis* used in this study were generated by Dr. Castillo's lab at Eastern Washington University. *Lactococcus lactis* (IL1403) was transformed with with the pAC plasmid alone, pGh9:ISS1 (Maguin et al., 1996), which contains the gene for erythromycin resistance (pAC *L. lactis*) or pAC with the *L. lactis* (IL1403) genes for glutamic acid decarboxylase (*gadB*) and the GABA/glutamate antiporter (gadC) regulated by the constitutive promoter sequence P5, P2, or P8. Of these differing promoters, P8 has been identified as the strongest with the greatest transcriptional efficiency followed by P5 and P2 (Zhu et al., 2015). All *L. lactis* strains used in this study were grown at 30°C without shaking in M17 broth or agar plates (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose (GM17) and, in the case of plasmid containing strains, 5ug/mL erythromycin (ERM GM17). Strains were archived for long term storage in GM17 media supplemented with 15% glycerol and frozen at -80°C.

GABA quantitation

A preliminary assay was done with supernatants from each strain of *L. lactis* (P8-*L. lactis*, pAC-*L. lactis*, WT-*L. lactis,* P2-*L. lactis*, and P5-*L. lactis;* grown overnight) after samples were centrifuged to remove cells. The supernatants from the bacterial strain were tested in triplicate with a GABA-specific enzyme-linked immunosorbent assay (ELISA) using a GABA ELISA (LDN BA E-2500) at Washington State University, with assay controls provided by the ELISA kit.

Next, we compared GABA levels produced by the different strains at different growth phases. To create a growth curve, we took stationary phase bacteria, grown overnight in GM17 or GM17 ERM broth and diluted 2 replicates of each strain except P2 *L. lactis* (which due to low GABA production levels was removed) to a OD_{600} 0.2 (optical density) with GM17 media. After 1 hour of growth, their OD_{600} levels were tested and every ½ hour afterwards. Samples were plated to obtain colony forming unit counts (CFUs) and supernatants were collected and centrifuged (to remove cells) for ELISA's at OD_{600} 0.5, 1.0, 1.5 and 2 with two replicates for each strain. At OD_{600} 1.5 there were 8.5 x 10^8 CFUs/mL of bacteria.

Collected samples along with GM17 media as a control were centrifuged and cell pellets removed then supernatants were tested with a GABA ELISA kit (AVIVA Systems Biology, GABA ELISA Kit OKEH02564). The supernatant samples of P8-*L. lactis*, pAC-*L. lactis*, P5-*L. lactis,* and the GM17 media were all in triplicate. WT-*L. lactis* was run in duplicate. The ELISA was run twice with both replicates from the growth curve experiment and results were combined.

Treatment for $GABA_A$ receptor modulator EAE Experiment

To determine the impact of $GABA_A$ receptor modulator farnesol on EAE progression, we randomly separated 28 mice into 3 treatment groups; a farnesol treatment group (FOL-EAE; n=10), a vehicle control group (CO-EAE; n=9), and an untreated control group (U-EAE; n=9). In the FOL group; mice were treated with 100 mg/kg/day FOL (trans-trans-farnesol, sigma-Aldrich, cat.#277541) emulsified in corn oil. The vehicle control group received 100 µL corn oil with no farnesol added. The untreated control group were induced with EAE with no treatments. Corn oil and farnesol treatments were given by gavage. Mice were weighed weekly, and treatments were adjusted for mouse weight. Animals were euthanized on day 19, when the EAE scores are highest in untreated animals.

Treatment for GAD L. lactis EAE Experiments

Four separate EAE experiments were conducted to determine the impact of GAD-*L. lactis* (P8-*L. lactis*) on EAE severity though only the first worked. In each experiment, we randomly divided mice into 3 treatment groups: a medium only group (medium), a pAC-*L. lactis* group (pAC), and a GAD-*L. lactis* group (P8). The medium group was treated with 0.1 mL of GM17 media. The pAC and GAD groups were treated with 5 x 10^8 CFU a day of their respective strains of bacteria suspended in 0.1 mL of GM17 media. Mice were treated from day 0 to end of the experiment, 5 times a week (Robert et al., 2014). Treatments were administered via oral gavage. Mice were weighed weekly and EAE clinical scores were measured daily.

The P8 and pAC bacterial cultures for treatment were started weekly on GM17 agar plates containing 5 μg/mL erythromycin from -80° C archived strains. Incubated at 30° C, these plates were then used to inoculate overnight cultures of ERM GM17 media. New ERM GM17 media was made every 3 days and it was stored at ~3°C. Morning cultures were diluted to OD_{600} 0.2. The bacteria were incubated for 3 hours to OD_{600} 1.5 at 30°C. The ERM GM17 media was removed by centrifugation and the cell pellets were resuspended in GM17 media to achieve $5X10^8$ CFU per 0.1 mL media. New overnight cultures were made daily with a dilution of 2 mL broth to 25 μL previous overnight culture.

Experimental setup for comparing EAE severity between mice providers

To explore the differences in EAE severity between mice from different providers, we divided 14 C57BL/6 mice from Envigo (Envigo RMS, Inc., Indianapolis, IN, USA), and 15 Jackson mice (The Jackson Laboratory, Inc., Bar Harbor, ME, USA) into a naïve group (Envigo-naïve, n=5; Jackson-naïve, n=5) and an EAE induction group (Envigo-EAE, n=9; Jackson-EAE, n=10). EAE clinical scores were taken daily, stool samples and mouse weights were collected weekly. The experiment lasted for 21 days.

Statistical analyses

Repeated measures and mixed-effect ANOVA followed by Tukey's multiple comparison post-hoc tests were used to estimate group differences for EAE clinical scores, body weights, and body weight changes. Group differences in spinal cord and spleen immune cell infiltration, disease onset, and disease severity were evaluated using non-parametric Kruskal-Wallis followed by Dunn's multiple comparisons tests. GABA

level means quantified by ELISA were compared by mixed-effect ANOVA followed by Tukey's multiple comparison post-hoc test. *P* values below 0.05 were considered significant.

RESULTS

Treatment with $GABA_A$ receptor modulator reduces immune cell infiltration into spinal cords at the peak of EAE disease.

Farnesol is an allosteric positive modulator of $GABA_AR$. Due to farnesol's reported anti-inflammatory and neuroprotective activities, we sought to determine whether those protective effects correlated with reduced immune cell infiltration of the CNS at the peak of the EAE disease (day 19). We observed a significant treatment effect ($p < 0.05$) on clinical scores comparing the 3 treatment groups; farnesol (FOL-EAE; n=10), corn oil (CO-EAE; n=9), and untreated (U-EAE; n=9) (Fig. 1A).

Next, we examined whether the observed protective effects of farnesol correlated with a reduction in inflammatory immune cell infiltration of the CNS. In preparation for the flow cytometry analyses, we extracted and processed spinal cords from each mouse at peak EAE severity on day 19. We discriminated doublets ??and selected singlets from each spinal cord preparation isolated from the EAE mice (FOL-treated: n=10; vehicle-treated: n=9; untreated: n=9), to ensure only singlets were counted to avoid false positives. We used a previously published gating strategy (Weiss et al., 2015).

The spinal cords' macrophage subpopulations were compared using the cell markers; CD11b, CD11c, F4/80 positive and negative markers (Figure 1B). Spinal cords from mice treated with farnesol had significantly reduced percentages of granulocyte/monocyte subsets ($CD11b^{+}F4/80^{-}$ and $CD11b^{+}F4/80^{int}$), as well as monocyte derived macrophages ($CD11b^{+}F4/80^{+}$) in their spinal cords in comparison to untreated and vehicle treated groups (Figure 1C).

We also compared spinal cord CD4+ T cell and Treg frequencies between groups using the cell markers of CD4+ (CD4+ T cells) and CD25+Foxp3+ (Treg cells). We found that treatment with farnesol significantly reduced the CD4+ T cell percentage (Figure 2A) and increased the proportion of anti-inflammatory Treg cells within the CD4+ T cell population (Figure 2B), compared to the untreated and vehicle treated control groups.

We further examined whether these cell frequency results were the same in the periphery by analyzing immune cell numbers of the spleens taken from the mice used in the study. Single-cell suspensions were prepared from the mouse spleens and analyzed. CD4+ T and Treg (CD25+Foxp3+) cell frequencies and proportions did not show significant differences between groups (Figure 3B). Splenic monocyte/macrophages and dendritic cell percentages were similarly analyzed. We did not see significant differences between groups in the percentage of dendritic cells ($CD11b^{+}F4/80^{-}CD11c^{+}$). We did see a significant increase in percentage of $F4/80^{int}$ granulocyte/monocytes and monocyte derived macrophages ($CD11b^{+}F4/80^{+}$) in farnesol treated mice when compared to untreated animals. We also saw a significant reduction in $F4/80^{neg}$ granulocyte/monocytes in cells isolated from corn oil treated (CO-EAE) mice in

comparison to untreated mice (U-EAE), but there were no significant differences between CO-EAE and FOL-EAE groups or FOL-EAE and U-EAE (Figure 3A).

The results of our EAE experiment and flow cytometry data confirm our hypothesis. Farnesol, a positive allosteric $GABA_AR$ modulator, confers protection to EAE mice as shown by a decrease in EAE clinical scores. This protection correlates with a decrease in pro-inflammatory infiltration of immune cells into their spinal cords. Due to farnesol being a positive allosteric $GABA_A$ receptor modulator, we examined whether intestinally produced GABA had similar effects on the disease severity of EAE in mice.

Characterization of GAD L. lactis

To examine the effects of intestinally produced GABA, we first characterized the amount of GABA produced by the modified *L. lactis* strains. To do this, we preformed three ELISAs. The first preliminary GABA ELISA (LDN BA E-2500), looking at the GABA content of supernatants taken from each strain cultured to the stationary growth phase, showed an increase in GABA levels in supernatants from P8 and P5 GAD- *L. lactis,* while P2 had the same GABA levels as the wild type and pAC plasmid *L. lactis*. Which aligns with other researchers findings showing increased transcriptional efficiency when using the P8 promoter, with P5 having slightly less, and P2 the least (Zhu et al., 2015).

We collected supernatants from bacteria grown to various OD600 levels that had previously been correlated for cell count. These supernatants were tested by ELISA (see methods). The ELISA was run twice with both replicates from the original experiment and the results were combined. Unfortunately, the controls provided with the kit did

not provide a signal that could be quantified, and we were unable to determine a standard curve for this ELISA. We therefore used the standard curve generated from the previous ELISA looking at the stationary phase GABA. We did observe a significant difference between pAC and P8 *L. lactis* strains at OD 1.5 after controlling for GM17 media (Figure 4B, $p = 0.0445$). Despite the uncontrollable issues with these ELISAs and based on the initial results obtained in the first and second set of ELISAs done (Figure 4), we selected P8 as a strain of *L. lactis* with enhanced capability to produce GABA for the in EAE experiments. We determined that P8 *L. lactis* grown to OD_{600} 1.5 would be used for these *in vivo* studies, as the P8 *L. lactis* supernatant grown to OD 1.5 had the most GABA production in comparison to others. Furthermore, reverse transcription qPCR experiments demonstrated that *gadB* expression in P8 *L. lactis* strain is consistent with its increased production of GABA shown in the ELISAs (Castillo, unpublished results).

In vivo effects of GAD L. lactis on EAE

Here we sought to determine if P8 *L. lactis* producing high levels of GABA would decrease disease severity in EAE induced mice. We administered P8 *L. lactis* (GAD *L. lactis*), a modified *L. lactis* strain that produced significantly more GABA compared to *L. lactis* with plasmid alone (pAC *L. lactis*). In our first experiment (GAD *L. lactis* Experiment #1) that ran for 25 days, we observed a significant treatment effect on clinical scores comparing the *P8 L. lactis* group *(*n=10) to the *L. lactis* control (pAC *L. lactis*; n=10), and medium control (Medium; n=10) (Figure 5A). We also observed a significant decrease in weights between mice treated with P8 *L. lactis*, those treated with pAC *L. lactis*, and those treated solely with GM17 medium (Figure 5B). We also saw a significant increase

in weight comparing the *P8 L. lactis* group (; n=10) to the pAC *L. lactis* control (n=10), and medium control (Medium; n=10) (Figure 5B). Due to the loss of the sickest and thereby lightest animals, 2 in the medium and 3 in the pAC group, the weights went up towards the end of the experiment. The distribution of scores showed a clear decrease in disease severity of P8 mice group with 6 mice not getting sick at all (Figure 5C). Our multiple attempts to repeat these experiments were met with challenges inducing disease in EAE mice (Figure 6). To address this issue, we conducted induction experiments on EAE mice from different animal providers.

Comparing EAE severity in mice from different providers

EAE induction can be easily impacted by numerous factors, including microbiome diversity. It is possible that the mice from Envigo have a modified intestinal microbiota because of a change of the microbial environment at the provider. This change in the microbiota could therefore change their susceptibility to EAE induction.

Due to the issues with the repeat EAE experiments using C57BL/6 mice obtained from Envigo, we sought to see if mice sourced from a different provider (Jackson) would have the same level of EAE disease severity and if differences in the microbiome were responsible. We did not see significant differences between the EAE induction groups (C57BL/6 Jackson EAE; n=10: and C57BL/6 Envigo EAE; n=9), this may have been due to the low *n* number (Figure 7). Yet, we did observe differences in disease severity. The Jackson group had 8 mice that had to be sacrificed due to high clinical scores (≥3.5 for 2 consecutive days) by the end of the experiment (day 21), while only one mouse from

the Envigo group had to similarly be sacrificed. Both groups had their first clinical scores on day 11, with two 0.5 scores in the Envigo group, while the Jackson group had three 0.5 scores and a 1.0 score. We collected stool samples and have sent them to be analyzed. We hope to farther explore the possibility of differences in intestinal microbiota of mice between the two providers once we receive the results of the analysis. In future experiments with EAE, we plan to use Jackson mice due to their increased susceptibility to the EAE induction.

DISCUSSION

Multiple sclerosis is a neuroinflammatory disease of the central nervous system (CNS). Although the origins of the disease remain to be deciphered, it has been proposed that environmental factors, as well as genetics are all involved in disease initiation and progression. Unfortunately, although several drugs for the treatment of the disease have emerged over the last decades, no definitive cure exists. Most treatments for MS are expensive, lack effectiveness, and/or have dangerous side effects. Novel strategies for dealing with MS disease progression are needed. Despite the advances in the understanding of the etiology but also the pathogenesis and progression of the disease, many questions about MS remain unanswered. As described in the introduction, one striking feature of the disease is the unbalanced levels of GABA observed in MS patients. Similarly, recent findings indicate that the composition of the gut microbiota plays a significant role in modulating the immune, metabolic and neuronal pathways, including GABA production and function. The goal of this study was to look at the effects of intestinal $GABA_A$ receptor activation on murine EAE.

In our investigation, we first examined the effects of farnesol, a positive allosteric $GABA_A$ receptor modulator. Farnesol had been previously described as an anti-inflammatory, anti-oxidative, and neuroprotective natural compound, produced by many organisms. Prior laboratory experiments have identified Farnesol as a potential novel treatment for CNS inflammatory demyelination diseases, such as MS.

In the experiments shown here, we observed a significant decrease in disease severity between farnesol treated animals and untreated animals. Using flow cytometry, we were able to correlate this decrease in disease severity to a decrease in immune cell infiltration of the CNS when the severity of the disease is at the highest level (peak of disease). Previous experiments on farnesol by Christina Ramelow and Lacey Sell, graduate students from our lab, identified the protective effects of farnesol in the EAE model. They found through experiments with the same three treatment groups we used; (FOL-EAE, n =18; CO-EAE, n = 23; U-EAE, n = 24), a significant decrease in EAE score severity between the farnesol treated group and the others ($p < 0.001$) from induction (day 0) to the end of treatment (day 26). They also found that disease onset on average was significantly delayed in farnesol treated mice (14.9 ± 0.54) in comparison to untreated mice (12.6 ± 0.37; $p = 0.0004$). Flow cytometry analysis of the spinal cords of surviving treated and untreated EAE mice at the end of the experiment (day 26) (untreated: $n = 4$; vehicle-treated: $n = 9$; FOL-treated: $n = 8$) showed a significant reduction of infiltrated CD4+ T cells in comparison to the untreated EAE mice. Unfortunately, due to high animal loss of the most severe EAE animals by day 26 in their experiments, the sickest animals were not included. We attempted the same experiment using flow cytometry results from mice taken at the peak of disease (day 19), allowing a greater n number, due to a decrease in animal loss from severe EAE later in the disease.

The results provided in this thesis were obtained not on surviving animals but on EAE mice during full blown disease, providing even more relevant information regarding

the effects of farnesol, a $GABA_AR$ modulator, correlating reduced immune cell infiltration with disease protection. Furthermore, the results shown in this thesis provide evidence that the oral treatment with farnesol promotes the accumulation of Tregs in the CNS of EAE mice. As described in the introduction, Tregs are a key anti-inflammatory cell subpopulation of T cells associated with protection against inflammatory and autoimmune disorders, such MS. We describe a decrease in CNS of inflammatory immune cell percentage (granulocyte/monocyte, monocyte derived macrophage, and CD4+ T cell percentages) in the spinal cords of mice treated with farnesol, in comparison to untreated and vehicle treated groups. We also saw an increase in the percent of anti-inflammatory Treg cells. It may be tempting to conclude that farnesol works through Treg accumulation in the CNS. As a limitation of the study, we must remember that these differences are based on cell percentages not absolute cell count, and the small number of cells counted through flow cytometry. As we observed no corresponding increase in peripheral (spleen) Treg numbers (Figure 3), farnesol protection may not be associated with Treg mediated immunomodulation. The decrease in inflammatory immune cells were not seen in the periphery either. This observation could imply that treatment of farnesol does not cause immunodeficiency. Simultaneously, it implies that the mechanism by which farnesol is protective against CNS inflammatory demyelination needs to be determined.

The difficulty with ascribing this effect to $GABA_A$ receptor modulation, is that farnesol's anti-inflammatory and neuroprotective properties may be caused through mechanisms other than $GABA_A$ receptors. Farnesol is known to block Ca^{2+} channels (Luft

et al., 1999; Beedle and Zamponi, 2000) and intraneuronal Ca^{2+} overload has been linked to MS pathogenesis (Kattimani and Veerappa, 2018; Verma et al., 2018). Farnesol is also a potent antioxidant and has been shown to decrease pro-inflammatory cytokine secretion. These or other mechanisms may be the underlying reason for the neuroprotective activity of the orally administered farnesol, not activation of $GABA_A$ receptors. To further elucidate the specific effect of intestinal $GABA_A$ receptor activation, we used modified *L. lactis* strains provided by Dr. Castillo's lab at EWU as a mechanism to increase intestinal GABA levels.

The first step of the validation of the probiotic *L. lactis* constructs was to determine the production of GABA by the genetically modified strains. We performed three ELISAs to quantify the amount of GABA produced by the strains. The first ELISA used supernatant from strains grown till they saturated the media in their stationary phase. Since they were all grown to the same stage, we were able to compare between strains to a degree, and although we observed a trend to increased production, no significance was observed. The colony forming units (CFU) of the original bacterial samples used for the ELISA analysis were not collected. Thus, and despite the positive ELISA outcome, with a standard curve (correlation of 0.9887 and R squared 97.75%). we were unable to normalize GABA levels with CFU of bacteria per volume (CFU/ml). The lack of CFU/ml normalization could explain why no significance was achieved. GABA production can also vary by growth phase as you see with the second set of ELISAs. The findings from this ELISA align with other researchers findings that the P8 promoter has

the greatest transcriptional efficiency, followed by the P5, and of the series of promoters the P2 promoter is the least efficient (Zhu et al., 2015).

For the next GABA quantification experiments, the ELISA were performed with supernatants taken from strains grown from an OD_{600} of 0.2 for the same amount of time, with known CFU levels (based on growth curves determined as described in the methods section). The GABA characterization by ELISA was then performed using a different kit from (AVIVA Systems Biology, GABA ELISA Kit OKEH02564). Unfortunately, the standard controls provided with the kit did not provide a signal that could be quantified and were oversaturated. We were unable to determine a standard curve for this ELISA. We used the standard curve generated from the previous ELISA on stationary phase substrates to generate approximate GABA levels. Strain comparison showed a significant difference between pAC and P8 *L. lactis* strains at OD 1.5. Furthermore, RT-qPCR data indicate that the P8 *L. lactis* strain expresses *gadB* (encoding for glutamic acid decarboxylase responsible for synthesizing GABA) at a significantly higher level than wild type *L. lactis* and pAC (Castillo, unpublished results). Additional commercially available GABA ELISAs will need to be used to validate our preliminary results, normalizing them to CFU/ml of cultured *L. lactis*.

GABA produced naturally by the wild-type strain IL1403 changes depending on the environment. The expression levels of *gadBC* genes in *Lactobacillus brevis* have been found to increase in response to moderately acidic environments (pH 5.2), while declining in neutral environments (pH 6.8). Expression is highest in the exponential growth phase, while sharply declining once the bacteria enter stationary growth phase.

Expression of *gadBC* can also change depending on temperature (Lyu et al., 2018; Gong et al., 2019). The exact amount of GABA produced by our strains would vary depending on the intestinal environment of the mice. We attempted to control for this as well as for non-GABA related effects by treating one group of mice with an *L. lactis* strain that did not contain the extra g*adCB* genes. As the engineered strains express their extra copies of *gadCB* constitutively; those extra copies are not under the endogenous promoter controls. Another variable is the ability of *L. lactis* to survive. In the first hour of ingestion, *L. lactis* decreases sharply due to poor survival in low pH, pH 2 is considered lethal (Tan et al., 2019). Stomach pH can vary widely, depending on how recently and what the subject ate, as well as how close to the mucosa it is measured. Humans range from pH 1-2 in the gastric lumen to pH 6-7 at the mucosal surface (Hunt et al., 2015). All these variables may result in differing levels of intestinal GABA production in treated mice. Future projects/experiments from the lab will attempt to encapsulate the GABA-producing GAD L. lactis in biodegradable microparticles that facilitate their survival through their gastrointestinal tract journey.

One of the most limiting stumbling blocks of our project was that in our GAD *L. lactis in vivo* experiments, the severity of EAE disease was reduced in mice. Even the first experiment, where a significant treatment effect was observed, there were mice from each group that did not show clinical scores (6 in P8-treated, 3 in pAC-treated, 2 in medium-treated groups). At first, we thought the reduced severity may have been the result of increased chronic stress due constant handling of the mice (stool collection, weights, daily scores, and oral treatments by gavage). Chronic stress before EAE onset,

can lead to a decrease in EAE disease severity, but if exposed to chronic stress later in the course of EAE it exacerbates the disease (Correa et al., 1998). Though some studies have shown contradictory results (Gerrard et al., 2017). Reducing human contact did not affect EAE severity in our studies.

An alternative to the stress hypothesis as a modifier of EAE severity is the microbiota and the origins of the mice used for the studies. Previous experiments, and the studies shown in figures 1-3 with farnesol, used the same mice strain, mouse provider, EAE induction kit, and living situation (EWU vivarium), as well as similar handling levels. However, no problems with EAE induction and disease progression were observed. EAE induction can be easily impacted by numerous factors, including microbiome diversity. The microbiome is needed for the appropriate development of the immune system (Kiyono et al., 1982; Wannemuehler et al., 1982; Sudo et al., 1997) and numerous studies point to a link between microbiome dysbiosis and MS (Ochoa-Repáraz et al., 2018; Kohl et al., 2020). Moreover, previous studies have shown that mouse breeding conditions significantly affect the composition of mouse microbiota, with the presence or absence of bacterial species associated with the induction of Th17 proinflammatory responses, such as segmented filamentous bacteria SFB (Ivanov et al., 2009). SFB monocolonization has been shown to restore EAE susceptibility in germ-free mice by increasing proinflammatory Th17 cell function (Lee et al., 2011). Due to the coronavirus pandemic of 2020, numerous lab protocols changed worldwide, with modifications in numbers and age of mice available, it is possible that the mice received harbor a modified microbiota because of a change of the microbial environment at the

provider. The change in the microbiota could therefore change their susceptibility to disease, as the microbiota is key to modulating disease severity.

To test whether the problem was our vivarium or a difference in the providers mice, we compared EAE induced C57BL/6 female mice from one commercial provider (Envigo) to C57BL/6 mice from a second provider (Jackson Laboratory), independent of treatments. We observed a difference, though it was not statistically significant possibly due to the low n number. However, 8 mice from Jackson died or had to be euthanized due high disease scores (>3.5 for two consecutive days) while only one mouse was euthanized due high score in the Envigo group. Overall, the incidence of the disease was high, as expected for C57BL/6 mice. This could imply that the reduced incidence and severity of EAE is not due to changes in EWU's vivarium's environment. We hope to farther explore the possibility of differences in the microbiome causing changes in EAE severity between the same mouse strains from different providers. We have collected stool samples from all mice and sent them for microbiota sequencing and analysis.

While we were able to show a decrease in EAE severity in mice treated with farnesol, a positive allosteric $GABA_A$ receptor modulator, it is not clear if those effects were due to the activation of the $GABA_A$ receptor or due to one of farnesol's other effects. The difficulties in inducing EAE prevented us from confirming our hypothesis that intestinal $GABA_A$ receptor activation by intestinally produced bacteria ameliorates the progression of murine EAE. It may be possible in future studies to repeat this experiment with Jackson mice and have stronger EAE disease induction.

TABLES AND FIGURES

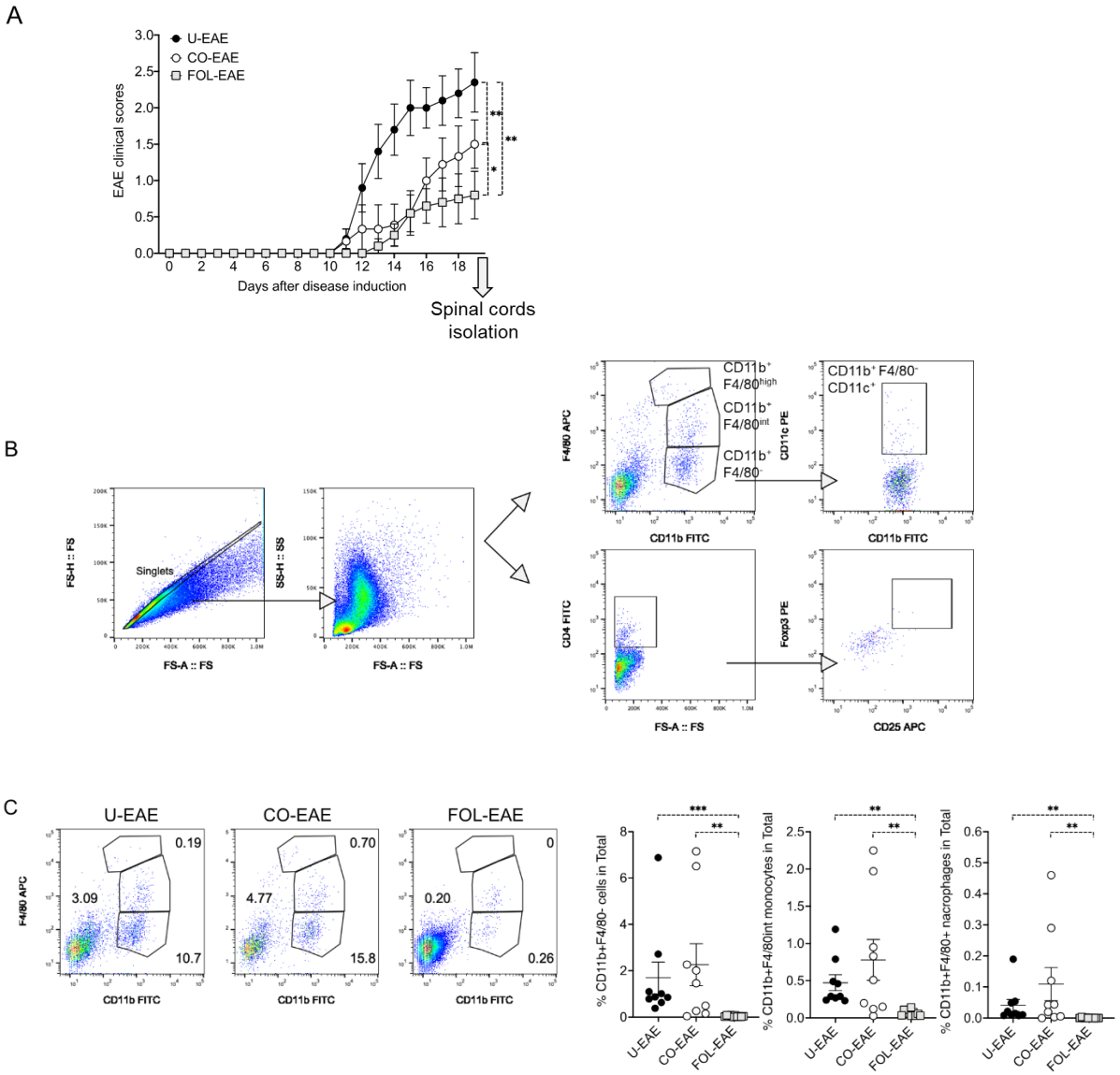
Table 1 Most common FDA approved treatments for MS

FDA approved Treatments for Multiple Sclerosis			
Injectable Medications			
Chemical Name	Limitations	Common Side Effects	Mechanism of Action
Interferon beta-1a	Can lead to flu-like symptoms	Flu-like symptoms, injection site pain and inflammation	Synthetic Interferon beta an anti-inflammatory cytokine
Interferon beta-1b	Can lead to flu-like symptoms	Flu-like symptoms injection site pain and inflammation skin breakdown low white blood cell count	Synthetic Interferon beta an anti-inflammatory cytokine
Glatiramer acetate	Weakens immune system	Injection site inflammation and pain, flushing, shortness of breath, rash, and chest pain	Kills immune cells
Oral Treatments			
Teriflunomide	Weakens Immune system	Headache, hair thinning, diarrhea, nausea, abnormal liver tests	Dampens Immune System
Fingolimod	Weakens the Immune system Bradycardia	Headache, flu, diarrhea, back pain, abnormal liver tests, sinusitis, abdominal pain, pain in extremities, cough	Stops T and B cells from leaving the lymph nodes (Mehling et al., 2008).
Cladribine	Weakens the immune system	Upper respiratory infection, headache, low	Kills T and B cells by interfering with DNA synthesis and

		white blood cell count	repair (Alroughani et al., 2019)
Dimethyl fumarate	Weakens immune System	Flushing and gastrointestinal issues	Dampens immune system
Intravenous Infusion Treatments			
Alemtuzumab	Weakens Immune System Can lead to serious complications with lungs, thyroid, and blood	Flu like symptoms, rash, increased risk of infections, hives, itching, thyroid gland disorders, pain in joints, extremities and back, diarrhea, vomiting, flushing and infusion reactions	Kills T and B cells (Alroughani et al., 2019)
Ocrelizumab	Weakens Immune System	Infusion reactions which in rare instances may be life-threatening; increased risk of infections; possible increased risk of cancer	It is an antibody that targets B cells (Alroughani et al., 2019)
Natalizumab	Weakens immune system Increases risk of progressive multifocal leukoencephalopathy (PML)	Flue like symptoms, increased risk of infection, depression, pain in extremity, abdominal discomfort, diarrhea, and rash	It is a monoclonal antibody that targets T cells preventing them from activating and migrating to sites of inflammation (Rice et al., 2005).
Other treatments			
Hematopoietic stem cell	Expensive	Side effects from chemotherapy	Uses chemotherapy

transplantation (HSCT)	Requires chemotherapy		to kill off immune cells and replaces them with the patients' hematopoietic stem cells from the their bone marrow

Figure 1: Oral treatment of FOL decreases mouse EAE clinical scores and macrophage infiltration of the CNS



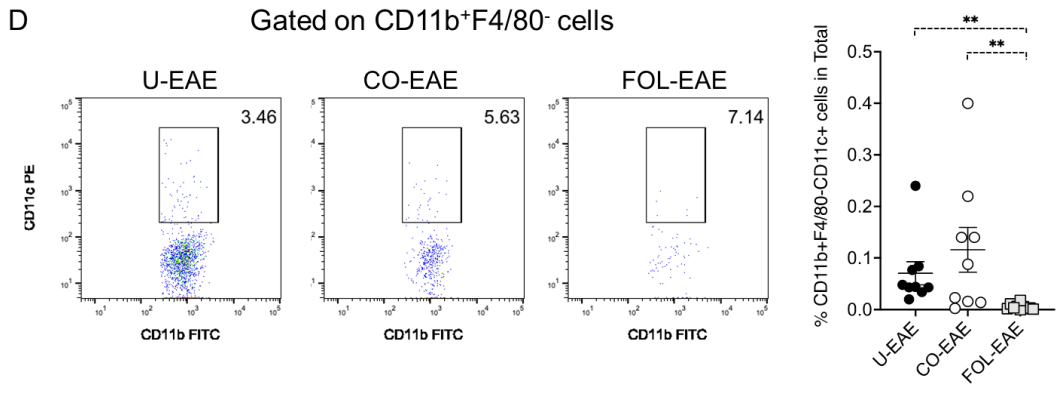


Figure 1. Oral treatment of FOL decreases mouse EAE clinical scores and macrophage infiltration of the CNS. Granulocyte/monocyte subsets ($CD11b^{+}F4/80^{-}$ and $CD11b^{+}F4/80^{int}$), as well as monocyte derived macrophages ($CD11b^{+}F4/80^{+}$) were measured by flow cytometry at day 19 post-EAE induction in untreated EAE mice (U-EAE; *n* = 9), corn oil vehicle control mice (CO-EAE; *n* = 9), and farnesol treated mice (FOL-EAE; *n* = 10) mice. Data are presented as mean ± 1 SEM. **A.** EAE scores from EAE induction, day 0, until peak of disease, day 19 (*, p< 0.05, **, *p* < 0.01). **B.** The gating strategy used to identify $CD11b^{+}F4/80^{-}$ (granulocyte/monocytes), $CD11b^{+}F4/80^{int}$ (granulocyte/monocytes), $CD11b^{+}F4/80^{+}$ (monocyte-derived macrophages), $CD11b^{+}F4/80^{-}CD11c^{+}$ (monocyte-derived dendritic cells) subpopulations, and $CD4^{+}$ T cells **C.** Percentage of $CD11b^{+}F4/80^{-}$, $CD11b^{+}F4/80^{int}$, and $CD11b^{+}F4/80^{+}$ subpopulations (**, p < 0.01; ***, p < 0.001). **D.** Percentage of $CD11c^{+}$ cells in gated CD11b+F4/80- cells (**, *p* < 0.01). The percentages displayed in the plots signify the positive cell frequency in pre-gated populations.

Figure 2: CD4+ T cell frequency decreases and the proportion of Treg cells increase in FOL treated EAE mice

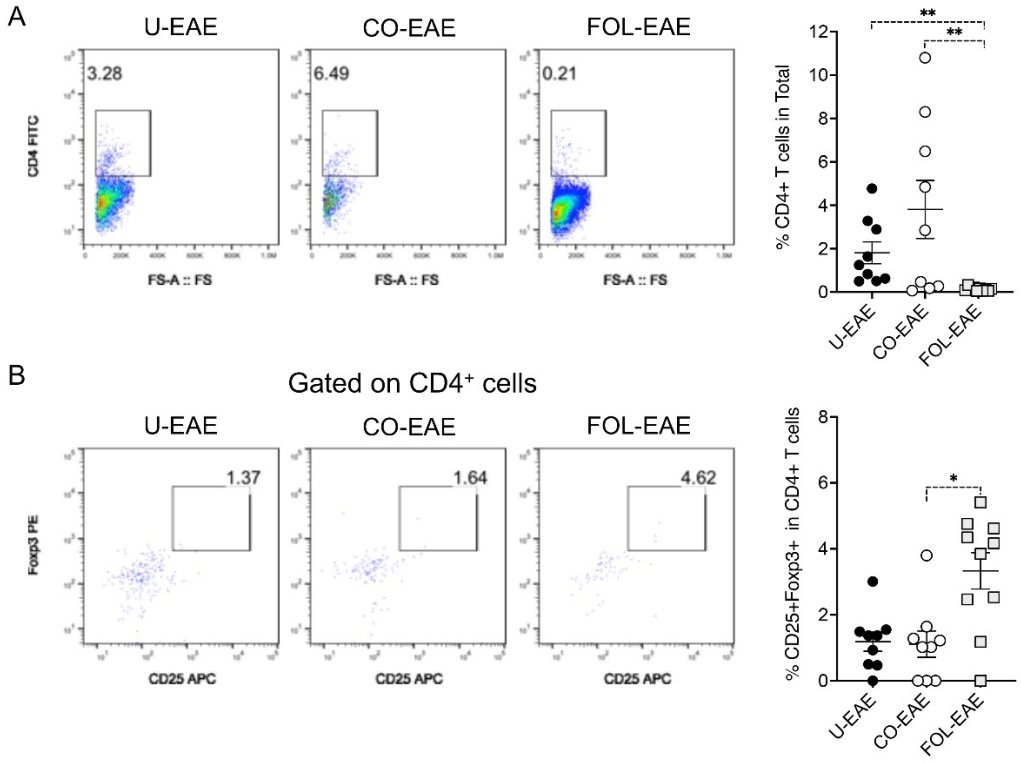


Figure 2: CD4+ T cell frequency decreases and proportion of Treg cells increase in FOL treated EAE. Flow cytometry measurements show the percentages of $CD4^+$ T cells and CD4+ T cells expressing $CD25^+Foxp3^+$ (Tregs) taken from mouse spinal cords at day 19 post-EAE induction in U-EAE (*n* = 10), CO-EAE (*n* = 9), and FOL-EAE (*n* = 10) mice. Data are presented as mean ± 1 SEM. **A.** Percentage of $CD4^+$ T cells in singlets and total of the spinal cords. **B.** Percentage of $CD25^+Foxp3^+$ cells in gated CD4+ T cells. (*, $p < 0.05$; **, $p < 0.01$).

Figure 3: FOL treatment modifies splenic monocyte/macrophage frequencies in EAE mice, but not splenic CD4+ T cell infiltration

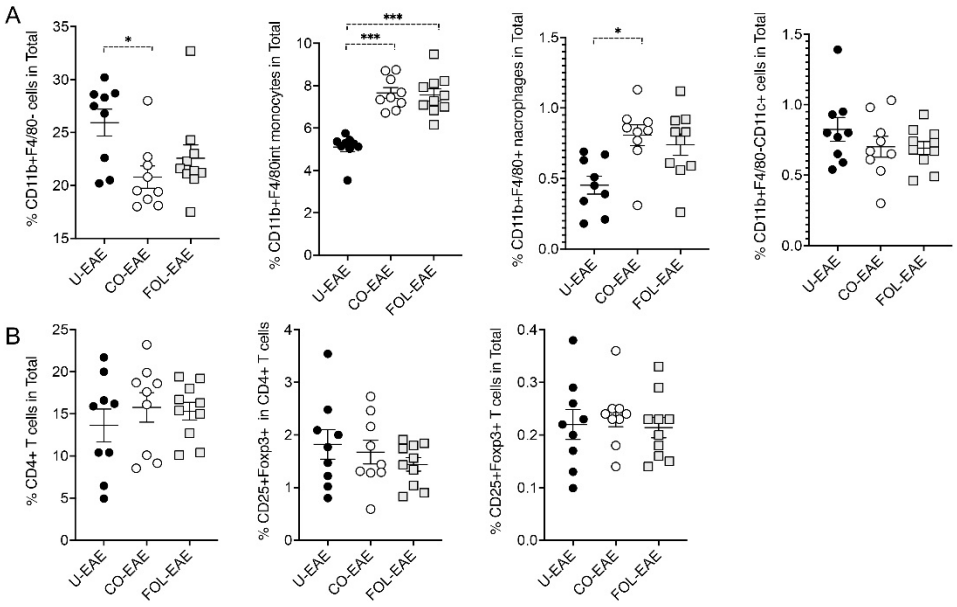


Figure 3: Oral treatment with FOL modifies the splenic monocyte/macrophage frequencies in mice at the peak of EAE but does not change splenic infiltration by $CD4^+$ T cells or dendritic cells. Splenic cell analyses were performed by flow cytometry at day 19 (end of treatment) in U-EAE (n = 10), CO-EAE (n = 9), and FOL-EAE (n = 10) mice. **A**. Percentages of $F4/80^{neg}$ granulocyte/monocytes, $F4/80^{int}$ granulocyte/monocytes, monocyte-derived macrophages, and monocyte-derived dendritic cells subpopulations in splenic population (individual data and mean ± 1 SEM). **B**. Percentages of CD4+ T cells in total splenic population, $CD25^+Foxp3^+$ cells in gated $CD4^+$ T cells, and $CD25^+Foxp3^+CD4^+$ T cells in total splenic population.

Figure 4: Increased GABA production in P8 L. lactis strain at OD 1.5

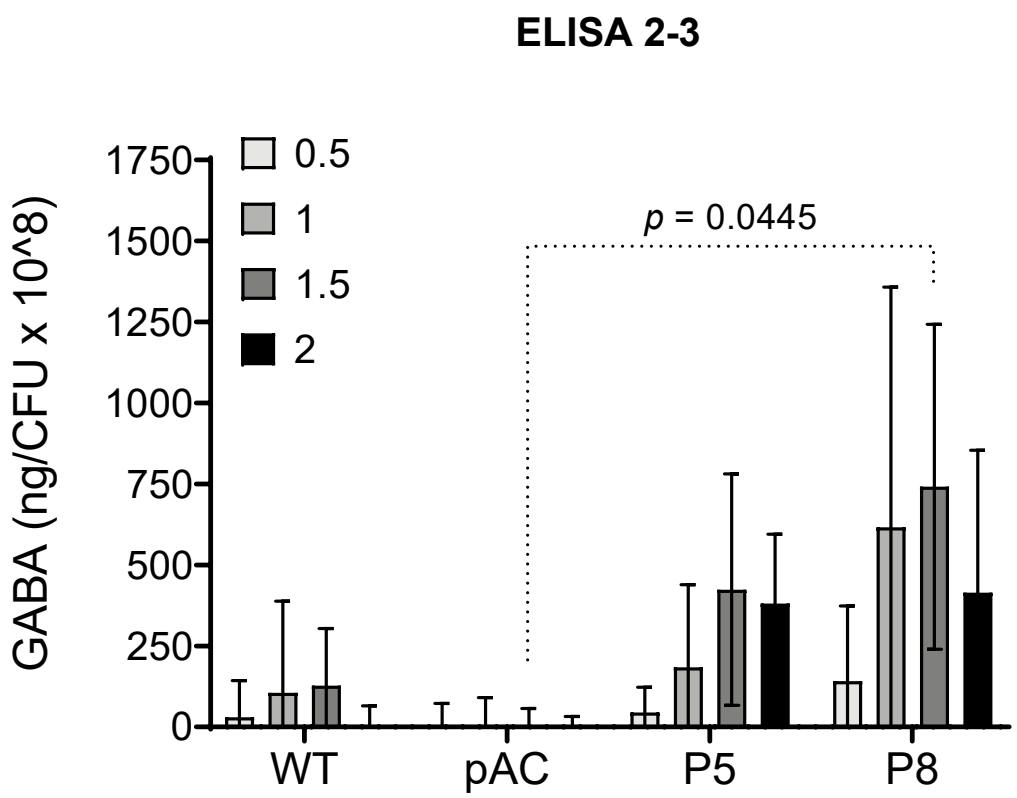


Figure 4: ELISA test results showing increased GABA levels in supernatants from *P8 L. lactis* compared to supernatants from wild type *L. lactis* (WT), pAC *L. lactis*, and P5 *L. lactis*. ELISA results for GABA levels of both replicates of supernatants taken from cultures grown to OD_{600} 0.5, 1.0, 1.5, and 2.0 after controlling for GM17 media GABA levels. The experiments were conducted on 2 separate occasions with 3 replicates per experiment.

Figure 5: GAD L. lactis experiment shows P8 L. lactis strain decreases EAE disease severity

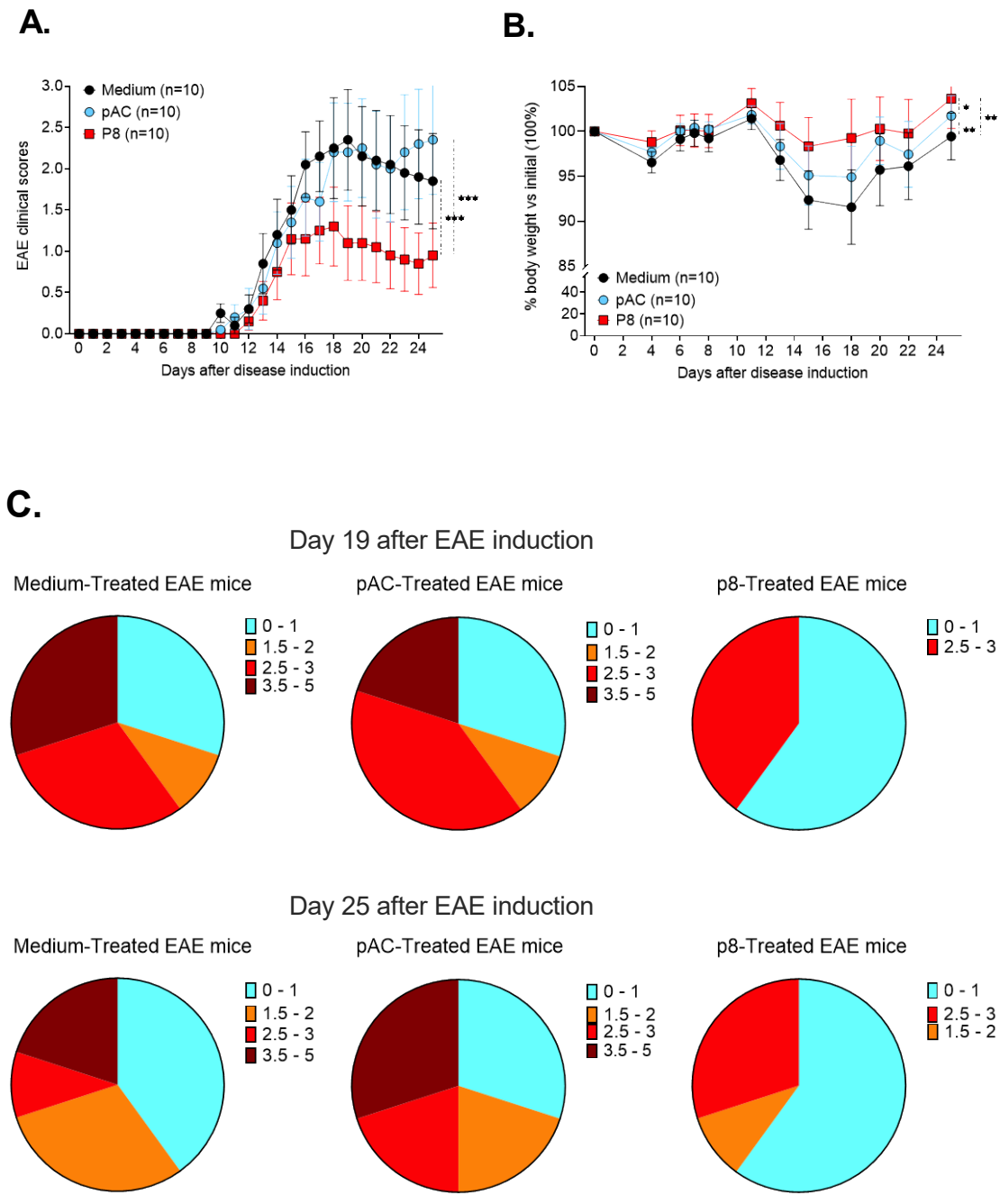


Figure 5: GAD *L. lactis* oral administration of P8 *L. lactis* strain decreases EAE disease severity (P8 *L. lactis*, n =10; pAC *L. lactis*, n = 10; Medium, n = 10). **A.** Clinical severity score from induction (day 0) to end of treatment (day 25) (treatments 5X week). **B.** Percent body weight vs. initial body weight. Data are presented as mean ±1 SEM. **C.** EAE clinical score distribution at peak of disease (day 19) and end of experiment (day 25).(*, $p < 0.05$, ***, $p < 0.001$)**.**

Figure 6: Mice failed to develop EAE in repeat GAD L. lactis Experiments

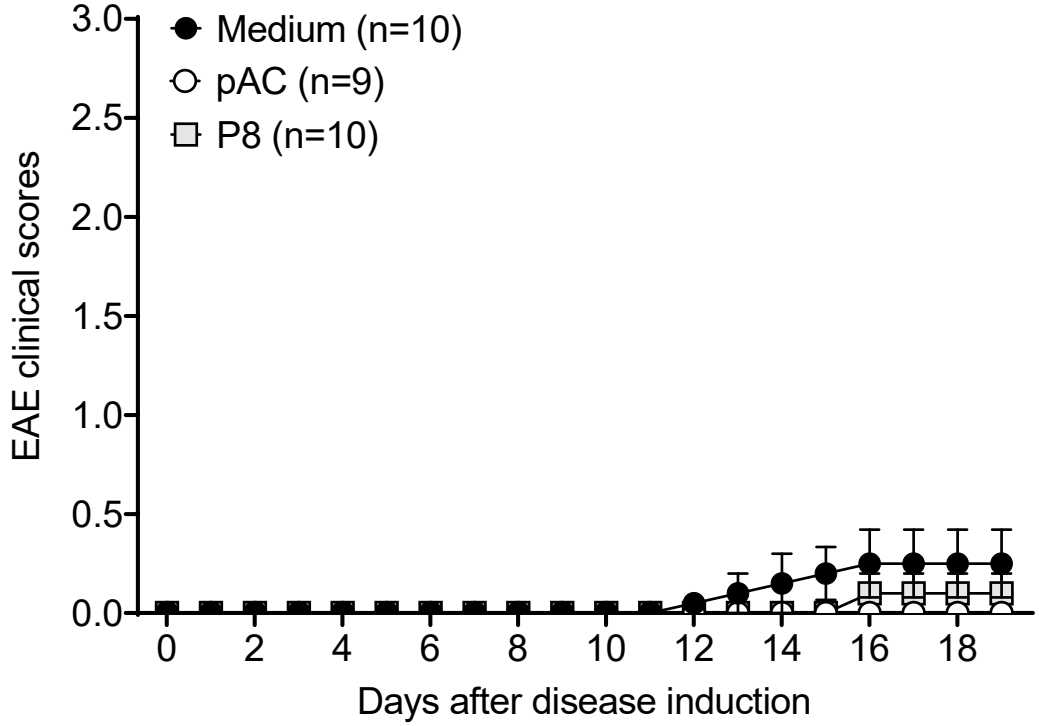


Figure 6: Mice failed to develop EAE in GAD *L. lactis* repeat EAE experiments. Above is the results of one of 3 repeat experiments. In all of them the mice were did not develop enough EAE severity to use the experiment.

Figure 7: Differential EAE disease severity seen between C57BL/6 mice from different providers

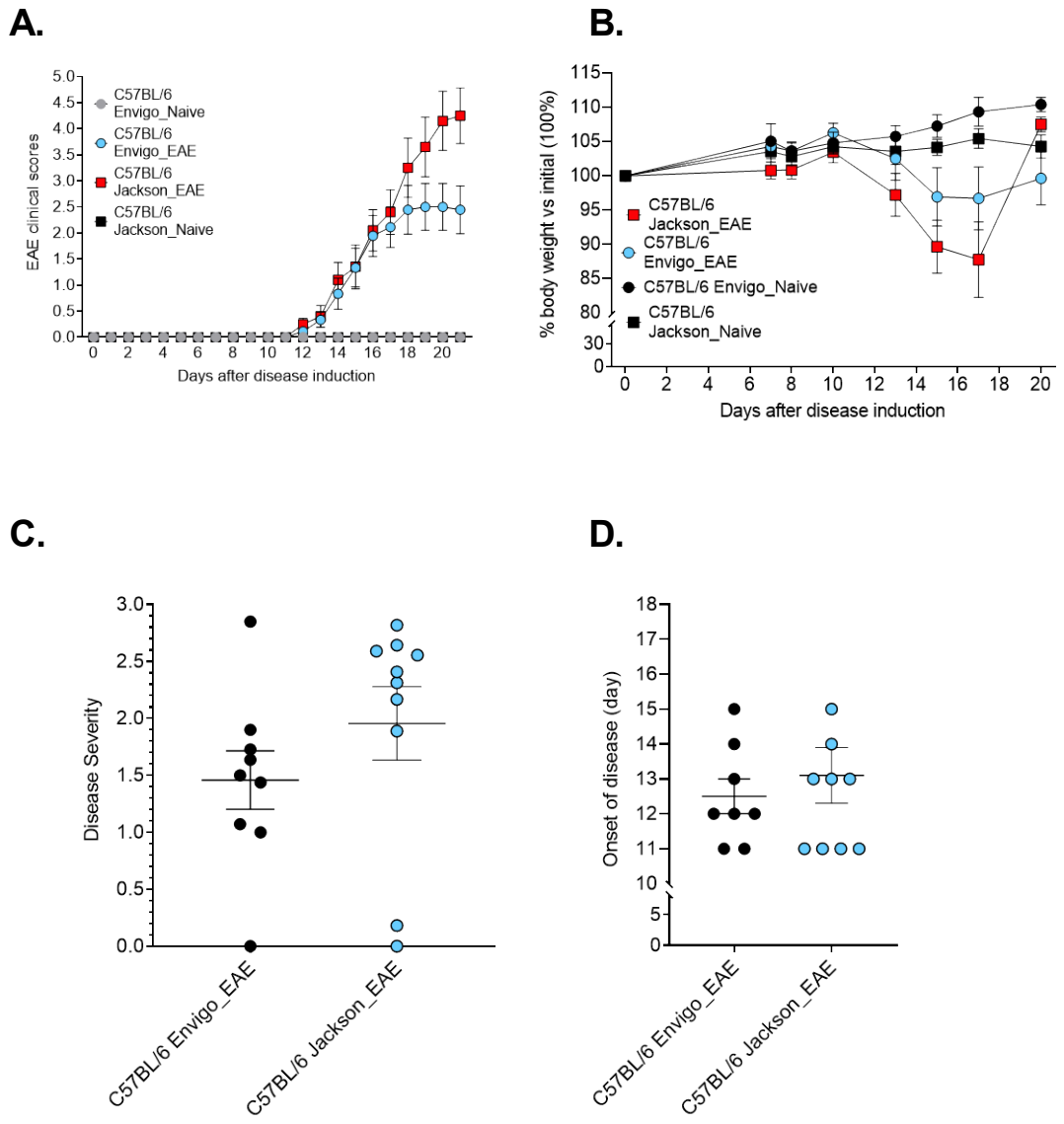


Figure 7: Differential EAE disease severity seen between C57BL/6 mice from different providers. C57BL/6 female Envigo mice (Envigo-EAE, n=9) and C57BL/6 female Jackson mice (Jackson-EAE, n=10) were induced with EAE with no treatments, EAE clinical scores

were collected daily, and mice were weighed weekly. Naïve C57BL/6 mice from each provider were used as controls (Envigo-naïve, n=5; Jackson-naïve, n=5). **A.** Clinical severity score from induction (day 0) to the end of experiment (day 25) **B.** Percent body weight vs. initial body weight. **C.** Disease severity comparing the two EAE induction groups (C57BL/6 Jackson EAE; n=10: and C57BL/6 Envigo EAE; n=9) **D.** Day of disease onset comparing the two EAE induction groups. No significant differences between the EAE induction groups (C57BL/6 Jackson EAE; n=10: and C57BL/6 Envigo EAE; n=9) were observed.

REFERENCES

Alroughani, R., Inshasi, J. S., Deleu, D., Al-Hashel, J., Shakra, M., Elalamy, O. R., et al. (2019). An Overview of High-Efficacy Drugs for Multiple Sclerosis: Gulf Region Expert Opinion. *Neurol Ther* 8, 13–23. doi:10.1007/s40120-019-0129-0.

Ascherio, A., and Munger, K. L. (2007). Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Annals of Neurology* 61, 288–299. doi:10.1002/ana.21117.

Auteri, M., Zizzo, M. G., and Serio, R. (2015). GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation. *Pharmacological Research* 93, 11–21. doi:10.1016/j.phrs.2014.12.001.

Bar-Or, A., Hintzen, R. Q., Dale, R. C., Rostasy, K., Brück, W., and Chitnis, T. (2016). Immunopathophysiology of pediatric CNS inflammatory demyelinating diseases. *Neurology* 87, S12-19. doi:10.1212/WNL.0000000000002821.

Beedle, A. M., and Zamponi, G. W. (2000). Block of Voltage-Dependent Calcium Channels by Aliphatic Monoamines. *Biophysical Journal* 79, 260–270. doi:10.1016/S0006-3495(00)76288-6.

Berer, K., Gerdes, L. A., Cekanaviciute, E., Jia, X., Xiao, L., Xia, Z., et al. (2017). Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10719–10724. doi:10.1073/pnas.1711233114.

Bhat, R., and Steinman, L. (2009). Innate and adaptive autoimmunity directed to the central nervous system. *Neuron* 64, 123–132. doi:10.1016/j.neuron.2009.09.015.

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., et al. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 405, 458–462. doi:10.1038/35013070.

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., et al. (2014). The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med* 6, 263ra158. doi:10.1126/scitranslmed.3009759.

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., et al. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16050–16055. doi:10.1073/pnas.1102999108.

Browne, P., Chandraratna, D., Angood, C., Tremlett, H., Baker, C., Taylor, B. V., et al. (2014). Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology* 83, 1022–1024. doi:10.1212/WNL.0000000000000768.

Burrows, D. J., McGown, A., Jain, S. A., De Felice, M., Ramesh, T. M., Sharrack, B., et al. (2019). Animal models of multiple sclerosis: From rodents to zebrafish. *Mult Scler* 25, 306–324. doi:10.1177/1352458518805246.

Cavaillon, J. M. (1994). Cytokines and macrophages. *Biomedicine & Pharmacotherapy* 48, 445–453. doi:10.1016/0753-3322(94)90005-1.

Cawley, N., Solanky, B. S., Muhlert, N., Tur, C., Edden, R. A. E., Wheeler-Kingshott, C. A. M., et al. (2015). Reduced gamma-aminobutyric acid concentration is associated with physical disability in progressive multiple sclerosis. *Brain* 138, 2584–2595. doi:10.1093/brain/awv209.

Cekanaviciute, E., Yoo, B. B., Runia, T. F., Debelius, J. W., Singh, S., Nelson, C. A., et al. (2017). Gut bacteria from multiple sclerosis patients modulate human t cells and exacerbate symptoms in mouse models. *PNAS* 114, 10713–10718. doi:10.1073/pnas.1711235114.

Chebib, M., and Johnston, G. A. R. (1999). The 'Abc' of Gaba Receptors: A Brief Review. *Clinical and Experimental Pharmacology and Physiology* 26, 937–940. doi:https://doi.org/10.1046/j.1440-1681.1999.03151.x.

Cheli, V. T., Santiago González, D. A., Spreuer, V., and Paez, P. M. (2015). Voltage-gated Ca2+ entry promotes oligodendrocyte progenitor cell maturation and myelination in vitro. *Exp Neurol* 265, 69–83. doi:10.1016/j.expneurol.2014.12.012.

Colpitts, S. L., Kasper, E. J., Keever, A., Liljenberg, C., Kirby, T., Magori, K., et al. (2017). A bidirectional association between the gut microbiota and CNS disease in a biphasic murine model of multiple sclerosis. *Gut Microbes* 8, 561–573. doi:10.1080/19490976.2017.1353843.

Correa, S. G., Rodriguez-Galán, M. C., Rivero, V. E., and Riera, C. M. (1998). Chronic varied stress modulates experimental autoimmune encephalomyelitis in Wistar rats. *Brain Behav Immun* 12, 134–148. doi:10.1006/brbi.1998.0519.

Danikowski, K. M., Jayaraman, S., and Prabhakar, B. S. (2017). Regulatory T cells in multiple sclerosis and myasthenia gravis. *J Neuroinflammation* 14, 117. doi:10.1186/s12974-017-0892-8.

De Angelis, F., Plantone, D., and Chataway, J. (2018). Pharmacotherapy in Secondary Progressive Multiple Sclerosis: An Overview. *CNS Drugs* 32, 499–526. doi:10.1007/s40263-018-0538-0.

Dhakal, R., Bajpai, V. K., and Baek, K.-H. (2012). Production of gaba (γ - aminobutyric acid) by microorganisms: a review. *Brazilian Journal of Microbiology* 43, 1230–1241. doi:10.1590/S1517-83822012000400001.

Dobson, R., and Giovannoni, G. (2019). Multiple sclerosis – a review. *European Journal of Neurology* 26, 27–40. doi:10.1111/ene.13819.

Enders, M., Heider, T., Ludwig, A., and Kuerten, S. (2020). Strategies for Neuroprotection in Multiple Sclerosis and the Role of Calcium. *Int J Mol Sci* 21. doi:10.3390/ijms21051663.

Evrensel, A., and Ceylan, M. E. (2016). Fecal Microbiota Transplantation and Its Usage in Neuropsychiatric Disorders. *Clin Psychopharmacol Neurosci* 14, 231–237. doi:10.9758/cpn.2016.14.3.231.

Fang, Y., Wu, C., Wang, Q., and Tang, J. (2019). Farnesol contributes to intestinal epithelial barrier function by enhancing tight junctions via the JAK/STAT3 signaling pathway in differentiated Caco-2 cells. *J Bioenerg Biomembr* 51, 403–412. doi:10.1007/s10863-019-09817-4.

Farooqi, N., Gran, B., and Constantinescu, C. S. (2010). Are current disease-modifying therapeutics in multiple sclerosis justified on the basis of studies in experimental autoimmune encephalomyelitis? *Journal of Neurochemistry* 115, 829–844. doi:10.1111/j.1471-4159.2010.06982.x.

Feng, X., Hu, Y., Zheng, Y., Zhu, W., Li, K., Huang, C.-H., et al. (2014). Structural and Functional Analysis of Bacillus subtilis YisP Reveal a Role of its Product in Biofilm Production. *Chem Biol* 21, 1557–1563. doi:10.1016/j.chembiol.2014.08.018.

Garret, M., Du, Z., Chazalon, M., Cho, Y. H., and Baufreton, J. (2018). Alteration of GABAergic neurotransmission in Huntington's disease. *CNS Neuroscience & Therapeutics* 24, 292–300. doi:10.1111/cns.12826.

Geffen, Y., Nudelman, A., Gil-Ad, I., Rephaeli, A., Huang, M., Savitsky, K., et al. (2009). BL-1020: a novel antipsychotic drug with GABAergic activity and low catalepsy, is efficacious in a rat model of schizophrenia. *Eur Neuropsychopharmacol* 19, 1–13. doi:10.1016/j.euroneuro.2008.07.002.

Gerrard, B., Singh, V., Babenko, O., Gauthier, I., Wee Yong, V., Kovalchuk, I., et al. (2017). Chronic mild stress exacerbates severity of experimental autoimmune encephalomyelitis in association with altered non-coding RNA and metabolic biomarkers. *Neuroscience* 359, 299–307. doi:10.1016/j.neuroscience.2017.07.033.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., et al. (2006). Metagenomic Analysis of the Human Distal Gut Microbiome. *Science* 312, 1355–1359. doi:10.1126/science.1124234.

Gold, R., Linington, C., and Lassmann, H. (2006). Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129, 1953–1971. doi:10.1093/brain/awl075.

Gong, L., Ren, C., and Xu, Y. (2019). Deciphering the crucial roles of transcriptional regulator GadR on gamma-aminobutyric acid production and acid resistance in Lactobacillus brevis. *Microb Cell Fact* 18. doi:10.1186/s12934-019-1157-2.

Guan, Y., Jakimovski, D., Ramanathan, M., Weinstock-Guttman, B., and Zivadinov, R. (2019). The role of Epstein-Barr virus in multiple sclerosis: from molecular pathophysiology to in vivo imaging. *Neural Regen Res* 14, 373–386. doi:10.4103/1673-5374.245462.

Hanisch, U.-K. (2002). Microglia as a source and target of cytokines. *Glia* 40, 140–155. doi:https://doi.org/10.1002/glia.10161.

Hemmer, B., Kerschensteiner, M., and Korn, T. (2015). Role of the innate and adaptive immune responses in the course of multiple sclerosis. *Lancet Neurol* 14, 406–419. doi:10.1016/S1474-4422(14)70305-9.

Henderson, A. P. D., Barnett, M. H., Parratt, J. D. E., and Prineas, J. W. (2009). Multiple sclerosis: distribution of inflammatory cells in newly forming lesions. *Ann Neurol* 66, 739–753. doi:10.1002/ana.21800.

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155, 1451–1463. doi:10.1016/j.cell.2013.11.024.

Hunt, R. H., Camilleri, M., Crowe, S. E., El-Omar, E. M., Fox, J. G., Kuipers, E. J., et al. (2015). The stomach in health and disease. *Gut* 64, 1650–1668. doi:10.1136/gutjnl-2014-307595.

Huseby, E. S., Huseby, P. G., Shah, S., Smith, R., and Stadinski, B. D. (2012). Pathogenic CD8 T Cells in Multiple Sclerosis and Its Experimental Models. *Front. Immunol.* 3. doi:10.3389/fimmu.2012.00064.

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139, 485–498. doi:10.1016/j.cell.2009.09.033.

Jabra-Rizk, M. A., Meiller, T. F., James, C. E., and Shirtliff, M. E. (2006). Effect of Farnesol on Staphylococcus aureus Biofilm Formation and Antimicrobial Susceptibility. *Antimicrob Agents Chemother* 50, 1463–1469. doi:10.1128/AAC.50.4.1463-1469.2006.

Jahangir, T., Khan, T. H., Prasad, L., and Sultana, S. (2005). Alleviation of free radical mediated oxidative and genotoxic effects of cadmium by farnesol in Swiss albino mice. *Redox Report* 10, 303–310. doi:10.1179/135100005X83671.

Janik, R., Thomason, L. A. M., Stanisz, A. M., Forsythe, P., Bienenstock, J., and Stanisz, G. J. (2016). Magnetic resonance spectroscopy reveals oral Lactobacillus

promotion of increases in brain GABA, N-acetyl aspartate and glutamate. *NeuroImage* 125, 988–995. doi:10.1016/j.neuroimage.2015.11.018.

Jeevan, B. G., Szlenk, C. T., Gibson, K. M., Roullet, J.-B., and Natesan, S. (2019). Membrane-Facilitated Allosteric Modulation of GABAA Receptor by Farnesol: An In Silico Modeling and Simulation Study. *The FASEB Journal* 33, 809.8-809.8. doi:https://doi.org/10.1096/fasebj.2019.33.1_supplement.809.8.

Joscelyn, J., and Kasper, L. H. (2014). Digesting the emerging role for the gut microbiome in central nervous system demyelination. *Mult Scler* 20, 1553–1559. doi:10.1177/1352458514541579.

Jung, Y. Y., Hwang, S. T., Sethi, G., Fan, L., Arfuso, F., and Ahn, K. S. (2018). Potential Anti-Inflammatory and Anti-Cancer Properties of Farnesol. *Molecules* 23. doi:10.3390/molecules23112827.

Karni, A., Bakimer-Kleiner, R., Abramsky, O., and Ben-Nun, A. (1999). Elevated Levels of Antibody to Myelin Oligodendrocyte Glycoprotein Is Not Specific for Patients With Multiple Sclerosis. *Arch Neurol* 56, 311–315. doi:10.1001/archneur.56.3.311.

Kattimani, Y., and Veerappa, A. M. (2018). Dysregulation of NRXN1 by mutant MIR8485 leads to calcium overload in pre-synapses inducing neurodegeneration in Multiple sclerosis. *Mult Scler Relat Disord* 22, 153–156. doi:10.1016/j.msard.2018.04.005.

Kennel De March, A., De Bouwerie, M., Kolopp-Sarda, M. N., Faure, G. C., Béné, M. C., and Bernard, C. C. A. (2003). Anti-myelin oligodendrocyte glycoprotein B-cell responses in multiple sclerosis. *Journal of Neuroimmunology* 135, 117–125. doi:10.1016/S0165-5728(02)00434-4.

Khalil, M., Reindl, M., Lutterotti, A., Kuenz, B., Ehling, R., Gneiss, C., et al. (2006). Epitope specificity of serum antibodies directed against the extracellular domain of myelin oligodendrocyte glycoprotein: Influence of relapses and immunomodulatory treatments. *Journal of Neuroimmunology* 174, 147–156. doi:10.1016/j.jneuroim.2006.01.012.

Kiyono, H., McGhee, J. R., Wannemuehler, M. J., and Michalek, S. M. (1982). Lack of oral tolerance in C3H/HeJ mice. *J. Exp. Med.* 155, 605–610. doi:10.1084/jem.155.2.605.

Kohl, H. M., Castillo, A. R., and Ochoa-Repáraz, J. (2020). The Microbiome as a Therapeutic Target for Multiple Sclerosis: Can Genetically Engineered Probiotics Treat the Disease? *Diseases* 8, 33. doi:10.3390/diseases8030033.

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., et al. (2006). IL-17 Plays an Important Role in the Development of Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology* 177, 566–573. doi:10.4049/jimmunol.177.1.566.

Ku, C.-M., and Lin, J.-Y. (2015). Farnesol, a Sesquiterpene Alcohol in Herbal Plants, Exerts Anti-Inflammatory and Antiallergic Effects on Ovalbumin-Sensitized and -Challenged Asthmatic Mice. *Evid Based Complement Alternat Med* 2015. doi:10.1155/2015/387357.

Lavasani, S., Dzhambazov, B., Nouri, M., Fåk, F., Buske, S., Molin, G., et al. (2010). A Novel Probiotic Mixture Exerts a Therapeutic Effect on Experimental Autoimmune Encephalomyelitis Mediated by IL-10 Producing Regulatory T Cells. *PLoS One* 5. doi:10.1371/journal.pone.0009009.

Lee, Y. K., Menezes, J. S., Umesaki, Y., and Mazmanian, S. K. (2011). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* 108 Suppl 1, 4615–4622. doi:10.1073/pnas.1000082107.

Lucas, R. M., Hughes, A. M., Lay, M.-L. J., Ponsonby, A.-L., Dwyer, D. E., Taylor, B. V., et al. (2011). Epstein–Barr virus and multiple sclerosis. *Journal of Neurology, Neurosurgery & Psychiatry* 82, 1142–1148. doi:10.1136/jnnp-2011-300174.

Luft, U. C., Bychkov, R., Gollasch, M., Gross, V., Roullet, J.-B., McCarron, D. A., et al. (1999). Farnesol Blocks the L-Type Ca2+ Channel by Targeting the α1C Subunit. *Arteriosclerosis, Thrombosis, and Vascular Biology* 19, 959–966. doi:10.1161/01.ATV.19.4.959.

Lyu, C., Zhao, W., Peng, C., Hu, S., Fang, H., Hua, Y., et al. (2018). Exploring the contributions of two glutamate decarboxylase isozymes in Lactobacillus brevis to acid resistance and γ-aminobutyric acid production. *Microb Cell Fact* 17. doi:10.1186/s12934-018-1029-1.

Maguin, E., Prévost, H., Ehrlich, S. D., and Gruss, A. (1996). Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *Journal of Bacteriology* 178, 931–935. doi:10.1128/jb.178.3.931-935.1996.

Marchesi, J. R., and Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome* 3. doi:10.1186/s40168-015-0094-5.

Martin, I., Bowery, N., and Dunn, S. (2020). An Overview of GABA Receptor Pharmacology. *News-Medical.net*. Available at: https://www.news-medical.net/whitepaper/20200408/An-Overview-of-GABA-Receptors.aspx [Accessed May 21, 2021].

Mehling, M., Brinkmann, V., Antel, J., Bar-Or, A., Goebels, N., Vedrine, C., et al. (2008). FTY720 therapy exerts differential effects on T cell subsets in multiple sclerosis. *Neurology* 71, 1261–1267. doi:10.1212/01.wnl.0000327609.57688.ea.

Mendu, S. K., Bh, A., Jin, Z., and Birnir, B. (2012). Different subtypes of GABA-A receptors are expressed in human, mouse and rat T lymphocytes. in *Article ID e42959*.

Miki, Y. (2019). Magnetic resonance imaging diagnosis of demyelinating diseases: An update. *Clinical and Experimental Neuroimmunology* 10, 32–48. doi:10.1111/cen3.12501.

Milosevic, E., Dujmovic, I., Markovic, M., Mesaros, S., Rakocevic, G., Drulovic, J., et al. (2015). Higher expression of IL-12Rβ2 is associated with lower risk of relapse in relapsing-remitting multiple sclerosis patients on interferon-β1b therapy during 3-year follow-up. *J. Neuroimmunol.* 287, 64–70. doi:10.1016/j.jneuroim.2015.07.011.

Muñoz-Culla, M., Irizar, H., and Otaegui, D. (2013). The genetics of multiple sclerosis: review of current and emerging candidates. *Appl Clin Genet* 6, 63–73. doi:10.2147/TACG.S29107.

Nomura, M., Nakajima, I., Fujita, Y., Kobayashi, M., Kimoto, H., Suzuki, I., et al. (1999). Lactococcus lactis contains only one glutamate decarboxylase gene. *Microbiology,* 145, 1375–1380. doi:10.1099/13500872-145-6-1375.

Nouri, M., Bredberg, A., Weström, B., and Lavasani, S. (2014). Intestinal barrier dysfunction develops at the onset of experimental autoimmune encephalomyelitis, and can be induced by adoptive transfer of auto-reactive T cells. *PLoS ONE* 9, e106335. doi:10.1371/journal.pone.0106335.

Ochoa-Repáraz, J., and Kasper, L. H. (2014). Gut microbiome and the risk factors in central nervous system autoimmunity. *FEBS Lett* 588, 4214–4222. doi:10.1016/j.febslet.2014.09.024.

Ochoa-Repáraz, J., Kirby, T. O., and Kasper, L. H. (2018). The Gut Microbiome and Multiple Sclerosis. *Cold Spring Harb Perspect Med* 8. doi:10.1101/cshperspect.a029017.

Ochoa-Repáraz, J., Mielcarz, D. W., Ditrio, L. E., Burroughs, A. R., Foureau, D. M., Haque-Begum, S., et al. (2009). Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 183, 6041–6050. doi:10.4049/jimmunol.0900747.

Olsen, R. W., and DeLorey, T. M. (1999). GABA Receptor Physiology and Pharmacology. *Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition*. Available at: https://www.ncbi.nlm.nih.gov/books/NBK28090/ [Accessed May 21, 2021].

Olszak, T., An, D., Zeissig, S., Vera, M. P., Richter, J., Franke, A., et al. (2012). Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. *Science* 336, 489–493. doi:10.1126/science.1219328.

Ortiz, G. G., Pacheco-Moisés, F. P., Macías-Islas, M. Á., Flores-Alvarado, L. J., Mireles-Ramírez, M. A., González-Renovato, E. D., et al. (2014). Role of the blood-brain

barrier in multiple sclerosis. *Arch Med Res* 45, 687–697. doi:10.1016/j.arcmed.2014.11.013.

Ponomarev, E. D., Shriver, L. P., Maresz, K., Pedras-Vasconcelos, J., Verthelyi, D., and Dittel, B. N. (2007). GM-CSF Production by Autoreactive T Cells Is Required for the Activation of Microglial Cells and the Onset of Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology* 178, 39–48. doi:10.4049/jimmunol.178.1.39.

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J.-S., et al. (2007). Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration. *Glia* 55, 453–462. doi:10.1002/glia.20467.

Rajda, C., Pukoli, D., Bende, Z., Majláth, Z., and Vécsei, L. (2017). Excitotoxins, Mitochondrial and Redox Disturbances in Multiple Sclerosis. *Int J Mol Sci* 18. doi:10.3390/ijms18020353.

Ramage, G., Saville, S. P., Wickes, B. L., and López-Ribot, J. L. (2002). Inhibition of Candida albicans Biofilm Formation by Farnesol, a Quorum-Sensing Molecule. *Appl Environ Microbiol* 68, 5459–5463. doi:10.1128/AEM.68.11.5459-5463.2002.

Reindl, M., Linington, C., Brehm, U., Egg, R., Dilitz, E., Deisenhammer, F., et al. (1999). Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* 122, 2047–2056. doi:10.1093/brain/122.11.2047.

Rice, G. P. A., Hartung, H.-P., and Calabresi, P. A. (2005). Anti-alpha4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology* 64, 1336–1342. doi:10.1212/01.WNL.0000158329.30470.D0.

Robert, S., Gysemans, C., Takiishi, T., Korf, H., Spagnuolo, I., Sebastiani, G., et al. (2014). Oral Delivery of Glutamic Acid Decarboxylase (GAD)-65 and IL10 by Lactococcus lactis Reverses Diabetes in Recent-Onset NOD Mice. *Diabetes* 63, 2876–2887. doi:10.2337/db13-1236.

Sánchez de Medina, F., Romero-Calvo, I., Mascaraque, C., and Martínez-Augustin, O. (2014). Intestinal inflammation and mucosal barrier function. *Inflamm Bowel Dis* 20, 2394–2404. doi:10.1097/MIB.0000000000000204.

Santhanasabapathy, R., and Sudhandiran, G. (2015). Farnesol attenuates lipopolysaccharide-induced neurodegeneration in Swiss albino mice by regulating intrinsic apoptotic cascade. *Brain Res* 1620, 42–56. doi:10.1016/j.brainres.2015.04.043.

Santhanasabapathy, R., Vasudevan, S., Anupriya, K., Pabitha, R., and Sudhandiran, G. (2015). Farnesol quells oxidative stress, reactive gliosis and inflammation during acrylamide-induced neurotoxicity: Behavioral and biochemical evidence. *Neuroscience* 308, 212–227. doi:10.1016/j.neuroscience.2015.08.067.

Scalfari, A., Knappertz, V., Cutter, G., Goodin, D. S., Ashton, R., and Ebers, G. C. (2013). Mortality in patients with multiple sclerosis. *Neurology* 81, 184–192. doi:10.1212/WNL.0b013e31829a3388.

Secher, T., Kassem, S., Benamar, M., Bernard, I., Boury, M., Barreau, F., et al. (2017). Oral Administration of the Probiotic Strain Escherichia coli Nissle 1917 Reduces Susceptibility to Neuroinflammation and Repairs Experimental Autoimmune Encephalomyelitis-Induced Intestinal Barrier Dysfunction. *Front Immunol* 8. doi:10.3389/fimmu.2017.01096.

Sintzel, M. B., Rametta, M., and Reder, A. T. (2017). Vitamin D and Multiple Sclerosis: A Comprehensive Review. *Neurol Ther* 7, 59–85. doi:10.1007/s40120-017-0086-4.

Smolentseva, O., Gusarov, I., Gautier, L., Shamovsky, I., DeFrancesco, A. S., Losick, R., et al. (2017). Mechanism of biofilm-mediated stress resistance and lifespan extension in C. elegans. *Sci Rep* 7. doi:10.1038/s41598-017-07222-8.

Sokovic Bajic, S., Djokic, J., Dinic, M., Veljovic, K., Golic, N., Mihajlovic, S., et al. (2019). GABA-Producing Natural Dairy Isolate From Artisanal Zlatar Cheese Attenuates Gut Inflammation and Strengthens Gut Epithelial Barrier in vitro. *Front. Microbiol.* 10. doi:10.3389/fmicb.2019.00527.

Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C., and Koga, Y. (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J. Immunol.* 159, 1739–1745.

Tan, E. W., Tan, K. Y., Phang, L. V., Kumar, P. V., and In, L. L. A. (2019). Enhanced gastrointestinal survivability of recombinant Lactococcus lactis using a double coated mucoadhesive film approach. *PLoS One* 14. doi:10.1371/journal.pone.0219912.

Thompson, A. J., Baranzini, S. E., Geurts, J., Hemmer, B., and Ciccarelli, O. (2018). Multiple sclerosis. *Lancet* 391, 1622–1636. doi:10.1016/S0140-6736(18)30481-1.

Tian, J., Chau, C., Hales, T. G., and Kaufman, D. L. (1999). GABAA receptors mediate inhibition of T cell responses. *Journal of Neuroimmunology* 96, 21–28. doi:10.1016/S0165-5728(98)00264-1.

Tian, J., Dang, H., Wallner, M., Olsen, R., and Kaufman, D. L. (2018). Homotaurine, a safe blood-brain barrier permeable GABA A -R-specific agonist, ameliorates disease in mouse models of multiple sclerosis. *Sci Rep* 8, 1–8. doi:10.1038/s41598-018-32733-3.

Tian, J., Lu, Y., Zhang, H., Chau, C. H., Dang, H. N., and Kaufman, D. L. (2004). γ-Aminobutyric Acid Inhibits T Cell Autoimmunity and the Development of Inflammatory Responses in a Mouse Type 1 Diabetes Model. *The Journal of Immunology* 173, 5298–5304. doi:10.4049/jimmunol.173.8.5298.

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mörk, S., and Bö, L. (1998). Axonal Transection in the Lesions of Multiple Sclerosis. *New England Journal of Medicine* 338, 278–285. doi:10.1056/NEJM199801293380502.

Treiman, D. M. (2001). GABAergic Mechanisms in Epilepsy. *Epilepsia* 42, 8–12. doi:10.1046/j.1528-1157.2001.042suppl.3008.x.

Tvrdik, P., and Kalani, M. Y. S. (2017). In Vivo Imaging of Microglial Calcium Signaling in Brain Inflammation and Injury. *Int J Mol Sci* 18. doi:10.3390/ijms18112366.

Van Leusden, J. W. R., Sellaro, R., and Colzato, L. S. (2015). Transcutaneous Vagal Nerve Stimulation (tVNS): a new neuromodulation tool in healthy humans? *Front Psychol* 6. doi:10.3389/fpsyg.2015.00102.

Verma, M., Wills, Z., and Chu, C. T. (2018). Excitatory Dendritic Mitochondrial Calcium Toxicity: Implications for Parkinson's and Other Neurodegenerative Diseases. *Front Neurosci* 12. doi:10.3389/fnins.2018.00523.

Voskuhl, R. R. (2020). The Effect Of Sex on Multiple Sclerosis Risk And Disease Progression. *Mult Scler* 26, 554–560. doi:10.1177/1352458519892491.

Wallin, M. T., Culpepper, W. J., Campbell, J. D., Nelson, L. M., Langer-Gould, A., Marrie, R. A., et al. (2019). The prevalence of MS in the United States: A population-based estimate using health claims data. *Neurology* 92, e1029–e1040. doi:10.1212/WNL.0000000000007035.

Wannemuehler, M. J., Kiyono, H., Babb, J. L., Michalek, S. M., and McGhee, J. R. (1982). Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J. Immunol.* 129, 959–965.

Watters, C., Fleming, D., Bishop, D., and Rumbaugh, K. P. (2016). Host Responses to Biofilm. *Prog Mol Biol Transl Sci* 142, 193–239. doi:10.1016/bs.pmbts.2016.05.007.

Weiss, M., Byrne, A. J., Blazek, K., Saliba, D. G., Pease, J. E., Perocheau, D., et al. (2015). IRF5 controls both acute and chronic inflammation. *Proc Natl Acad Sci U S A* 112, 11001–11006. doi:10.1073/pnas.1506254112.

Wekerle, H. (2017). B cells in multiple sclerosis. *Autoimmunity* 50, 57–60. doi:10.1080/08916934.2017.1281914.

Wilkins, L. W. & (2019). The prevalence of MS in the United States: A population-based estimate using health claims data. *Neurology* 93, 688–688. doi:10.1212/WNL.0000000000007915.

Wilson, Q. N., Wells, M., Davis, A. T., Sherrill, C., Tsilimigras, M. C. B., Jones, R. B., et al. (2018). Greater Microbial Translocation and Vulnerability to Metabolic Disease

in Healthy Aged Female Monkeys. *Sci Rep* 8, 11373. doi:10.1038/s41598-018-29473-9.

Wong, C. G. T., Bottiglieri, T., and Snead, O. C. (2003). GABA, γ-hydroxybutyric acid, and neurological disease. *Annals of Neurology* 54, S3–S12. doi:10.1002/ana.10696.

Yacyshyn, B., Meddings, J., Sadowski, D., and Bowen-Yacyshyn, M. B. (1996). Multiple sclerosis patients have peripheral blood CD45RO+ B cells and increased intestinal permeability. *Dig. Dis. Sci.* 41, 2493–2498. doi:10.1007/BF02100148.

Yalçınkaya, N., Akcan, U., Örçen, A., Küçükali, C. İ., Türkoğlu, R., Kürtüncü, M., et al. (2016). Reduced fecal GABA levels in multiple sclerosis patients. *Multiple Sclerosis and Related Disorders* 9, 60–61. doi:10.1016/j.msard.2016.06.009.

Zhu, D., Liu, F., Xu, H., Bai, Y., Zhang, X., Saris, P. E. J., et al. (2015). Isolation of strong constitutive promoters from Lactococcus lactis subsp. lactis N8. *FEMS Microbiol Lett* 362. doi:10.1093/femsle/fnv107.

CURRICULUM VITAE

Hannah Marie Kohl

208-691-7765

4707 E. Upriver Dr. Apt J202, Spokane, WA 99217

hkohl7@gmail.com

EDUCATION & TRAINING

Masters of Biology, will graduate in Spring 2021, Eastern Washington University, Cheney, WA

Bachelors of Science, Summa Cum Laude, Animal and Veterinary Sciences, University of Idaho, Moscow, ID; May 2017 GPA 4.0

Associate of Science, General Studies, North Idaho College, Coeur D'Alene, ID; December 2012 GPA 4.0

Wilderness First Responder (Desert Mountain Medicine, University of Idaho); 2014

CPR and AED (Desert Mountain Medicine, University of Idaho); 2014

PUBLISHED WORKS and PRESENTATIONS

Kohl, H.M.; Castillo, A.R.; Ochoa-Repáraz, J. The Microbiome as a Therapeutic Target for Multiple Sclerosis: Can Genetically Engineered Probiotics Treat the Disease? *Diseases* **2020**, *8*, 33.

Lacey B. Sell, Christina C. Ramelow, Hannah M. Kohl, Kristina Hoffman, Jasleen K. Bains, William J. Doyle, Kevin D. Strawn, Theresa Hevrin, Trevor O. Kirby, K. Michael Gibson, Jean-Baptiste Roullet, and Javier Ochoa-Repáraz. Farnesol induces protection against murine CNS inflammatory demyelination and modifies gut microbiome*. Clin Immunol.* **2021**. In Press.

Kohl, H. M., Hoffman, K., Staben, K., Shi, X., Long, T., Castillo, A., Gibson, K. M., Roullet, J., and Ochoa-Repáraz, J. (2021, May 10-15). Evaluating the Effects of Intestinal Bacteria's Production of GABA Neurotransmitter on an Animal Model of Multiple Sclerosis [Conference presentation] AAI Virtual Immunology 2021, Online

PROFESSIONAL EXPERIENCE

Research Assistant, Eastern Washington University, *Sept 2019-Present*

Worked with research mice in Dr. Ochoa's Lab, prepared and gave treatments to mice via oral gavage, and helped research, design, and implement experiments studying the effects of intestinal bacteria on multiple sclerosis using EAE mice.

Microbiology Lab Teacher's Assistant, Eastern Washington University, *Spring 2020*

Helping grade lab reports and answer questions.

Youth Group Mentor-Christ Redeemer Church- *Sept 2019-Spring 2020*

Mentored a group of 6-15 Junior High Girls, and led discussion groups weekly.

Office Manager, Intermountain Fabricators*, April 2018-Sept 2019*-Left to go to school

Did payroll, researched and created company policy regarding new sick leave law, and created job applications. Ran accounts payable-recorded invoices, checked against purchases orders, ordered materials and office supplies. Created vendors on our computer system. Printed checks and made certain our bills were paid on time.

Logged hours and materials for each job, created and sent bills to customers, wrote remainder emails for late payments, made certain companies had everything they needed to pay us, including lien releases and job specific payment applications.

Paid taxes, researched regulations, updated webpage, answered and screened phone calls, and greeted customers

Temporary- Humanix Frontier Behavior Health-*2 weeks March 2018*

Called patients for appointment reminders.

Temporary- Office Team-*April 2018*

Entered data, shredded papers, including confidential information, containing health data, social security numbers, financial data, etc

Records Secretary, Candle Hill Shepherds, LLC; *2004-Dec 2017*-Left due to moving to Spokane

Registered German Shepherd dogs and litters with national registries, including the American Kennel Club, kept detailed financial and dog health records, also updated the website and photographed dogs, gave shots, and helped develop and maintain kennel health protocols.

Dog Trainer, Candle Hill Shepherds, LLC; *2004-Dec 2017*- Left due to Moving

Trained and competed with adult German Shepherds in a variety of disciplines. Trained puppies from birth to five months old in an intensive and comprehensive puppy enrichment program and taught clients how to properly train and interact with their German Shepherd Dog.

Volunteer Teacher's Assistant, West Park Elementary School, Moscow, ID; Feb-May 2017

Helped children with homework, reading, and science projects, as well as helping the teacher prepare the classroom.

Volunteer Children's Church Helper, Christ the Redeemer Church; 2016-September 2019

Helped setup, monitor, organize activities, and teach a class with up to 70 students.

Office Clerk, Farragut State Park; *2014*-Summer Job

Registered campers, kept records, sold merchandise, filed complaints, resolved tickets, and ensured a positive customer experience

Maintenance Personnel, Farragut State Park; *2014*- Summer Job

Kept the park clean, cleaned toilets, campsites, and showers, mowed lawns, split and stacked firewood.

Search and Rescue Dog Handler, Kootenai County Sheriff's Office; *2013*

Trained a Search and Rescue dog for the Kootenai County Sheriff's Office Search and Rescue Dog Unit, Assisted in the training and certification of multiple dogs, and acted as record keeper for the unit.

MEMBERSHIPS, HONORS, AWARDS & COMPUTER SKILLS

Phi Theta Kappa Honor Society Member

Dean's List, University of Idaho, 2013-2017, GPA 4.0

Dean's List, North Idaho College 2010-2012, GPA 4.0

Numerous Training Awards, titled numerous dogs in multiple disciplines including Agility, Obedience, and Schutzhund

Lake City Toastmasters Club Member; 2015, Completed 6 Speeches

Microsoft Office, Efficient with Microsoft Office Suite, creating and successfully completing multiple college projects

Construction Partner-Construction Accounting Software-efficient with it.