Spring 2022

Effects of GABA on inflammation and intestinal barrier disruption

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EFFECTS OF GABA ON INFLAMMATION AND INTESTINAL BARRIER DISRUPTION

A Thesis

Presented To

Eastern Washington University

Cheney, Washington

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science in Biology

By

Tyrel W. Long

Spring 2022

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MASTER'S THESIS

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ABSTRACT

Effects of GABA on Inflammation and Intestinal Barrier Disruption

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Gut dysbiosis and intestinal barrier disruption have been linked to multiple sclerosis (MS). Our previous works show that experimental autoimmune encephalomyelitis (EAE) induction modifies the gut's microbiota composition, reducing frequencies of gamma-aminobutyric acid (GABA)-producing bacteria. GABA levels are reduced in the brains and circulation of MS patients. We engineered *Lactococcus lactis* with extra copies of *gadB* (glutamic acid decarboxylase) and *gadC* (glutamate/GABA antiporter) to increase GABA levels produced by the bacterium (GAD-*L. lactis*). EAE studies showed that the treatment with GAD-*L. lactis* and not with a *L. lactis* control expressing an empty plasmid (P-*L. lactis*) reduced the severity of the disease. We hypothesized that the increased levels of GABA produced by GAD-*L. lactis* would restore the permeability in the intestinal epithelia of EAE mice and in a monolayer composed of Caco-2 cells exposed to inflammatory mediators. Intestinal permeability of the *in vivo* model was measured by the oral administration of 4-kDa fluorescein isothiocyanate (FITC)-labeled dextran 19 days post-EAE induction. Results showed increased trend of intestinal integrity when EAE with were treated with GAD-*L. lactis* vs. P-*L. lactis* (not significant). *In vitro,* Caco-2 cells were plated on tissue culture trans-well plates creating a monolayer. The Caco-2 cells were exposed to TNF-α, a known barrier disruptor, and to increasing concentrations of GABA (0 – 10 mM). Transepithelial electrical resistance (TER) measurements and the flux of (FITC)-labeled dextran were quantified (0-48 hrs).

Our results showed dose- and time-dependent effects of GABA exposure on monolayer integrity. Exposure of cells to 0.5 – 1 mM, but not higher, of GABA resulted in significant increases in monolayer integrity compared with TNF-α controls and unexposed Caco-2 cells over the first (p = 0.0115) and second hour (p = 0.0006). Continuing work with GAD-*L. lactis* and the P-*L. lactis,* in conjunction with Caco-2 cell monolayers, will help us understand the role GABA has on permeability.

ACKNOWLEDGEMENTS:

It is a genuine pleasure to express my deepest gratitude to my advisor, Dr. Javier Ochoa-Repáraz, for his guidance, invaluable patience, and feedback. I also could not have undertaken this journey without my defense committee, Dr. Andrea Castillo, for her mentorship in electrophoresis and for allowing me to use the GAD *L. lactis* strain in my experiments. I also thank Dr. Hani Saad for taking the time to be my third committee member.

I would also like to thank and acknowledge Dr. K. Michael Gibson and Dr. Jean-Baptiste Roullet at Washington State University for allowing me to access their facility and for providing assistance when needed. Additionally, I would like to thank Xutong Shi for the patience and guidance given on creating monolayers and performing ELISAs and PCR.

I am also grateful to fellow graduate colleagues who provided support throughout my project, Hannah Kohl, Kristina Hoffman, and Jake Doyle. I also thank the undergraduate assistants who helped in various capacities; Zack Brownell, Bethlehem Yohannes, Andrew Flaig, and Molly Ristig.

I thank Rick Barido for his mentorship in experimental animal care and the use of the EWU vivarium. I thank David French and Alan Tipert for their patience and help with ordering materials and reagents needed for my projects.

Finally, this endeavor would not have been possible without the generous support from the National Institutes of Health (NIH) for their financial aid through their grant (1R15NS107743).

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Introduction

Multiple sclerosis (MS)

Multiple sclerosis (MS) is one of the most common neurodegenerative diseases affecting nearly 2.3 million people worldwide. Diagnosis rates vary greatly between regions and demographics. Prevalence is higher in developed or developing countries (Dobson & Giovannoni, 2019). MS is generally detected in individuals between the ages of 20 and 40, and women are two times more likely to be affected by the disease than men (Compston & Coles, 2008; Doshi & Chataway, 2016; Mendibe Bilbao et al., 2019). The affected group's life expectancy is five to ten years lower than the general population.

Also known as encephalomyelitis disseminata, MS is a demyelinating disease in which the insulating cover of nerve cells called myelin sheath in the brain and spinal cord is damaged (Compston & Coles, 2008). MS arises when autoreactive T-cells cause chronic inflammation to break down the myelin sheath of axons in the central nervous system (CNS) (Basso et al., 2008; Schumacher, 1957). During initial stages of MS, the degradation of the myelin is sporadic and produces characteristic “sclerosed plaques” along the CNS (Dobson & Giovannoni, 2019). The pathology of MS transpires due to T cell-mediated inflammatory response (Lassmann, 1999). Myelin-specific autoreactive T cells cross the blood-brain barrier (BBB) to enter the CNS and release proinflammatory cytokines that promote demyelination (Liu et al., 2018). Ultimately the inflammatory process results in the degradation of the axon and even neuronal death (Basso et al., 2008; Schumacher, 1957).

As the myelin sheath is damaged, neurons are incapable of effectively transmitting electrical signals, resulting in various signs and symptoms, including physical, mental, and

debilitating psychiatric effects (Doshi & Chataway, 2016). Double vision, blindness in one eye, muscle weakness, and problems with sensation or coordination are also possible symptoms (Kalincik, 2015). MS manifests itself in various ways, with new symptoms appearing in isolated bouts or over time. Symptoms may go away entirely between attacks, but persistent neurological issues are common, especially as the condition progresses (Baecher-Allan et al., 2018; Pan et al., 2018). These include autonomic, visual, motor, and sensory difficulties are among the most prevalent neurological symptoms and signs in people with MS (Kalincik, 2015), including muscle spasms, or trouble moving, challenges with coordination and balance; problems with speaking or swallowing, vision problems, fatigue, acute or chronic pain, and bladder and bowel disorders, among others (Kalincik, 2015).Additional symptoms include concentration and thinking difficulties, and emotional issues such as sadness or mood swings are also expected. The expanded Disability Status Scale (EDSS) is used to quantify disability in MS patients, who also have an increased tendency to fall. Women with MS who become pregnant have fewer relapses; nonetheless, the chance of recurrence rises in the first few months after delivery (Wingerchuk & Carter, 2014). Symptoms can differ drastically from one individual to another.

MS is categorized according to the progressive stages of the disease (Kalincik, 2015). The most common form of MS is the relapsing-remitting MS (RRMS) and is classified by intervals of remission followed by relapses where neurological symptoms exacerbate (Lublin, 2014). The continued demyelination and repair are characterized clinically as RRMS and produce the identifiable repeating loss of sensory and motor function (Baecher-Allan et al., 2018). Approximately 85% of the newly diagnosed MS patients are RRMS. Within 10-15 years, 50% of RRMS patients will emerge into secondary progressive MS (SPMS) with worsening neurological

symptoms and few to no remissions. About 15% of the newly diagnosed MS patients with primary progressive MS (PPMS) have gradually worsened neurological symptoms with slight remission from the onset of MS (De Angelis et al., 2018).

Identifying the subtypes of MS is vital when deciding on the best treatment plan. RRMS is characterized by unpredictable relapses followed by periods of months to years of relative remission with no new signs of disease activity. Deficits that occur during attacks may either resolve or leave problems, the latter in about 40 % of attacks and being more common the longer the person has had the disease. 30 to 70 % of persons who experience CIS late develop MS. It is characterized by progression of disability from onset, with no, or only occasional and minor remissions and improvements.

Etiology of MS

Although the etiology of MS remains uncertain, many studies suggest that genetic and environmental factors influence the onset of MS (Ghasemi et al., 2017; Jangi et al., 2016). The genetic component indicates that some individuals are more vulnerable than others. Polymorphisms in the human leukocyte antigen (HLA) loci— groups of genes on chromosome 6 that encode the major histocompatibility complex (MHC) molecules MHC class I and MHC class II, required for antigen presentation to T cell lymphocytes —have been linked to MS. The same region has also been related to the development of other autoimmune diseases, such as diabetes type I and systemic lupus erythematosus (Borchers et al., 2014; Maghbooli et al., 2020). A rare mendelian genetic variants including KIF5A p.Ala361Val, MLC1p.pro92S and REEP1 c.606 + 43G>T contribute directly to developing the phenotypes of MS (Jia et al., 2018).

In addition to genetics, the environment also contributes to the known cause of MS. There is increasing evidence linking Epstein-Barr virus infection (EBV) to MS (Bjornevik et al., 2022; W. H. Robinson & Steinman, 2022). The longitudinal analysis provided by Bjornevik et al. looked at 955 military personnel from a cohort of 10 million who were diagnosed with MS. They found that EBV positive patients had a 32-fold increase in the risk of developing MS (Bjornevik et al., 2022). Other factors include exposure to human virus type 6, mycoplasma, smoking, pneumonia, vitamin deficiency, and exposure to UV radiation that may lead to MS development (Ghasemi et al., 2017).

The geographical location, including latitude, where an individual reside could play a role in the development of MS. Those who lived in higher latitudes during the primary years have an increased chance of MS due to a lack of vitamin D (Sintzel et al., 2018). Evidence also found that people who relocate to a different location of the world before the age of 15 acquire the new region's risk of MS (Kotzamani et al., 2012). However, if a person migrates beyond the age of 15, they are still exposed to the risks of their original location (Kotzamani et al., 2012). A relative of an affected person has an increased risk of developing the condition, with a higher risk among those who are more closely related. If both parents are affected, their children's risk is ten times higher than the general population. Several additional potential risk factors, such as food and hormone consumption, have been investigated, but insufficient evidence links them to the condition. Genetics and environmental factors such as viral infections have been suggested as possible reasons.

Experimental Autoimmune Encephalomyelitis (EAE)

Animal models are an essential part of understanding diseases such as Multiple sclerosis. Mice are largely used because of the wide availability of transgenic and knockout mice accessible for specific studies (A. P. Robinson et al., 2014). There are a variety of models used to study the pathogenesis of diseases effectively. One model that proved successful in studying MS is the experimental autoimmune encephalomyelitis (EAE). It is effective because it models the autoimmune-mediated inflammatory demyelination and closely mirrors the pathology and symptoms of MS (A. P. Robinson et al., 2014). Due to EAE's substantial utilization and accessibility, it works well when comparing treatments.

EAE is a condition induced in mice where external immunizations are administered, resulting in changes in the immunopathological and neuropathological mechanisms to mimic the pathological features of MS (Constantinescu et al., 2011). EAE can be induced in several ways. In one method, EAE uses transgenic mice explicitly bred for EAE to spontaneously develop symptoms (Gold, 2006; Burrows et al., 2019). EAE can also be generated by transplanting the microbiota of MS patients into germ-free mice (Berer et al., 2017; Cekanaviciute et al., 2017). The last EAE induction method involves injecting intravenously myelin antigens, including myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55})(Burrows et al., 2019). This causes myelin-specific CD4+ T cells to cross the blood-brain barrier, resulting in inflammation in the CNS, eventually leading to damage comparable to MS.

The conditions in which EAE develops likewise plays a significant role. For example, mice given antibiotics before induction to remove the gut microbiota impaired the development of EAE (J. Ochoa-Repáraz et al., 2010; Javier Ochoa-Repáraz et al., 2009; Yokote et al., 2008). Germ-

free mice who also did not develop EAE, produced lower levels of proinflammatory cytokines (Lee et al., 2011). Under the same research, the germ-free mice were given segmented filamentous bacteria and EAE developed like conventionally colonized animals (Lee et al., 2011). Additional protective result has included using *Bacteroides fragilis* that produced polysaccharide A (J. Ochoa-Repáraz et al., 2010; Wang, Begum-Haque, et al., 2014).

The EAE model is a good indicator of the effects of MS, and it also comes with some challenges. One of the main concerns with using EAE is the impact of the microbiome on the development of the immune system (Adamczyk-Sowa et al., 2017). Here at EWU, we worked on a protocol for analyzing the microbiome of mice that have been induced by EAE (Daberkow et al., 2021). Data indicated that mice retrieved from different facilities had differences in disease induction (Daberkow et al., 2021). The results of this protocol have shown the need of having other methods supporting in vivo studies.

Diagnosis and Current Treatments of MS

MS is typically diagnosed based on the signs and symptoms present and by supporting medical imaging and laboratory testing. The McDonald criteria, the most widely used methodology for the diagnosis of MS focuses on clinical, laboratory, and radiologic evidence of lesions at different times and in distinct regions (Thompson et al., 2018). However, no single test can provide a definitive diagnosis for MS, and diagnosis may be complex, especially in the early stages, because the indications and symptoms can be confusing. Additionally, long-term outcomes are difficult to predict; more prolonged periods of remission are more common in women, those who have the condition early in life, those who have a relapsing history, and those who have had few episodes at first (Wingerchuk & Carter, 2014).

Magnetic resonance imaging of the brain may show areas of demyelination (Zivadinov & Leist, 2005). The metal gadolinium can be administered intravenously as a contrast agent to highlight active plaques and by elimination, demonstrate the existence of lesions not associated with symptoms at the moment of the evaluation (Zivadinov & Leist, 2005). Central brain lesion signs have been proposed as a good indicator of MS compared to other conditions causing white lesions (Cortese et al., 2019). In addition, brain atrophy is seen as an indicator of MS (Zivadinov & Leist, 2005). Testing of cerebrospinal fluid obtained from a lumbar puncture can provide evidence of chronic inflammation in the CNS. Cerebrospinal fluid is tested for oligoclonal bands of IgG on electrophoresis, which are inflammation markers found in 75-85% of people with MS. Despite the challenges that come with MS diagnosis, early treatment can help lessen the progression of MS.

Although there is no known cure for MS, a variety of treatments are available to decrease the progression of MS. The focal point of treatments is to weaken the immune system, therefore reducing inflammation (Chu et al., 2018; Doshi & Chataway, 2016). Such treatments include immunotherapeutic drugs that target effector T cells, regulatory T cells, B cells, and cell trafficking into the CNS (Baecher-Allan et al., 2018). Several therapies have proven beneficial to the improvement of MS. The primary aims of therapy are returning function after an attack, preventing new attacks, and preventing disability (Baecher-Allan et al., 2018). Starting medications is generally recommended in people after the first attack when more than two lesions are seen on MRI (Baecher-Allan et al., 2018). As with any medical treatment, medications used in the management of MS have several adverse effects. Academic laboratories and industries are currently pursuing alternative treatments.

Probiotics may be an alternative treatment approach against the inflammatory response of MS. (Colpitts et al., 2017; Dolpady et al., 2016; Tankou et al., 2018). The health benefits of probiotics have been acknowledged throughout history and appear to impact the immune system, both experimentally *in vivo* using animal models and *in vitro* (Dolpady et al., 2016; Liu et al., 2018). Probiotics may consist of one or various microbial species. Examples of some bacteria used in probiotic formulations are members of the family *Bifidobacteriaceae* (*B. longum*, *B. infantis*, and *B. breve*), *Lactobacillaceae* (*L. acidophilus*, *L. paracasei*, L*. delbrueckii* subsp. *Bulgaricus*, and *L. plantarum*), or *Streptococcus thermophile*. These species of bacteria are considered commensal (so would they be mutualistic) bacteria and play a positive role in the individual's health. Other bacteria might be directly involved in the pathogenesis of MS (Hedblom et al., 2018). Many of these mechanisms are still unknown. For example, the bacterial family of *Verrucomicrobiaceae* has an unknown function in autoimmunity (Colpitts et al., 2017). Future studies will be needed to fully comprehend the functions of bacteria in the gut.

Furthermore, individuals with MS demonstrated a notable change in gut microbial abundance compared to healthy individuals. This indicates that there is communication between the host and the gut microbiota (Javier Ochoa-Repáraz & Kasper, 2018). The bi-directional association between microbiota and those with MS can be shown in the role microbiota has with the body's immune cells. Altering the gut microbiota by using probiotics can affect the severity of MS.

Inflammation as a mediator of CNS demyelination

Apart from demyelination, inflammation is another symptom of MS. T cells, thymus-derived lymphocytes that play a crucial part in the body's defenses, are in part responsible for

the inflammatory process (Khaibullin et al., 2017). Inflammation is a necessary response to microbial infections and tissue damage in the short run. It is the body's natural healing system in which the immune system defends the body from foreigners. The body reacts to harmful stimuli like pathogens, damaged cells, toxic compounds, or irradiation (Chen et al., 2018). The challenge arises when autoimmune diseases like MS emerge from the immune cells like helper T cells (T_h), ultimately damaging the host (Kaskow & Baecher-Allan, 2018).

The pathology of MS transpires due to T_h cell-mediated inflammatory response (Lassmann, 1999). T cells play a critical role in managing the immune response and pathogenesis of MS (Kaskow & Baecher-Allan, 2018). When an antigen is presented by antigen-presenting cells to antigen-specific T-cell receptors (TCRs), the T cell bearing the antigen-specific TCR is activated. Activation results in clonal expansion and differentiation into T cell subsets with the ability to respond differently depending on the nature of the insult. Differentiation into different subsets that include Th1, Th2, Th17, Tregs, and T follicular helper cells is regulated by polarizing cytokines. Each of these effector T cell subsets produces different cytokines, including inflammatory cytokines that will further promote inflammation and damage the myelin sheath (MacLeod & Wetzler, 2007; Reynolds et al., 2012).

Inflammatory cytokines associated with MS include IL-6, TNF-α, IL-1b, IL-17, and IFN-γ [18]. IL-1*β* is produced by macrophages and promotes differentiation of T cells into Th17 cells via the STAT3 pathway, promoting and aggravating the CNS's inflammatory environment (Allam et al., 2018; Lin & Edelson, 2017). Th17 cells promote and aggravate the CNS's inflammatory environment (Allam et al., 2018; Lin & Edelson, 2017). Th17 cells found in the CNS of EAE mice

have been found to send out cytokines including IL-17 that recruit myeloid cells that perpetuate an inflammatory cascade (Rostami & Ciric, 2013).

Another cytokine is tumor necrosis factor (TNF-α). TNF-α is produced by macrophages, natural killer cells, and Th1 cells. Its primary role is to regulate immune cells (Carswell et al., 1975). In the gastrointestinal tract, TNF-α exacerbates inflammation that ultimately changes the gene expression in tight epithelial junction proteins (Roulis et al., 2011). The intracellular mechanism behind this process is still unclear (T. Y. Ma et al., 2005), but it has been described that intestinal epithelial cells express the receptor for TNF-α, and that TNF-α interaction with the receptor can modulate the expression of tight junction proteins (T. Y. Ma et al., 2004). A decrease in tight junction protein expression is believed to be correlated with the progression of many autoimmune disorders (Allam et al., 2018; H. Di Ma et al., 2018). The impact of the changes of tight junction protein expression on MS is described in further detail below.

The gastrointestinal tract and multiple sclerosis

The gastrointestinal (GI) tract is a muscular tube covered in mucus and an epithelium (Dieterich et al., 2018). The gut-associated lymphoid tissue (GALT) is a secondary lymphoid structures surrounding the GI tract and displays both innate and adaptive immune responses essential for the control of infections, interactions with the beneficial microbiota, and defenses against microbe translocation (Brenchley & Douek, 2012). Humans are born with a sterile gastrointestinal tract colonized by bacteria at birth, although pre-birth colonization through the placenta has also been proposed with conflicting views (Gil et al., 2020). The hygiene hypothesis states that the infant's environment and genetics can influence the microbiome and affect the

probability of developing childhood and adult diseases (Houghteling & Walker, 2015). Recent analysis indicates that MS patients' gut microbiota composition decreases potentially beneficial bacteria (Chu et al., 2018; Cox et al., 2021; Jia et al., 2018).

On the contrary, an increase in proinflammatory bacteria is related to autoimmunity regulation (Kadowaki et al., 2019). Based on these findings, it has been proposed that an altered composition of the microbiota can lead to exacerbated inflammation. Under this hypothesis, intestinal dysbiosis changes in the gut microbiome regulate systemic immune responses by changing the phenotype, proliferation, and functional capacity of inflammatory or regulatory cells, eventually leading to neuroinflammation and CNS disease (Pröbstel & Baranzini, 2018). Individuals with MS demonstrated a notable change in gut microbial abundance compared to healthy individuals, and this indicates that there is communication between the host and the gut microbiota (Javier Ochoa-Repáraz & Kasper, 2018).

Leaky gut syndrome

Leaky gut syndrome occurs when inflammation in the GI tract causes tight junctions found in the intestinal epithelia to loosen, allowing bacterial translocation (Anders et al., 2013). The translocation of gut microbes or microbial products to the lamina propria, tissue underneath the gut epithelium, may activate inflammatory responses locally and systemically. Systemic inflammation, characterized by exacerbated production of proinflammatory cytokines, could cause both T cell immune imbalance and dysfunction and increased bacterial translocation (Andersen et al., 2017). TNF-α, increased in CNS and circulation in EAE mice and MS patients, can disrupt an *in vitro* monolayer of intestinal epithelial cells (Watari et al., 2017). Intestinal barrier

disruption can cause bacterial translocation, resulting in various complications for those with MS due to increased inflammation. Leaky gut caused by inflammation could be considered a pathway that bacteria use to get past the barrier provided by the epithelial layer, but moreover, it could contribute to the expansion of the pathogenic effects of inflammation in MS (Kinashi & Hase, 2021; Nouri et al., 2014)

γ-aminobutyric acid (GABA)

Anti-inflammatory cytokines that downregulate the impact of proinflammatory responses may play a positive role in stopping bacterial translocation and decreasing permeability in the epithelial cells. Other host factors, such as neurotransmitters, could play a similar role. Gamma-aminobutyric acid (GABA) is a neurotransmitter found in the CNS and produced in the gut by bacterial members of the microbiota. GABAergic systems are composed of four primary parts: the GABA-A receptors, the GABA-B receptor, the GABA transporters, and the enzymes that make or degrade GABA (Jin et al., 2013). GABA transporters, also known as GAT, help regulate GABA concentrations and artificial GAT has been used to help change GABA concentrations (Guastella et al., 1990).

There are many ways in which GABA helps with the regulation of inflammation. It was found in cultured $CD4^+$ T cells, a concentration-dependent amount of GABA regulated the secretion of 37 cytokines involved with inflammation (Bhandage et al., 2018). Another study looking at a mouse model of rheumatoid arthritis found that the T cells of the mice treated with GABA had a reduced proliferative response and a decrease in inflammation (Tian et al., 2011). Additionally, GABAergic compounds were found to have a protective effect with EAE by reducing proinflammatory cytokines, including IL12 and INFγ (Bhat et al., 2010).

During neuroinflammation, GABA transport is linked with decreased function (Paul et al., 2014). One area not thoroughly studied is GABA's role in controlling gastrointestinal function (Auteri et al., 2015). It is proposed that GABA affects the extracellular space in the gut and prevents pathogenetic T lymphocytes from causing inflammation (Bjurstöm et al., 2008). Many different bacteria also produce GABA in the GI tract. *Lactococcus lactis* is a gram-positive bacterium with a distinct pathway called the GAD pathway, which produces GABA (Anders et al., 2013). GABAergic agents used by researchers showed decreased cytokine production of proinflammatory cytokines (Reynolds et al., 2012; Wang, Telesford, et al., 2014). For those suffering from MS, there appears to be a correlation between GABA concentrations and inflammation (De Stefano & Giorgio, 2015).

Previous studies highlighted the benefits of GABAergic agents (Wang, Telesford, et al., 2014) displaying that TNF-α can increase monolayer permeability (Watari et al., 2017). Therefore, this study will culture Caco-2 cells, a commercially available immortal human intestinal cell line, and insert them into Transwell plates, creating a monolayer. The monolayer will then be exposed to TNF-α and gut contents of various EAE mice to observe the permeability effects of TNF-α and GABA.

In vitro studies applicable to MS research

Several methods can be used to study the effects of MS, including *in vivo* (EAE model, described previously) *in vitro*, and *ex vivo*. There is no "one size fits all" method, and each method has its advantages and disadvantages. Despite the differences, each method can be useful in explaining various parts of the etiology of MS.

Numerous *in vitro* models are used for the study of specific processes associated with human diseases, including those that impact the CNS (Gogolla et al., 2006). However, due to the complexity of the pathology of MS *in vitro* models have been explored less (Ponz-Sarvise et al., 2020; Schlachetzki et al., 2013). Emerging studies have tried to create *in vitro* methods primarily in the way of trying to replicate neuronal tissue. *Ex vivo* studies have been performed where slice cultures of the brain and spinal cords were used to observe the methylation process (Zhang et al., 2011). The slides were subsequently used *in vitro* to find the methylation mechanism in MS (Birgbauer et al., 2004). The study of stem cells is also an emerging strategy for exploring MS. Neuronal cells can be cultured from fibroblasts and used in vitro (Hu et al., 2015). Because MS is a neurological disease, it should be no surprise that there is an abundance of studies completed on the CNS (Birgbauer et al., 2004; Zhang et al., 2011). The importance of gut microbiota translocation has been emphasized. However, few studies failed to clearly demonstrate the mechanism behind translocation within MS.

In vitro methods have previously been used to demonstrate the translocation of the epithelia (Grothaus et al., 2018; Mir et al., 2016). The creation of a monolayer can aid in determining the translation of epithelia in MS patients (T. Y. Ma et al., 2004). Creating the monolayer requires culturing Caco-2 epithelial cells (immortal line of cells derived from human

colorectal adenocarcinoma cells) into a single layer that is interconnected. The monolayer replicates the function of the GI tract by creating a barrier of interconnected cells (T. Y. Ma et al., 2004; Mattar et al., 2001). Using a monolayer is a valid in vitro model to evaluate specific in vivo parameters. The monolayer cells are grown using appropriate media and an assay to test their integrity. To measure the monolayer's integrity, the Transepithelial Electrical Resistance (TER) can be used. TER is quantified by placing one electrode on the top of the monolayer and another electrode underneath the monolayer (Srinivasan et al., 2015). An electric current will pass through the monolayer, where the integrity of the barrier can be quantitatively measured (Srinivasan et al., 2015). Using an epithelial monolayer *in vitro* model, even considering the simplicity of the method and its limitations when considering the complexity of a human condition, might help understand some specific aspects of bacterial translocation in MS.

Changes in the gut microbiota have been observed in individuals with MS. Gut microbiota tends to play a leading role in an individual's health. Bacterial translocation has been demonstrated to cause various health concerns. The entire functions of bacteria in the gut are still relatively unknown. There are inflammatory and anti-inflammatory molecules that play a key role in bacterial translocation, and this has been observed mainly by the effects shown in EAE mice. It will be necessary to perform studies in vivo and perform *in vitro* work. Creating a monolayer will be essential for studying the role of MS pathogenesis. Using inflammatory mediators associated with leaky gut to disrupt the integrity of the cell monolayer is an alternative approach to animal models to address specific questions regarding inflammation observed in the MS intestinal microenvironment and leaky gut. Furthermore, establishing an *in*

vitro model of leaky gut will facilitate the exploration of new treatment approaches against intestinal dysfunction and systemic inflammation.

Research Hypothesis

Hypothesis #1

The addition of GABA to a caco-2 cell monolayer will result in a decrease in permeability compared to an untreated cell monolayer, as measured by trans epithelial resistance and FITC dextran.

Hypothesis #2

Giving the Caco-2 cell monolayer a GABA producing probiotic will replicate the results found by the addition of pure concentrations of GABA and decrease the permeability of the monolayer compared to an untreated cell monolayer.

Materials and Methods

Cell culture

Caco-2 cells are an immortal line of cells derived from human colorectal adenocarcinoma cells. It models the intestinal epithelial barrier by spontaneously differentiating once confluent and includes the formation of tight junction proteins. The Caco-2 (HTB-37) cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in a growth media composed of Eagle's minimum essential medium (Nissui, Japan) with 10% fetal bovine serum (FBS) and 0.01 % penicillin and streptomycin. Caco-2 cells were incubated under 5% CO_2 at 37 ◦C. When the caco-2 cells reach 80% confluency, cells were subcultured using trypsin and growth media to appropriate seeding concentrations, as shown below.

Generation of a Caco-2 cell monolayer

The Caco-2 cells were cultured in 24 Transwell chambers (Corning, NY, USA) at a density of 6 x 10^4 for each well (Jin et al., 2013). The total amount of cells required for each plate is 1.5 x 10^6, which required two confluent petri dishes of the Caco-2 cells. Once the Caco-2 cells were combined, they were added to the apical chamber with growth media and incubated under 5% CO_2 at 37 ◦C.

Transepithelial electrical resistance

The permeability of the monolayer was measured using transepithelial electrical resistance (TER). The TER measurements were quantified in Ω•cm^2 every day for 16 days using a Millicell ERS-2 voltammeter (Millicore corporation, Billerica, MA, USA). Experiments continued

once the TER values plateau between 500 to 800 Ω•cm^2. Low TER values are correlated more permeability and high TER values are correlated less permeability.fs

Disruption of Caco-2 cell monolayer

Disruption of the Caco-2 cell monolayer was performed by the addition of TNF-α. Once the Caco-2 cell monolayer plateaus between 500 to 800 Ω•cm^2, 10 ng/mL of TNF-α is added to the basolateral side of the monolayer with 1 mL of growth media. The monolayer was then incubated under 5% CO_2 at 37 ◦C, and the TER measured every 24 hours for two days. A permeable monolayer was considered when TER measurements dropped below 400 Ω•cm^2 (Anders et al., 2013; Watari et al., 2017)

***Lactococcus lactis* strains**

Wild type and engineered strains of *Lactococcus lactis* (IL1403) were used for this study (Castillo, unpublished). The *L. lactis* (IL1403) was first modified with a pAC plasmid that provided erythromycin resistance (pAC *L. lactis*). Genes were then added to the plasmid that encode a glutamic acid decarboxylase (gadB) and GABA/glutamate antiporter (gadC) under the control of a constitutive promoter (P8s) (Zhu et al., 2015 and Castillo unpublished). The strains used on the monolayers included the pAC-*L. lactis* and GAD-*L. lactis* (the P8s promoter modified *L. lactis*). The bacterial strains were grown at 30◦C without shaking in M17 broth or agar plates (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose (GM17) and 5ug/mL erythromycin. GABA levels for each strain were quantified from cell supernatants from each strain of *L. lactis* (WT-*L. lactis*, pAC-*L. lactis*, P2-*L. lactis*, and P5-*L. lactis,* and P8-*L. lactis*) at stationary growth phase using a GABA ELISA kit (Khol, unpublished).

Animals

Ten-week-old male and female C57BL/6 mice used in this experiment were obtained from the Jackson Laboratory (The Jackson Laboratory, Inc., Bar Harbor, ME, USA). The mice arrived at Eastern Washington University at eight weeks old. Over the course of the next two weeks, the mice were given time to acclimate before being induced at ten weeks. All mice were housed at Eastern Washington University's vivarium in wire-top cages. All animals had access to water and food. All aspects of animal care and well-being followed Eastern Washington University's Institutional Animal Care and Use Committee's (IACUC) policies and procedures.

Induction of EAE

The C57BL/6 mice were induced using the Hook Kit™, a commercial EAE induction kit (Hooke Labs, Lawrence, MA). Per manufacturer's guidelines, mice were induced by flank subcutaneous injection of an emulsion of 250 µg myelin oligodendrocyte glycoprotein peptide in complete Freund's adjuvant. That same day and two to four hours later, the mice received intraperitoneal injections of 100 ng/dose of pertussis toxin provided with the kit.

GAD L. lactis treatment

GAD *L. lactis* is a modified strain of bacteria that has an increased expression of the GAD gene and produced GABA at levels higher than the wild-type strain (Castillo, unpublished). Specifically, the P8 strain produced more GABA (4000 ng/mL) than the vector only control (2100 ng/mL)(Khol, unpublished) The mice were housed in separated cages forming four groups of 12. For controls, one group had no treatment and no disease induction (Naïve; Group 1) and one with induced disease, but no treatment (Group 2 EAE control). The third group was induced with EAE and

treated with *L. lactis*-wt (Group 3). The final group was EAE induced and treated modified *L. lactis* – GAD (Group 4).

Each mouse was orally gavaged with 5 x 10^8 CFU/day of their treatment bacteria. CFU/mL were previously correlated with optical density at 600nm (Kohl and Castillo, unpublished). Control groups were dosed with a sham sterile GM17+erythromycin medium to control for the added stress of being gavaged. This will be done five days weekly for 21-day. On the 21st day, the animals were euthanized to avoid animal loss and sample size reduction due to the disease progression.

Stool processing and cell culturing

50 mg of fecal material was collected from each mouse, once a week, for a total of 21 days. The fecal material was resuspended in 0.5 mL of phosphate buffered saline (PBS) in a maltodextrin-trehalose solution and stored at -80 ˚C for future use in microbiome studies (Burz et al., 2019). In addition, the supernatant from the gut content of treated and untreated EAE mice were stored at -80 ˚C, after centrifugation.

Caco-2 cells were exposed to the gut microbiota and fecal content supernatants from treated and untreated EAE mice. The monolayer was then incubated under 5% CO_2 at 37 ˚C, and the TER was measured every 24 hours for two days. To determine the effects of the supernatants, different dilutions of the supernatants were co-cultured with the Caco-2 cells. The supernatants were diluted 1:5, 1:10, 1:20, and 1:40 (v/v) with growth media. After the Caco-2 cell monolayer was grown for seven days, 0.2 mL of the diluted supernatant samples were added to the apical chamber of the monolayer. The monolayer was then given the diluted supernatant samples and observed for 48 hours (Geirnaert et al., 2017).

After the Caco-2 cell monolayers were exposed to the microbiome of untreated and treated EAE mice, TNF- α or sterile PBS was added for 48 hours as described before. After 48 hours, the cells were washed with PBS. 10 µL of the supernatant was added to the apical side of the monolayer along with 0.5 mL of growth media (Bhat et al., 2010). The basolateral chamber received 1 mL of growth media.

4-kDa fluorescein isothiocyanate (FITC)-labeled dextran

An additional permeability test was performed after the addition of the gut contents of the mice. A fluorescent probe called 4-kDa fluorescein isothiocyanate (FITC)-labeled dextran was used. The protocol for FITC-labeled dextran starts with the washing of the upper and lower compartment of the Transwell chambers with Hanks' Balanced Salt solution (HBSS). An additional 1 mL of HBSS was added to the basolateral side of the monolayer, and 0.5 mL of FITC-labeled dextran (1 mg/mL in HBSS) was added to the apical side of the monolayer. The Transwell plate was incubated under 5% CO_2 at 37 ◦C for 15 minutes. Protecting the samples from light, the transwell will be transferred to a clear bottom black-walled microplate assessed using a plate reader with 485 nm excitation and 520 nm emission.

Statistical analysis

All were analyzed using GraphPad Prism (GraphPad Inc., La Jolla California, USA). All permeability measurements were analyzed by a two-way ANOVA followed by a multiple comparisons test. A statistical significance of $p<0.05$ was used for all tests.

Results

Quantification of *in vitro* epithelial cell integrity

The generation of a monolayer starts with properly culturing cells that will adhere to one another. The cells chosen for this experiment were human colorectal adenocarcinoma cells (Caco-2). We first investigated how these cells grew in a transwell plate for 16 days. A gradual increase in TER measurements was observed during the allotted time, indicating continued growth (Figure 1A). Two different concentrations of TNF-α, 10 ng/mL and 20 ng/mL, were added to the transwell plate and compared to non-treated controls (Figure 1B). The addition of TNF-α was intended to increase the permeability of the monolayer. We observed a significant treatment effect ($P < 0.05$) on the TER measurements over both a 24 and 48 hour period (10 ng/mL n=4, 20 ng/mL n=4) (Figure 1B).

Effects of GABA on epithelial cell integrity

After correctly establishing the proper protocol for generating a monolayer, the next step was to determine the amount of GABA concentration required to maintain integrity of cell monolayers treated with TNF-a. This was conducted by completing three experiments where each transwell of monolayers (n=4) was treated with various GABA concentrations and 10 ng/mL TNF-α to compare with nontreated controls. All three experiments (n=4) showed no significant differences in TER measurements when dosed with any concentration of GABA. Monolayer 2 (Figure 2A) (n= 3, 1 mM, 10 mM) showed the opposite results, where permeability significantly increased at both the 24 and 48-hour mark. The experiment was repeated for monolayers three (Figure 2B) and four (Figure 2C)(n= 3, 1 µM, 10 µM) to affirm the results. These two monolayers again with the same concentrations of GABA resulted in a decrease in TER, indicating an increase in permeability.

After some consideration, it was decided the effects of GABA might occur within the first hours of treatment and at lower concentrations. We decided to conduct additional trials and measure treatment effects at hours 1, 2, 3, 12, 24, and 48. Monolayer 5 (1 mM, 500 µM, 100 µM, 10 µM) showed an increase in TER during the first 3 hours for the 1 mM and 500 µM GABA concentration (Figure 3A). We wanted to further confirm the difference in permeability by using the FITC-dextran test. The results indicated lower concentrations of FITC-dextran in the GABA treated wells compared to the non-treated control. (Figure 3B).

Next, we compared higher concentrations of GABA with the 1 mM and 500 µM amounts. Monolayer 7 (n=4; 10 mM, 5 mM, 1 mM and 500 µM) was observed for three hours, where previous results indicated the highest increase in TER with treatment. There was a significant difference between the 1 mM (P=0.0072) and 500 µM (P=0.0099) amounts compared to controls and other treatments within the first hour (Figure 5A). FITC-dextran was then administered and again showed treated groups had lower concentrations when compared to the control wells (Figure 5B). Monolayer 8 (n=4; 10 mM, 5 mM, 1 mM, and 500 µM) was performed at the same time as Monolayer 7 with the intent of having the same concentrations observed for 48 hours. This again showed a significant increase in TER for GABA concentrations 500 µM (P=0.0346) (Figure 6).

Effects of *L. lactis* and GAD *L. lactis*

With the proper concentrations of GABA known from previous ELISA results (Khol, unpublished), we wanted to examine how monolayers treated the stool of EAE mice, EAE mice given pAC, and EAE mice given GAD *L. lactis* that produces high amounts of GABA would react. Monolayer 6 (Figure 4) (n=4) was generated, and stool samples were homogenized with growth media and added directly to the monolayer. We observed no significant increase in TER. Additionally, there was a big drop in TER after 24 hours, suggesting Caco-2 cell death (figure 4).

Following Monolayer 6, a direct addition of the pAC L. lactis (empty plasmid control) and P8 GAD-L were used rather than stool. Monolayer 9 (n=6) was established and exposed to TNF-a. We observed a significant difference in TER between the P8 and the pAC control ($P < 0.0001$), and TNF-α ($P=0.0259$) after 24 hours (Figure 7A). The treatment was repeated for Monolayer 10 (n=6) and had results comparable to monolayer 9 except for hour 48, where there was a significant drop in TER (Figure 7B).

In Vivo FITC-Dextran

The results from the in vitro experiments prompted further studies testing the permutability of mice treated with GAD *L. lactis*. We administered the P8 (GAD *L. lactis*) that produces high amounts of GABA and the control (pAC *L. lactis*) to C57BL/6 mice (n=10) over a 23 day period. This experiment aimed to determine whether leaky gut would be observed in vivo: in those animals, with increased intestinal permeability FITC-dextran detected in serum would be higher than in animals with unaffected intestinal permeability, due to lack of dextran translocation. The FITC-dextran treatments were given to the C57BL/6 mice on the final day via

oral gavage. We collected the serum and observed some fluorescence, however, there was no significance between the P8, P, and controls (Figure 8).

Discussion

Multiple sclerosis is a neurodegenerative disease that targets the CNS. Although there is no definitive cause of MS, it was proposed that genetics, viral infection, or other environmental factors increase the risk of disease. Despite the advantages made towards the pathology and etiology of MS, there is no cure. Many avenues of study are underway and are essential in dealing with the progression of MS. The symptoms noted include gastrointestinal challenges including leaky gut syndrome. Leaky gut syndrome results from an increase in the permeability of the intestinal epithelial barrier that leads to subsequent bacterial translocation. It was hypothesized that leaky gut exacerbates the severity and progression of MS. Recent findings have uncovered lower levels of GABA in the CNS and gut of MS patients. It is believed that changes in the gut microbiota will aid in modulating the immune system and metabolic pathways, including GABA production. The purpose of this study was to test the effects of GABA on a monolayer created from Caco-2 cells to generate a leaky gut model *in vitro*.

To study the effects of GABA, we had to establish if a monolayer created from Caco-2 cells could be a viable model to test inflammation and permeability. In addition to replicating previous studies (Ye et al., 2006), the monolayer with Caco-2 cells was grown and treated with the known proinflammatory cytokine TNF-α. The first monolayer demonstrated a significant decrease in the transepithelial resistance (TER) for the 10 ng/mL ($n=4$, $p=0.0101$) and 20 ng/mL ($n=4$, $p=0.0171$) doses of TNF-α. This was comparable to the results found in previous studies.

The first step following the successful creation of a monolayer included the addition of GABA to the monolayer to determine the effects. Three of the six monolayers (monolayers 2, 3, and 4)

treated with GABA appeared to either have no effect or significantly increase permeability. We considered that the duration of exposure and GABA concentrations would affect the validity of the model. The uptake of too much GABA across the membrane of the Caco-2 cellscould have resulted in cell death leading to the decrease of TER observed. We proposed obtaining TER measurements during shorter times (hours versus days), and titrated down the concentrations of GABA used.

In Monolayer 5, we observed an increase in TER during the first three hours of treatment with concentrations of 1 mM and 500 µM (n=4). This trend was also observed in the FITC-dextran results. These results suggested specific concentrations of GABA have time-dependent benefits to intestinal epithelia. This was confirmed in monolayer 7 (n=4) and monolayer 8 (n=4), where at 3 hours, statistically significant results between the control and 1 mM and 500 µM concentrations of GABA were observed. Although the results were encouraging, we consider that using a simple monolayer system only composed of Caco-2 cells exposed to GABA might not translate well to living tissue found in an organism.

We next addressed whether treating the cells with a probiotic capable of producing enhanced levels of GABA would restore the integrity of the monolayer exposed to TNF-α. The synthesis of GABA by the genetically engineered strains was the first step in validating the probiotic *L. lactis* constructs. Previous ELISAs performed by Hannah Khol and our lab were used to determine the quantity of GABA generated by the strains. Supernatant from strains cultivated until they saturated the media in their stationary phase was utilized in the first ELISA. We were able to compare strains to some extent because they were all developed to the same stage, and while there was a trend toward increased production, there was no statistical significance. The

colony forming units (CFU) of the initial bacterial samples that were used in the ELISA study were not obtained. With a standard curve, notwithstanding the positive ELISA result (correlation of 0.9887 and R squared 97.75 percent). GABA levels could not be normalized using CFU per volume (CFU/ml). The lack of CFU/ml standardization could explain no statistical significance. You might change the way you talk about.

For the following GABA quantification tests, supernatants from strains grown to an OD_{600} of 0.2 for the same amount of time and with known CFU levels (based on growth curves calculated as reported in the methodology section) were used in the ELISA. The prior ELISA kit used (LDN BA E-2500) expired, and replacement kits from the same vendor failed to perform, in addition to the existing limitations of our investigation (observations made by our colleagues at WSU College of Pharmacy). The GABA ELISA characterization was then carried out with a separate kit from (AVIVA Systems Biology, GABA ELISA Kit OKEH02564). Unfortunately, the kit's controls failed to work, and we were unable to confirm the ELISA results, despite a significant difference between the pAC and P8 *L. lactis* strains at OD 1.5 in a strain comparison.

The addition of pAC and P8 to a caco-2 cell monolayer had a beneficial effect (restored monolayer integrity after exposure to TNF-α for the first 24 hours. This could be due to the changes in GABA production of *L. lactis* that happens even in wild-type strains depending on the environment. All the bacteria used to treat the monolayers were taken from their M17 media and put in a well that contained the eukaryotic cell growth media. As indicated in the results section, Dr. Castillo's lab later confirmed the ability of P8 GAD *L. lactis* to produce increased GABA levels compared to pAC and WT *L. lactis.* This introduced a change in the environment that could have potentially changed gene expression levels and GABA production. It has been found that

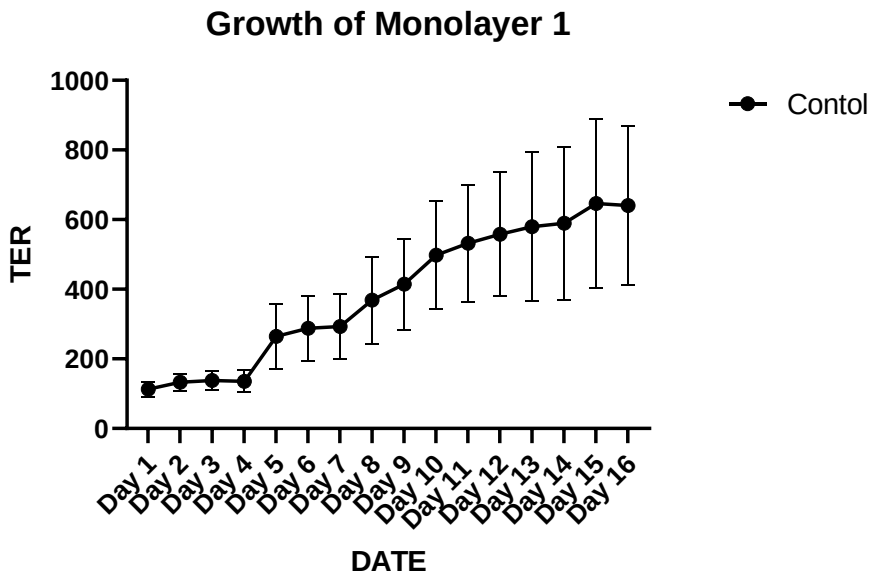
Lactobacillus brevis, a similar commensal bacterium, had changes in expression levels of *gadBC* genes depending on environment and growth phase (Lyu et al., 2018). This could imply the changes in media could negatively impact pAC and P8 GAD *L. lactis.* Future experiments would include measuring the expression of *gadBC* genes of pAC and P8 GAD *L. lactis* after its addition to a Caco-2 cell monolayer and seeing if changes in Media and pH negatively impact these bacteria.

Many challenges arose in this thesis project. The main problem was identifying the suitable treatment concentrations and timeframe of measurements. We initially followed what was found in the literature but had to change due to unsuccessful results. This was exacerbated by the limited amount of transwell plates and supplies available due to shipping issues. This thesis was able to show the positive effect GABA has on an intestinal epithelial model in the form of a Caco-2 cell monolayer.

Additionally, we were able to show any addition of commensal bacteria is beneficial in the first 24 hours of treatment. It was unclear whether this was due to GABA production or other effects created from L. lactis, like the increased lactic acid production that might also have anti-inflammatory effects. Potential future studies would include running more monolayers with lower concentrations of *L. lactis* and performing Qt-PCR to examine the expression of tight junction proteins.

Figures

A



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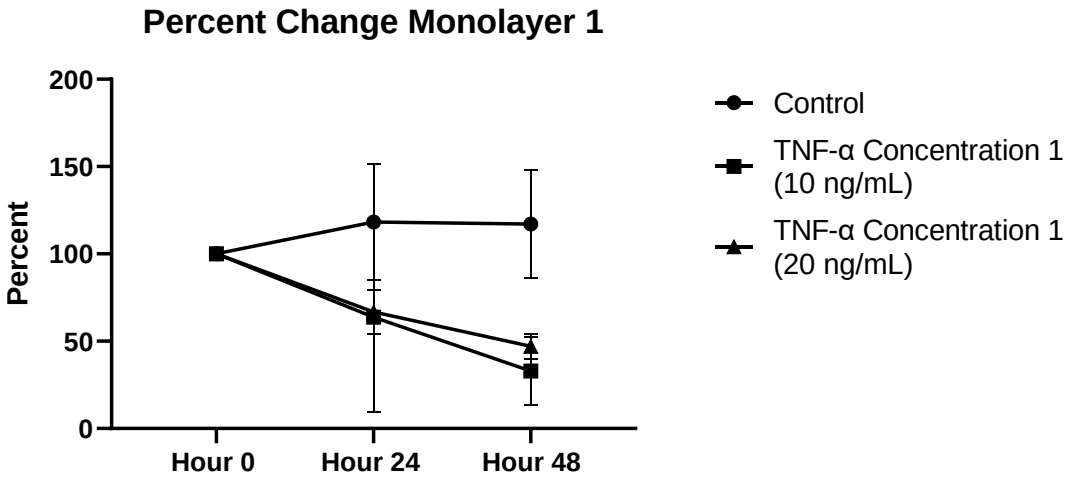


Figure 1. Percent change from TNF-α treatment to Caco-2 cell monolayer. The percent change of Caco-2 cell monolayer was treated with 10 ng/mL TNF-α (n=3) and 20 ng/mL TNF-α (N=3).

5. TER measurements of the first 14 days of Caco-2 cells grown in transwell plates without treatment. **B.** Change in TER percent from T=0 reading for TNF-α treatment of 10 ng/mL (n=4) and 20 ng/mL (n=4) at 24 and 48 hrs

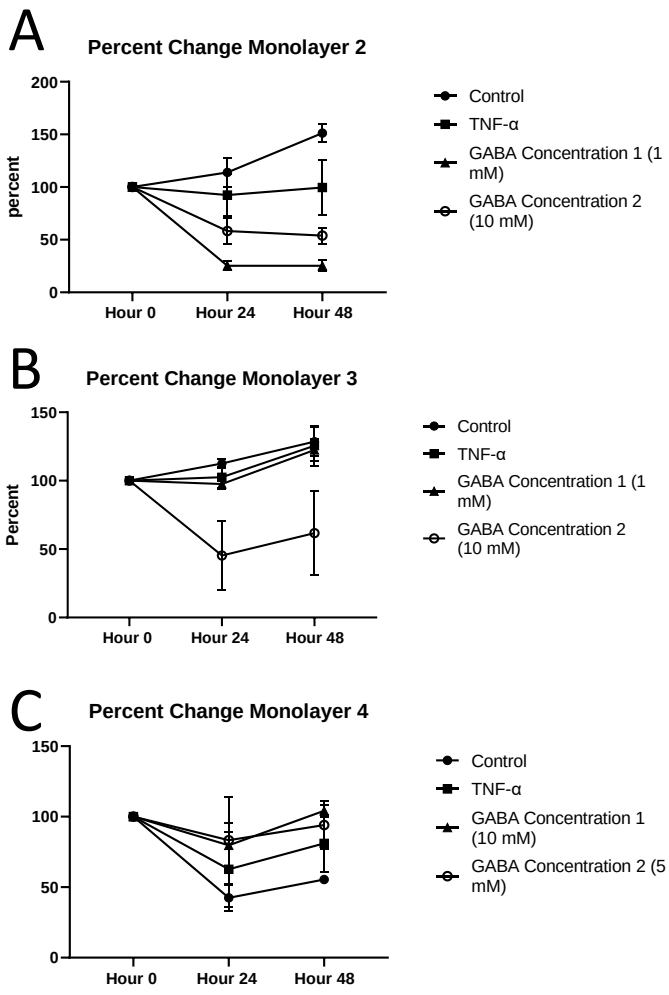


Figure 2. GABA treatment of Monolayers 2, 3, and 4 does not increase TER measurements in 24 and 48 hours. TER measurements show the change in percent from initial at hours 24 and 48. **A.** Percent change in TER for Monolayer 2 with GABA concentrations of 1 mM (n=4), and 10 mM (n=4). **B.** Percent change in TER for Monolayer 3 with GABA concentrations of 1 mM (n=4), and 10 mM (n=4). **C.** Percent change in TER for Monolayer 4 with GABA concentrations of 10 mM (n=4), and 5 mM (n=4).

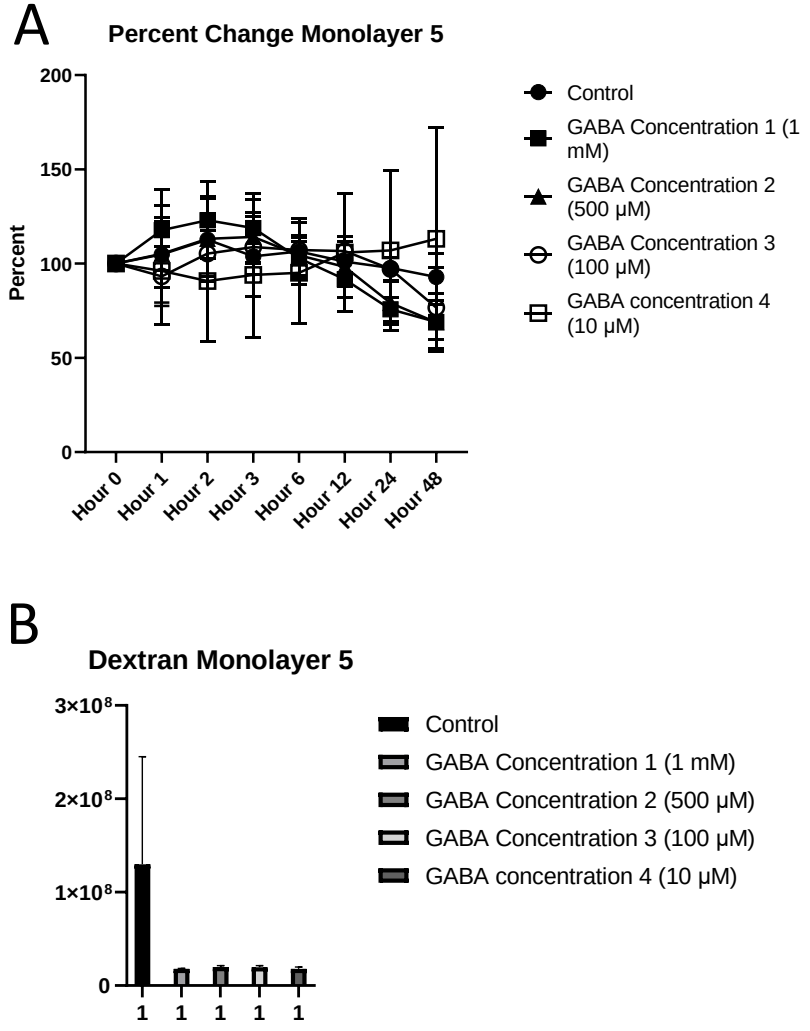


Figure 3. Treatment of GABA increases TER in the first three hours and has lower FITC-dextran. Treatment of GABA increases TER in the first three hours and has lower FITC-dextran. TER and FITC-dextran measurements show a decrease in permeability from GABA treatments of 1 mM (n = 5), 500 µM (n = 5), 100 µM (n = 5), and 10 µM (n = 5) µM. **A.** Monolayer 5 change in percent from initial of TER measurements **B.** FITC-dextran Measurements in total fluorescence.

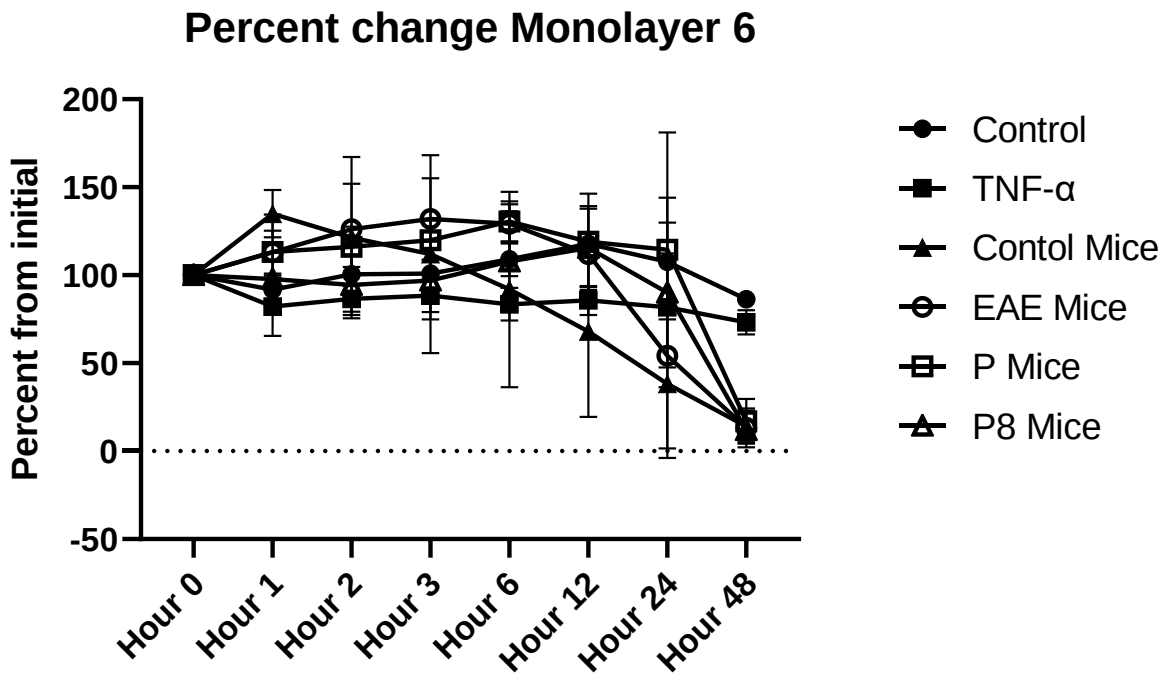
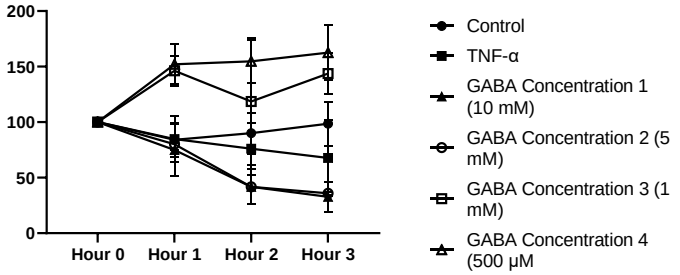


Figure 4. Stool treatment does not significantly change permeability. Stool treatment does not significantly change permeability. Permeability was measured using TER and recorded as percent from initial. Stool was taken from control mice (n = 3), EAE induced mice (n = 3), Mice with the oral administration of pAC *L. lactis* (n = 3), and Mice with the oral administration of P8 *L. lactis* This was compared to wells that only had Caco-2 cells (n=3) and Caco-2 cells treated with 10 ng/mL TNF-α (n=3).

A



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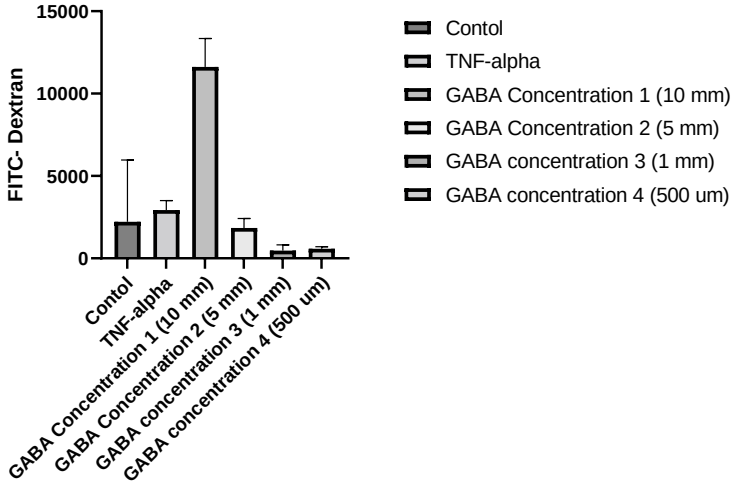

Figure 5. GABA treatment of Monolayer 7 decreases permeability with higher TER and lower FITC-dextran. GABA treatment decreases Permeability with higher TER and lower FITC-dextran. TER and FITC-dextran measurements for Monolayer 7 with GABA treatments of 10 mM (n = 4), 5 mM (n = 4), 1 mM (n = 4), and 500 µM (n = 4). **A.** Changes in percent from initial of Monolayer 7 over a period of 3 hours. **B.** FITC-dextran measured in total fluorescence.

Figure 6. GABA treatment of Monolayer 8 increases TER in the first three hours

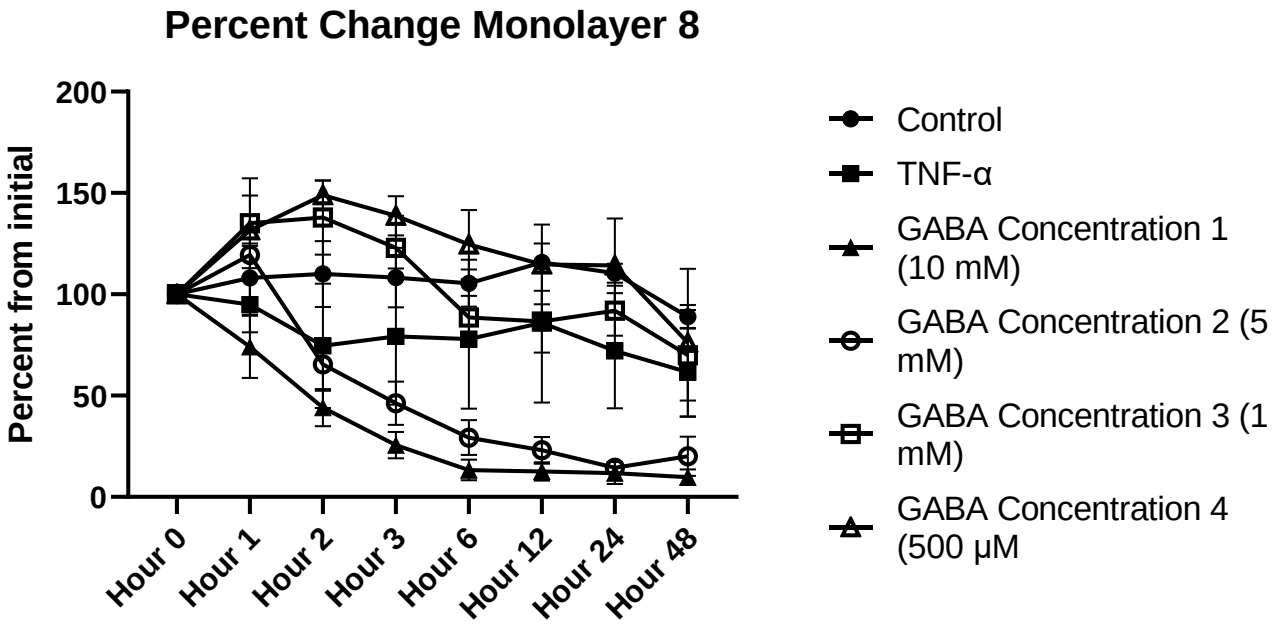

Figure 6. GABA treatment of Monolayer 8 increases TER in the first three hours. TER and FITC-dextran measurements for Monolayer 8 with GABA treatments of 10 mM (n = 4), 5 mM (n = 4), 1 mM (n = 4), and 500 µM (n = 4). Changes were recorded in percent from initial of Monolayer 7 over a period of 48 hours.

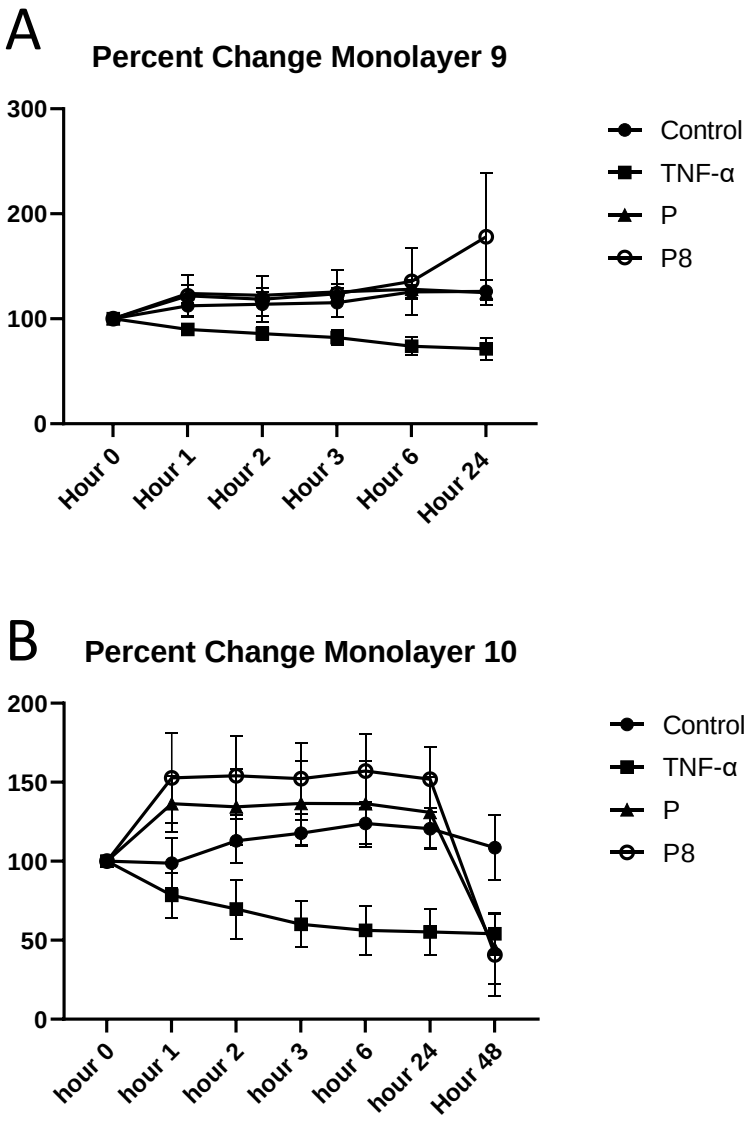

Figure 7 Permeability decrease with treatment of probiotics. Permeability decreases with treatment of probiotics. Monolayers treated with pAC *L. lactis* (n = 6) and p8 *L. lactis* (n = 6). **A.** Monolayer 9 TER percent from initial measurements over a period of 24 hours **B.** Monolayer 10 TER percent from initial measurements over a 48 hour period

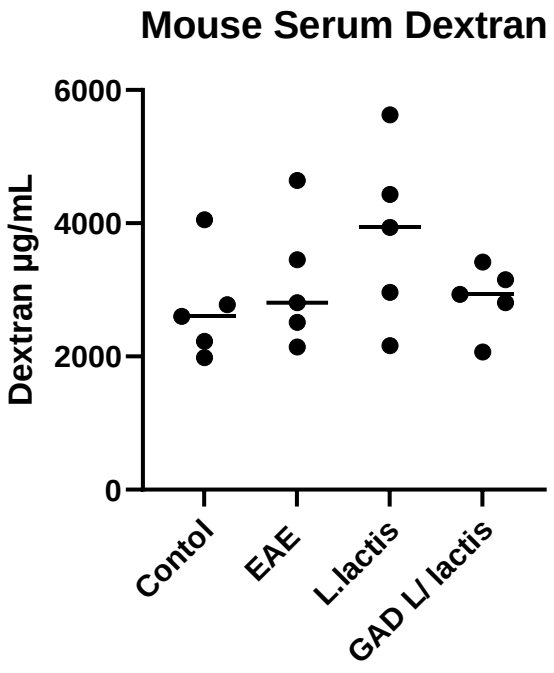

Figure 8 *In vivo* Dextran experiment. *In vivo* Dextran experiment. Blood serum collected from control mice (n = 5), EAE induced mice (n = 5), mice with oral treatment of pAC *L. lactis* (n = 5) and p8 *L. lactis* (n = 5). FITC dextran measured in total amount ug/mL

References

Adamczyk-Sowa, M., Medrek, A., Madej, P., Michlicka, W., & Dobrakowski, P. (2017). Does the gut microbiota influence immunity and inflammation in multiple sclerosis pathophysiology? *Journal of Immunology Research*, *2017*. https://doi.org/10.1155/2017/7904821

Allam, O., Samarani, S., Mehraj, V., Jenabian, M. A., Tremblay, C., Routy, J. P., Amre, D., & Ahmad, A. (2018). HIV induces production of IL-18 from intestinal epithelial cells that increases intestinal permeability and microbial translocation. *PLoS ONE*, *13*(3). https://doi.org/10.1371/journal.pone.0194185

Anders, H.-J., Andersen, K., & Stecher, B. (2013). The intestinal microbiota, a leaky gut, and abnormal immunity in kidney disease. *Kidney International*, *83*(6), 1010–1016. https://doi.org/10.1038/ki.2012.440

Andersen, K., Kesper, M. S., Marschner, J. A., Konrad, L., Ryu, M., Kumar, S. V. R., Kulkarni, O. P., Mulay, S. R., Romoli, S., Demleitner, J., Schiller, P., Dietrich, A., Müller, S., Gross, O., Ruscheweyh, H. J., Huson, D. H., Stecher, B., & Anders, H. J. (2017). Intestinal dysbiosis, barrier dysfunction, and bacterial translocation account for CKD-related systemic inflammation. *Journal of the American Society of Nephrology*, *28*(1), 76–83. https://doi.org/10.1681/ASN.2015111285

Auteri, M., Zizzo, M. G., & Serio, R. (2015). GABA and GABA receptors in the gastrointestinal tract: From motility to inflammation. In *Pharmacological Research* (Vol. 93, pp. 11–21). Academic Press. https://doi.org/10.1016/j.phrs.2014.12.001

Baecher-Allan, C., Kaskow, B. J., & Weiner, H. L. (2018). Multiple Sclerosis: Mechanisms and Immunotherapy. In *Neuron* (Vol. 97, Issue 4, pp. 742–768). Cell Press. https://doi.org/10.1016/j.neuron.2018.01.021

Basso, A. S., Frenkel, D., Quintana, F. J., Costa-Pinto, F. A., Petrovic-Stojkovic, S., Puckett, L., Monsonego, A., Bar-Shir, A., Engel, Y., Gozin, M., & Weiner, H. L. (2008). Reversal of axonal loss and disability in a mouse model of progressive multiple sclerosis. *Journal of Clinical Investigation*, *118*(4), 1532–1543. https://doi.org/10.1172/JCI33464

Berer, K., Gerdes, L. A., Cekanaviciute, E., Jia, X., Xiao, L., Xia, Z., Liu, C., Klotz, L., Stauffer, U., Baranzini, S. E., Kümpfel, T., Hohlfeld, R., Krishnamoorthy, G., & Wekerle, H. (2017). Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(40), 10719–10724. https://doi.org/10.1073/pnas.1711233114

Bhandage, A. K., Jin, Z., Korol, S. V., Shen, Q., Pei, Y., Deng, Q., Espes, D., Carlsson, P. O., Kamali-Moghaddam, M., & Birnir, B. (2018). GABA Regulates Release of Inflammatory Cytokines From Peripheral Blood Mononuclear Cells and CD4+ T Cells and Is Immunosuppressive in Type 1 Diabetes. *EBioMedicine*, *30*, 283–294. https://doi.org/10.1016/J.EBIOM.2018.03.019

Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R. W., & Steinman, L. (2010). Inhibitory role for GABA in autoimmune inflammation. *Proceedings of the National*

Academy of Sciences of the United States of America, *107*(6), 2580–2585. https://doi.org/10.1073/pnas.0915139107

Birgbauer, E., Rao, T. S., & Webb, M. (2004). Lysolecithin induces demyelination in vitro in a cerebellar slice culture system. *Journal of Neuroscience Research*, *78*(2), 157–166. https://doi.org/10.1002/jnr.20248

Bjornevik, K., Cortese, M., Healy, B. C., Kuhle, J., Mina, M. J., Leng, Y., Elledge, S. J., Niebuhr, D. W., Scher, A. I., Munger, K. L., & Ascherio, A. (2022). Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science*, *375*(6578), 296–301. https://doi.org/10.1126/SCIENCE.ABJ8222/SUPPL_FILE/SCIENCE.ABJ8222_MDAR_REPRODUCIBILITY_CHECKLIST.PDF

Bjurstöm, H., Wang, J. Y., Ericsson, I., Bengtsson, M., Liu, Y., Kumar-Mendu, S., Issazadeh-Navikas, S., & Birnir, B. (2008). GABA, a natural immunomodulator of T lymphocytes. *Journal of Neuroimmunology*, *205*(1–2), 44–50. https://doi.org/10.1016/j.jneuroim.2008.08.017

Borchers, C. H., Kast, J., Foster, L. J., Siu, K. W. M., Overall, C. M., Binkowski, T. A., Hildebrand, W. H., Scherer, A., Mansoor, M., & Keown, P. A. (2014). The Human Proteome Organization Chromosome 6 Consortium: Integrating chromosome-centric and biology/disease driven strategies. *Journal of Proteomics*, *100*, 60–67. https://doi.org/10.1016/J.JPROT.2013.08.001

Brenchley, J. M., & Douek, D. C. (2012). Microbial translocation across the GI tract. In

Annual Review of Immunology (Vol. 30, pp. 149–173). NIH Public Access. https://doi.org/10.1146/annurev-immunol-020711-075001

Burrows, D. J., McGown, A., Jain, S. A., De Felice, M., Ramesh, T. M., Sharrack, B., & Majid, A. (2019). Animal models of multiple sclerosis: From rodents to zebrafish. *Multiple Sclerosis (Houndmills, Basingstoke, England)*, *25*(3), 306–324. https://doi.org/10.1177/1352458518805246

Burz, S. D., Abraham, A. L., Fonseca, F., David, O., Chapron, A., Béguet-Crespel, F., Cénard, S., Le Roux, K., Patrascu, O., Levenez, F., Schwintner, C., Blottière, H. M., Béra-Maillet, C., Lepage, P., Doré, J., & Juste, C. (2019). A Guide for Ex Vivo Handling and Storage of Stool Samples Intended for Fecal Microbiota Transplantation. *Scientific Reports 2019 9:1*, *9*(1), 1–16. https://doi.org/10.1038/s41598-019-45173-4

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(9), 3666–3670. https://doi.org/10.1073/PNAS.72.9.3666

Cekanaviciute, E., Yoo, B. B., Runia, T. F., Debelius, J. W., Singh, S., Nelson, C. A., Kanner, R., Bencosme, Y., Lee, Y. K., Hauser, S. L., Crabtree-Hartman, E., Sand, I. K., Gacias, M., Zhu, Y., Casaccia, P., Cree, B. A. C., Knight, R., Mazmanian, S. K., & Baranzini, S. E. (2017). Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proceedings of the National Academy of*

Sciences of the United States of America, *114*(40), 10713–10718. https://doi.org/10.1073/PNAS.1711235114

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. In *Oncotarget* (Vol. 9, Issue 6, pp. 7204–7218). Impact Journals LLC. https://doi.org/10.18632/oncotarget.23208

Chu, F., Shi, M., Lang, Y., Shen, D., Jin, T., Zhu, J., & Cui, L. (2018). Gut microbiota in multiple sclerosis and experimental autoimmune encephalomyelitis: current applications and future perspectives. In *Mediators of Inflammation* (Vol. 2018). Hindawi Limited. https://doi.org/10.1155/2018/8168717

Colpitts, S. L., Kasper, E. J., Keever, A., Liljenberg, C., Kirby, T., Magori, K., Kasper, L. H., & Ochoa-Repáraz, J. (2017). A bidirectional association between the gut microbiota and CNS disease in a biphasic murine model of multiple sclerosis. *Gut Microbes*, *8*(6), 561–573. https://doi.org/10.1080/19490976.2017.1353843

Compston, A., & Coles, A. (2008). Multiple sclerosis. In *The Lancet* (Vol. 372, Issue 9648, pp. 1502–1517). Lancet. https://doi.org/10.1016/S0140-6736(08)61620-7

Constantinescu, C. S., Farooqi, N., O'Brien, K., & Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British Journal of Pharmacology*, *164*(4), 1079. https://doi.org/10.1111/J.1476-5381.2011.01302.X

Cortese, R., Collorone, S., Ciccarelli, O., & Toosy, A. T. (2019). Advances in brain imaging in multiple sclerosis. *Therapeutic Advances in Neurological Disorders*, *12*, 1756286419859722. https://doi.org/10.1177/1756286419859722

Cox, L. M., Maghzi, A. H., Liu, S., Tankou, S. K., Dhang, F. H., Willocq, V., Song, A., Wasén, C., Tauhid, S., Chu, R., Anderson, M. C., De Jager, P. L., Polgar-Turcsanyi, M., Healy, B. C., Glanz, B. I., Bakshi, R., Chitnis, T., & Weiner, H. L. (2021). Gut Microbiome in Progressive Multiple Sclerosis. *Annals of Neurology*, *89*(6), 1195–1211. https://doi.org/10.1002/ANA.26084

Daberkow, D. P., Hoffman, K., Kohl, H. M., Long, T., Kirby, T. O., & Ochoa-Repáraz, J. (2021). Microbiome Methods in Experimental Autoimmune Encephalomyelitis. *Current Protocols*, *1*(12), e314. https://doi.org/10.1002/CPZ1.314

De Angelis, F., Plantone, D., & Chataway, J. (2018). Pharmacotherapy in Secondary Progressive Multiple Sclerosis: An Overview. *CNS Drugs*, *32*(6), 499–526. https://doi.org/10.1007/S40263-018-0538-0

De Stefano, N., & Giorgio, A. (2015). GABA: A new imaging biomarker of neurodegeneration in multiple sclerosis? In *Brain* (Vol. 138, Issue 9, pp. 2467–2468). https://doi.org/10.1093/brain/awv213

Dieterich, W., Schink, M., & Zopf, Y. (2018). Microbiota in the Gastrointestinal Tract. *Medical Sciences 2018, Vol. 6, Page 116*, *6*(4), 116. https://doi.org/10.3390/MEDSCI6040116

Dobson, R., & Giovannoni, G. (2019). Multiple sclerosis – a review. *European Journal of Neurology*, *26*(1), 27–40. https://doi.org/10.1111/ENE.13819

Dolpady, J., Sorini, C., Di Pietro, C., Cosorich, I., Ferrarese, R., Saita, D., Clementi, M., Canducci, F., & Falcone, M. (2016). Oral probiotic VSL#3 prevents autoimmune diabetes by modulating microbiota and promoting indoleamine 2,3-dioxygenase-enriched tolerogenic intestinal environment. *Journal of Diabetes Research*, *2016*. https://doi.org/10.1155/2016/7569431

Doshi, A., & Chataway, J. (2016). Multiple sclerosis, a treatable disease. In *Clinical medicine (London, England)* (Vol. 16, Issue Suppl 6, pp. s53–s59). Clin Med (Lond). https://doi.org/10.7861/clinmedicine.16-6-s53

Geirnaert, A., Calatayud, M., Grootaert, C., Laukens, D., Devriese, S., Smagghe, G., De Vos, M., Boon, N., & Van De Wiele, T. (2017). Butyrate-producing bacteria supplemented in vitro to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Scientific Reports 2017 7:1*, *7*(1), 1–14. https://doi.org/10.1038/s41598-017-11734-8

Ghasemi, N., Razavi, S., & Nikzad, E. (2017). Multiple Sclerosis: Pathogenesis, Symptoms, Diagnoses andCell-Based Therapy. *Cell Journal (Yakhteh)*, *19*(1), 1. https://doi.org/10.22074/CELLJ.2016.4867

Gil, A., Rueda, R., Ozanne, S. E., van der Beek, E. M., van Loo-Bouwman, C., Schoemaker, M., Marinello, V., Venema, K., Stanton, C., Schelkle, B., Flourakis, M., & Edwards, C. A. (2020). Is there evidence for bacterial transfer via the placenta and any role in

the colonization of the infant gut?–a systematic review. *Critical Reviews in Microbiology*, *46*(5), 493–507. https://doi.org/10.1080/1040841X.2020.1800587/SUPPL_FILE/IMBY_A_1800587_SM9235.DOCX

Gogolla, N., Galimberti, I., DePaola, V., & Caroni, P. (2006). Preparation of organotypic hippocampal slice cultures for long-term live imaging. *Nature Protocols*, *1*(3), 1165–1171. https://doi.org/10.1038/nprot.2006.168

Grothaus, J. S., Ares, G., Yuan, C., Wood, D. R., & Hunter, C. J. (2018). Rho kinase inhibition maintains intestinal and vascular barrier function by upregulation of occludin in experimental necrotizing enterocolitis. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *315*(4), G514–G528. https://doi.org/10.1152/ajpgi.00357.2017

Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A., & Kanner, B. I. (1990). Cloning and Expression of a Rat Brain GABA Transporter. In *New Series* (Vol. 249, Issue 4974).

Hedblom, G. A., Reiland, H. A., Sylte, M. J., Johnson, T. J., & Baumler, D. J. (2018). Segmented filamentous bacteria - metabolism meets immunity. *Frontiers in Microbiology*, *9*(AUG), 1991. https://doi.org/10.3389/FMICB.2018.01991/BIBTEX

Houghteling, P. D., & Walker, W. A. (2015). Why is initial bacterial colonization of the intestine important to infants' and children's health? In *Journal of Pediatric Gastroenterology and Nutrition* (Vol. 60, Issue 3, pp. 294–307). Lippincott Williams

and Wilkins. https://doi.org/10.1097/MPG.0000000000000597

Hu, W., Qiu, B., Guan, W., Wang, Q., Wang, M., Li, W., Gao, L., Shen, L., Huang, Y., Xie, G., Zhao, H., Jin, Y., Tang, B., Yu, Y., Zhao, J., & Pei, G. (2015). Direct Conversion of Normal and Alzheimer's Disease Human Fibroblasts into Neuronal Cells by Small Molecules. *Cell Stem Cell*, *17*(2), 204–212. https://doi.org/10.1016/j.stem.2015.07.006

Jangi, S., Gandhi, R., Cox, L. M., Li, N., Von Glehn, F., Yan, R., Patel, B., Mazzola, M. A., Liu, S., Glanz, B. L., Cook, S., Tankou, S., Stuart, F., Melo, K., Nejad, P., Smith, K., Topçuolu, B. D., Holden, J., Kivisäkk, P., … Weiner, H. L. (2016). Alterations of the human gut microbiome in multiple sclerosis. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms12015

Jia, X., Madireddy, L., Caillier, S., Santaniello, A., Esposito, F., Comi, G., Stuve, O., Zhou, Y., Taylor, B., Kilpatrick, T., Martinelli-Boneschi, F., Cree, B. A. C., Oksenberg, J. R., Hauser, S. L., & Baranzini, S. E. (2018). Genome sequencing uncovers phenocopies in primary progressive multiple sclerosis. *Annals of Neurology*, *84*(1), 51–63. https://doi.org/10.1002/ANA.25263

Jin, Z., Mendu, S. K., & Birnir, B. (2013). GABA is an effective immunomodulatory molecule. In *Amino Acids* (Vol. 45, Issue 1, pp. 87–94). https://doi.org/10.1007/s00726-011-1193-7

Kadowaki, A., Saga, R., Lin, Y., Sato, W., & Yamamura, T. (2019). Gut microbiota-dependent CCR9+ CD4+ T cells are altered in secondary progressive multiple

sclerosis. *Brain*, *142*(4), 916–931. https://doi.org/10.1093/brain/awz012

Kalincik, T. (2015). Multiple sclerosis relapses: Epidemiology, outcomes and management. A systematic review. In *Neuroepidemiology* (Vol. 44, Issue 4, pp. 199–214). S. Karger AG. https://doi.org/10.1159/000382130

Kaskow, B. J., & Baecher-Allan, C. (2018). Effector t cells in multiple sclerosis. *Cold Spring Harbor Perspectives in Medicine*, *8*(4). https://doi.org/10.1101/cshperspect.a029025

Khaibullin, T., Ivanova, V., Martynova, E., Cherepnev, G., Khabirov, F., Granatov, E., Rizvanov, A., & Khaiboullina, S. (2017). Elevated levels of proinflammatory cytokines in cerebrospinal fluid of multiple sclerosis patients. *Frontiers in Immunology*, *8*(MAY). https://doi.org/10.3389/fimmu.2017.00531

Kinashi, Y., & Hase, K. (2021). Partners in Leaky Gut Syndrome: Intestinal Dysbiosis and Autoimmunity. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/FIMMU.2021.673708

Kotzamani, D., Panou, T., Mastorodemos, V., Tzagournissakis, M., Nikolakaki, H., Spanaki, C., & Plaitakis, A. (2012). Rising incidence of multiple sclerosis in females associated with urbanization. *Neurology*, *78*(22), 1728–1735. https://doi.org/10.1212/WNL.0B013E31825830A9

Lassmann, H. (1999). The Pathology of Multiple Sclerosis and Its Evolution. In *Philosophical Transactions: Biological Sciences* (Vol. 354, Issue 1390). Progress and

Problems. https://about.jstor.org/terms

Lee, Y. K., Menezes, J. S., Umesaki, Y., & Mazmanian, S. K. (2011). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 1*(Suppl 1), 4615–4622. https://doi.org/10.1073/PNAS.1000082107

Lin, C.-C., & Edelson, B. T. (2017). New Insights into the Role of IL-1β in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *The Journal of Immunology*, *198*(12), 4553–4560. https://doi.org/10.4049/jimmunol.1700263

Liu, Y., Alookaran, J. J., & Rhoads, J. M. (2018). Probiotics in autoimmune and inflammatory disorders. In *Nutrients* (Vol. 10, Issue 10). MDPI AG. https://doi.org/10.3390/nu10101537

Lublin, F. D. (2014). New multiple sclerosis phenotypic classification. *European Neurology*, *72*, 1–5. https://doi.org/10.1159/000367614

Lyu, C., Zhao, W., Peng, C., Hu, S., Fang, H., Hua, Y., Yao, S., Huang, J., & Mei, L. (2018). Exploring the contributions of two glutamate decarboxylase isozymes in Lactobacillus brevis to acid resistance and γ-aminobutyric acid production. *Microbial Cell Factories*, *17*(1), 1–14. https://doi.org/10.1186/S12934-018-1029-1/TABLES/3

Ma, H. Di, Zhao, Z. Bin, Ma, W. T., Liu, Q. Z., Gao, C. Y., Li, L., Wang, J., Tsuneyama, K.,

Liu, B., Zhang, W., Zhou, Y., Gershwin, M. E., & Lian, Z. X. (2018). Gut microbiota translocation promotes autoimmune cholangitis. *Journal of Autoimmunity*, *95*, 47–57. https://doi.org/10.1016/j.jaut.2018.09.010

Ma, T. Y., Boivin, M. A., Ye, D., Pedram, A., & Said, H. M. (2005). Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: Role of myosin light-chain kinase protein expression. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *288*(3 51-3). https://doi.org/10.1152/ajpgi.00412.2004

Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., Boivin, M. A., & Said, H. M. (2004). TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *286*(3 49-3). https://doi.org/10.1152/ajpgi.00173.2003

MacLeod, H., & Wetzler, L. M. (2007). T cell activation by TLRs: a role for TLRs in the adaptive immune response. *Science's STKE : Signal Transduction Knowledge Environment*, *2007*(402). https://doi.org/10.1126/stke.4022007pe48

Maghbooli, Z., Sahraian, M. A., & Naser Moghadasi, A. (2020). Multiple sclerosis and human leukocyte antigen genotypes: Focus on the Middle East and North Africa region. *Multiple Sclerosis Journal - Experimental, Translational and Clinical*, *6*(1), 2055217319881775. https://doi.org/10.1177/2055217319881775

Mattar, A. F., Drongowski, R. A., Coran, A. G., & Harmon, C. M. (2001). Effect of probiotics on enterocyte bacterial translocation in vitro. *Pediatric Surgery*

International, *17*(4), 265–268. https://doi.org/10.1007/s003830100591

Mendibe Bilbao, M., Boyero Durán, S., Bárcena Llona, J., & Rodriguez-Antigüedad, A. (2019). Multiple sclerosis: Pregnancy and women's health issues. In *Neurologia* (Vol. 34, Issue 4, pp. 259–269). Spanish Society of Neurology. https://doi.org/10.1016/j.nrl.2016.06.005

Mir, H., Meena, A. S., Chaudhry, K. K., Shukla, P. K., Gangwar, R., Manda, B., Padala, M. K., Shen, L., Turner, J. R., Dietrich, P., Dragatsis, I., & Rao, R. (2016). Occludin deficiency promotes ethanol-induced disruption of colonic epithelial junctions, gut barrier dysfunction and liver damage in mice. *Biochimica et Biophysica Acta - General Subjects*, *1860*(4), 765–774. https://doi.org/10.1016/j.bbagen.2015.12.013

Nouri, M., Bredberg, A., Weström, B., & 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*(9), e106335. https://doi.org/10.1371/JOURNAL.PONE.0106335

Ochoa-Repáraz, J., Mielcarz, D. W., Wang, Y., Begum-Haque, S., Dasgupta, S., Kasper, D. L., & Kasper, L. H. (2010). A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. *Mucosal Immunology*, *3*(5), 487–495. https://doi.org/10.1038/mi.2010.29

Ochoa-Repáraz, Javier, & Kasper, L. H. (2018). The Microbiome and Neurologic Disease: Past and Future of a 2-Way Interaction. In *Neurotherapeutics* (Vol. 15, Issue 1). https://doi.org/10.1007/s13311-018-0604-9

Ochoa-Repáraz, Javier, Mielcarz, D. W., Ditrio, L. E., Burroughs, A. R., Foureau, D. M., Haque-Begum, S., & Kasper, L. H. (2009). Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. *Journal of Immunology (Baltimore, Md. : 1950)*, *183*(10), 6041–6050. https://doi.org/10.4049/JIMMUNOL.0900747

Pan, Z., Li, S., Liu, Q., Wang, Z., Zhou, Z., Di, R., Miao, B., Hu, W., Wang, X., Hu, X., Xu, Z., Wei, D., He, X., Yuan, L., Guo, X., Liang, B., Wang, R., Li, X., Cao, X., … Li, Y. (2018). Whole-genome sequences of 89 Chinese sheep suggest role of RXFP2 in the development of unique horn phenotype as response to semi-feralization. In *GigaScience* (Vol. 7, Issue 4). https://doi.org/10.1093/gigascience/giy019

Paul, A. M., Branton, W. G., Walsh, J. G., Polyak, M. J., Lu, J. Q., Baker, G. B., & Power, C. (2014). GABA transport and neuroinflammation are coupled in multiple sclerosis: Regulation of the GABA transporter-2 by ganaxolone. *Neuroscience*, *273*, 24–38. https://doi.org/10.1016/j.neuroscience.2014.04.037

Ponz-Sarvise, M., Alberto, P.-M., Slanzi, A., Iannoto, G., Rossi, B., Zenaro, E., & Constantin, G. (2020). *In vitro Models of Neurodegenerative Diseases*. https://doi.org/10.3389/fcell.2020.00328

Pröbstel, A. K., & Baranzini, S. E. (2018). The Role of the Gut Microbiome in Multiple Sclerosis Risk and Progression: Towards Characterization of the “MS Microbiome.” In *Neurotherapeutics* (Vol. 15, Issue 1, pp. 126–134). Springer New York LLC. https://doi.org/10.1007/s13311-017-0587-y

Reynolds, J. M., Martinez, G. J., Chung, Y., & Dong, C. (2012). Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Source*, *109*(32), 13064–13069. https://doi.org/10.1073/pnas.1120585109

Robinson, A. P., Harp, C. T., Noronha, A., & Miller, S. D. (2014). The experimental autoimmune encephalomyelitis (EAE) model of MS. utility for understanding disease pathophysiology and treatment. In *Handbook of Clinical Neurology* (Vol. 122, pp. 173–189). Elsevier BV https://doi.org/10.1016/B978-0-444-52001-2.00008-X

Robinson, W. H., & Steinman, L. (2022). Epstein-Barr virus and multiple sclerosis. *Science*, *375*(6578), 264–265. https://doi.org/10.1126/SCIENCE.ABM7930

Rostami, A., & Ciric, B. (2013). Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. *Journal of the Neurological Sciences*, *333*(0), 76. https://doi.org/10.1016/J.JNS.2013.03.002

Roulis, M., Armaka, M., Manoloukos, M., Apostolaki, M., & Kollias, G. (2011). Intestinal epithelial cells as producers but not targets of chronic TNF suffice to cause murine Crohn-like pathology. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(13), 5396–5401. https://doi.org/10.1073/pnas.1007811108

Schlachetzki, J. C. M., Saliba, S. W., & Pinheiro De Oliveira, A. C. (2013). *Studying neurodegenerative diseases in culture models*. https://doi.org/10.1590/1516-4446-2013-1159

Schumacher, G. A. (1957). Multiple Sclerosis. In *Source: The American Journal of Nursing* (Vol. 57, Issue 6).

Sintzel, M. B., Rametta, M., & Reder, A. T. (2018). Vitamin D and Multiple Sclerosis: A Comprehensive Review. *Neurology and Therapy*, *7*(1), 59–85. https://doi.org/10.1007/S40120-017-0086-4

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER Measurement Techniques for In Vitro Barrier Model Systems. In *Journal of Laboratory Automation* (Vol. 20, Issue 2, pp. 107–126). SAGE Publications Inc. https://doi.org/10.1177/2211068214561025

Tankou, S. K., Regev, K., Healy, B. C., Cox, L. M., Tjon, E., Kivisakk, P., Vanande, I. P., Cook, S., Gandhi, R., Glanz, B., Stankiewicz, J., & Weiner, H. L. (2018). Investigation of probiotics in multiple sclerosis. *Multiple Sclerosis*, *24*(1), 58–63. https://doi.org/10.1177/1352458517737390

Thompson, A. J., Banwell, B. L., Barkhof, F., Carroll, W. M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M. S., Fujihara, K., Galetta, S. L., Hartung, H. P., Kappos, L., Lublin, F. D., Marrie, R. A., Miller, A. E., Miller, D. H., Montalban, X., … Cohen, J. A. (2018). Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *The Lancet Neurology*, *17*(2), 162–173. https://doi.org/10.1016/S1474-4422(17)30470-2

Tian, J., Yong, J., Dang, H., & Kaufman, D. L. (2011). Oral GABA treatment downregulates inflammatory responses in a mouse model of rheumatoid arthritis.

Http://Dx.Doi.Org/10.3109/08916934.2011.571223, *44*(6), 465–470. https://doi.org/10.3109/08916934.2011.571223

Wang, Y., Begum-Haque, S., Telesford, K. M., Ochoa-Repáraz, J., Christy, M., Kasper, E. J., Kasper, D. L., Robson, S. C., & Kasper, L. H. (2014). A commensal bacterial product elicits and modulates migratory capacity of CD39+ CD4 T regulatory subsets in the suppression of neuroinflammation. *Gut Microbes*, *5*(4), 552–561. https://doi.org/10.4161/gmic.29797

Wang, Y., Telesford, K. M., Ochoa-Repáraz, J., Haque-Begum, S., Christy, M., Kasper, E. J., Wang, L., Wu, Y., Robson, S. C., Kasper, D. L., & Kasper, L. H. (2014). An intestinal commensal symbiosis factor controls neuroinflammation via TLR2-mediated CD39 signalling. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms5432

Watari, A., Sakamoto, Y., Hisaie, K., Iwamoto, K., Fueta, M., Yagi, K., & Kondoh, M. (2017). Rebeccamycin Attenuates TNF-α-Induced Intestinal Epithelial Barrier Dysfunction by Inhibiting Myosin Light Chain Kinase Production. *Cellular Physiology and Biochemistry*, *41*(5), 1924–1934. https://doi.org/10.1159/000472367

Wingerchuk, D. M., & Carter, J. L. (2014). Multiple sclerosis: Current and emerging disease-modifying therapies and treatment strategies. In *Mayo Clinic Proceedings* (Vol. 89, Issue 2, pp. 225–240). Elsevier Ltd. https://doi.org/10.1016/j.mayocp.2013.11.002

Ye, D., Ma, I., & Ma, T. Y. (2006). Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier. *American Journal of*

Physiology - Gastrointestinal and Liver Physiology, *290*(3). https://doi.org/10.1152/ajpgi.00318.2005

Yokote, H., Miyake, S., Croxford, J. L., Oki, S., Mizusawa, H., & Yamamura, T. (2008). NKT cell-dependent amelioration of a mouse model of multiple sclerosis by altering gut flora. *The American Journal of Pathology*, *173*(6), 1714–1723. https://doi.org/10.2353/AJPATH.2008.080622

Zhang, H., Jarjour, A. A., Boyd, A., & Williams, A. (2011). Central nervous system remyelination in culture - A tool for multiple sclerosis research. *Experimental Neurology*, *230*(1), 138–148. https://doi.org/10.1016/j.expneurol.2011.04.009

Zhu, D., Liu, F., Xu, H., Bai, Y., Zhang, X., Saris, P. E. J., & Qiao, M. (2015). Isolation of strong constitutive promoters from Lactococcus lactis subsp. lactis N8. *FEMS Microbiology Letters*, *362*(16). https://doi.org/10.1093/FEMSLE/FNV107

Zivadinov, R., & Leist, T. P. (2005). Clinical–Magnetic Resonance Imaging Correlations in Multiple Sclerosis. *Journal of Neuroimaging*, *15*(4 SUPPL.), 10S-21S. https://doi.org/10.1177/1051228405283291

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PUBLICATIONS

Daberkow, D. P., Hoffman, K., Kohl, H. M., **Long, T.**, Kirby, T. O., & Ochoa-Repáraz, J. (2021). Microbiome Methods in Experimental Autoimmune Encephalomyelitis. *Current Protocols*, *1*(12), e314.

PRESENTATIONS

Tyrel Long, Xutong Shi, Jake Doyle, Kristina Hoffman, Andrea Castillo, Jean-Baptiste Roullet, K. Michael Gibson, and Javier Ochoa-Repáraz. Effects of GABA on Inflammation and Intestinal Barrier Disruption. 2022 Keystone Symposia Conference: Neuro-Immune Interactions in the Central Nervous System. June 5 – 9, 2022; Keystone Resort, Keystone, CO.

Kohl, H. M., Hoffman, K., Staben, K., Shi, X., **Long, T.**, Castillo, A., ... & Ochoa-Repáraz, J. (2021). Evaluating the Effects of Intestinal Bacteria's Production of GABA Neurotransmitter on an Animal Model of Multiple Sclerosis.

Javier Ochoa-Repáraz, Kristina Hoffman, **Tyrel Long**, William J. Doyle, Hannah M.

Kohl, Kendall Staben, Alivia Sargent, Rachel Linton, Molly Ristig, Rylee Harris, Xutong Shi, Andrea Castillo, K. Michael Gibson, and Jean-Baptiste Roullet. A GABA-producing probiotic for the protection of CNS demyelinating inflammation. Immunology 2022, AAI Annual Meeting; May 6 -10, 2022; Oregon Convention Center, Portland, OR.

William J. Doyle, Lacey B. Sell, Christina C. Ramelow, Hannah M. Kohl, Kristina Hoffman, Jasleen K. Bains, Kevin D. Strawn, Theresa Hevrin, Trevor O. Kirby, K. Michael Gibson, Jean-Baptiste Roullet, Javier Ochoa-Reparaz. Farnesol induces protection against CNS inflammatory demyelination and decreases spinal infiltration of CD4+ T-Cells. *Immunology 2022, AAI Annual Meeting; May 6 -10, 2022; Oregon Convention Center, Portland, OR.*

Kristina Hoffman, David P. Daberkow, Hannah M. Kohl, **Tyrel Long**, Trevor O. Kirby, and Javier Ochoa-Repáraz. Microbiome methods in experimental autoimmune encephalomyelitis. *Immunology 2022, AAI Annual Meeting; May 6 -10, 2022; Oregon Convention Center, Portland, OR.*

PROFESSIONAL EXPERIENCE

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

- Instructed students in performing and completing assigned tasks.
- Evaluated student responses and provided constructive feedback

Microbiology Lab Assistant, Providence Sacred Heart Medical Center, Spokane, WA

- Maintained strict aseptic fields when collecting biological samples, minimizing staff and patient infection risks.
- Organized, maintained and verified sterility of lab equipment and tools.
- Assembled growth media plates with collected biological samples

Prep Chemist, Vista Analytical – El Darado Hills, CA

- Executed technical laboratory functions in compliance with regulatory agencies and safety requirements.
- Operated standardized tests on organic and inorganic compounds to observe fundamental differences in properties.
- Prepared waste drums for pick-up and delivery to comply with local, state and federal regulations for treatment and disposal of hazardous waste.

Organic Prep Supervisor, Eurofins TestAmerica – Sacramento, CA

- Mediated and resolved challenges to enhance team production and performance and maintained consistent quality levels.
- Oversaw inventory and product stock to develop and maintain inventory controls resulting in cost savings and reduced overages.
- Led staff training and supervision and managed staff hiring.

SKILLS and TECHINIQUES

- Strong skills in preparing Caco-2 cells into monolayers to test for permeability
- Experience with standard molecular and cellular techniques (ELISA, qPCR, SDS-PAGE, cell culturing)
- Experience with in vivo mouse experiments
- Strong team player with the ability to take initiative and work independently including inducing EAE.