The gut microbiome: a contributing mechanism to the anti-seizure effect of topiramate

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ABSTRACT

Epilepsy is one of the most common neurological disorders worldwide. This neurological disorder is characterized by spontaneous recurrent seizures and impacts about 65 million people globally. As there is no cure for epilepsy, the treatment goal for patients is seizure management, and ultimately seizure freedom. The first line of defense in seizure management is anti-epileptic drugs, which aim to restore the excitatory and inhibitory balance in the brain. Unfortunately, about 30% of people with epilepsy are drug resistant, a number which has remained unchanged despite the increasing amount of anti-epileptic drugs. This leads patients to seek alternative treatments, which include surgery, vagus nerve stimulation, or diet alterations such as the ketogenic diet. Due to the invasiveness of surgeries, difficulty to maintain specialty diets, or lack of effectiveness of these treatments in some patients, additional therapies are needed.

The gut-brain axis is a bidirectional communication network connecting the central and enteric nervous systems. Part of this network includes communication via the gut microbiota. The gut microbiota consists of all the microorganisms living in the gut, including bacteria, viruses, and fungi. It is involved in aiding nutrient absorption, promoting the maturation of immune cells and functions, and protection against pathogens. There is growing interest in the role of the gut microbiome in human health and disease. Studies have shown that patients with epilepsy have altered gut microbiomes compared to healthy controls, and that gut microbiome alteration can impact seizure frequencies. These exciting findings have ignited research on the potential therapeutic role of the gut microbiome in epilepsy. Although studies have explored the impact of alterations in the gut microbiome on seizure activity, they have not studied how anti-epileptic drugs may contribute to this relationship. Thus, this dissertation explores the role of the commonly prescribed anti-epileptic drug topiramate on the gut microbiome. Fecal samples of mice treated with topiramate were analyzed using 16S ribosomal RNA gene sequencing. Analysis revealed that topiramate ingestion increased the probiotic bacteria *Lactobacillus johnsonii* in the gut microbiome. In addition, cotreatment of topiramate and *Lactobacillus johnsonii* reduced seizure susceptibility in a pentylenetetrazol-kindling seizure model. Moreover, cotreatment increased the butyrate producing family *Lachnospiraceae* and subsequently increased the neuroprotective SCFA, butyrate in the gut microbiome. Importantly, cotreatment also resulted in an increased GABA/glutamate ratio in the cortex of mice that underwent pentylenetetrazol-kindling.

These results are the first to demonstrate that the anti-seizure effect of topiramate may be facilitated by the modulation of the gut microbiota via increasing butyrate and altering the GABA/glutamate ratio in the cortex. Lastly, this work highlights the potential for probiotics as an adjuvant therapy in seizure management.

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GENERAL AUDIENCE ABSTRACT

Epilepsy is one of the most common neurological disorders worldwide. This neurological disorder is characterized by spontaneous recurrent seizures and impacts about 65 million people globally. As there is no cure for epilepsy, the treatment goal for patients is seizure management, and ultimately seizure freedom. The first line of defense in seizure management is anti-epileptic drugs, which aim to restore the excitatory and inhibitory balance in the brain. Unfortunately, about 30% of people with epilepsy are drug resistant, a number which has remained unchanged despite the increasing amount of anti-epileptic drugs. Due to this unmet need, epilepsy patients utilize alternative treatments, which include surgery, vagus nerve stimulation, or diet modifications such as the ketogenic diet. Due to the invasiveness of surgeries, difficulty to maintain specialty diets, or lack of effectiveness of these treatments in some patients, additional therapies are needed.

The gut microbiota consists of all the microorganisms living in the gut, including bacteria, viruses, and fungi, which can be both harmful and helpful. In healthy individuals, the gut microbiota coexists in a balance that prevents diseases and helps the host, however, disruptions in this balance can lead to susceptibility to several diseases. As a result, researchers are increasingly interested in the role of the gut microbiota in human health and disease. In epilepsy, the gut microbiome is altered compared to healthy individuals, and gut microbiome alterations can impact seizure activity. This has led researchers to investigate the potential therapeutic role of the gut microbiome in epilepsy. Although studies have explored the impact of alterations in the gut microbiome on seizure activity, they have not studied how anti-epileptic drugs may contribute to this relationship. Thus, this dissertation explores the role of the commonly prescribed anti-epileptic drug topiramate on the gut microbiome. The results demonstrate that topiramate increases probiotic bacteria in the gut microbiome of mice. Moreover, this probiotic bacterium facilitates topiramate in reducing the susceptibility to seizures in a mouse model by resulting in a beneficial gut microbiome and restoring excitatory and inhibitory balance to the brain.

These results are the first to demonstrate that the anti-seizure effect of topiramate may be facilitated by the gut microbiome. Lastly, this work highlights the potential for probiotics as an adjuvant therapy in seizure management.

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Abbreviations

AED- anti-epileptic drug
AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASV- amplicon sequence variant
AUC- area under the curve
bp- base pairs
cDNA- complementary deoxyribonucleic acid
CNS- central nervous system
CTLA- cytotoxic T-lymphocyte associated protein
DNA- deoxyribonucleic acid
FDA- U.S. Food and Drug Administration
FMT- fecal matter transplant
GABA- gamma-aminobutyric acid
HIV- human immunodeficiency virus
HPA- hypothalamic-pituitary-adrenal
IBS- irritable bowel syndrome
LDA- linear discriminant analysis
MRS- De Man, Rogosa, and Sharpe
NMDA- *N*-methyl-D-aspartate receptor
OTU- operational taxonomic unit
PCoA- principal coordinate analysis
PCR- polymerase chain reaction
PTZ- pentylenetetrazol
qPCR- quantitative polymerase chain reaction
RNA- ribonucleic acid
rRNA- ribosomal ribonucleic acid
SCFA- short-chain fatty acid
TBI- traumatic brain injury
TNF- tumor necrosis factor
TPM- topiramate
US- United States
ZO- zonula occluden

Chapter 1: Introduction

1.1 Epilepsy

Epilepsy is one of the most common neurological disorders worldwide (Beghi, 2020). It is a disorder characterized by spontaneous recurrent seizures which impacts 1% of people in the United States (US) alone and around 65 million people globally (Kanner & Bicchi, 2022). This disorder is caused by numerous factors, including central nervous system (CNS) infection, traumatic brain injury (TBI), stroke, brain tumors, genetics, and unknown causes. Epilepsy slightly impacts men more than women and is heavily concentrated in lower- and middle-income countries (80% of cases), however this is likely attributed to the increased risk factors affecting these groups (Beghi, 2020). Epilepsy affects people of all ages but is more commonly skewed to younger and older populations, with highest prevalence during the first year of life and over the age of 85 (Beghi, 2020).

While epilepsy encompasses a wide range of conditions, these conditions all share the common characteristic of seizure activity. Seizures are bursts of uncontrolled electrical activity in the brain, often due to an imbalance of excitation and inhibition. Seizures can be focal, originating in one location, or generalized, occurring simultaneously throughout the cortex. Additionally, seizures may or may not impact motor systems. Given that seizures are the hallmark of epilepsy, the primary treatment goal is to reduce or rid patients of seizures. The first line of defense against seizures is anti-epileptic drugs (AEDs). If two tolerated and appropriately chosen AEDs fail to achieve seizure freedom, the patient is deemed drug resistant (Kanner & Bicchi, 2022). Drug-resistance requires patients to seek alternative treatments including surgery, vagal nerve stimulation, and/or the ketogenic diet (Kanner & Bicchi, 2022).

AEDs are a group of medications that aim to reduce seizure occurrence typically by either decreasing excitation, primarily mediated by glutamate, or increasing inhibition, primarily mediated by gamma-aminobutyric acid (GABA), in the brain. There are over 30 major AEDs approved for use in the United States (Rudzinski et al., 2016) (Table 1). These drugs span vast and various mechanisms of action which include increasing inhibition by either increasing GABA release in the synapse or activating GABA receptors or decreasing excitation by decreasing glutamate release in the synapse or blocking glutamate receptors. AEDs are prescribed based on seizure type and epilepsy syndrome (Kanner & Bicchi, 2022). Treatment usually begins as a monotherapy at a low dose, then slowly increasing the dosage to a moderate dose with minimal adverse events (Kanner & Bicchi, 2022). This dose may be increased until there are no presentations of seizures, without surpassing the maximum dose or resulting in adverse events, and if seizures continue to occur, a different or additional AED will be chosen and implemented (Kanner & Bicchi, 2022).

Drug Name	Year	Generation
Bromides	Early 1900	First
Phenobarbital	1912	First
Phenytoin	1937	First
Corticotropin/ACTH	1952	First
Primidone	1954	First
Methsuximide	1957	First
Ethotoin	1957	First
Diazepam	1958	First
Ethosuximide	1960	First
Clorazepate	1972	First
Carbamazepine	1974	First
Clonazepam	1975	First
Valproate	1978	First
Lorazepam	1980	First
Felbamate	1993	Second
Gabapentin	1993	Second
Lamotrigine	1994	Second
Topiramate	1996	Second
Tiagabine	1997	Second
Levetiracetam	1999	Second
Oxcarbazepine	2000	Second
Zonisamide	2000	Second
Pregabalin	2004	Second
Lacosamide	2008	Third
Rufinamide	2008	Third
Vigabatrin	2009	Third
Clobazam	2011	Third
Ezogabine	2011	Third
Perampanel	2012	Third
Eslicarbazepine acetate	2013	Third
Brivaracetam	2016	Third
Cannibadiol	2018	Third
Stiripentol	2018	Third
Cenobamate	2019	Third
Fenfluramine	2020	Third

Table 1: Introduction of major AEDs in the United States and their corresponding generations. (Information derived from Rudzinski et al. 2016 and http://www.cureepilepsy.org).

1.2 Microbiome

The human microbiota encompasses all the microorganisms living on and within the human body. These microorganisms include but are not limited to bacteria, viruses, and fungi. There are at least as many microbial cells in the human microbiome as there are human cells in the body (Sender et al., 2016). The genome of these microbiota is known as the microbiome and is often how microbiota are studied. There are many body sites of which microbiota can colonize, including the skin, oral cavity, esophagus, urogenital tract, and gut. The gut microbiome has become a major topic of study as it can play a large role during human development and in disease.

The gut microbiota is involved in aiding nutrient absorption, promoting the maturation of immune cells and functions, and protection against pathogens. The development of the gut microbiota begins at birth and can be affected by mode of birth (Dominguez-Bello et al., 2010). The human gut microbiota becomes stable at two to three years of age and remains stable until elderly age (Clemente et al., 2012). Many factors can impact the gut microbiota throughout an individual's life, including but not limited to diet, exercise, medications, and biological sex (Figure 1). An altered composition of the gut microbiota as well as specific pathogens have been linked to a variety of diseases including gastric cancer, autism, obesity, type 2 diabetes, and irritable bowel diseases (Clemente et al., 2012). These links have pushed researchers to determine if the gut microbiota can play a causal role in human health and disease.

Although many microorganisms makeup the gut microbiome, bacteria are the most commonly studied. Bacterial classification follows the Linnean taxonomy structure, starting with kingdom, followed by phylum, class, order, family, genus, and ending with species. The human gut microbiota is comprised of 6 major phyla, Firmicutes and Bacteroidetes being the dominate phyla, followed by Actinobacteria, Verrucomicrobia, Proteobacteria, and Fusobacteria.

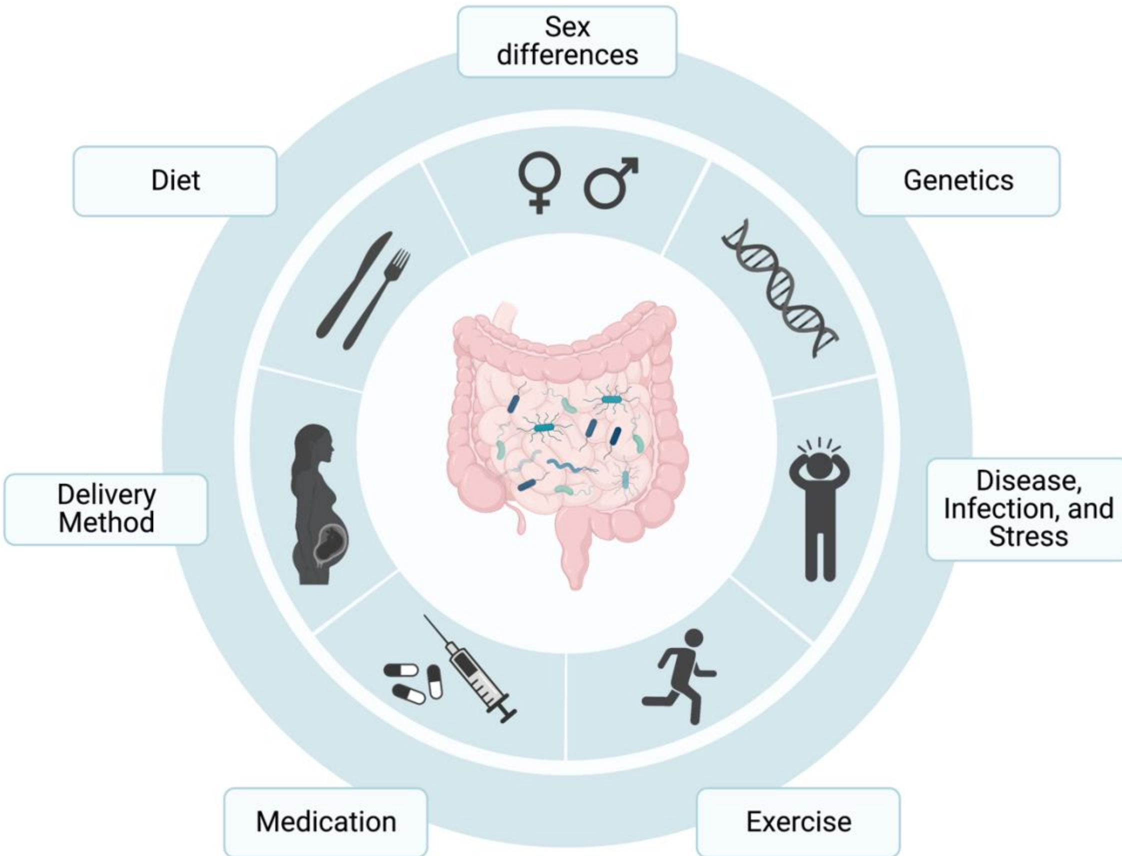

Figure 1: Factors impacting the gut microbiome. Biological sex, genetics, disease, infection, stress, exercise, medication, delivery method, and diet can all impact the gut microbiome. Adapted from "Gut-Brain Axis Regulators", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

1.3 Epilepsy and the gut microbiome

Gut-brain axis describes the bidirectional communication between the gastro-intestinal tract and the CNS (Figure 2). This communication is mediated by direct and indirect pathways, including the enteric nervous system, the vagus nerve, the neuroendocrine system, the hypothalamic-pituitary adrenal (HPA) axis, the immune system, and metabolic pathways (Morais et al., 2021). The gut microbiome is thought to contribute to the gut-brain axis by impacting the immune system, stimulating the vagus nerve, and releasing metabolites. Some metabolites released by the gut microbiota are short-chain fatty acids (SCFAs) which are lipids produced by bacteria via fermentation of dietary fiber. SCFAs have been shown to impact behavior and disease in animal studies (Silva et al., 2020; Tan et al., 2014). In addition to SCFAs, some bacteria can also produce GABA and serotonin, both of which are able to impact the enteric nervous system and possibly CNS levels of these two neurotransmitters, although neither readily cross the blood brain barrier (Morais et al., 2021). In addition to this direct impact from the gut microbiota, beneficial bacteria can have anti-inflammatory and immunomodulatory effects (Cristofori et al., 2021).

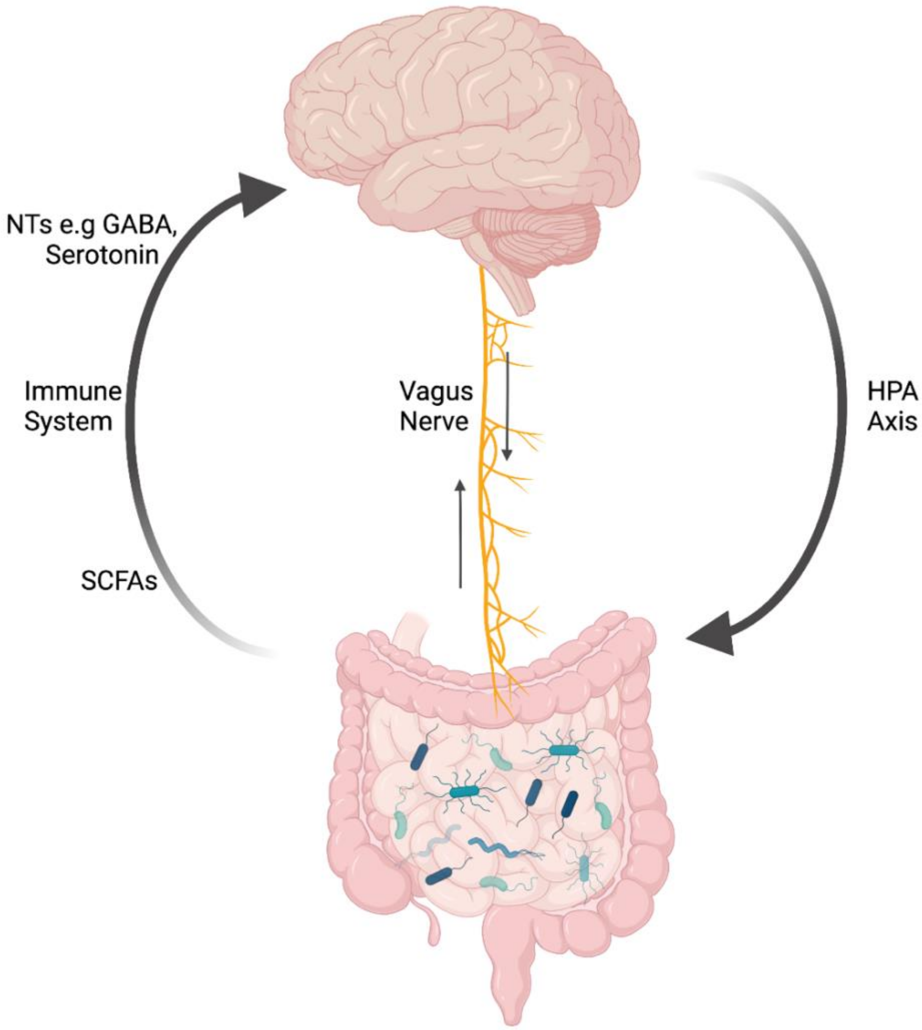

Figure 2: The gut-brain axis. The gut-brain axis is a bidirectional communication network between the enteric and central nervous system. The enteric nervous system communicates with the brain via metabolites and neurotransmitters released from the gut microbiota. In addition, the gut microbiota can interact with the immune system and stimulate the vagus nerve. In turn, the HPA axis can impact the enteric nervous system. Created with BioRender.com.

The gut microbiome is not only responsible for peripheral development, but CNS development as well. In the absence of the gut microbiome, mice have shown reduced blood-brain barrier integrity, increased myelination in the pre-frontal cortex, and reduced maturity of microglia, the resident immune cell of the brain (Sharon et al., 2016). This has led researchers to pursue the possible impact gut microbiota may have on CNS diseases. Due to the growing body of evidence that the gut microbiome can impact CNS diseases, researchers have become interested in exploring the relationship of the gut microbiome and epilepsy.

1.4 16S rRNA gene sequencing and analysis

The most common way to analyze the gut microbiome is to extract and sequence DNA from the bacteria. There are a few ways to sequence bacterial DNA, including shotgun metagenomic sequencing and 16S rRNA gene sequencing (Table 2). I utilized 16S rRNA gene sequencing as it was most suitable to address these experimental questions. The benefits of 16S rRNA gene sequencing is its accessibility and affordability compared to other sequencing methods. In addition, 16S rRNA gene sequencing produces more manageable amounts of data to analyze, which makes it easier to process than the other sequencing methods that produce larger amounts of data. This is because 16S rRNA gene sequencing use PCR amplification to target the 16S rRNA gene in bacteria, which is then sequenced, rather than sequencing the entire genome.

16S rRNA gene sequencing	Shotgun metagenomic sequencing

Advantages:
• Low cost
• Fast
• Small data set
  • Requires beginner to intermediate experience to analyze
	Advantages:
	• High resolution
	  • Identifies species and even strain level resolution
	  • Identifies genes, mutations, and SNPs

Disadvantages:
• Low resolution
  • Lacks species level resolution
  • Cannot identify changes in the genome (e.g., antibiotic resistance genes)
	Disadvantages:
	• High cost
	• Slow
	• Large data set
	  • Requires intermediate to advanced experience to analyze

Table 2: Advantages and disadvantages of DNA sequencing. The two main sequencing methods to analyze the gut microbiome are 16S rRNA gene sequencing and shotgun metagenomic sequencing. As time progresses, these sequencing methods will become even more cost effective and accessible, in addition, new technology continues to emerge, such as that of long-read sequencing (i.e. Nanopore), an intermediary approach to gut microbiome analysis.

The 16S rRNA gene is unique to bacteria and made up of constant and variable regions (Fukuda et al., 2016) (Figure 3). These characteristics allow for universal primers to be made targeting the constant regions, but bacterial identification possible analyzing the variable regions as these can be unique to each bacterium. This process generates less data than the other sequencing methods which requires sequencing the whole bacterial genome. Unfortunately, it is these characteristics that lead to the low resolution associated with 16S rRNA gene sequencing. 16S rRNA gene sequencing only leads to the identification of bacteria, with reliable resolution down to the genus level, unlike whole genome sequencing which can not only identify down to the species level, but even down to the strain level while also evaluating specific genes within the bacterial genome, such as antibiotic resistance genes. In summation, 16S rRNA gene sequencing is accessible and affordable, but results in lower resolution data when analyzing the gut microbiome.

Figure 3: 16S rRNA gene. The 16S rRNA gene is ~1500 bp and consists of 9 variable regions. The 16S rRNA gene encodes for the 30S ribosomal subunit in prokaryotes. The green boxes represent the variable regions while the blue portions are the conserved regions. The solid lines show the forward and reverse primers used to amplify the V4 and V5 regions, the dotted line demonstrates the sequence obtained during amplification. Image is meant to be representative and is not to exact scale. Created with BioRender.com.

Another benefit of 16S rRNA gene sequencing is the fast and streamlined process from sample collection to sequencing (Figure 4). For microbiome analysis, it is important to prevent any bacterial contamination during the collection and processing of fecal samples, this will limit variability in the data as any bacterial contamination will also be sequenced with the bacteria present in the fecal sample. In addition to reducing bacterial contamination during collection and processing, focus is aimed at reducing DNA extraction bias, which occurs as some bacteria are easier to lyse than others. This can be avoided by using an extraction method that reduces lysing bias, as well as utilizing controls to measure any potential biases.

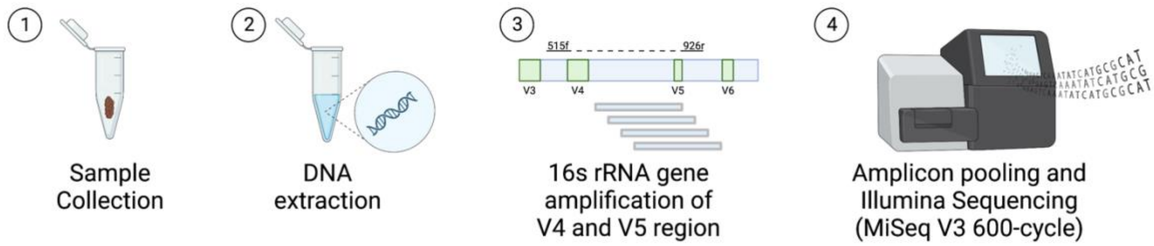

Figure 4: 16S rRNA gene sequencing process. The 16S rRNA gene sequencing process begins with appropriate sample collection. Next, DNA is extracted from the samples and used for amplification of the 16S rRNA gene. Amplicons are then pooled and used for sequencing in the Illumina MiSeq V3 600-cycle. Created with BioRender.com.

1.5 Development of QIIME2 pipeline

QIIME2 is a microbiome informatics platform that is free, open source, and community developed. I developed an in-house pipeline that is used to analyze microbiome data. The pipeline starts from sample collection, using sterile tubes to ensure there is no bacterial contamination. Samples are then processed with the ZymoBIOMICS MiniPrep Kit, which has a low lysing bias. In addition, during the sample processing, two controls are introduced, a known microbial cell control to determine if contamination occurred during DNA extraction, and a known microbial DNA control to determine if the sequencing and analysis pipeline contain any biases. The pipeline uses DADA2 to clean, trim, and denoise the sequences. Parameters used during trimming include trimming if Phred (Q scores) drop below 20, meaning there is an error rate in every 1 out of 100 base pairs (bp). Next, amplicon sequence variants (ASVs) are generated using the SILVA database to build a phylogenetic tree. Then, a SILVA classifier that has been trained to the 515F and 926R primer set is used to compare the ASVs to, to determine the bacteria. Using a trained classifier in the pipeline, rather than the entire SILVA database, allows for less non-specific results, as in theory, the only amplicons available to analyze should be between the 515th-926th base pairs in the 16S rRNA gene.

1.6 Microbiome analysis

Before exploring the results of this dissertation, it would be useful to understand some of the analyses performed on the microbiome data. The most common and core analysis of microbiome data utilizes the following metrics: alpha diversity, beta diversity, and relative abundance. These metrics have been used by ecology researchers to study communities and ecosystems, so naturally as the microbiome is a community and ecosystem, these metrics were easily extended to studying the microbiome.

Alpha diversity is a metric that is used to measure the species richness or evenness within one sample (Pyron, 2010). This would mean that for one given fecal sample, the evenness or the richness of that sample would be measured. Species evenness refers to the distribution of abundance of species within the sample, increasing when species are evenly distributed, and decreasing when there are larger variances between species abundance. Species evenness can be measured in several ways; for this data, the Shannon index was used to measure species evenness. Next, species richness refers to the number of species in a sample. This can be measured using operational taxonomic units (OTUs), which can be used as a proxy for the number of types of bacteria. Since the gut microbiome is a complex ecosystem, there may not be a set evenness or richness measurement associated with a “normal” gut microbiome, leading to the importance of controls in gut microbiome analysis, as the data will often be in reference to how it compares to the controls.

Beta diversity is a metric that is used to measure the composition of the sample in relation to other samples (Pyron, 2010). There are many metrics that measure beta diversity, each metric considers different measurements of importance. This data was analyzed using the UNIFRAC beta diversity metric, which considers the phylogenetic relatedness of bacteria in its compositional analysis. Moreover, it contains two statistical tests, unweighted UNIFRAC, which only considers the presence and absence of bacterial species, and weighted UNIFRAC, which considers the abundance of each of those species. Both metrics are useful in their own ways when assessing beta diversity in a population. This allows us to determine if there are species differences as well as differences in abundance among the species in the populations. The output of UNIFRAC tests are principal coordinate analysis (PCoA) plots. These plots are 3-dimensional and show the amount of variance in the population. The axes are in percentages, each axis shows the amount of variance in the population, along that axis. What factors belong to the variance explained along an axis is unknown. In addition, the variance along an axis is calculated for maximum explanatory value. To further elucidate potential variance, additional analysis is required, usually by analyzing relative abundance.

Relative abundance is a metric used in gut microbiome analysis that compares the percent of a bacteria within a sample to the total composition within that sample. Relative abundance is an important metric in microbiome research in contrast to absolute abundance. Absolute abundance shows the total amounts of each bacterium, which can be important if the bacteria have effects if certain amounts are reached, such of which can occur with quorum sensing or infection dose required for virulence. However, relative abundance can be used to describe the ecosystem in which bacteria interact with each other, which considers how much bacteria are present in relation to other bacteria, an important metric when determining the impact bacteria have on their ecosystem. Lastly, there is a technological limitation with sequencing data that reduces the ease of obtaining absolute abundance data, but can output relative abundance, resulting in this metric being of more common use.

Chapter 2: Review of Literature

2.1 Epilepsy and the gut microbiome

Over the last decade, research has expanded our understanding of the relationship between the gut microbiome and epilepsy. This interest was prompted by data showing that the ketogenic diet is an effective alternative therapy to reduce seizures in drug-resistant epilepsy. In addition, there are numerous reported comorbidities with epilepsy and irritable bowel syndrome (IBS), which has been associated with alterations in the gut microbiome (Aydemir et al., 2020; Camara-Lemarroy et al., 2016). These and other studies led researchers to start questioning the contribution of gut microbiota and the gastrointestinal tract in epilepsy.

The ketogenic diet is a diet that was born out of the original treatment for epilepsy developed in the early 1900s, starvation, which reduced seizure frequency (Kossoff et al., 2011). Due to the impracticality of this treatment, the ketogenic diet was designed to mimic the state of starvation, where the body switches to fat sources for fuel instead of the preferred source of carbohydrates. The ketogenic diet allows minimal carbohydrates and high amounts of fat, with moderate to low amounts of protein. By maintaining low levels of carbohydrates, the body will enter a state of ketosis, where it produces ketone bodies from the processing of fatty acids by the liver. As some forms of epilepsy are considered metabolic, this alternative diet was thought to change the metabolic state to aid in seizure reduction (Lin Lin Lee et al., 2018). In addition to this diet related link to epilepsy, analysis of a large cohort of people diagnosed with IBS revealed that individuals with IBS had a greater cumulative incidence of epilepsy than those without IBS (C.-H. Chen et al., 2015). Together, these findings led researchers to pursue studies investigating the possibility that the gut microbiota may play a role in the development of epilepsy.

2.1.1 Human studies

As the cost of sequencing decreased and the accessibility of 16S rRNA gene sequencing and analysis increased, there is now easier access to utilize this technique to study the potential links between the gut microbiome and epilepsy. Since 2017, there have been a multitude of studies assessing the gut microbiome in patients with epilepsy. One of the first assessments of the gut microbiome in patients with epilepsy was conducted in a pediatric population with drug-resistant epilepsy using 16S rRNA gene sequencing and although the focus of the study was on the impact of the ketogenic diet, it showed that pediatric patients with drug-resistant epilepsy had altered gut microbiomes compared to healthy controls (Xie et al., 2017). In 2018, Peng and colleagues investigated the relationship between drug-resistant patients, drug-sensitive patients, and healthy controls using 16S rRNA gene sequencing (Peng et al., 2018). Evaluating 42 patients with drug-resistance, 49 drug-sensitive patients, and 65 healthy controls, they found that the gut microbiomes of drug-sensitive patients with epilepsy were more like that of healthy controls without epilepsy than they were to drug-resistant patients with epilepsy. Not only did they observe this in the evenness metric of alpha diversity, but they also saw specific bacterial differences between the drug-resistant and drug-sensitive groups. Moreover, patients with four seizures or less per year had more similar alpha diversity metrics than those who had more than four seizures per year. Along with other bacterial changes, they also showed that patients with four or fewer seizures per year had more *Bifidobacteria* and *Lactobacillus,* two genera of bacteria that are known for their potential beneficial effects. To build on the results from this study, Gong and colleagues also investigated the gut microbiome of patients with epilepsy, but this time included healthy controls from the same household (Gong et al., 2020). This allowed for further control of environmental factors that do contribute to the composition of the gut microbiome. They found similar results that showed that the gut microbiome of epileptic patients differed from healthy controls. One striking similarity to the study conducted by Peng and colleagues was the increase in the rarer phylum, Verrucomicrobia in the disease population. Since these initial studies, more studies have been conducted to assess changes in the gut microbiome in patients with epilepsy and found similar and differing results to the previously published studies. Similarities among the studies were that the epileptic population were more like themselves and different than healthy controls, as well as an alteration in the relative abundance of major phyla and reductions in beneficial bacteria. To date, there are over 10 studies documenting the alterations in the gut microbiome in patients with epilepsy, most of which have been well summarized for further reading (Ding et al., 2021; Lum et al., 2020).

Although many of these studies are observations, there have been some case studies that led researchers to infer a stronger link between the impact of the gut microbiome on seizures in humans. Braakman and van Ingen reported 6 case studies from their practice, wherein patients with epilepsy achieved temporary seizure freedom during antibiotic treatment (Braakman & van Ingen, 2018). Specifically, 5 out of 6 reached 100% seizure freedom while the remaining patient achieved substantial but not complete seizure freedom, however, seizures returned for all patients within 2 weeks after ending antibiotic treatment. Another group also presented a patient who was diagnosed with autism and epilepsy in which seizure episodes were reported to be dramatically decreased following antibiotic treatment (Ghanizadeh & Berk, 2015). Lastly, a patient with a 17-year history of epilepsy received a fecal matter transplant (FMT) with the goal of treating symptoms of Crohn’s Disease (He et al., 2017). After receiving the first FMT, the patient stopped taking sodium valproate, the AED used for seizure treatment, and remained seizure free throughout a 20-month follow-up. As of today, there is an on-going clinical trial using FMTs to treat seizures in people with epilepsy (NCT02889627). In addition to FMTs, researchers have shown in clinical trials that the administration of probiotics caused a greater than 50% reduction of seizures in 28.9% of patients treated (Gómez-Eguílaz et al., 2018). These case studies and clinical trial further strengthen the idea that the gut microbiome may play a role in seizure occurrence.

2.1.2 Animal studies

Although human studies showed consistent correlation for the gut microbiome's role in epilepsy, there was a lack of a causal relationship. To address this, animal models were utilized to better determine if the gut microbiome played a role in epilepsy, specifically focusing on the use of the ketogenic diet as a treatment. The mechanism involved in the ketogenic diet's effectiveness via altered metabolic processes, potential anti-seizure effect of ketone bodies, or perhaps the gut microbiome has been previously debated with no conclusive data to support one mechanism. However, in 2018, Olson and colleagues conducted a study showing that the ketogenic diet's efficacy in decreasing seizure susceptibility may also work by increasing the gut bacteria, *Akkermansia muciniphila* and *Parabacteroides*, which ultimately altered the levels of GABA and glutamate in the brain of mice (Olson et al., 2018). Following this seminal paper, Medel-Matus et al. showed that transplant of the gut microbiome of stressed rats, increased the susceptibility to seizures in naïve rats (Medel-Matus et al., 2018). Most recently it was shown that the gut microbiome is altered in a genetic epilepsy rat model prior to seizure development, suggesting the possibility that in a genetic model, the gut microbiome plays a role in epileptogenesis, versus being altered due to seizures (Citraro et al., 2021).

2.2 AEDs and the gut microbiome

As there is no cure for epilepsy, the first line of treatment remains symptom management. Epilepsy being hallmarked by seizures, means that this symptom management is the complete reduction in seizure occurrence. The goal of AEDs is to render a patient seizure free, however, about one third of the epileptic population is deemed drug resistant. Of interest, this number remains unchanged even with the increasing number of AEDs that have come to market (Z. Chen et al., 2018). Due to this persistent drug resistance, several outstanding questions remain, leading researchers to identify alternative therapies in replacement or in conjunction with AEDs in hopes of alleviating seizures in this population. One possible avenue for therapeutic intervention for patients with drug-resistant epilepsy may be the gut microbiome. However, due to the complexity of AED usage, the relationship of AEDs and the gut microbiome have been understudied, despite the increase in studies examining the impact of non-antibiotic drugs on the gut microbiome (Maier et al., 2018; Zimmermann et al., 2019).

2.2.1 In vitro

Earlier studies established the possibility that AEDs could interact with some bacteria in culture, particularly the antimicrobial effects of sodium valproate (Esiobu & Hoosein, 2003). In addition, other studies revealed that ammonium derivatives of lamotrigine have antimicrobial properties and lamotrigine can inhibit bacterial ribosome biogenesis in *Escherichia coli* (Qian et al., 2009; Stokes et al., 2014). In 2018, a landmark paper by Maier and colleagues conducted a large scale study showing the possibility that many non-antibiotic drugs had antimicrobial effects (Maier et al., 2018). Unfortunately, of the more than 1000 drugs screened, only a few were antiepileptic drugs, none of which showed antimicrobial activity in the chosen gut isolates

(Maier et al., 2018). However, this study did re-ignite interest into the potential antimicrobial impact of antiepileptic drugs. In 2022, Ilhan and colleagues embarked on a large scale study examining the potential antimicrobial effects of nine antiepileptic drugs and four syrup formulations often used when delivering the AEDs to children (Ilhan et al., 2022). The group found that carbamazepine, lamotrigine, and topiramate reduced the growth of ten selected strains (Ilhan et al., 2022). Lastly, carbamazepine was shown to impact the gut microbiome of soil collembolan (Y.-F. Wang et al., 2020).

2.2.2 In vivo

There has been only one study that examined the impact of AEDs on the gut microbiome in humans, and a few studies that have observed the potential impact in rodents. In 2022, Gong and colleagues recruited 10 patients who were newly diagnosed with epilepsy and given valproic acid to treat their seizures. Their fecal samples were collected before the start of the treatment and three months after treatment to compare the potential impact of an AED on the gut microbiome in humans (Gong et al., 2022). The researchers found an alteration in the ratio of two major phyla, *Firmicutes* and *Bacteroidetes* (Gong et al., 2022). While this is an interesting finding and a first step into uncovering the potential impact of AEDs on the gut microbiota in humans, it is difficult to separate the other potential contributing factor, which is that valproic acid reduced seizures at least 90% and in some cases, 100%. Previous human studies have shown that amount of seizures per year may contribute to gut microbiota alteration, meaning it would be necessary to determine if the alteration in the gut microbiome seen by Gong et al. were due to the drug itself or the effects of the drug on seizure occurrence (Peng et al., 2018).

Early studies in rodents showed that the AEDs, clonazepam and zonisamide, were being metabolized by the gut microbiota of rats, and that *Clostridium sporogenes* was the culturable species with the most reductase activity towards zonisamide (Elmer & Remmel, 1984; Kitamura et al., 1997). It was not until two decades later that another study by Cussotto and colleagues looked at the impact of psychotropics on the gut microbiome. Although this study focused on drugs primarily used for psychiatric disorders, it included the AED valproate, which did not inhibit bacterial growth *in vitro* but did alter the alpha and beta diversity of rodents when supplemented in their food (Cussotto et al., 2019). Specifically, valproate decreased S24-7 and increased a *Ruminococcaceae* species as well as altered the levels of SCFAs, propionate, butyrate, and isovalerate. Lastly, as previously discussed, Citraro and colleagues demonstrated that gut microbiome alterations precede disease onset in a genetic model of absence epilepsy in rats, but more specifically they also showed that transplantation of the gut microbiome of epileptic rats treated with the AED ethosuximide was sufficient in reducing seizures in untreated epileptic rats. The researchers demonstrated that there was no drug present in the transplanted gut microbiome and that the gut microbiome itself was the cause, rather than remnants of the AED. These researchers theorize that the AED might alter the gut microbiome, which aids in its anti-seizure effect, however, they were unable to separate the possibility that the reduction in seizures drove the gut microbiome alteration (Citraro et al., 2021).

2.3 Gaps in knowledge

To date, the field of research examining the relationship of the gut microbiome and epilepsy has made major strides in determining the link between how the gut microbiome may play a role in the development of seizures in animal models and exploring the compositional differences in the gut microbiome of patients with epilepsy. However, with these major strides in teasing out the mechanisms in which the gut microbiome may impact epilepsy, little research has focused on how AEDs impact the gut microbiome *in vivo*. Many studies have looked at the impact of AEDs *in vitro* but these present limitations as they remove the complex ecosystem of the gut microbiome by singly culturing gut bacterial isolates, while also creating a selection bias for culturable bacteria. In addition, these studies have mainly focused on the antimicrobial impact of AEDs and not the potential beneficial effects, if any, they may have on the gut microbiome. My work aims to fill the gaps in knowledge of whether AEDs impact the gut microbiome *in vivo*, and whether those changes impact the development of epilepsy.

Chapter 3: Experimental Design

3.1 Experiment 1: How do AEDs impact the gut microbiome in naïve mice

The first experiment was designed to determine if AEDs alter the gut microbiome in naïve mice. AED-induced microbial changes were first assessed in naïve mice and not in a seizure model to distinguish AED-induced microbial changes from seizure associated changes. Topiramate was chosen to evaluate as it is a widely prescribed AED. Topiramate was placed in the drinking water as it would be the easier and less stressful experimental choice, although the disadvantage is that the amount of AED ingested would be an estimate based on the normal drinking patterns of mice. Five weeks were chosen as the length of time for topiramate exposure, as it typically takes two weeks for alterations in the gut microbiome to occur in response to antibiotic treatment. With this in mind, I wanted to allow for longer time for changes to occur with a non-antibiotic drug. In addition, I chose to house the mice in pairs of two, as I believe this would minimize cage effect and maximize n's, this is because mice are coprophagic and eat their own feces, therefore, animals within the same cage will typically share the same gut microbiome and can often be evaluated as a single n. After the 5 weeks, I evaluated the final fecal sample collected and compared the microbial composition to the controls. The results from experiment one showed that ingestion of topiramate in the drinking water increases *Lactobacillus johnsonii* in the gut microbiome of naïve mice.

3.2 Experiment 2: How does *Lactobacillus johnsonii* impact seizure susceptibility

Due to the increase of *Lactobacillus johnsonii* in naïve animals from topiramate ingestion, I wanted to determine if *Lactobacillus johnsonii* played a role in seizure susceptibility. I chose the pentylenetetrazol (PTZ)-kindling model because it is commonly used to evaluate seizure activity in response to novel anti-epileptic drugs. PTZ is a chemical kindling agent that acts as a $GABA_A$ receptor antagonist, meaning that it blocks $GABA_A$ receptor activity, ultimately resulting in the loss of inhibitory signals (Shimada & Yamagata, 2018). The loss of inhibitory signals results in an imbalance in the control of excitatory signals, which can cause seizures. This model measures the susceptibility to seizures by using repeat injections of a sub-convulsive dose of PTZ, resulting in seizures over time. Using this model, an efficacious AED would ultimately result in no seizure development over the course of injections.

I chose to evaluate PTZ-induced seizures in four groups, control saline, topiramate alone, *Lactobacillus johnsonii* alone, and cotreatment of topiramate and *Lactobacillus johnsonii*. These groups would allow me to determine what changes may be due to the drug, bacteria, or synergistic effect. The groups that were to receive *Lactobacillus johnsonii* were pretreated for one week to allow time for the bacteria to colonize in the gut, all other groups received saline as a control. After one week of pretreatment, all animals received a gavage once a day with their assigned treatment and were injected with a sub-convulsive dose of PTZ one hour after gavage, every other day, allowing for topiramate to be at peak levels in the system for maximum efficacy. After injection, animals were video monitored and scored for 30 minutes. Data collected from this experiment would determine if *Lactobacillus johnsonii* could reduce susceptibility to seizures.

3.3 Experiment 3: How does topiramate impact the growth of *Lactobacillus johnsonii in vivo*

As an increase in *Lactobacillus johnsonii* was observed in the gut of naïve mice after topiramate ingestion, further studies were needed to elucidate if topiramate had a direct impact on the growth of *Lactobacillus johnsonii*. To do this, *Lactobacillus johnsonii* was grown with differing concentrations of topiramate, including physiological relevant doses, and higher, non-physiologically relevant doses. There were trends in the rate of growth of *Lactobacillus johnsonii* when exposed to topiramate, however, further studies are now needed to determine if topiramate acts as a fuel source for the bacteria, or if it impacts the growth through other mechanisms.

Chapter 4: Methods

4.1 Animals

Animals were housed and handled according to the guidelines of the National Institutes of Health Committee on Laboratory Animal Resources. Prior approval of the Virginia Polytechnic Institute and State University institutional animal care and use committee was obtained for all experimental protocols. C57BL/6J mice aged 7-8 weeks were purchased from Jackson Laboratory. Mice were allowed to acclimate for 7 days prior to the start of experiments and were provided water and chow ad libitum. Animals were housed in a facility with ambient temperature providing a 12-h light/dark cycle. All efforts were made to minimize animal pain and number of animals.

4.2 Topiramate treatment and fecal collection

Topiramate (Sigma Aldrich 1672206) (80 mg/kg) was dissolved into the drinking water of mice and given ad libitum for 5 weeks. Water was changed every 2-3 days. Once a week, mice were weighed, and fecal samples were collected in DNA-free Eppendorf tubes (Eppendorf 022600028). Fecal samples were immediately put on dry ice and stored at -80°C until processing. Mice were housed two per cage to reduce cage effect.

4.3 16S rRNA sequencing and analysis

DNA was isolated from fecal samples using the ZymoBIOMICS DNA Miniprep Kit (Zymo D4300) following the manufacturer’s protocol. 16S rRNA sequencing analysis was performed as previously described (Gallucci et al., 2021). The universal primers 515F and 926R were used to amplify the V4-V5 region of the 16S rRNA gene per the Earth Microbiome Project protocol (https://www.earthmicrobiome.org/). The V4-V5 regions were sequenced using the MiSeq v3 600-cycle kit on the MiSeq platform (Illumina) resulting in 2 x 300 paired-end sequences.

The raw sequences were processed and analyzed with QIIME2 v2020.2 (Bolyen et al., 2019). Forward and reverse reads were quality filtered, trimmed, and joined with DADA2, as well as used to denoise joined reads to amplicon sequence variants (ASVs) (via q2-dada2) (Callahan et al., 2016). ASVs were aligned to a phylogenetic tree using an insertion method to the SILVA 128 SEPP reference database (via-q2-fragment-insertion-sepp) (Janssen et al., 2018). Alpha and beta diversity metrics were calculated using samples rarified to 45,881 and 26,986 sequences per sample, respectively (via q2-diversity).

4.4 SCFA analysis

Thawed samples were weighed and diluted in distilled water in a ratio of 1 g cecal content to 2 mL of distilled water, vortexed for 3 minutes at max speed using the Vortex Genie 2 and left to rest overnight at 4°C. Samples were then centrifuged at 1000 × g for 5 min and the supernatant was collected. Next, 0.1 mL of supernatant was acidified with 0.17 mL of metaphosphoric acid (25%, w/v), and 0.13 mL of internal standard (5 mmol, 4-methyl-valeric acid, Sigma, St. Louis, MO) was added, vortexed, and the solution was left to rest for 30 min at 4°C. Samples were then centrifuged at 3000 × g for 15 min. The supernatant was collected and used for SCFAs determination using a 6890 N Network GC System gas chromatograph (Agilent Technologies) equipped with a flame ionization detector as previously described (Izuddin et al., 2019). One microliter of the sample was injected at split 1:30, at a temperature of 230°C. Separation of SCFA profile was determined using Quadrex 007-10 Series (Quadrex Corp., New

Haven, CT 06525, USA) bonded phase fused silica capillary column (15 m, 0.250 mm internal diameter, 0.25 μm film thickness). The temperature of the column was set at 60°C held for 2 min; increased to 100 °C (10 °C/min), increased to 200 °C (20 °C/min), and held for 5 min. Nitrogen gas was supplied as carrier gas at 1 mL/min. The temperature of the detector was set at 230°C. Commercial standards (Sigma-Aldrich, St. Louis, MO) of acetic (45997), propionic (94425), iso-butyric (46935), butyric (19215), iso-valeric (78651), valeric (75054), and caproic (21529) acids were used as external standards for peak identification. The molar concentration of SCFA was identified based on a single point of internal standard and calibration curve with external standards.

4.5 *Lactobacillus johnsonii* isolation, colony PCR, qPCR

One fecal pellet from a C57BL/6J mouse was vortexed in 1 mL of sterile 1X PBS for 15 minutes at max speed. Next, the sample was centrifuged for 1 minute at 500 × g. Glycerol was added to the supernatant to make a 20% solution and stored at -80°C. This solution was sub-cultured overnight in MRS broth at 37°C. The sub-culture was plated on MRS agar and left to grow overnight at 37°C. Colonies were chosen at random for colony PCR, which were split to continue growth in MRS broth and for colony PCR. This process was repeated until one culture had all six randomly selected colonies positive for *Lactobacillus johnsonii*. This solution was then grown in MRS broth overnight at 37°C, centrifuged for 1 minute at 500 × g, and glycerol was added to make a 20% glycerol solution.

qPCR was performed using the DNA extracted from the fecal pellets as described in section 4.3. qPCR was performed using the Biorad SsoAdvanced Universal SYBR Green Supermix. 8 μL of DNA at 50 ng was used in a 20 μL reaction consisting of 1 μL of forward primer at 10 μM, 1 μL of reverse primer at 10 μM, and 10 μL of SYBR Green Master Mix. Reactions were performed using the Biorad CFX96 Touch Real-time PCR Detection System. The reaction was run at 98°C for 3 minutes and then 49 cycles at 98°C for 10 seconds, 60°C for 40 seconds for plate reading. Primers were synthesized by Invitrogen and are listed in Table 3.

4.6 PTZ-kindling and seizure scoring

C57BL/6J mice ~9 weeks of age were administered sub-convulsive doses of PTZ (Sigma Aldrich P6500) (35mg/kg) i.p. every other day for a total of 12 injections. PTZ was freshly dissolved in saline (0.9% NaCl). Mice were video monitored and scored for 30 minutes following each injection. Seizure scores were determined based on a modified Racine scale as follows: 0= no change; 1=immobilization; 2=head nodding, partial myoclonus; 3= continuous whole-body myoclonus, whole-body jerks; 4= rearing and falling, tonic seizure; 5=tonic-clonic seizure with wild rushing and jumping; 6= death (Shimada & Yamagata, 2018).

Prior to the start of PTZ injections, mice that were to receive *Lactobacillus johnsonii* (10^9 CFUs) as a treatment, either alone or in combination with topiramate, were given a once daily oral gavage for 7 days, all other mice received saline (0.9% NaCl). Mice were divided into four groups with 7 mice per group, saline (0.9% NaCl), *Lactobacillus johnsonii* (10^9 CFUs), topiramate (50 mg/kg), and *Lactobacillus johnsonii* (2.5×10^9 CFUs) + topiramate (50 mg/kg). Mice received an oral gavage daily (Braintree Scientific N-PK 010) of their respective treatments one hour prior to PTZ administration on PTZ injection days.

Topiramate was freshly dissolved in saline (0.9% NaCl). *Lactobacillus johnsonii* was sub-cultured in MRS broth at 37°C every morning from an overnight culture at a 1:100 ratio. The sub-culture was centrifuged after 4-5 hours of growth, at which the bacteria were in the exponential phase. 200 μL of the sub-culture was added to a 96-well plate to read the optical density at 600 nm on a microplate reader. CFUs were determined using a previously established standard curve.

4.7 Sample processing and Western blotting

Hippocampal samples were removed from the brain, immediately frozen, and stored at -80°C. Intestinal samples were dissected and contents were gently removed before freezing and storage at -80°C. Ileum samples were cut just before the cecum and measured 2 cm long, while proximal colon samples were cut just below the cecum and measured 1-2 cm long. Hippocampal and intestinal samples were processed using RIPA buffer. RIPA buffer contained 50mM Tris pH 8.0, 150 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, with 1% protease and phosphatase inhibitors added just before use. Samples were homogenized in a glass homogenizer and sat on ice for 30 minutes. Samples were centrifuged at 10,000 × g for 10 minutes at 4°C. The supernatant was collected and used for Western blotting.

As previously described (Orsi et al., 2019), samples (15-30 μg) were loaded on 7-20% acrylamide gels, ran through SDS-PAGE and transferred using a Turbo Transfer System (Biorad). Membranes were incubated in a 50:50 blocking buffer consisting of 50% Licor tris-buffered saline (TBS) blocking buffer and 50% 1X TBS + 0.1% Tween-20 (TBSt). Membranes were then incubated overnight at 4°C with primary antibody diluted in 50% Licor TBS blocking buffer and 50% TBSt. Primary antibodies included ZO-1 (Invitrogen 61-7300) (1:1000), Claudin-5 (Invitrogen 34-1600) (1:1000), MUC2 (Abcam ab272692) (1:1000), and β-actin (Cell signaling 4967S) (1:1000) the next day, membranes were washed 3 times for 10 minutes at room temperature with TBSt and incubated at room temperature in secondary antibody (1:40,000, Licor goat anti-rabbit 800CW) diluted in 50% Licor TBS blocking buffer and 50% TBSt for 45 minutes protected from light. Following the secondary antibody incubation, membranes were washed twice for 10 minutes each, protected from light. Lastly, membranes were soaked in 1X TBS and imaged using the LI-COR Odyssey Fc. Visualized proteins were analyzed using Image Studio Ver 5.2.

4.8 qPCR for hippocampal mRNA

RNA was extracted from hippocampal samples using the Qiagen AllPrep DNA/RNA Mini Prep Kit following manufacturer's instructions. RNA concentrations were measured via NanoDrop, normalized to 200 ng in a 20 μL reaction, and converted to cDNA using the Biorad iScript cDNA Synthesis Kit following manufacturer's instructions, final product was diluted in 60 μL of nuclease-free water for a final volume of 80 μL. qPCR was performed using the Biorad SsoAdvanced Universal SYBR Green Supermix. 2 μL of cDNA was used in a 20 μL reaction consisting of 0.5 μL of forward primer at 5 μM, 0.5 μL of reverse primer at 5 μM, 10 μL of SYBR Green Master Mix, and 7 μL of nuclease-free water. Reactions were performed using the Biorad CFX96 Touch Real-time PCR Detection System. The reaction was run at 95°C for 30 seconds and then 40 cycles at 95°C for 10 seconds, 60°C for 30 seconds for plate reading. Primers were synthesized by IDT and dissolved at 200 μM in nuclease-free water. Primer sequences are listed in Table 3.

Gene/Target	Primer	Sequence
β-actin	Forward	GCTCTTTTCCAGCCTTCCTT
	Reverse	GATGTCAACGTCACACTT
Bdnf	Forward	GTGTTTCTGGTGACAGCGTG
	Reverse	CCCACTTCCTCCCGCTTTAG
Caspase-3	Forward	ACATGGGAGCAAGTCAGTGG
	Reverse	CGTCCACATCCGTACCAGAG
IL-1β	Forward	TGGACCTTCCAGGATGAGGACA
	Reverse	GTTCATCTCGGAGCCTGTAGTG
IL-6	Forward	CTCTGGGAAATCGTGGAAAT
	Reverse	CCAGTTTGGTAGCATCCATC
IL-6R2	Forward	CCTGTGTGGGGTTCCAGAGGAT
	Reverse	CTGCCAGTATTCTCAGCAGCTG
Tnf-α	Forward	GGTGCCTATGTCTCAGCCTCTT
	Reverse	GCCATAGAACTGATGAGAGGGAG
Lactobacillus gasseri	Forward	TCAAGAGCTGTTAAGGCTGT
	Reverse	CTATCGCTTCAAGTGCTTTC
Lactobacillus johnsonii	Forward	AGAGAGAAACTCAACTTGAAATA
	Reverse	CCTTCATTAACCTTAACAGTTAA
Lactobacillus	Forward	TGGAAACAGRTGCTAATACCG
	Reverse	GTCCATTGTGGAAGATTCCC
Total bacteria	Forward	ACTCCTACGGGAGGCAGCAGT
	Reverse	ATTACCGCGGCTGCTGGT

Table 3: Primer sequences.

4.9 *In vitro* growth experiment

Lactobacillus johnsonii was cultured overnight in 5 mL of MRS broth at 37°C. The next day, the sample was sub-cultured into 20 mL of 10% MRS broth at 1:100 ratio with either no topiramate, 50 μM topiramate, 100 μM topiramate, 1000 μM, or 2000 μM topiramate. Samples were plated in a clear bottom 96-well plate with a PCR cover adhesive, incubated and shaking at 37°C for 40 hours in a microplate reader. OD600 was read every 30 minutes.

4.10 Statistics

All statistics were conducted in GraphPad Prism 9 and reported as mean $\pm$ SEM unless otherwise specified. Statistical tests used are reported with figures.

Chapter 5: Results

5.1 Topiramate alters the beta diversity of the gut microbiome in naïve mice

To evaluate the effect of topiramate on microbial composition, naïve C57BL/6J male mice (~9 weeks old) were treated with topiramate (80 mg/kg) or regular drinking water for five weeks. Their weight was monitored weekly, and there were no differences seen between control and topiramate-treated mice after five weeks, this is important as weight differences may lead to alterations in the gut microbiome (Figure 5). After five weeks of treatment, fecal samples were compared between the control and topiramate-treated mice using QIIME2 to analyze the V4 and V5 region of the 16S rRNA gene. Alpha diversity was measured with Shannon index for evenness and total Operational Taxonomic Units (OTUs) for richness. Beta diversity was measured using weighted and unweighted UNIFRAC statistical tests. Analyses of alpha diversity and beta diversity using an unweighted UNIFRAC statistical test were not altered due to topiramate treatment ($p=0.3450$; Figure 6A, $p=0.5481$; Figure 6B, $p=0.328$; Figure 6C). However, topiramate treatment did alter the beta diversity using a weighted UNIFRAC statistical test ($p=0.003$, 999 permutations, PERMANOVA; Figure 6D).

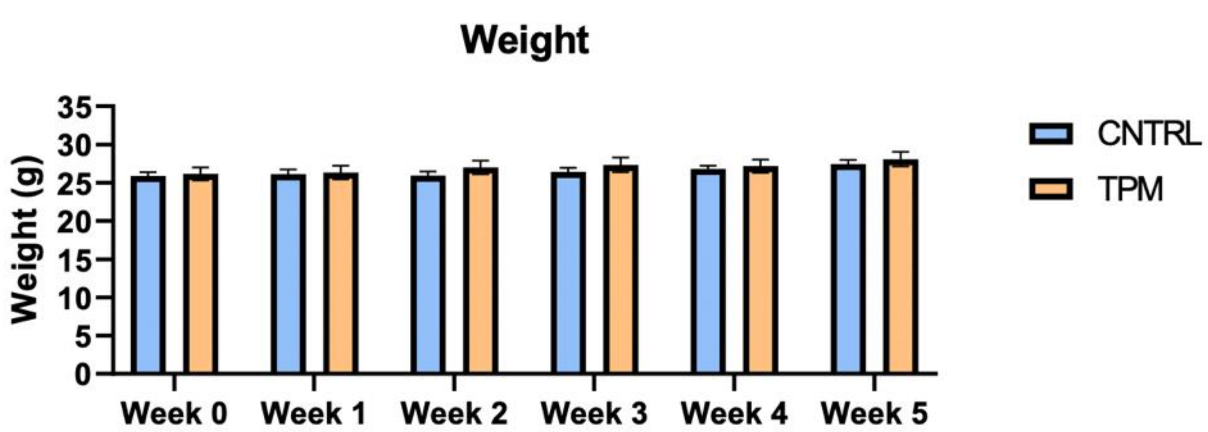

Figure 5: Weight of animals treated with topiramate. After 5 weeks, alterations in weight were not seen between control and topiramate treated mice. Data are shown as mean ±SEM. CNTRL n=8, TPM n=6

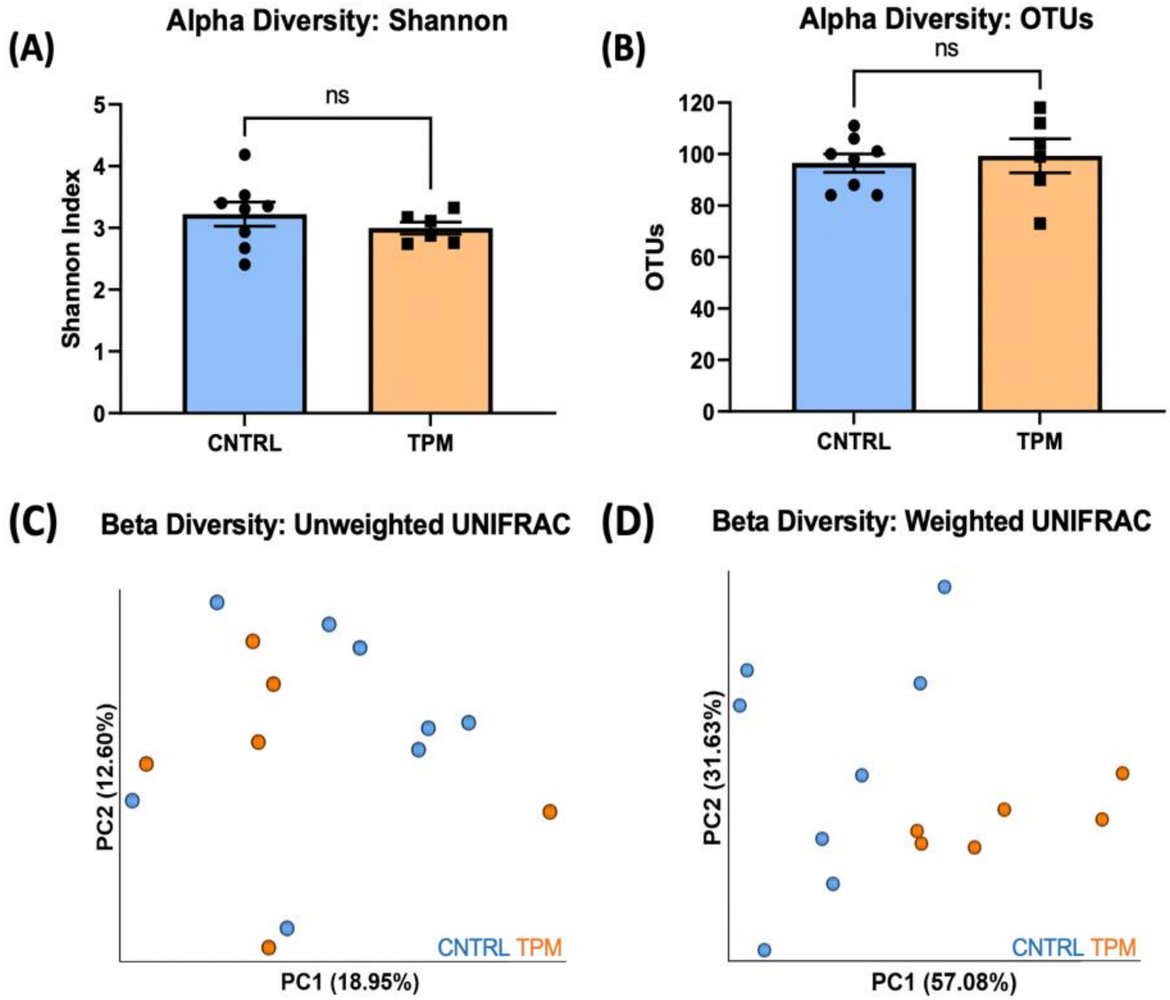

Figure 6: Alpha and beta diversity metrics comparing the gut microbiome of mice treated with topiramate or controls. No changes were seen in alpha diversity metrics using the (A) Shannon index or (B) total OTUs. No changes were seen in the (C) unweighted UNIFRAC statistical test to measure beta diversity, however there were significant differences in the (D) weighted UNIFRAC statistical test visualized by the principal coordinates (PC1 and PC2) analysis (PCoA). Each dot represents a fecal sample from an individual mouse, mice in the control group are blue and mice in the topiramate-treated group are orange. Data are shown as mean ±SEM.

5.2 Relative abundance of *Lactobacillus johnsonii* is increased in topiramate-treated mice

The significance in the weighted UNIFRAC but not unweighted UNIFRAC test suggested that there may not be differing bacteria between the two groups, but an alteration in abundance of specific microbes. Analysis of the relative abundance at the genus level revealed that topiramate treatment significantly increased the abundance of *Lactobacillus* (p=0.045971, multiple unpaired t tests with Welch Correction; Figure 7A). Due to the alteration of the genus *Lactobacillus* we sought to determine if there were any dominant species driving this change. Analysis of the four reported *Lactobacillus* species showed that *Lactobacillus johnsonii* was significantly different between the control and topiramate-treated groups (p=0.001196, multiple unpaired t tests with Welch Correction; Figure 7B). Phylogenetic mapping using a SILVA database originally listed this bacterium as *Lactobacillus gasseri* but was later confirmed to be *Lactobacillus johnsonii* via PCR (Figure 8). This was further established via qPCR and Linear Discriminant Analysis (LDA) effect size (LEfSe) method. Using validated primers, DNA extracted from fecal samples showed an increase in relative fold change of *Lactobacillus johnsonii* normalized to total bacteria in topiramate-treated mice (p=0.0375, unpaired t test; Figure 7C). In addition, LEfSe, using a linear discriminant analysis to identify significant features with an LDA score greater than 2, identified genus *Lactobacillus* as well as genus *Butyricicoccus* in the topiramate-treated group, and genus *Bacteroides* and *Turicibacter* and class *Erysipelotrichia* in controls (Figure 7D).

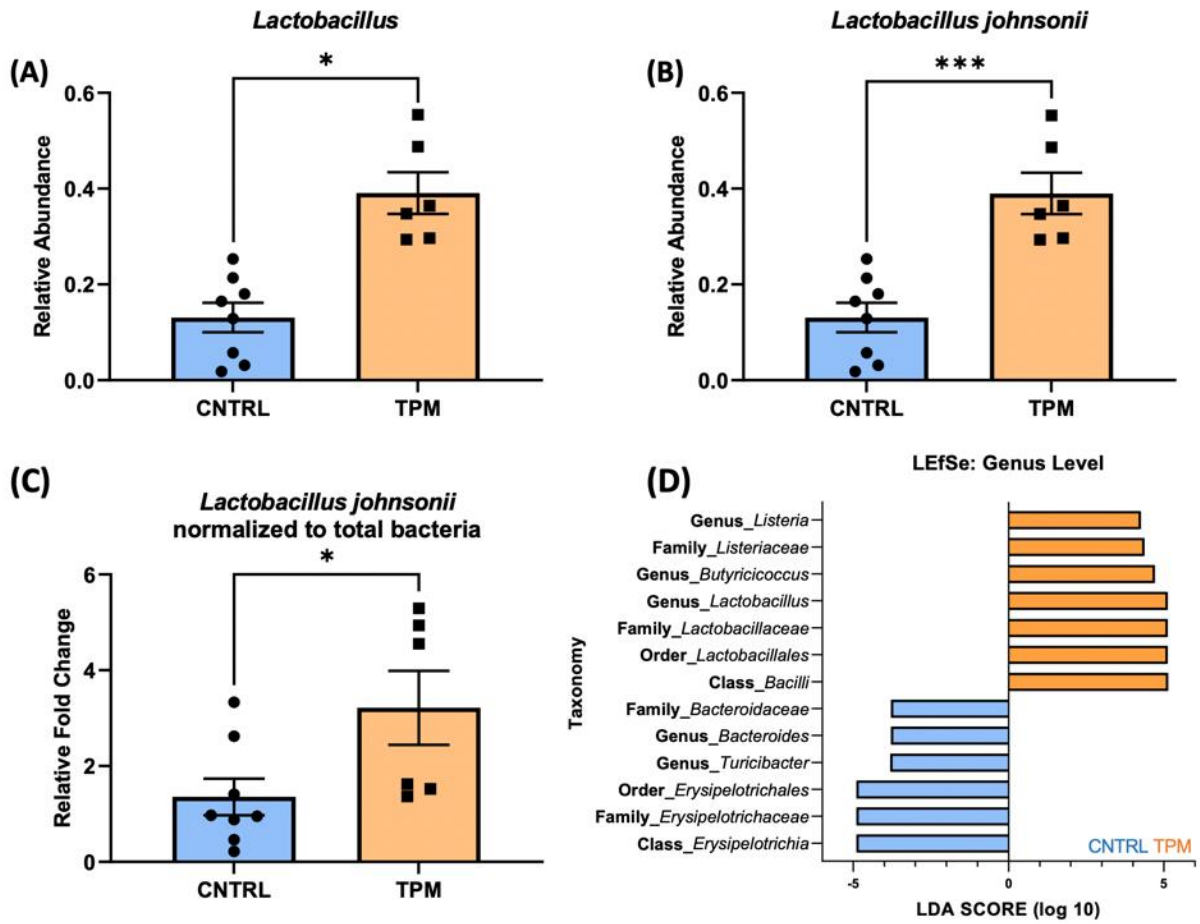

Figure 7: Topiramate increases *Lactobacillus johnsonii* in the gut microbiome of naïve mice. (A) The genus *Lactobacillus* is increased in the gut microbiome of naïve mice after 5 weeks of topiramate treatment. Further analysis revealed that at the species level, (B) *Lactobacillus johnsonii* is significantly increased due to topiramate treatment. This result is confirmed using (C) qPCR with primers targeting *Lactobacillus johnsonii* and total bacteria. (D) LEfSe also describes *Lactobacillus* as well as *Butyricocccus* and *Listeria* as defining features in topiramate treated mice. Lastly, LEfSe identifies *Bacteroides* and *Erysipelotrichaceae* as defining features of control mice. Bars in orange are topiramate features and bars in blue are control features. Data are shown as mean ±SEM. * $p < 0.05$, *** $p < 0.001$.

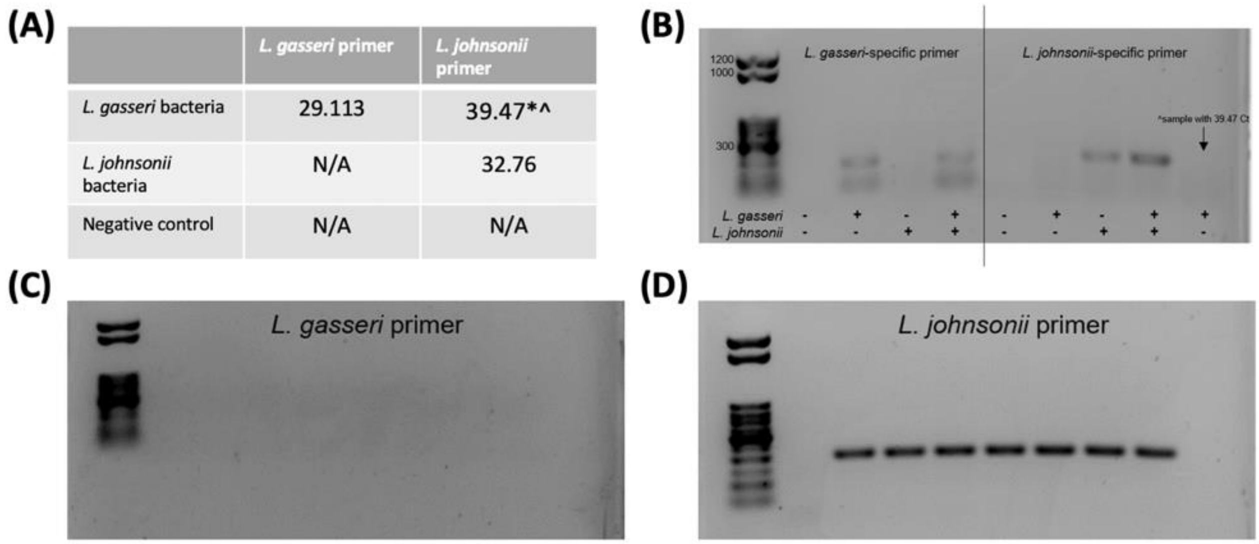

Figure 8: *Lactobacillus johnsonii* primer validation and confirmation of presence in fecal samples. (A) Average Ct of triplicates of qPCR samples of bacteria with specified primers. *Lactobacillus gasseri* primer has one out of three triplicates amplify at 39.47. (B) 2% DNA gel of *Lactobacillus gasseri* and *Lactobacillus johnsonii* products after qPCR with known bacterial samples showing that primers are specific to each species. Last lane is the product from the well containing *Lactobacillus gasseri* but using the *Lactobacillus johnsonii* primer, showing no product. (C) Product ran on 2% gel from *Lactobacillus gasseri* primer used on fecal samples of mice from Figure 2 showing no product. (D) Product ran on 2% gel from *Lactobacillus johnsonii* primer used on fecal samples of mice from Figure 2, suggesting *Lactobacillus johnsonii* is in the samples and not *Lactobacillus gasseri* as identified by QIIME2 analysis. *Lactobacillus gasseri* expected product size: 176 bp, *Lactobacillus johnsonii* expected product size: 195 bp.

5.3 SCFAs in the cecum are impacted by topiramate treatment

Cecal content of control and topiramate-treated mice were analyzed for SCFA composition. No differences in the amount of SCFAs were observed between the control and topiramate-treated mice in butyric acid, iso-butyric acid, acetic acid, valeric acid, iso-valeric acid, or propionic acid (Figure 9). However, a simple linear regression revealed a correlation between butyric acid, propionic acid, and acetic acid and the relative abundance of *Lactobacillus johnsonii* in the control and topiramate-treated mice. In control mice, these SCFAs had a negative or no correlation to the abundance of *Lactobacillus johnsonii* (p=0.4886; Figure 10A, p=0.6832; Figure 10B, p=0.0292; Figure 10C). However, in topiramate-treated mice, these

SCFAs were positively correlated or showed a positive trend with the abundance of *Lactobacillus johnsonii* (p=0.0364; Figure 10D, p=0.0006; Figure 10E, p=0.1383; Figure 10F). This suggests that the positive correlation seen only in the topiramate-treated mice between major SCFAs and *Lactobacillus johnsonii* may be due to the high abundance of *Lactobacillus johnsonii* in the topiramate-treated group, the ingestion of topiramate, or an interaction between topiramate and *Lactobacillus johnsonii*.

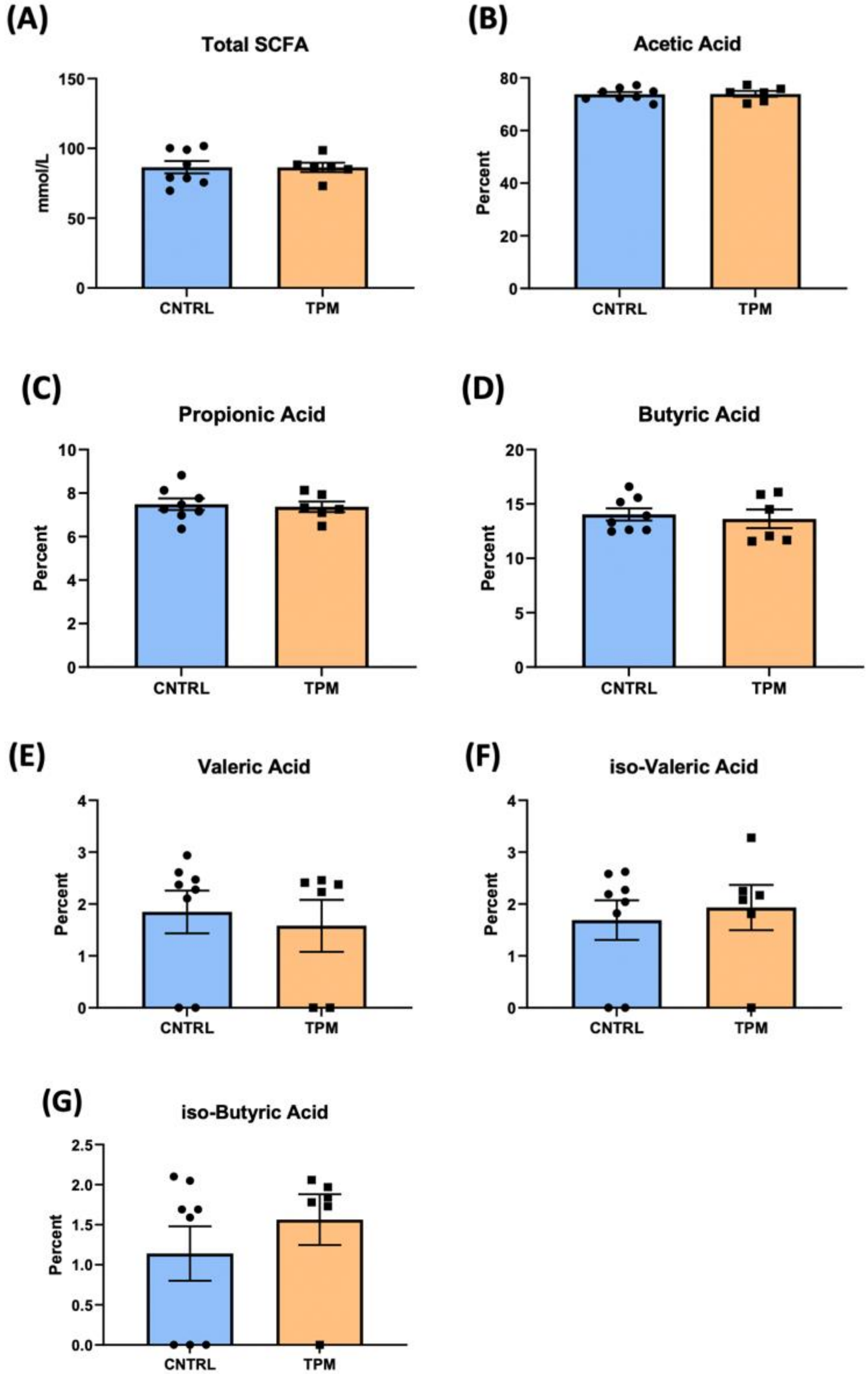

Figure 9: SCFA composition of the ceca of control or topiramate-treated mice. No significant differences were found in (A) total amount of SCFAs or (B-G) any SCFAs. Data are shown as mean ±SEM.

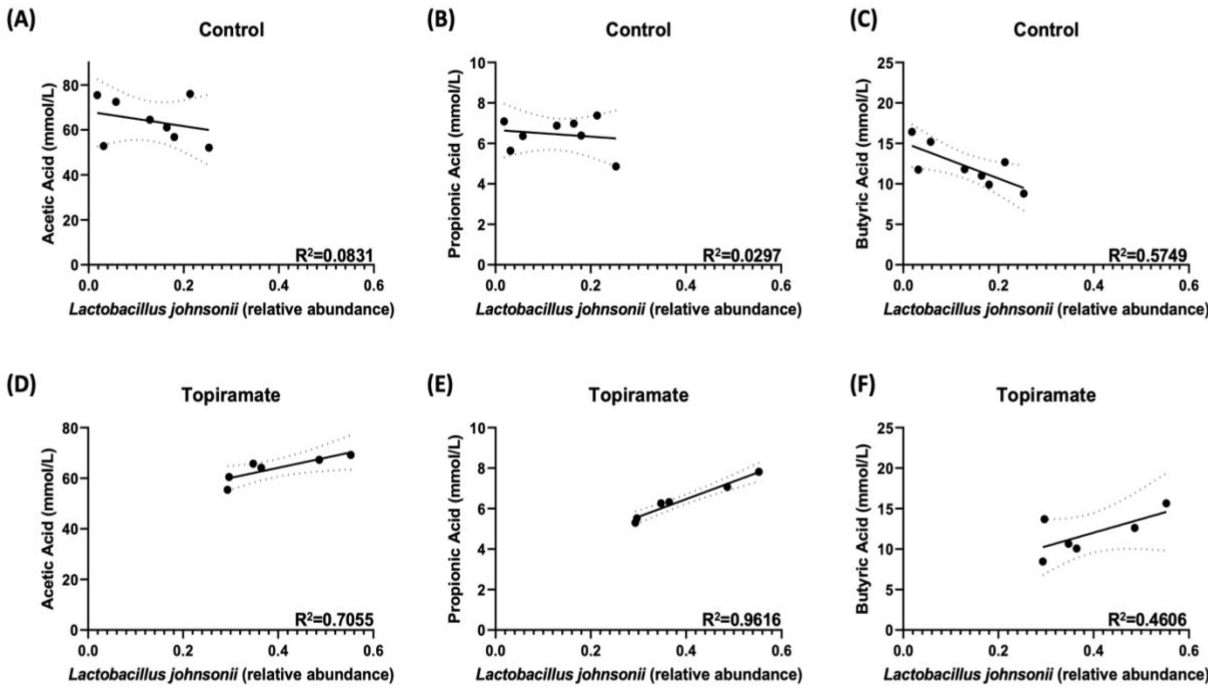

Figure 10: Abundance of *Lactobacillus johnsonii* is positively correlated with the major SCFAs. Acetic acid, propionic acid, and butyric acid, in topiramate treated, but not control, mice are positively correlated with *Lactobacillus johnsonii* abundance. Control animals have (A, B) no or (C) negative correlation with the major SCFAs in the ceca of naïve mice. However, in topiramate-treated mice, *Lactobacillus johnsonii* is either (D, E) positively correlated or trending (F) with the major SCFAs. Data is shown with regression line and 95% confidence bands.

5.4 *Lactobacillus johnsonii* aids topiramate in reducing PTZ-induced seizure susceptibility

Since topiramate increased the relative abundance of *Lactobacillus johnsonii*, we sought to determine if *Lactobacillus johnsonii* impacted topiramate's anti-seizure effect. C57BL/6J mice ~9 weeks old received a daily oral gavage of either saline, topiramate (50 mg/kg), *Lactobacillus johnsonii*, or a combination of topiramate (50 mg/kg) and *Lactobacillus johnsonii*. Mice administered *Lactobacillus johnsonii* were pre-treated for 7 days with 10^7 CFUs and all others received saline. After the 7 days, all mice received a sub-convulsive dose of PTZ every other day 1 hour after the daily gavage for a total of 12 injections. All mice were video monitored for 30 minutes after PTZ injection and seizure activity was scored using a modified Racine scale. We found that after 12 injections, mice treated with both topiramate and *Lactobacillus johnsonii* had significantly lower seizure susceptibility than the saline controls ($p=0.0339$, Friedman test with Dunn's correction for multiple comparisons; Figure 11A). Treatment with topiramate ($p=0.1062$) or *Lactobacillus johnsonii* ($p> 0.9999$) alone did not significantly impact seizure score compared to the saline controls, however, the use of topiramate was protective against mortality (Figure 11B). Moreover, the combination treatment of topiramate and *Lactobacillus johnsonii* reduced the number of mice that exhibited Stage 4 or greater convulsive seizures (Figure 11C).

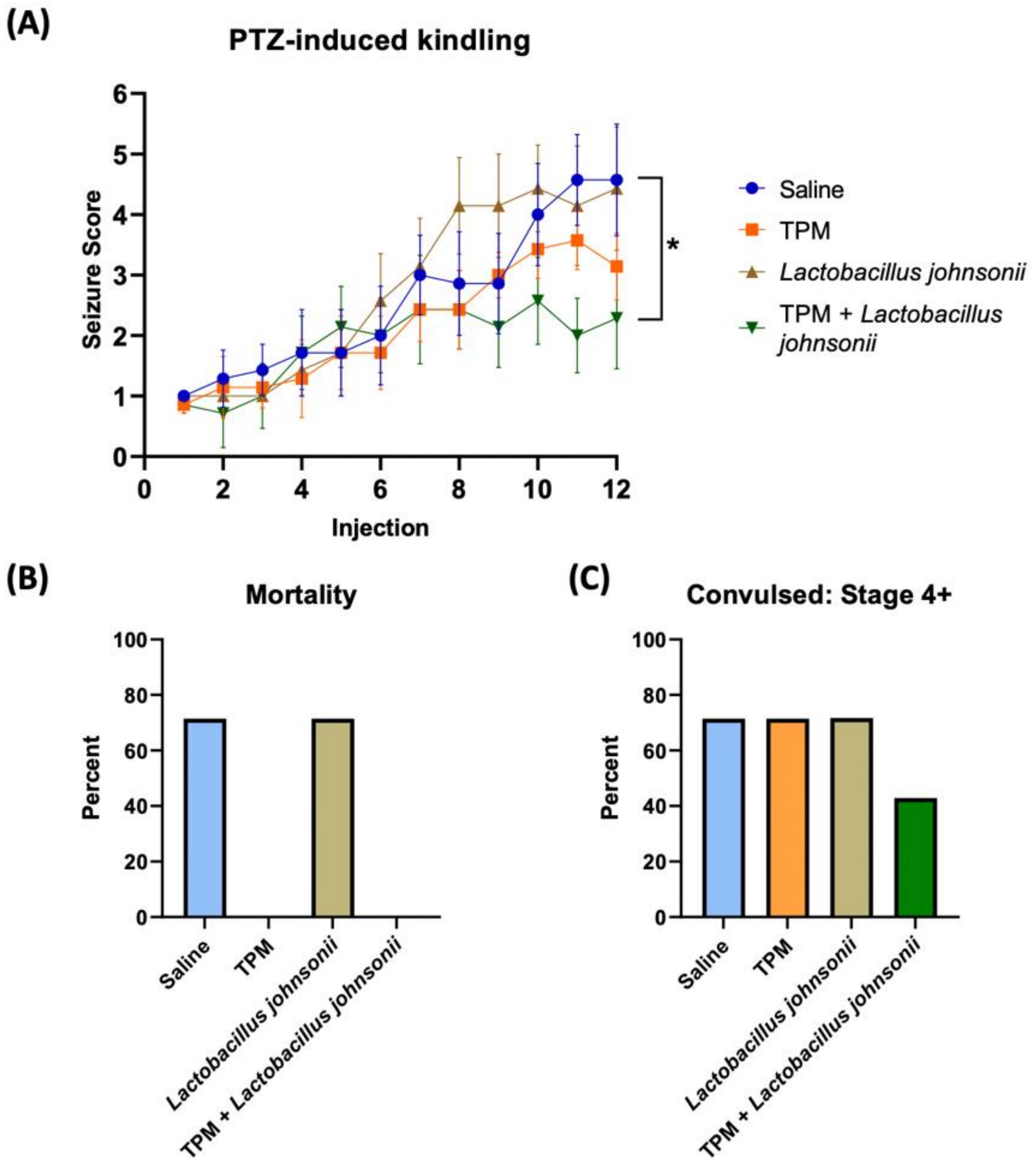

Figure 11: Cotreatment with topiramate and *Lactobacillus johnsonii* significantly reduces seizure susceptibility in PTZ-kindling. (A) Cotreatment of topiramate and *Lactobacillus johnsonii* significantly reduced seizure susceptibility when compared to saline-treated mice. Topiramate or *Lactobacillus johnsonii* alone was not enough to significantly reduce seizure susceptibility. (B) Topiramate treatment appears to reduce mortality due to PTZ-kindling. (C) Cotreatment with topiramate and *Lactobacillus johnsonii* appears to reduce the number of mice that develop convulsive seizures, measured by stage 4 or above on the modified Racine scale. Data are shown as mean $\pm$SEM. * $p < 0.05$. n=7

5.5 Topiramate and *Lactobacillus johnsonii* together, remodel the gut microbiome in response to PTZ-induced seizures

As our data showed that topiramate and *Lactobacillus johnsonii* cotreatment conferred protection against PTZ-induced seizures, we analyzed the fecal samples of mice that underwent PTZ-kindling with and without treatment to determine if there were any changes in microbial composition. We found no alteration in alpha diversity, Shannon index or OTUs (Figure 12) among PTZ-treated mice that were administered saline, topiramate, *Lactobacillus johnsonii*, or topiramate and *Lactobacillus johnsonii*. However, there was a significant difference in beta diversity in unweighted UNIFRAC (p=0.001, 999 permutations, PERMANOVA; Figure 13A) and weighted UNIFRAC (p=0.019, 999 permutations, PERMANOVA; Figure 13B) among the groups. These changes require further investigation to determine what may be driving the differences seen in beta diversity.

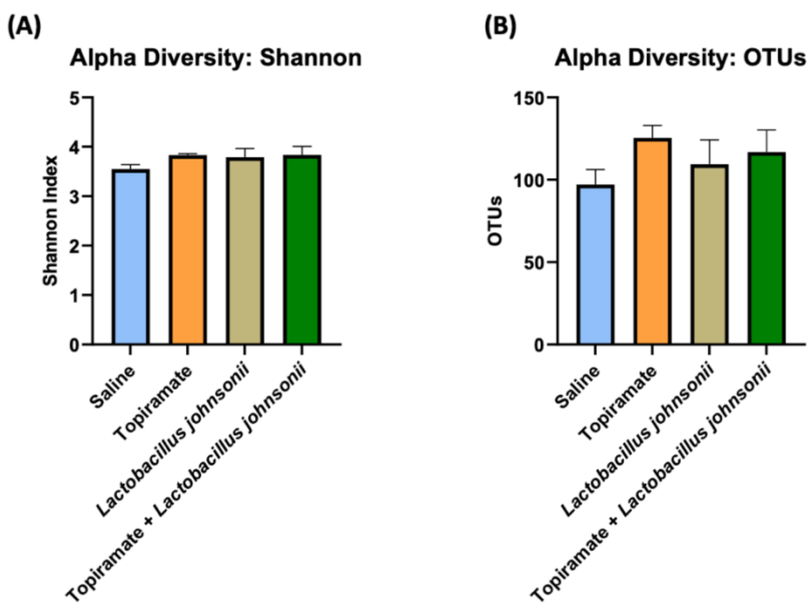

Figure 12: No alterations in alpha diversity in mice undergoing PTZ-kindling. (A) Shannon index was used to measure species evenness. (B) OTUs were used to measure species richness. Data are shown as mean ±SEM. * $p < 0.05$. Saline=6, TPM=7, *Lactobacillus johnsonii*=7, TPM + *Lactobacillus johnsonii*=7.

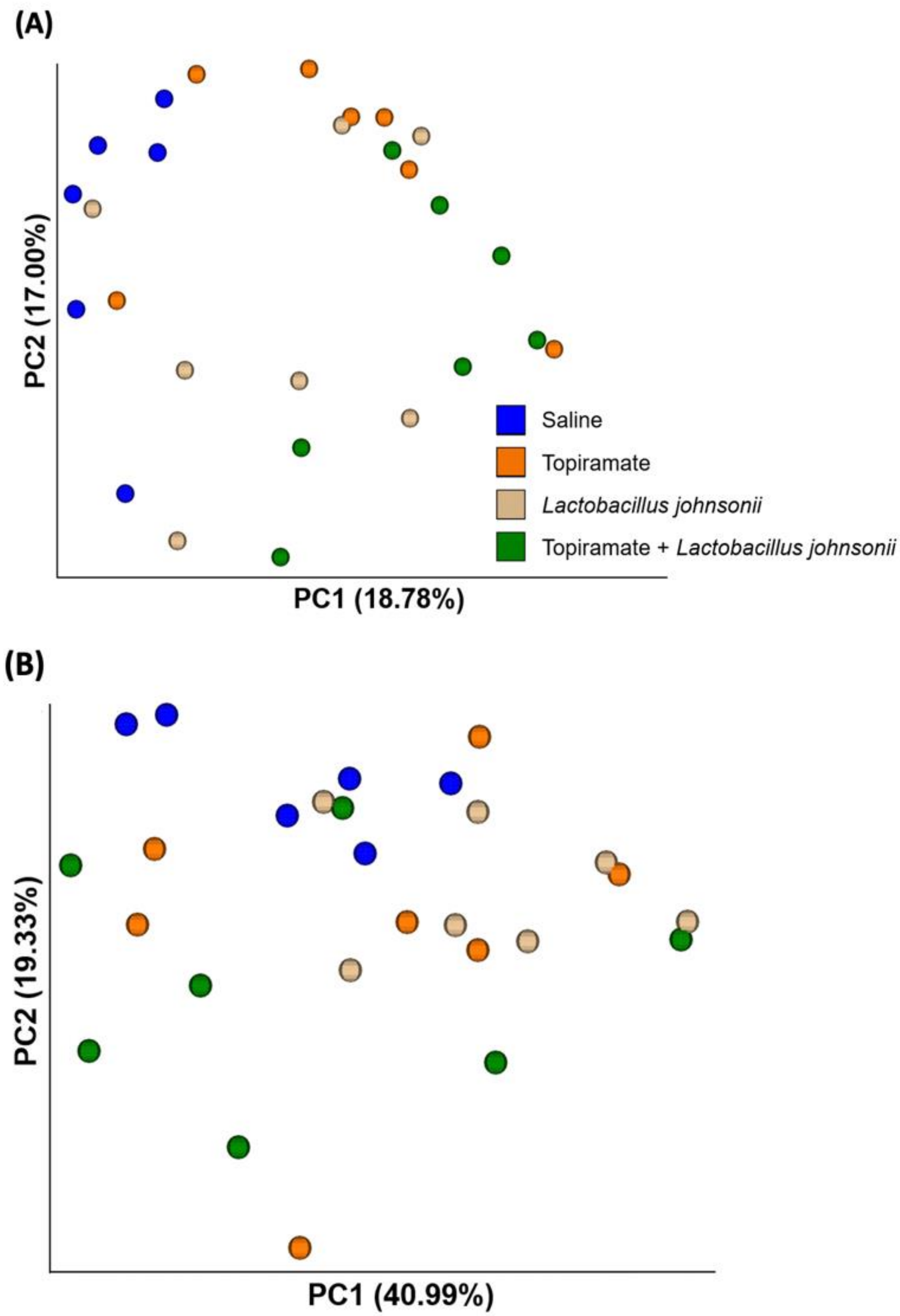

Figure 13: Treatments in mice undergoing PTZ-kindling significantly alter beta diversity. (A) unweighted UNIFRAC statistical test (B) weighted UNIFRAC statistical test visualized by the principal coordinates (PC1 and PC2) analysis (PCoA). Each dot represents a fecal sample from an individual mouse.

Differential abundance with ANCOM performed at the family level showed that *Erysipelotrichaceae* (W=4), *Lachnospiraceae* (W=3), *Peptostreptococcaceae* (W=1), *Bacteroidaceae* (W=1), and *Anaeroplasmataceae* (W=1) were significantly different. A Kruskal-Wallis with Dunn's multiple comparisons was used on each family identified by ANCOM to further elucidate the differences between the groups. The relative abundance of *Erysipelotrichaceae* and *Peptostreptococcaceae* were significantly lower in the topiramate and *Lactobacillus johnsonii* group compared to saline (p=0.0022; Figure 14A, p=0.0426; Figure 14C). *Lachnospiraceae* was significantly increased in the topiramate and *Lactobacillus johnsonii* cotreatment group compared to saline (p=0.0116; Figure 14B). In addition, *Bacteroidaceae* was significantly reduced in the topiramate and *Lactobacillus johnsonii* cotreatment group compared to the topiramate group (p=0.0063; Figure 14D). Lastly, there were no differences in *Anaeroplasmataceae* using a Kruskal-Wallis (p=0.3190; Figure 14E) despite ANCOM identifying it as differentially abundant. Interestingly, Figure 14F shows a trend towards an increase in the butyrate producing family *Ruminococcaceae* in the topiramate and *Lactobacillus johnsonii* cotreatment group, although it was not identified by ANCOM.

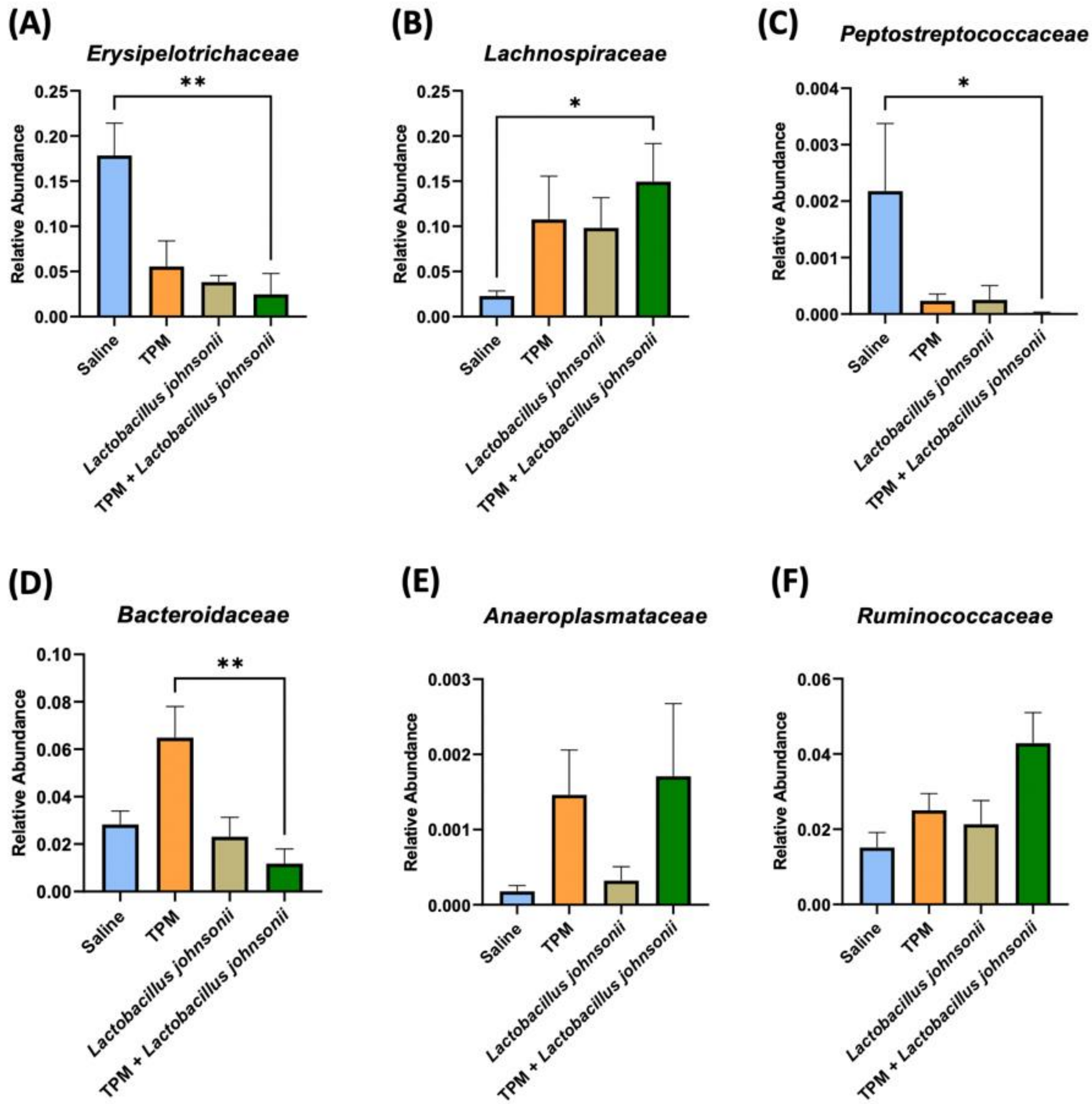

Figure 14: Remodeling of the gut microbiome composition by topiramate and *Lactobacillus johnsonii* cotreatment after PTZ-kindling. ANCOM identified (A-E) five families found significantly different across the groups. A Kruskal-Wallis with Dunn's multiple comparisons were run on the five families identified. (A, C) *Erysipelotrichaceae* and *Peptostreptococcaceae* are significantly reduced in the cotreatment group compared to saline treatment. (B) *Lachnospiraceae* is significantly increased in the cotreatment group when compared to saline treatment. (D) *Bacteroidaceae* is significantly decreased in the cotreatment group compared to the topiramate treated group. (E) Although identified by ANCOM, there were no significant differences between the groups in *Anaeroplasmataceae* when using further tests. (F) Not identified by ANCOM, the butyrate producing family, *Ruminococcaceae*, trends toward an increase in the cotreatment group. Data are shown as mean $\pm$SEM. * $p < 0.05$, ** $p < 0.01$. Saline=6, TPM=7, *Lactobacillus johnsonii*=7, TPM + *Lactobacillus johnsonii*=7.

5.6 Co-administration of topiramate and *Lactobacillus johnsonii* alters SCFA composition

The ceca of mice that underwent PTZ-kindling were analyzed for SCFA composition. One-way ANOVAs with Tukey's multiple comparisons test with a single pooled variance between all groups were performed on all SCFAs (Figure 15A-H). The data revealed no significant difference in the total amount of SCFAs ($p = 0.1201$; Figure 15A). Analysis of the percent of SCFAs showed no significant differences in acetic acid, caproic acid, or iso-valeric acid. However, propionic acid was reduced in the topiramate and *Lactobacillus johnsonii* treatment group compared to *Lactobacillus johnsonii* treatment alone ($p=0.0090$; Figure 15C). Butyric acid was increased (Figure 15D) in the topiramate and *Lactobacillus johnsonii* compared to saline ($p=0.0168$) and *Lactobacillus johnsonii* treatment alone ($p=0.0225$). *Lactobacillus johnsonii* treatment increased valeric acid compared to the topiramate treatment ($p=0.0317$; Figure 15E), and increased iso-butyric acid (Figure 15F) compared to topiramate treatment ($p=0.0126$) and topiramate and *Lactobacillus johnsonii* treatment group ($p=0.0429$).

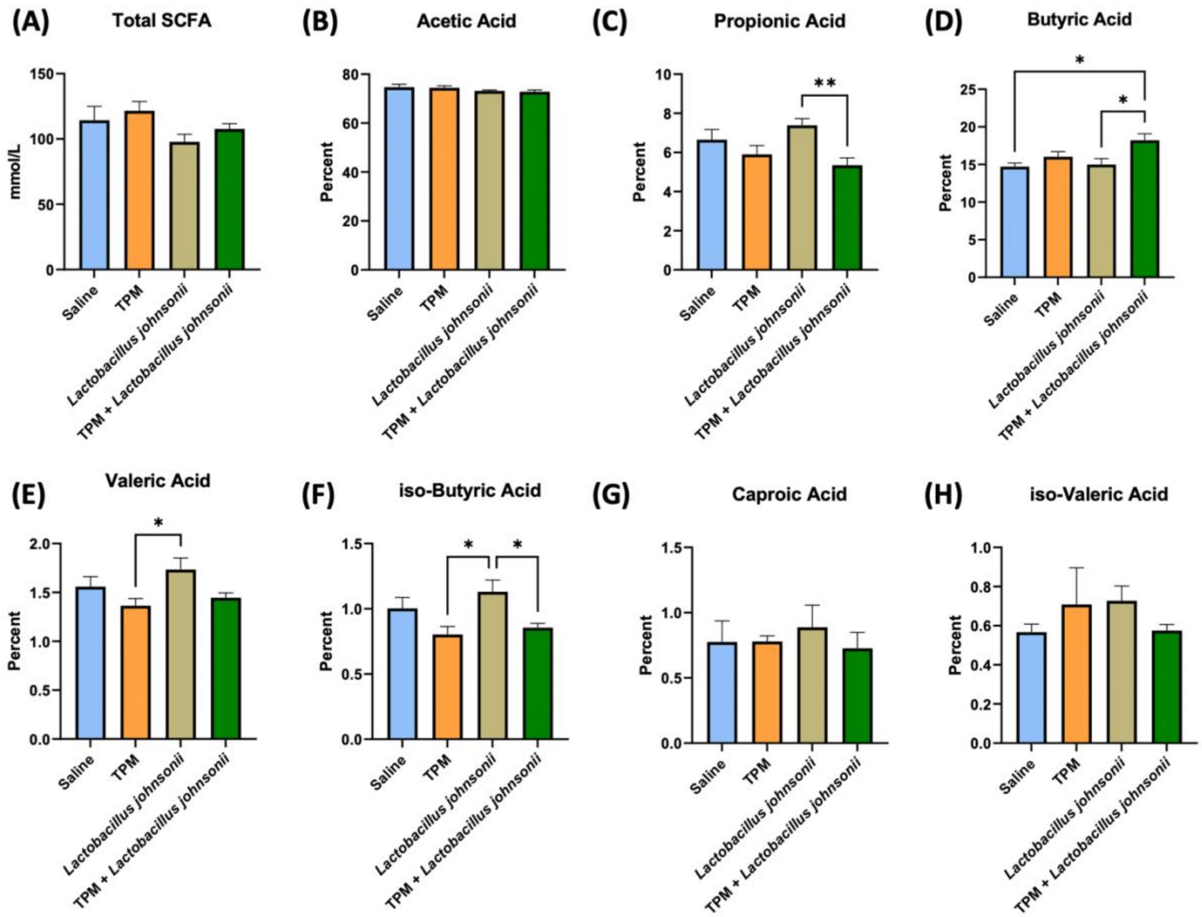

Figure 15: Butyric acid is altered due to cotreatment of topiramate and *Lactobacillus johnsonii*. (A, B) No significant changes were seen in total amount of SCFAs or the percentage of acetic acid in the ceca of mice after treatment and PTZ-kindling. (C) Propionic acid was significantly reduced in the cotreatment group compared to the *Lactobacillus johnsonii* treatment alone. (D) Butyric acid was significantly increased in the cotreatment group when compared to the saline or *Lactobacillus johnsonii* treatment alone. (E) Valeric acid was increased in the *Lactobacillus johnsonii* treatment when compared to the topiramate treatment. (F) Significant reductions in iso-butyric acid were seen in the topiramate and the topiramate and *Lactobacillus johnsonii* group compared to the *Lactobacillus johnsonii* group alone. No significant changes were seen in (G) caproic acid or (H) iso-Valeric acid. Data are shown as mean $\pm$SEM. * $p < 0.05$, ** $p < 0.01$. Saline=6, TPM=7, *Lactobacillus johnsonii*=7, TPM + *Lactobacillus johnsonii*=7.

As butyrate was increased in the ceca of mice treated with topiramate and *Lactobacillus johnsonii*, we performed a simple linear regression of the percentage of butyrate and the abundance of *Lachnospiraceae* and *Ruminococcaeae,* well known butyrate producers. We found that both families, *Lachnospiraceae* (p=0.0211, R^2= 0.1951; Figure 16A) and *Ruminococcaeae* (p=0.0008, R^2=0.3655; Figure 16B), were positively correlated with the percent of butyrate found in the mice.

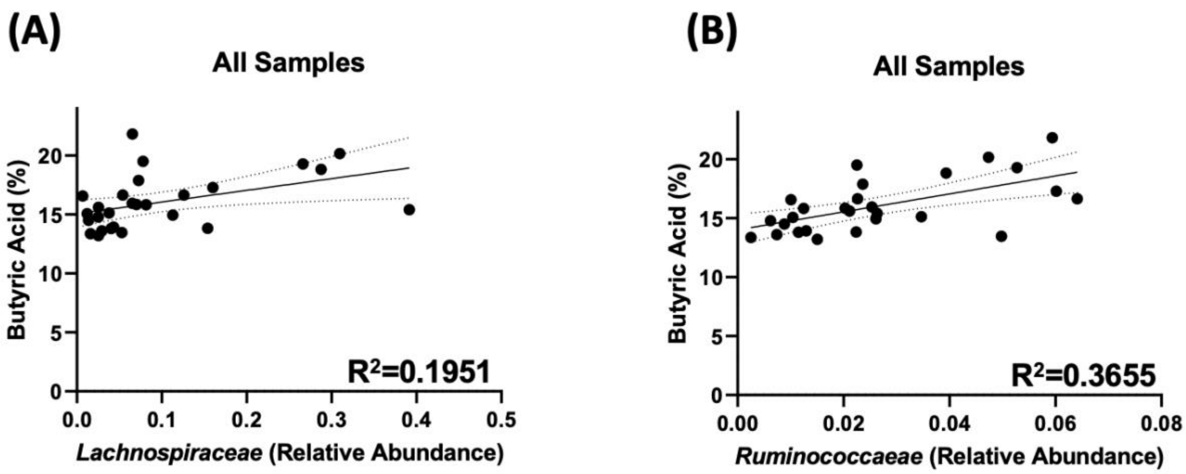

Figure 16: Butyric acid correlates with butyric acid-producing families. A simple linear regression showed a positive correlation amongst the abundance of butyrate producing families (A) *Lachnospiraceae* and (B) *Ruminococcaceae* and the percentage of butyrate across all treatments. Data are shown as regression line with 95% confidence bands.

5.7 Cotreatment of topiramate and *Lactobacillus johnsonii* increases GABA/glutamate ratio in the cortex of mice after PTZ-kindling

To further determine the mechanisms involved in the reduction of seizure susceptibility induced by cotreatment of topiramate and *Lactobacillus johnsonii,* we evaluated the cortical level of GABA and glutamate among the groups. The cortex of animals that underwent PTZ-kindling were analyzed for GABA and glutamate concentration. Kruskal-Wallis with Dunn's multiple comparisons test showed a significant decrease in glutamate in the cortex of mice treated with topiramate and *Lactobacillus johnsonii* together when compared with controls (p=0.0495; Figure 17A), but not in topiramate treatment alone (p=0.6071), or *Lactobacillus johnsonii* treatment alone (0.0943). We also saw an increase in GABA levels in mice that were treated with topiramate alone compared to saline controls (p=0.0388; Figure 17B), but not in the cotreatment group (p=0.0902). However, there was a significant increase in the GABA/glutamate ratio in the cotreatment group (p=0.0223; Figure 17C), but not in topiramate alone (p=0.1579), or *Lactobacillus johnsonii* treatment alone (p=0.0862).

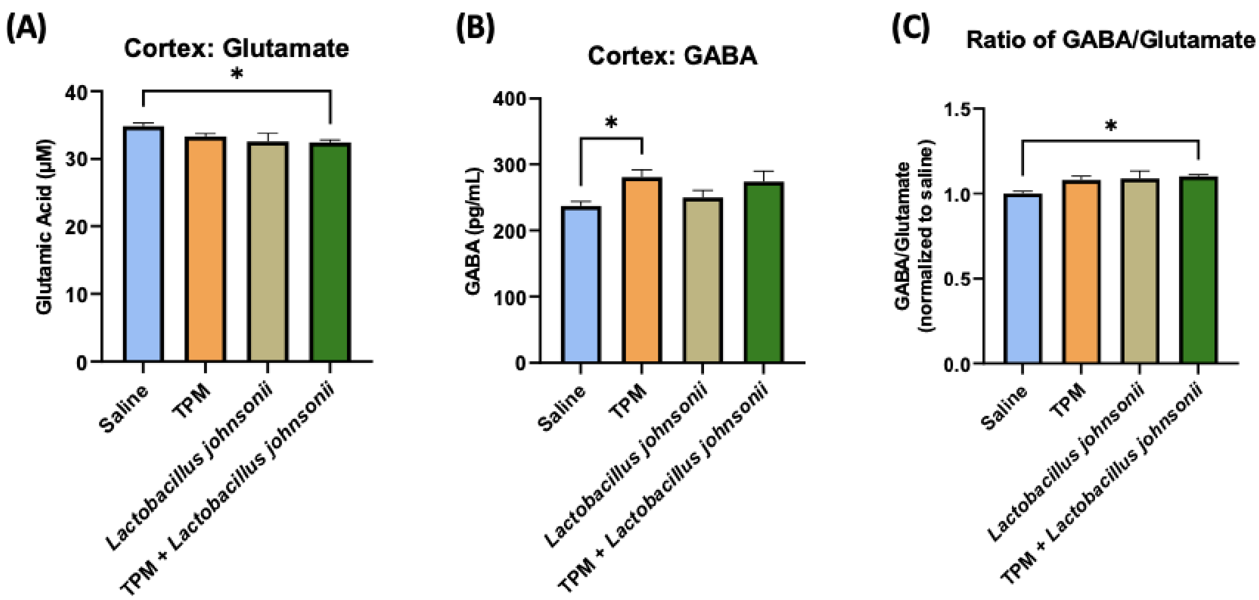

Figure 17: GABA/Glutamate ratio is increased due to cotreatment of topiramate and *Lactobacillus johnsonii*. (A) Glutamate was significantly decreased in the cortex of mice who received topiramate and *Lactobacillus johnsonii* together. (B) Topiramate treatment alone significantly increases GABA in the cortex of mice. (C) The ratio of GABA/glutamate is increased only in the combination treatment of topiramate and *Lactobacillus johnsonii*. Data are shown as mean ±SEM. * $p < 0.05$. Saline=6, TPM=7, *Lactobacillus johnsonii*=7, TPM + *Lactobacillus johnsonii*=7.

5.8 No alterations in tight junction or mucus proteins

The hippocampus and intestines of mice that underwent PTZ-kindling were analyzed for tight junction and mucus proteins. Tight junction proteins are involved in maintaining a regulatory barrier that can properly control what does and does not go through the barrier. These tight junctions can exist at the blood brain barrier, controlling passageway between the periphery and the central nervous system, and in the intestine, controlling passageway from the intestinal lumen to the lamina propria (Zihni et al., 2016). In addition, the intestinal lumen consists of a mucus layer that is primarily composed of MUC2, a mucus protein produced by intestinal goblet cells (Yao et al., 2021).

Alterations in tight junction proteins and mucus production are often linked to alterations in the gut microbiota, as inflammation can disrupt these proteins (Lee et al., 2018). The hippocampus was used to analyze tight junctions at the blood brain barrier, zonula occluden (ZO)-1 was measured as it is a major tight junction protein in which loss leads to decreased barrier integrity (Figure 18A). We did not see any significant differences in the ZO-1 in the hippocampus across the groups. Next, ZO-1 and claudin-5 were analyzed at the proximal colon, in which no significant differences were found across the groups (Figure 18C, D). Lastly, MUC2 was analyzed in the ileum, a prominent site for nutrient absorption in the intestine, where no significant differences were seen across the groups (Figure 18B).

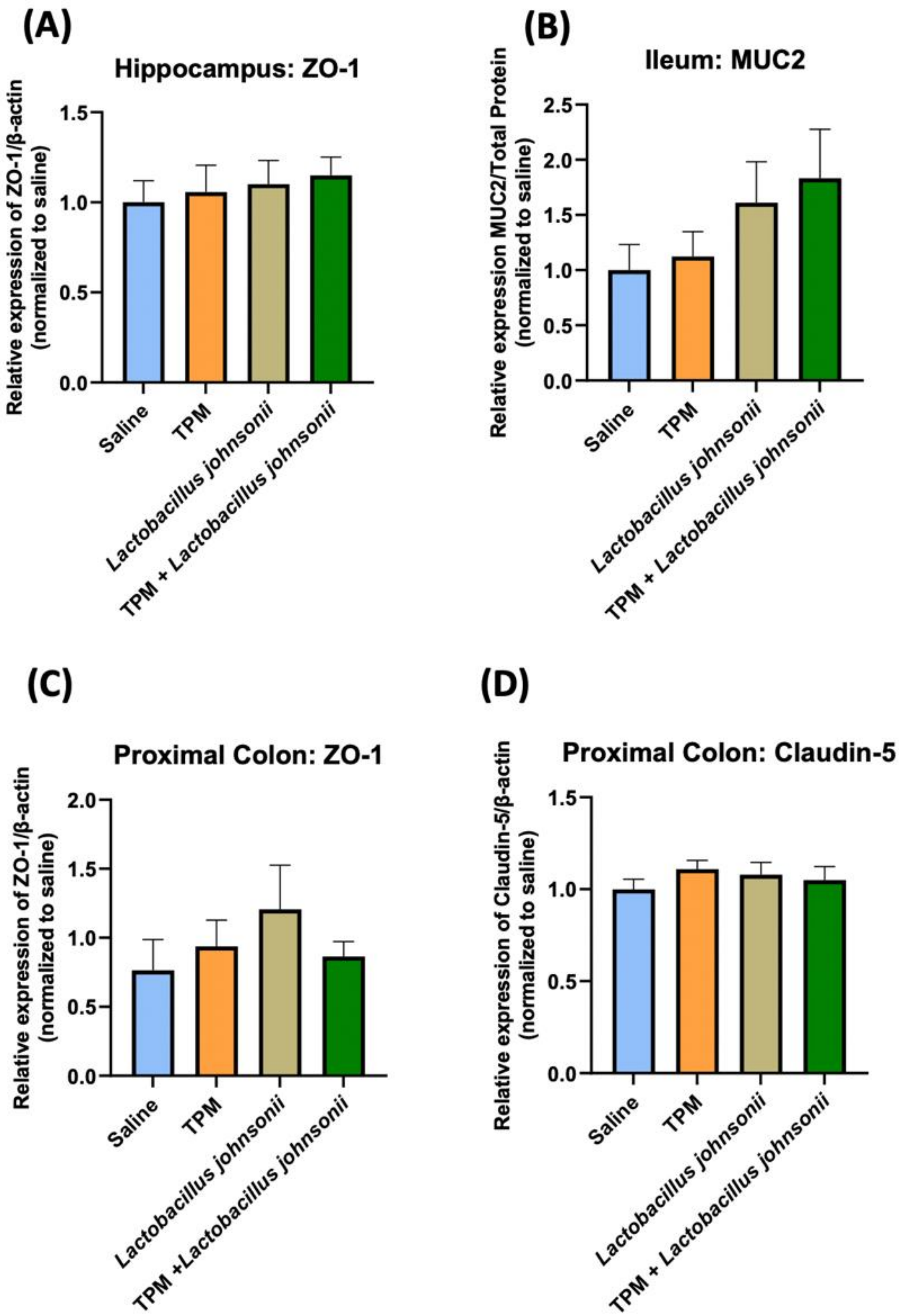

Figure 18: No significant differences in tight junction proteins. (A) ZO-1 in the hippocampus Saline=5, TPM=7, *Lactobacillus johnsonii*=7, TPM + *Lactobacillus johnsonii*=7. (B) MUC2 in the ileum Saline=6, TPM=7, *Lactobacillus johnsonii*=6, TPM + *Lactobacillus johnsonii*=7. (C) ZO-1 and (D) Claudin-5 in the proximal colon. Saline=7, TPM=7, *Lactobacillus johnsonii*=6, TPM + *Lactobacillus johnsonii*=7. Data are shown as mean ±SEM.

5.9 No alterations in immune markers

Due to the potential for immunomodulation by *Lactobacillus johnsonii* reported in the literature, the hippocampus of mice that underwent PTZ-kindling were used to measure the expression of pro-inflammatory, anti-inflammatory, neurotrophic, and cell death markers. No alterations were seen in any markers across the groups (Figure 19).

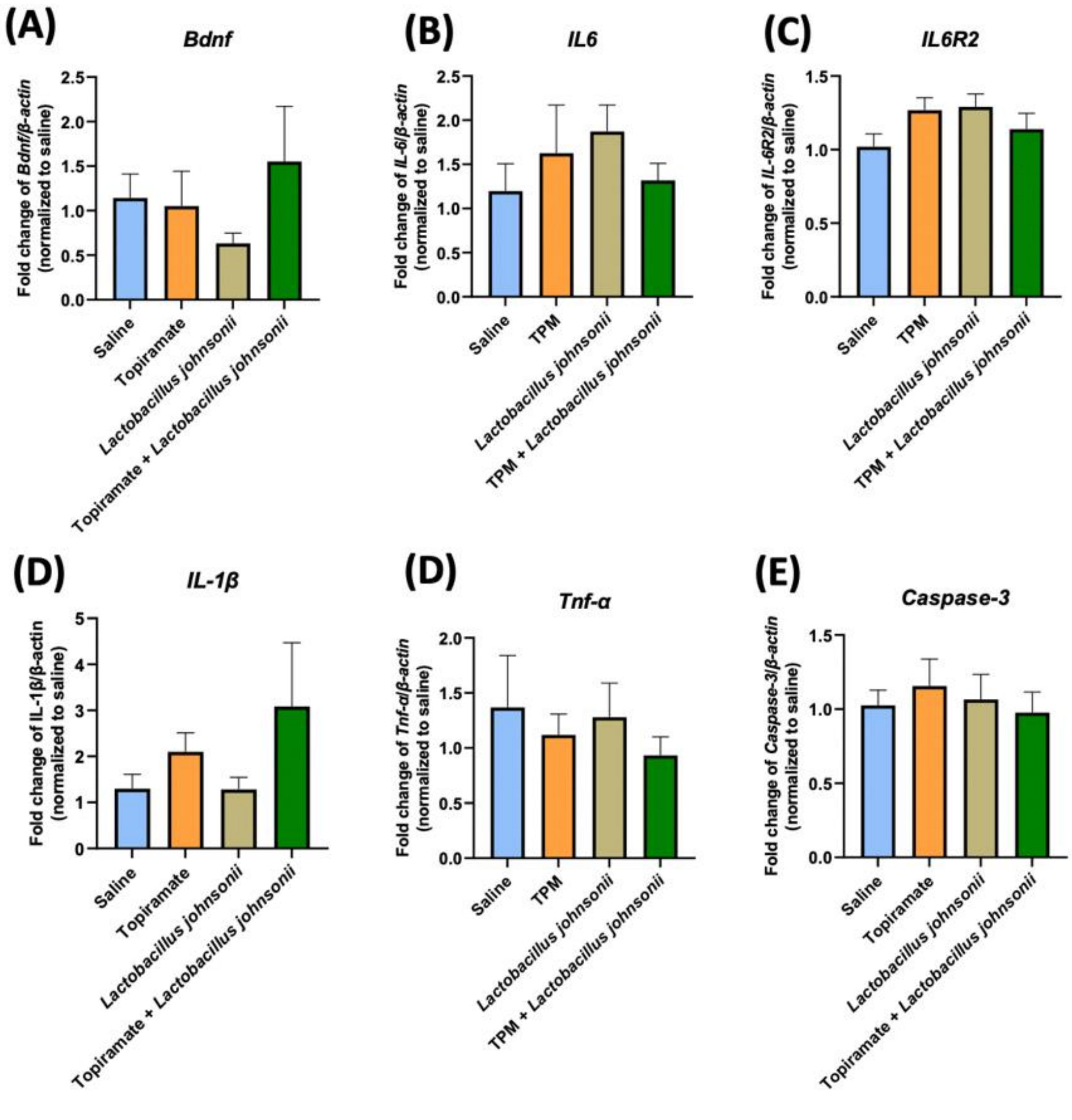

Figure 19: No significant differences in neurotrophic or immune markers in the hippocampus. All data are expressed as fold change relative to β-actin. Neurotrophic factor (A) *Bdnf*, anti-inflammatory markers (B) *IL6* and (C) *IL6 Receptor 2*, pro-inflammatory markers (D) *IL-1β* and (D) *Tnf-α*, and apoptosis marker (E) *Caspase-3*. Saline=6, TPM=7, *Lactobacillus johnsonii*=7, TPM + *Lactobacillus johnsonii*=7. Data are shown as mean $\pm$SEM.

5.10 Topiramate directly impacts the growth of *Lactobacillus johnsonii in vitro*

To determine if topiramate directly impacts the growth of *Lactobacillus johnsonii*, topiramate was added to the nutrient broth with *Lactobacillus johnsonii*. A range of concentrations were chosen including physiologically relevant concentrations seen in the intestines (50-100 μM), as well as non-physiological relevant concentrations (1000 and 2000 μM) (Maier et al., 2018). *Lactobacillus johnsonii* was plated with the various concentrations of topiramate in a 96-well plate, avoiding the outer edges due to increased risk of evaporation, and grown for 40 hours at 37ºC in a microplate reader (Figure 20A). The data was then fit to a logistic growth curve to further extrapolate information on the potential changes to bacterial growth (Figure 20B).

The data was processed using the Growthcurver package in R studio, from which we obtained information on growth rate, doubling time, area under the curve (AUC), and time to inflection point (Figure 21). Using an unpaired t test, we see that 2000 μM of topiramate resulted in a trending increase in growth rate (p= 0.0834; Figure 21A) and decrease in doubling time (p= 0.0838;Figure 21B) when compared with the control. We also found that 2000 μM significantly increased the area under the curve (p= 0.0059; Figure 21C). Lastly, we see that 1000 (p= 0.0096) and 2000 μM (p= 0.0010) decreased time to inflection point when compared to control (Figure 21D). From these results, it appears that 1000 and 2000 μM of topiramate can alter the growth characteristics of *Lactobacillus johnsonii in vitro.*

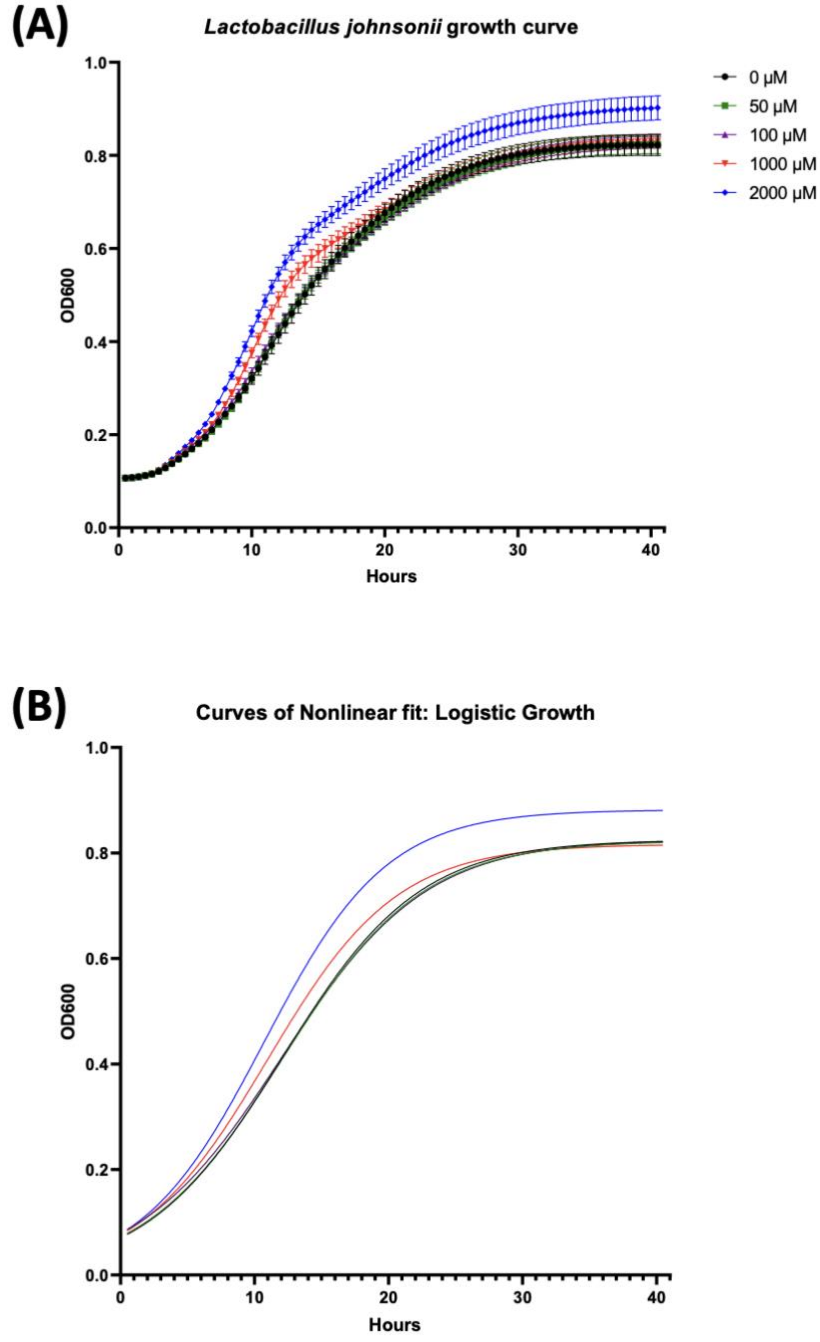

Figure 20: Growth curve of *Lactobacillus johnsonii* treated with topiramate. (A) OD600 was read every 30 minutes to develop a growth curve over 40 hours. n=10 per group. (B) Data was fit to a logistic growth curve using a nonlinear regression. Data are shown as mean ±SEM.

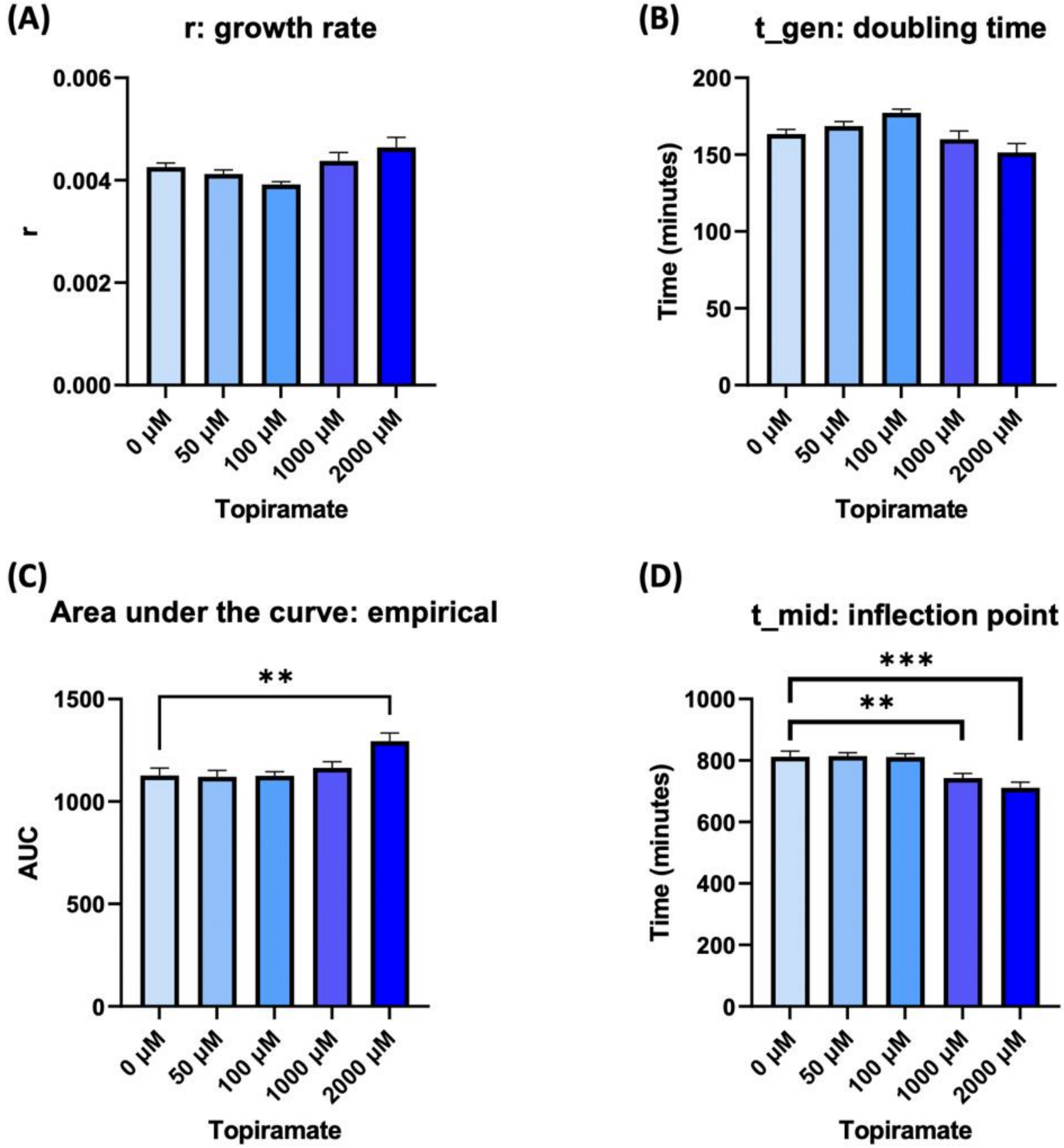

Figure 21: Measurements of *Lactobacillus johnsonii* growth with topiramate. (A) growth rate. (B) doubling time. (C) area under the curve. (D) inflection point. n=10 per group. ** $p < 0.01$, *** $p < 0.001$. Data are shown as mean $\pm$SEM.

Chapter 6: Discussion

6.1 Topiramate and its alteration of the gut microbiome

Topiramate is a second-generation AED used to treat both partial and generalized seizures and migraines (Fariba & Saadabadi, 2023). Several pharmacological mechanisms have been proposed in its anti-seizure effect, including enhancement of γ-aminobutyric acid type A ($GABA_A$) receptor activity, blockade of voltage-gated Na^+ channels, reduction of membrane depolarization via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/Kainate receptors, and weak carbonic anhydrase inhibitory activity (Fariba & Saadabadi, 2023) (Figure 22). In addition, topiramate is a unique AED as it is a sulfamate-substituted monosaccharide (Kim et al., 2023) (Figure 23). Topiramate is well absorbed from the gastro-intestinal tract and has peak plasma levels within 2-3 hours, with over 80% bioavailability. This AED is metabolized by hydroxylation, hydrolysis, and glucuronidation with a half-life of about 21 hours. We see that topiramate ingestion through drinking water increases the probiotic *Lactobacillus johnsonii*, and it is possible that these unique features of topiramate contribute to its gut microbiome altering effect. One of topiramate's unique features of being a weak carbonic anhydrase inhibitor has been thought to cause its side effects of metabolic acidosis, which would ultimately lead to an acidic environment. *Lactobacillus* species can often survive in low pH environments, even as low as a pH of 1, potentially leading to the selection for this naturally occurring species in the mouse gut (Aiba et al., 2015; Davoren et al., 2018). It is also unknown as to whether *Lactobacillus johnsonii* can use the sulfamate-substituted monosaccharide as a carbon source, contributing to its growth. Although it may seem counterintuitive for *Lactobacillus johnsonii* to ingest topiramate, leaving less for the host, there is likely an abundance of the drug as 70% of topiramate is excreted unchanged in the urine.

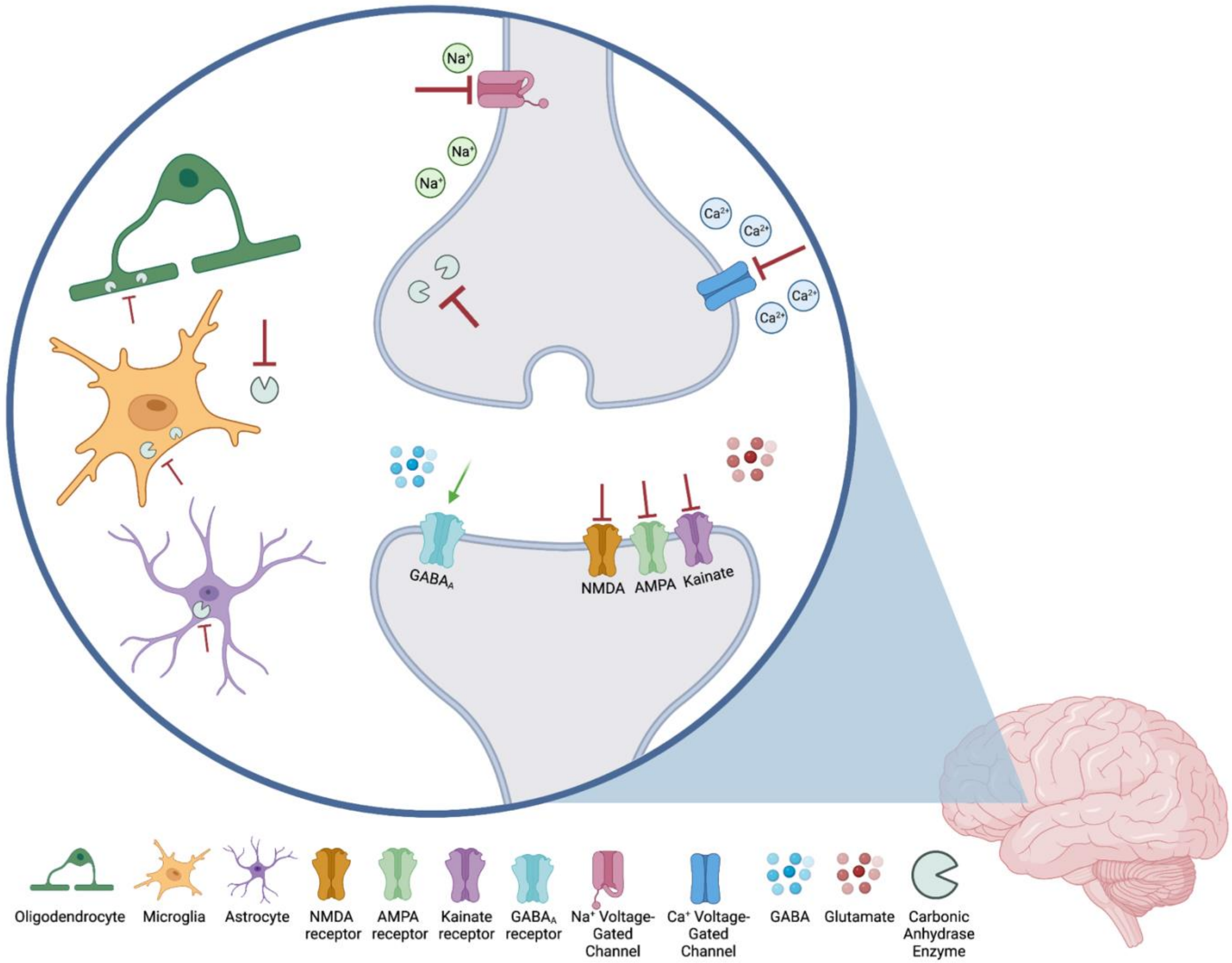

Figure 22: Topiramate's proposed mechanisms of action. Research has shown that topiramate can inhibit voltage-gated sodium channels and voltage-gated calcium channels on the presynaptic neuron to reduce excitation. In addition, topiramate can also inhibit NMDA, AMPA, and Kainate receptors on the post-synaptic neuron to also reduce excitation. To increase inhibition, topiramate has been shown to impact $GABA_A$ receptors on the post-synaptic neuron. Lastly, topiramate has been shown to have weak carbonic anhydrase inhibitor properties. Carbonic anhydrases are found in the brain and in the intestines, specifically in oligodendrocytes, microglia, astrocytes, and neurons. Created with BioRender.com.

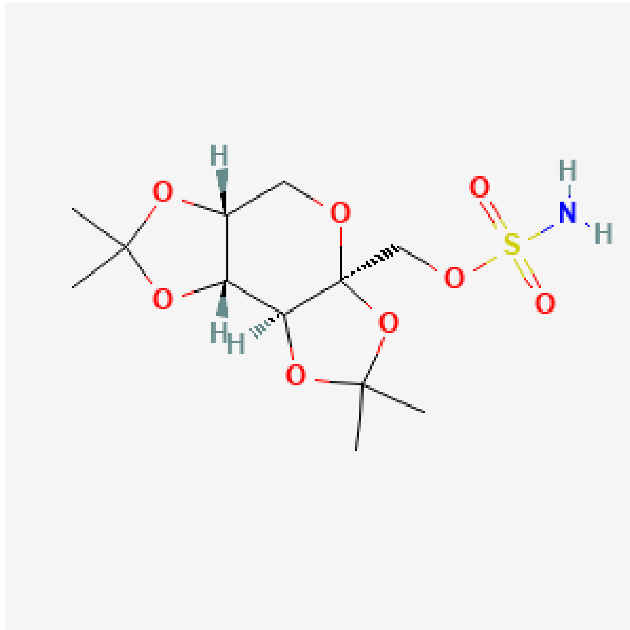

Figure 23: 2D Structure of Topiramate (Kim et al. 2023) 2D structure image of CID 5284627. https://pubchem.ncbi.nlm.nih.gov/compound/5284627#section=2D-Structure.

6.2 *Lactobacillus johnsonii* and its probiotic effects

Lactobacillus johnsonii has been shown to be a beneficial species despite being understudied when compared to its lactobacilli counterparts. *Lactobacillus johnsonii* belongs to the well-known genus *Lactobacillus*, which are gram-positive aerotolerant anaerobes (Zheng et al., 2020). Lactobacilli are rod-shaped and non-spore-forming but do often produce biofilms. They are known to have probiotic effects, in part due to their ability to protect the host against pathogens. In addition, they are lactic acid bacteria, hence the name, and produce lactic acid, which can be used and processed by other bacteria.

Research has shown that *Lactobacillus johnsonii* has immunomodulatory effects in which it can aid immune-checkpoint blockade therapy in tumor reduction, as well as increase neurotrophic factors in the brain, and increase tight-junction proteins at both the intestinal and blood-brain barrier (Mager et al., 2020; H. Wang et al., 2020; Bai et al., 2022). More specifically, Mager and colleagues showed that mice mono-colonized with *Lactobacillus johnsonii* in conjunction with anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) therapy resulted in tumor size reduction compared with germ-free mice or mice colonized with neutral bacteria serving as a control. In addition, the researchers showed that *Lactobacillus johnsonii*'s effect is likely due to the activation of anti-tumor T cells and the exact mechanism may be dependent on the T cell expression of the adenosine-A2 receptor ($A_{2A}R$) by increasing a ligand of $A_{2A}R$, hypoxanthine. In addition to its immunomodulatory potential, a group of researchers who have focused on the *Lactobacillus johnsonii* strain BS15, have displayed its ability to reduce psychological stress induced memory dysfunction in a water-avoidance stress model. Furthermore, after the 28 days of *Lactobacillus johnsonii* intake, they also saw increased levels of tight junction gene expression and decreased levels of pro-inflammatory gene expression in the intestinal tract. Lastly, they also found alterations in gene expression and antioxidant activity in the brain that favor neuroprotection.

In our study, we see that although *Lactobacillus johnsonii* administration alone is not sufficient to reduce seizure susceptibility, in combination with topiramate, *Lactobacillus johnsonii* reduces seizure susceptibility in a PTZ-kindling model. It is possible that this effect is due to its alteration of the gut microbiome by increasing butyrate, as well as the butyrate producing family, *Lachnospiraceae*. Of interest, Zhong and colleagues recently showed that *Lactobacillus johnsonii* alleviates the development of acute myocardial infarction (Zhong et al., 2023). In fact, they showed that administration of *Lactobacillus johnsonii* increases butyrate, but this effect is mitigated with prior treatment of antibiotics. This result coupled with our findings suggests that the administration of *Lactobacillus johnsonii* may alter the composition of the gut microbiome to favor butyrate production, and ultimately aid in reduced seizure susceptibility.

Another significant alteration seen due to *Lactobacillus johnsonii* treatment is the reduction in the family *Erysipelotrichaceae*. Of interest, current research points to the potential for members of this family to play a negative role in host health (Kaakoush, 2015). Research has seen that this family is increased in patients with colorectal cancer, as well as increased in a mouse model of colorectal cancer (W. Chen et al., 2012; Zhu et al., 2014). Moreover, studies show an increase in this family in patients with chronic human immunodeficiency virus (HIV) infection, in which it was positively correlated with levels of the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α (Dinh et al., 2015). Although some of the members of this family may play an inflammatory role in the gastrointestinal tract, the consensus on its commensal or pathogenic role is unclear, as *Erysipelotrichales* was lowered in patients with new onset Crohn's Disease (Gevers et al., 2014).

In addition to the significant increase in the cotreatment group of the butyrate producing family *Lachnospiraceae*, we looked at the correlation between two well-known butyrate producers and the percentage of butyrate in the ceca of mice. Overall, we see a significant positive correlation between the presence of *Lachnospiraceae* and *Ruminococcaceae* and the amount of butyrate in the mice. *Lachnospiraceae* contain metabolic pathways to use lactate, a product *Lactobacillus johnsonii* is capable of producing, to produce butyrate (Vacca et al., 2020; Vazquez-Munoz et al., 2022). It has been documented that butyrate producing bacteria from the family *Lachnospiraceae* can produce butyrate in the presence of lactate, and is even favored in low pH environments (Belenguer et al., 2007; Duncan et al., 2004).

6.3 Butyrate and its neuroprotective effect

Butyrate is one of the most abundant SCFAs in the gut microbiota, alongside acetate and propionate. This SCFA has been associated with many beneficial effects, ranging from an energy source for colonocytes to neuroprotection in the brain (Roediger, 1982; Sun et al., 2015; Jaworska et al., 2017). In addition, this SCFA is also capable of immunomodulatory effects that result in regulatory T cell differentiation (Arpaia et al., 2013). Regulatory T cells play a key role in immune system management, often ensuring the immune response is not overreactive. Moreover, butyrate has also been a noted histone-deacetylase (HDAC) inhibitor, regulating gene expression (Candido et al., 1978). Researchers have previously shown that chronic administration with sodium butyrate reduces seizure susceptibility in a PTZ-kindling as well as an electrical kindling model of mice, likely due to the reduction of mitochondrial dysfunction and oxidative stress in the former (Li et al., 2021; Reddy et al., 2018). These neuroprotective properties may be what confers protection against PTZ-induced seizures in our study.

6.4 Alteration of the GABA/glutamate ratio in the cortex

GABA and glutamate balance is imperative in seizure management. One of topiramate's mechanisms of action include enhancing $GABA_A$ receptor activity, in which it can increase GABA in the brain of patients (Petroff et al., 2001). This is seen in the topiramate only treatment with a significant increase in GABA in the cortex and a trending increase in the cotreatment group that received topiramate as well as *Lactobacillus johnsonii*. Glutamate reduction was only seen in the cotreatment group but was trending in the *Lactobacillus johnsonii* only group. This lends to the potential that the increase in GABA may be due to topiramate's effects and the decrease in glutamate may be due to *Lactobacillus johnsonii*'s effects, ultimately resulting in the significant increase in the GABA/glutamate ratio only seen in the cotreatment group. Further research needs to elucidate the role of *Lactobacillus johnsonii*'s effect on glutamate levels in the brain, as other research has shown that the gut microbiota are capable of altering the GABA/glutamate ratio in the hippocampus of mice, as well as impacting glutamine, a precursor to glutamate, levels in the brain of mice (Kawase et al., 2017; Olson et al., 2018).

Chapter 7: Summary and Future Directions

7.1 Summary

The gut microbiome can play a role in reducing seizure susceptibility; however, it is lesser known how AEDs may interact with the gut microbiome *in vivo*. Here, we show that ingestion of topiramate results in an abundant increase of the probiotic *Lactobacillus johnsonii.* In addition, only the combination treatment of topiramate and *Lactobacillus johnsonii* reduces seizure susceptibility in a PTZ-kindling mouse model compared with drug or bacteria treatment alone (Figure 24). This impact may be due to the remodeling of the gut microbiome to increase butyrate producing families, such as *Lachnospiraceae*, which in turn increases the neuroprotective SCFA, butyric acid. Lastly, the combination treatment alters the GABA/glutamate ratio in the cortex of mice that underwent PTZ-kindling. To our knowledge, this study is one of the first to show the ability for AEDs to alter the gut microbiome to aid in its anti-seizure effect. This study also highlights the potential for probiotics, such as *Lactobacillus johnsonii*, as therapeutic additions in seizure management.

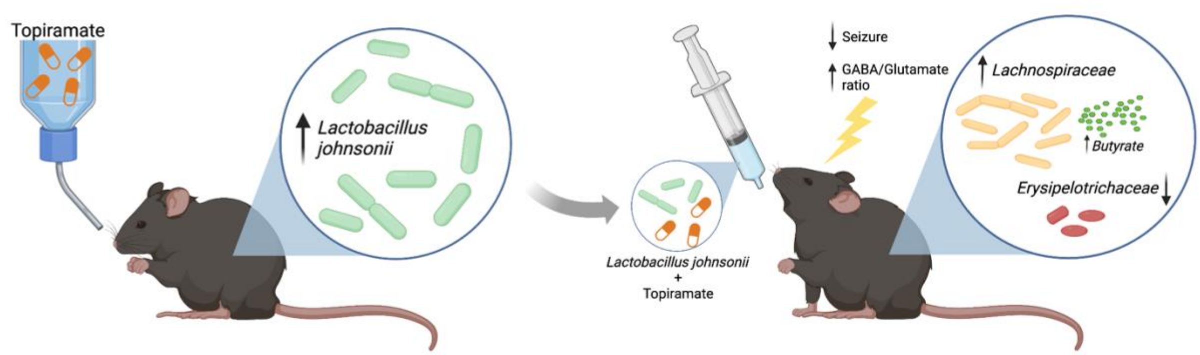

Figure 24: Summary of the role of *Lactobacillus johnsonii* aiding topiramate’s anti-seizure effect. Created with BioRender.com.

7.2 Future Directions

These results show the potential for AEDs to alter the gut microbiome to aid in decreasing seizures. Further studies are required to determine the relationship between topiramate and the gut microbiome. There are several unanswered questions regarding the interaction between topiramate and *Lactobacillus johnsonii*, including whether *Lactobacillus johnsonii* utilizes topiramate as a fuel source, and why the interaction between the probiotic and the drug results in increased levels of butyrate producing bacteria as well as butyrate. Further experiments could elucidate if *Lactobacillus johnsonii* uses topiramate as a fuel source, as well as explore the potential regulation of genes due to the use of this fuel source or the general interaction with topiramate. Perhaps these genes may contribute to the direct effect of increasing butyrate producing bacteria, as well as creating an environment that favors butyrate production. Another noteworthy study could determine if *Lactobacillus johnsonii* alters topiramates structure to a more effective anti-seizure molecule, or if it aids in topiramates bioavailability.

Due to increasing research on probiotics, there is a push to determine its use in medical treatment. These results show that probiotics, in conjunction with AEDs, have a strong role in reducing seizure susceptibility in rodent models, however, this may not as easily translate to human treatment as these effects could be unique to the mouse strain of *Lactobacillus johnsonii*. Despite the lack of direct translation, these studies do add to the body of work that the gut microbiome plays an important role in human health, including neurological diseases. Moreover, this work highlights the potential benefits of using probiotics as an adjuvant therapy with AEDs, to enhance their effect.

REFERENCES

Aiba, Y., Nakano, Y., Koga, Y., Takahashi, K., & Komatsu, Y. (2015). A highly acid-resistant novel strain of Lactobacillus johnsonii No. 1088 has antibacterial activity, including that against Helicobacter pylori, and inhibits gastrin-mediated acid production in mice. *MicrobiologyOpen*, *4*(3), 465–474. https://doi.org/10.1002/mbo3.252

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J. R., Pfeffer, K., Coffer, P. J., & Rudensky, A. Y. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*, *504*(7480), Article 7480. https://doi.org/10.1038/nature12726

Aydemir, Y., Carman, K. B., & Yarar, C. (2020). Screening for functional gastrointestinal disorders in children with epilepsy. *Epilepsy & Behavior*, *111*, 107267. https://doi.org/10.1016/j.yebeh.2020.107267

Bai, Y., Lyu, M., Fukunaga, M., Watanabe, S., Iwatani, S., Miyanaga, K., & Yamamoto, N. (2022). Lactobacillus johnsonii enhances the gut barrier integrity via the interaction between GAPDH and the mouse tight junction protein JAM-2. *Food & Function*, *13*(21), 11021–11033. https://doi.org/10.1039/D2FO00886F

Beghi, E. (2020). The Epidemiology of Epilepsy. *Neuroepidemiology*, *54*(2), 185–191. https://doi.org/10.1159/000503831

Belenguer, A., Duncan, S. H., Holtrop, G., Anderson, S. E., Lobley, G. E., & Flint, H. J. (2007). Impact of pH on Lactate Formation and Utilization by Human Fecal Microbial Communities. *Applied and Environmental Microbiology*, *73*(20), 6526–6533. https://doi.org/10.1128/AEM.00508-07

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), Article 8. https://doi.org/10.1038/s41587-019-0209-9

Braakman, H. M. H., & van Ingen, J. (2018). Can epilepsy be treated by antibiotics? *Journal of Neurology*, *265*(8), 1934–1936. https://doi.org/10.1007/s00415-018-8943-3

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), Article 7. https://doi.org/10.1038/nmeth.3869

Camara-Lemarroy, C. R., Escobedo-Zúñiga, N., Ortiz-Zacarias, D., Peña-Avendaño, J., Villarreal-Garza, E., & Díaz-Torres, M. A. (2016). Prevalence and impact of irritable bowel syndrome in people with epilepsy. *Epilepsy & Behavior*, *63*, 29–33. https://doi.org/10.1016/j.yebeh.2016.05.041

Candido, E. P. M., Reeves, R., & Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell*, *14*(1), 105–113. https://doi.org/10.1016/0092-8674(78)90305-7

Chen, C.-H., Lin, C.-L., & Kao, C.-H. (2015). Irritable Bowel Syndrome Increases the Risk of Epilepsy. *Medicine*, *94*(36), e1497. https://doi.org/10.1097/MD.0000000000001497

Chen, W., Liu, F., Ling, Z., Tong, X., & Xiang, C. (2012). Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. *PLOS ONE*, *7*(6), e39743. https://doi.org/10.1371/journal.pone.0039743

Chen, Z., Brodie, M. J., Liew, D., & Kwan, P. (2018). Treatment Outcomes in Patients With Newly Diagnosed Epilepsy Treated With Established and New Antiepileptic Drugs: A 30-Year Longitudinal Cohort Study. *JAMA Neurology*, *75*(3), 279–286. https://doi.org/10.1001/jamaneurol.2017.3949

Citraro, R., Lembo, F., De Caro, C., Tallarico, M., Coretti, L., Iannone, L. F., Leo, A., Palumbo, D., Cuomo, M., Buommino, E., Nesci, V., Marascio, N., Iannone, M., Quirino, A., Russo, R., Calignano, A., Constanti, A., Russo, E., & De Sarro, G. (2021). First evidence of altered microbiota and intestinal damage and their link to absence epilepsy in a genetic animal model, the WAG/Rij rat. *Epilepsia*, *62*(2), 529–541. https://doi.org/10.1111/epi.16813

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell*, *148*(6), 1258–1270. https://doi.org/10.1016/j.cell.2012.01.035

Cristofori, F., Dargenio, V. N., Dargenio, C., Miniello, V. L., Barone, M., & Francavilla, R. (2021). Anti-Inflammatory and Immunomodulatory Effects of Probiotics in Gut Inflammation: A Door to the Body. *Frontiers in Immunology*, *12*, 578386. https://doi.org/10.3389/fimmu.2021.578386

Cussotto, S., Strain, C. R., Fouhy, F., Strain, R. G., Peterson, V. L., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2019). Differential effects of psychotropic drugs on microbiome composition and gastrointestinal function. *Psychopharmacology*, *236*(5), 1671–1685. https://doi.org/10.1007/s00213-018-5006-5

Davoren, M. J., Liu, J., Castellanos, J., Rodríguez-Malavé, N. I., & Schiestl, R. H. (2018). A novel probiotic, Lactobacillus johnsonii 456, resists acid and can persist in the human gut beyond the initial ingestion period. *Gut Microbes*, *10*(4), 458–480. https://doi.org/10.1080/19490976.2018.1547612

Ding, M., Lang, Y., Shu, H., Shao, J., & Cui, L. (2021). Microbiota–Gut–Brain Axis and Epilepsy: A Review on Mechanisms and Potential Therapeutics. *Frontiers in Immunology*, *12*. https://www.frontiersin.org/articles/10.3389/fimmu.2021.742449

Dinh, D. M., Volpe, G. E., Duffalo, C., Bhalchandra, S., Tai, A. K., Kane, A. V., Wanke, C. A., & Ward, H. D. (2015). Intestinal Microbiota, Microbial Translocation, and Systemic Inflammation in Chronic HIV Infection. *The Journal of Infectious Diseases*, *211*(1), 19–27. https://doi.org/10.1093/infdis/jiu409

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences*, *107*(26), 11971–11975. https://doi.org/10.1073/pnas.1002601107

Duncan, S. H., Louis, P., & Flint, H. J. (2004). Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. *Applied and Environmental Microbiology*, *70*(10), 5810–5817. https://doi.org/10.1128/AEM.70.10.5810-5817.2004

Elmer, G. W., & Remmel, R. P. (1984). Role of the intestinal microflora in clonazepam metabolism in the rat. *Xenobiotica*, *14*(11), 829–840. https://doi.org/10.3109/00498258409151481

Esiobu, N., & Hoosein, N. (2003). An assessment of the in vitro antimicrobial effects of two antiepileptic drugs—Sodium valproate and phenytoin. *Antonie Van Leeuwenhoek*, *83*(1), 63–68. https://doi.org/10.1023/a:1022992224594

Fariba, K. A., & Saadabadi, A. (2023). Topiramate. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK554530/

Fukuda, K., Ogawa, M., Taniguchi, H., & Saito, M. (2016). Molecular Approaches to Studying Microbial Communities: Targeting the 16S Ribosomal RNA Gene. *Journal of UOEH*, *38*(3), 223–232. https://doi.org/10.7888/juoeh.38.223

Gallucci, A., Patel, D. C., Thai, K., Trinh, J., Gude, R., Shukla, D., & Campbell, S. L. (2021). Gut metabolite S-equol ameliorates hyperexcitability in entorhinal cortex neurons following Theiler murine encephalomyelitis virus-induced acute seizures. *Epilepsia*, *62*(8), 1829–1841. https://doi.org/10.1111/epi.16979

Gevers, D., Kugathasan, S., Denson, L. A., Vázquez-Baeza, Y., Van Treuren, W., Ren, B., Schwager, E., Knights, D., Song, S. J., Yassour, M., Morgan, X. C., Kostic, A. D., Luo, C., González, A., McDonald, D., Haberman, Y., Walters, T., Baker, S., Rosh, J., … Xavier, R. J. (2014). The Treatment-Naive Microbiome in New-Onset Crohn's Disease. *Cell Host & Microbe*, *15*(3), 382–392. https://doi.org/10.1016/j.chom.2014.02.005

Ghanizadeh, A., & Berk, M. (2015). Beta-Lactam Antibiotics as A Possible Novel Therapy for Managing Epilepsy and Autism, A Case Report and Review of Literature. *Iranian Journal of Child Neurology*, *9*(1), 99–102.

Gómez-Eguílaz, M., Ramón-Trapero, J. l., Pérez-Martínez, L., & Blanco, J. r. (2018). The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: A pilot study. *Beneficial Microbes*, *9*(6), 875–881. https://doi.org/10.3920/BM2018.0018

Gong, X., Liu, X., Chen, C., Lin, J., Li, A., Guo, K., An, D., Zhou, D., & Hong, Z. (2020). Alteration of Gut Microbiota in Patients With Epilepsy and the Potential Index as a Biomarker. *Frontiers in Microbiology*, *11*, 517797. https://doi.org/10.3389/fmicb.2020.517797

Gong, X., Liu, Y., Liu, X., Li, A. Q., Guo, K. D., Zhou, D., & Hong, Z. (2022). Analysis of gut microbiota in patients with epilepsy treated with valproate: Results from a three months observational prospective cohort study. *Microbial Pathogenesis*, *162*, 105340. https://doi.org/10.1016/j.micpath.2021.105340

He, Z., Cui, B.-T., Zhang, T., Li, P., Long, C.-Y., Ji, G.-Z., & Zhang, F.-M. (2017). Fecal microbiota transplantation cured epilepsy in a case with Crohn's disease: The first report. *World Journal of Gastroenterology*, *23*(19), 3565–3568. https://doi.org/10.3748/wjg.v23.i19.3565

Ilhan, Z. E., Brochard, V., Lapaque, N., Auvin, S., & Lepage, P. (2022). Exposure to anti-seizure medications impact growth of gut bacterial species and subsequent host response. *Neurobiology of Disease*, *167*, 105664. https://doi.org/10.1016/j.nbd.2022.105664

Izuddin, W. I., Loh, T. C., Samsudin, A. A., Foo, H. L., Humam, A. M., & Shazali, N. (2019). Effects of postbiotic supplementation on growth performance, ruminal fermentation and microbial profile, blood metabolite and GHR, IGF-1 and MCT-1 gene expression in post-weaning lambs. *BMC Veterinary Research*, *15*(1), 315. https://doi.org/10.1186/s12917-019-2064-9

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., Winker, K., Kado, D. M., Orwoll, E., Manary, M., Mirarab, S., & Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MSystems*, *3*(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Jaworska, J., Ziemka-Nalecz, M., Sypecka, J., & Zalewska, T. (2017). The potential neuroprotective role of a histone deacetylase inhibitor, sodium butyrate, after neonatal hypoxia-ischemia. *Journal of Neuroinflammation*, *14*(1), 34. https://doi.org/10.1186/s12974-017-0807-8

Kaakoush, N. O. (2015). Insights into the Role of Erysipelotrichaceae in the Human Host. *Frontiers in Cellular and Infection Microbiology*, *5*. https://www.frontiersin.org/articles/10.3389/fcimb.2015.00084

Kanner, A. M., & Bicchi, M. M. (2022). Antiseizure Medications for Adults With Epilepsy: A Review. *JAMA*, *327*(13), 1269–1281. https://doi.org/10.1001/jama.2022.3880

Kawase, T., Nagasawa, M., Ikeda, H., Yasuo, S., Koga, Y., & Furuse, M. (2017). Gut microbiota of mice putatively modifies amino acid metabolism in the host brain. *British Journal of Nutrition*, *117*(6), 775–783. https://doi.org/10.1017/S0007114517000678

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B. A., Thiessen, P. A., Yu, B., Zaslavsky, L., Zhang, J., & Bolton, E. E. (2023). PubChem 2023 update. *Nucleic Acids Research*, *51*(D1), D1373–D1380. https://doi.org/10.1093/nar/gkac956

Kitamura, S., Sugihara, K., Kuwasako, M., & Tatsumi, K. (1997). The Role of Mammalian Intestinal Bacteria in the Reductive Metabolism of Zonisamide. *Journal of Pharmacy and Pharmacology*, *49*(3), 253–256. https://doi.org/10.1111/j.2042-7158.1997.tb06790.x

Kossoff, E. H., Freeman, J. M., Turner, Z., & Rubenstein, J. E. (2011). *Ketogenic Diets: Treatments for Epilepsy and Other Disorders* (5th ed.). Demos Health.

Lee, B., Moon, K. M., & Kim, C. Y. (2018). Tight Junction in the Intestinal Epithelium: Its Association with Diseases and Regulation by Phytochemicals. *Journal of Immunology Research*, *2018*, 2645465. https://doi.org/10.1155/2018/2645465

Li, D., Bai, X., Jiang, Y., & Cheng, Y. (2021). Butyrate alleviates PTZ-induced mitochondrial dysfunction, oxidative stress and neuron apoptosis in mice via Keap1/Nrf2/HO-1 pathway. *Brain Research Bulletin*, *168*, 25–35. https://doi.org/10.1016/j.brainresbull.2020.12.009

Lin Lin Lee, V., Kar Meng Choo, B., Chung, Y.-S., P. Kundap, U., Kumari, Y., & Shaikh, Mohd. F. (2018). Treatment, Therapy and Management of Metabolic Epilepsy: A Systematic Review. *International Journal of Molecular Sciences*, *19*(3), 871. https://doi.org/10.3390/ijms19030871

Lum, G. R., Olson, C. A., & Hsiao, E. Y. (2020). Emerging roles for the intestinal microbiome in epilepsy. *Neurobiology of Disease*, *135*, 104576. https://doi.org/10.1016/j.nbd.2019.104576

Mager, L. F., Burkhard, R., Pett, N., Cooke, N. C. A., Brown, K., Ramay, H., Paik, S., Stagg, J., Groves, R. A., Gallo, M., Lewis, I. A., Geuking, M. B., & McCoy, K. D. (2020). Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy. *Science*, *369*(6510), 1481–1489. https://doi.org/10.1126/science.abc3421

Maier, L., Pruteanu, M., Kuhn, M., Zeller, G., Telzerow, A., Anderson, E. E., Brochado, A. R., Fernandez, K. C., Dose, H., Mori, H., Patil, K. R., Bork, P., & Typas, A. (2018). Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature*, *555*(7698), Article 7698. https://doi.org/10.1038/nature25979

Medel-Matus, J.-S., Shin, D., Dorfman, E., Sankar, R., & Mazarati, A. (2018). Facilitation of kindling epileptogenesis by chronic stress may be mediated by intestinal microbiome. *Epilepsia Open*, *3*(2), 290–294. https://doi.org/10.1002/epi4.12114

Morais, L. H., Schreiber, H. L., & Mazmanian, S. K. (2021). The gut microbiota–brain axis in behaviour and brain disorders. *Nature Reviews Microbiology*, *19*(4), Article 4. https://doi.org/10.1038/s41579-020-00460-0

Olson, C. A., Vuong, H. E., Yano, J. M., Liang, Q. Y., Nusbaum, D. J., & Hsiao, E. Y. (2018). The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet. *Cell*, *173*(7), 1728-1741.e13. https://doi.org/10.1016/j.cell.2018.04.027

Orsi, S. A., Devulapalli, R. K., Nelsen, J. L., McFadden, T., Surineni, R., & Jarome, T. J. (2019). Distinct subcellular changes in proteasome activity and linkage-specific protein polyubiquitination in the amygdala during the consolidation and reconsolidation of a fear memory. *Neurobiology of Learning and Memory*, *157*, 1–11. https://doi.org/10.1016/j.nlm.2018.11.012

Peng, A., Qiu, X., Lai, W., Li, W., Zhang, L., Zhu, X., He, S., Duan, J., & Chen, L. (2018). Altered composition of the gut microbiome in patients with drug-resistant epilepsy. *Epilepsy Research*, *147*, 102–107. https://doi.org/10.1016/j.eplepsyres.2018.09.013

Petroff, O. A. C., Hyder, F., Rothman, D. L., & Mattson, R. H. (2001). Topiramate Rapidly Raises Brain GABA in Epilepsy Patients. *Epilepsia*, *42*(4), 543–548. https://doi.org/10.1046/j.1528-1157.2001.18800.x

Pyron, M. (2010). Characterizing Communities. *Nature Education Knowledge*. https://www.nature.com/scitable/knowledge/library/characterizing-communities-13241173/

Qian, Y., Lv, P.-C., Shi, L., Fang, R.-Q., Song, Z.-C., & Zhu, H.-L. (2009). Synthesis, antimicrobial activity of lamotrigine and its ammonium derivatives. *Journal of Chemical Sciences*, *121*(4), 463–470. https://doi.org/10.1007/s12039-009-0055-2

Reddy, S. D., Clossen, B. L., & Reddy, D. S. (2018). Epigenetic Histone Deacetylation Inhibition Prevents the Development and Persistence of Temporal Lobe Epilepsy. *Journal of Pharmacology and Experimental Therapeutics*, *364*(1), 97–109. https://doi.org/10.1124/jpet.117.244939

Roediger, W. E. (1982). Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology*, *83*(2), 424–429.

Rudzinski, L. A., Vélez-Ruiz, N. J., Gedzelman, E. R., Mauricio, E. A., Shih, J. J., & Karakis, I. (2016). New Antiepileptic Drugs: Focus on Ezogabine, Clobazam, and Perampanel. *Journal of Investigative Medicine*, *64*(6), 1087–1101. https://doi.org/10.1136/jim-2016-000151

Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology*, *14*(8), e1002533. https://doi.org/10.1371/journal.pbio.1002533

Sharon, G., Sampson, T. R., Geschwind, D. H., & Mazmanian, S. K. (2016). The Central Nervous System and the Gut Microbiome. *Cell*, *167*(4), 915–932. https://doi.org/10.1016/j.cell.2016.10.027

Shimada, T., & Yamagata, K. (2018). Pentylenetetrazole-Induced Kindling Mouse Model. *Journal of Visualized Experiments : JoVE*, *136*, 56573. https://doi.org/10.3791/56573

Silva, Y. P., Bernardi, A., & Frozza, R. L. (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. *Frontiers in Endocrinology*, *11*. https://www.frontiersin.org/articles/10.3389/fendo.2020.00025

Stokes, J. M., Davis, J. H., Mangat, C. S., Williamson, J. R., & Brown, E. D. (2014). Discovery of a small molecule that inhibits bacterial ribosome biogenesis. *ELife*, *3*, e03574. https://doi.org/10.7554/eLife.03574

Sun, J., Wang, F., Li, H., Zhang, H., Jin, J., Chen, W., Pang, M., Yu, J., He, Y., Liu, J., & Liu, C. (2015). Neuroprotective Effect of Sodium Butyrate against Cerebral Ischemia/Reperfusion Injury in Mice. *BioMed Research International*, *2015*, 395895. https://doi.org/10.1155/2015/395895

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). Chapter Three—The Role of Short-Chain Fatty Acids in Health and Disease. In F. W. Alt (Ed.), *Advances in Immunology* (Vol. 121, pp. 91–119). Academic Press. https://doi.org/10.1016/B978-0-12-800100-4.00003-9

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., & De Angelis, M. (2020). The Controversial Role of Human Gut Lachnospiraceae. *Microorganisms*, *8*(4), 573. https://doi.org/10.3390/microorganisms8040573

Vazquez-Munoz, R., Thompson, A., Russell, J. T., Sobue, T., Zhou, Y., & Dongari-Bagtzoglou, A. (2022). Insights From the Lactobacillus johnsonii Genome Suggest the Production of Metabolites With Antibiofilm Activity Against the Pathobiont Candida albicans. *Frontiers in Microbiology*, *13*. https://www.frontiersin.org/articles/10.3389/fmicb.2022.853762

Wang, H., Sun, Y., Xin, J., Zhang, T., Sun, N., Ni, X., Zeng, D., & Bai, Y. (2020). Lactobacillus johnsonii BS15 Prevents Psychological Stress-Induced Memory Dysfunction in Mice by Modulating the Gut-Brain Axis. *Frontiers in Microbiology*, *11*, 1941. https://doi.org/10.3389/fmicb.2020.01941

Wang, Y.-F., Qiao, M., Zhu, D., & Zhu, Y.-G. (2020). Antibiotic Resistance in the Collembolan Gut Microbiome Accelerated by the Nonantibiotic Drug Carbamazepine. *Environmental Science & Technology*, *54*(17), 10754–10762. https://doi.org/10.1021/acs.est.0c03075

Xie, G., Zhou, Q., Qiu, C.-Z., Dai, W.-K., Wang, H.-P., Li, Y.-H., Liao, J.-X., Lu, X.-G., Lin, S.-F., Ye, J.-H., Ma, Z.-Y., & Wang, W.-J. (2017). Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. *World Journal of Gastroenterology*, *23*(33), 6164–6171. https://doi.org/10.3748/wjg.v23.i33.6164

Yao, D., Dai, W., Dong, M., Dai, C., & Wu, S. (2021). MUC2 and related bacterial factors: Therapeutic targets for ulcerative colitis. *EBioMedicine*, *74*, 103751. https://doi.org/10.1016/j.ebiom.2021.103751

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'Toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107

Zhong, X., Zhao, Y., Huang, L., Liu, J., Wang, K., Gao, X., Zhao, X., & Wang, X. (2023). Remodeling of the gut microbiome by Lactobacillus johnsonii alleviates the development of acute myocardial infarction. *Frontiers in Microbiology*, *14*, 1140498. https://doi.org/10.3389/fmicb.2023.1140498

Zhu, Q., Jin, Z., Wu, W., Gao, R., Guo, B., Gao, Z., Yang, Y., & Qin, H. (2014). Analysis of the Intestinal Lumen Microbiota in an Animal Model of Colorectal Cancer. *PLOS ONE*, *9*(3), e90849. https://doi.org/10.1371/journal.pone.0090849

Zihni, C., Mills, C., Matter, K., & Balda, M. S. (2016). Tight junctions: From simple barriers to multifunctional molecular gates. *Nature Reviews Molecular Cell Biology*, *17*(9), Article 9. https://doi.org/10.1038/nrm.2016.80

Zimmermann, M., Zimmermann-Kogadeeva, M., Wegmann, R., & Goodman, A. L. (2019). Mapping human microbiome drug metabolism by gut bacteria and their genes. *Nature*, *570*(7762), Article 7762. https://doi.org/10.1038/s41586-019-1291-3