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Effects of clinical ketogenic diet therapy for pediatric epilepsy on the gut microbiota and seizure resistance

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Effects of clinical ketogenic diet therapy for pediatric epilepsy on the gut microbiota and seizure resistance

A dissertation submitted in partial satisfaction of the requirements

for the degree Doctor of Philosophy in Molecular Biology

by

Gregory Ryan Tadashi Lum

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ABSTRACT OF THE DISSERTATION

Effects of clinical ketogenic diet therapy for pediatric epilepsy on the gut microbiota and seizure resistance

by

Gregory Ryan Tadashi Lum

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2023

Professor Elaine Yih-Nien Hsiao, Chair

The high-fat low-carbohydrate ketogenic diet (**KD**) is an established dietary therapy for individuals with refractory epilepsy, whose seizures are resistant to existing anti-epileptic drugs. However, use of the KD to treat refractory epilepsy is challenging because the diet is difficult for patients to implement, manage, and maintain due to its severe restrictiveness and adverse side effects. Exactly how the clinical KD reduces seizure symptoms when other anti-epileptic drugs are ineffective is poorly understood. The gut microbiome has emerged as a key intermediary between diet and host metabolism, neural activity, and behavior. The gut microbiome modulates seizure susceptibility and the anti-seizure effects of the ketogenic diet (KD) in animal models. This dissertation work seeks to understand if these relationships seen in animal models translate to KD therapies for human drug-resistant epilepsy. Herein we report that KD therapy in children with pediatric epilepsy alters the function of the human gut microbiome. In addition, colonizing mice with KD-associated human gut microbes confers increased resistance to 6-Hz

psychomotor seizures, as compared to colonization with gut microbes from matched pre-treatment controls. Parallel analysis of human donor and mouse recipient metagenomic and metabolomic profiles identifies subsets of shared functional features that are seen in response to KD treatment in humans and preserved upon transfer to mice fed a standard diet. These include enriched representation of microbial genes and metabolites related to anaplerosis, fatty acid beta-oxidation, and amino acid metabolism. Mice colonized with KD-associated human gut microbes further exhibit altered hippocampal and frontal cortical transcriptomic profiles relative to colonized pre-treatment controls, including differential expression of genes related to ATP synthesis, glutathione metabolism, oxidative phosphorylation, and translation. Integrative co-occurrence network analysis of the metagenomic, metabolomic, and brain transcriptomic datasets identifies features that are shared between human and mouse networks, and select microbial functional pathways and metabolites that are candidate primary drivers of hippocampal expression signatures related to epilepsy. Together, these findings reveal key microbial functions and biological pathways that are altered by clinical KD therapies for pediatric refractory epilepsy and further linked to microbiome-induced alterations in brain gene expression and seizure protection in mice.

For my loved ones --without their support this would not have been possible.

The dissertation of Gregory Ryan Tadashi Lum is approved.

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2023

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alterations in the human gut microbiome that confer seizure resistance in mice. *BioRxiv*, 2023.03.17.533243.

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Olson, C. A., **Lum, G. R.** and Hsiao, E. Y. (2020). Ketone bodies exert ester-ordinary suppression of bifidobacteria and Th17 Cells. *Cell Metabolism.* PMID: 32492391 PMCID: N/A

Lum, G. R., Olson, C. A. and Hsiao, E. Y. (2019). Emerging roles for the intestinal microbiome in epilepsy. *Neurobiology of Disease* 104576. PMID: 31445165; PMCID: N/A

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- *Chicago, IL; Dec. 2nd- 7th, 2021*

UCLA MBIDP Retreat (poster, invited) **September 2021**
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LA BEST - LA Bioscience Ecosystem Summit 2021 (poster) **May 2021**
- *Virtual; May 25th – 27th, 2021*

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Chapter 1: Overview

My dissertation research is focused on understanding human-gut microbe interactions that are shaped by the clinical ketogenic diet (KD) in the context of pediatric refractory epilepsy. More specifically my dissertation seeks to improve our understanding of the following questions: (i) how does the human microbiota change in composition and function in response to clinical KD therapy? (ii) Do previous findings from similar studies using the KD in rodent epilepsy models apply to human epilepsy, the human gut microbiome, and the clinical KD therapies used to treat pediatric epilepsy patients? (iii) What are the features of the clinical KD-associated gut microbiome seen in both human donors and inoculated mouse recipients that correlate with microbiome-dependent seizure protection? (iv) How does colonization with microbes derived from the fecal microbiota of clinical KD-treated individuals affect brain function and seizure susceptibility?

The foundation of my doctoral research is based on the need for tractable and effective treatments to control epilepsy that are not based on usage of anti-epileptic drugs (AEDs). An estimated one in three individuals out of 50 million epilepsy patients worldwide have refractory epilepsy, or pharmacoresistant epilepsy, wherein seizures cannot be controlled with AEDs (WHO, 2019). While the KD has been used to treat refractory epilepsy for nearly 100 years, the mechanisms by which the KD works to provide its seizure protection are still not completely understood. The restrictive high-fat low-carbohydrate nutritional requirement, difficult implementation, and side effects of the KD continue to hinder its use to treat refractory epilepsy. Increasing our understanding of which seizure modulating pathways the KD affects will inform the refinement of novel KD-based therapeutics to treat refractory epilepsy.

The gut microbiome has emerged as an essential mediator of diet on host metabolism, neurological activity, and behavior. There are several recent clinical studies reporting alterations in the gut microbiota of epileptic individuals and in response to treatment with the clinical KD. Additionally previous work from the Hsiao lab has also demonstrated in both an inducible and genetic model of refractory epilepsy that the microbiome and the KD are both necessary for the

host to receive KD-mediated seizure protection (Olson et. al., 2018). Thus, my dissertation research hopes to determine the mechanisms by which the human clinical KD-associated microbiome modifies seizure risk, and to further evaluate microbiome-based interventions that can increase the overall efficacy of KD treatment, ease clinical implementation, and/or eliminate dietary side effects.

In **Chapter 2**, I present a version of the chapter titled “Interactions between the gut microbiome and ketogenic diet in refractory epilepsy” from *Microbiome in Neurological Disease*. In this work, we first discuss the origins of the KD as a treatment for epilepsy and the recent renewed interest in refining the classical KD, and other variations of the KD, as an effective treatment for refractory epilepsy. We then compile the results of existing clinical and animal studies reporting microbiome differences in epileptic individuals compared to healthy controls, and cross-compare the results. Additionally, we discuss studies comparing the classical KD to other variations of the KD, and survey effects of these diets on host physiology, host gut microbiota, and effects on seizure outcomes. This work has been published as:
Özcan, E., **Lum, G. R.** and Hsiao, E. Y. (2022). Interactions between the gut microbiome and ketogenic diet in refractory epilepsy. International Review of Neurobiology. PMID: 36427956

In **Chapter 3**, I present a version of a literature review focused on increasing evidence linking the gut microbiota and gut microbiota-related factors with epilepsy and seizure susceptibility. I discuss microbiome-related factors such as infection and antibiotic treatment that affect seizure susceptibility in animal models of epilepsy and in humans with epilepsy. I highlight several recent clinical studies reporting changes in the gut microbiota of epileptic patients compared to healthy controls and in response to epilepsy treatments. Lastly, I discuss remaining open questions on the roles for the microbiome in epilepsy and addressing these questions can reveal novel approaches for treating refractory epilepsy. This work has been published as:

Lum, G. R., Olson, C. A. and Hsiao, E. Y. (2019). Emerging roles for the intestinal microbiome in epilepsy. *Neurobiology of Disease* 104576. PMID: 31445165; PMCID: N/A

In **Chapter 4**, I describe recent work where we investigate the effects of the clinical KD on the human gut microbiota and seizure susceptibility in mice. Furthermore, we identify microbial functions and metabolites that impact seizure susceptibility and that display significant network interactions with the brain transcriptome that may contribute to seizure protection in recipient mice inoculated with the clinical KD-associated human gut microbiome from children with refractory epilepsy. In collaboration with UCLA's Ketogenic dietary therapies program, we recruited a cohort of children with refractory epilepsy to study the human gut microbiome before (pre-KD) and after (post-KD) implementation of the clinical KD and the impact of the clinical KD-associated gut microbiota on seizure susceptibility in mice. In this study I first assessed compositional and functional differences in the microbiomes of our cohort of refractory epileptic children before and after treatment with the clinical KD. Despite the varied subtypes of refractory epilepsy and KD ratio and specific nutritional composition, there were shared changes in gut microbiota function related to GDP-mannose biosynthesis, 2-methylcitrate cycle, glycol metabolism and degradation, polyamine biosynthesis and biotin biosynthesis in response to the clinical KD. To understand if the clinical KD-associated gut microbes affect seizure susceptibility, I colonized paired cohorts of germ-free mice with matched pre-KD and post-KD stool samples collected from children with refractory epilepsy and performed cohort-level seizure testing using the 6-Hz psychomotor seizure model. I demonstrated that oral inoculation of mice with the clinical KD-associated human gut microbiota increased 6-Hz seizure threshold levels compared to the matched pre-KD control, a threshold increase similar to that of mice fed KD chow. We demonstrated the necessity of an intact clinical-KD associated microbiota for increased seizure threshold through oral inoculation of mice with clinical KD associated microbiota followed by depletion of gut bacteria using broad-spectrum antibiotics and seizure

threshold testing. Additionally, administration of clinical KD-associated intestinal small molecules to mice was only able to increase seizure threshold acutely, with seizure protection decreasing over 4 days. I next wanted to identify any microbial functions or metabolites that contribute to the seizure protective effects seen in our mouse model. Through parallel metagenomic analysis of both children with refractory epilepsy and inoculated recipient mice I identified fatty acid β-oxidation, glycol metabolism and degradation, methylcitrate cycle I, methylcitrate cycle II, and proline biosynthesis as clinical KD-associated microbiome functions appearing in both that may affect seizure susceptibility. Analysis of differentially abundant fecal metabolites in human and mouse samples revealed enrichment of chemical subclasses and metabolic pathways including amino acid, hydroxy fatty acid, sugar acid, phenylpropanoic acid, and monosaccharide-related metabolites and methionine metabolism, glycine and serine metabolism, and betaine metabolism. To investigate of changes in the gut microbiota may alter seizure susceptibility in the brain, I analyzed hippocampal transcriptomic from recipient mice colonized with clinical KD-associated microbes. These mice displayed distinct hippocampal gene expression patterns with the set of differentially expressed genes enriched for biological processes relating to RNA processing, translation, cellular stress response, TORC1 signaling, regulation of long-term synaptic potentiation, neuronal development, and response to nutrient levels. To identify additional gut microbial functions that may drive frontal cortical or hippocampal gene expression, we collaborated with Dr. Daniel Ha and Xia Yang to perform parallel network analysis of human donor and mouse recipient ‘omics datasets which were linked by metagenomic key driver nodes related to branched chain amino acid (BCAA) biosynthesis, CoA biosynthesis, L-alanine fermentation, and L-arginine biosynthesis and linked to hippocampal transcript modules enriched for genes related to neurogenesis and Wnt signaling, both linked to epileptogenesis. Lastly, we integrated data from genome-wide association studies (GWAS) of epilepsy which revealed that alterations in hippocampal transcripts that co-occurred with microbial metagenomic and metabolomic features may

contribute to the microbiome-dependent increases in seizure threshold of post-KD recipient mice compared to matched pre-KD controls.

A version of this work has been submitted for publication and is currently available as a pre-print on Biorxiv as:

Lum, G. R., Ha, S. M., Olson, C. A., Blencowe, M., Paramo, J., Reyes, B., Matsumoto, J. H., Yang, X., & Hsiao, E. Y. (2023). Ketogenic diet therapy for pediatric epilepsy is associated with alterations in the human gut microbiome that confer seizure resistance in mice. *BioRxiv*, 2023.03.17.533243.

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Chapter 2: Intersections between the gut microbiome and ketogenic diet in refractory epilepsy

Abstract

Epilepsy is one of the most common neurological diseases globally, afflicting approximately 50 million people worldwide. While many antiepileptic drugs exist, an estimated one-third of individuals do not respond to available medications. The high fat, low carbohydrate ketogenic diet (KD) has been used to treat refractory epilepsy in cases when existing antiepileptic drugs fail. However, there are many variations of the KD, each of which varies greatly in its efficacy and side effects. Increasing evidence suggests that interactions between the KD and gut microbiome may modulate the effects of the diet on host physiology. Herein, we review existing evidence of microbiome differences in epileptic individuals compared to healthy controls. We highlight in particular both clinical and animal studies revealing effects of the KD on the composition and function of the microbiome, as well as proof-of-concept animal studies that implicate the microbiome in the antiseizure effects of the KD. We further synthesize findings suggesting that variations in clinical KD formulations may differentially influence host physiology and discuss the gut microbial interactions with specific dietary factors that may play a role. Overall, understanding interactions between the gut microbiota and specific nutritional components of clinical KDs could reveal foundational mechanisms that underlie the effectiveness, variability, and side effects of different KDs, with the potential to lead to precision nutritional and microbiome-based approaches to treat refractory epilepsy.

1.Introduction to epilepsy

Since the dawn of recorded history, epilepsy has been a burden on human health. Documented in ancient Mesopotamian medical texts as early as 2000 B.C., and later acknowledged by the ancient Egyptians and Greeks, epilepsy was originally attributed to being possessed by an ill-intentioned spirit or deity (World Health Organization, 2005). Despite these early ideas, epilepsy was not completely distanced from its divine beginnings and was not fully

accepted as a brain disorder until the early 19th century (Magiorkinis, Sidiropoulou, & Diamantis, 2010).

Presently, epilepsy is the fourth most common neurological disorder worldwide and is broadly defined by recurring epileptic seizures. The general mechanism of an epileptic seizure event originates from unprovoked abnormal or excessive synchronous neuronal activity, commonly originating in the temporal lobe. A patient is considered epileptic if they have two or more unprovoked or reflex seizure events within a span of time greater than 24h apart (Fisher et al., 2014). Both genetic factors, such as mutations in voltage- or ligand-gated ion channels, and environmental factors, such as brain trauma or infection, contribute to risk for epilepsy (Stafstrom & Carmant, 2015).

Although epileptic seizure events and associated risk factors are well characterized, the fundamental mechanisms underlying epileptogenesis remain poorly understood. The World Health Organization reports over 50 million cases of epilepsy worldwide, accounting for 0.5% of the global health burden (WHO, 2019). Modern antiepileptic drugs (AEDs) are the frontline treatment reducing seizures in approximately 60–70% of cases by blocking sodium or calcium ion channels to decrease the release of the excitatory neurotransmitter glutamate or enhance signaling of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Xia, Ou, & Pan, 2017).

For the remaining one in three epilepsy patients who do not respond to available AEDs, alternative forms of seizure management must be considered, each with their own caveats, or the patient faces a three- to sixfold increase in premature death (Sperling, 2004). First, invasive surgery to resect portions of the brain where seizure activity originates can be used to eliminate seizure activity. However, it must be feasible to accurately locate and safely resect the portion of the brain from which the seizures originate. Success rates vary from 40% to 70% and are highly dependent on seizure etiology (Health Quality Ontario, 2012). Second, refractory (drug-resistant) epilepsy patients can also consider surgical implantation of a neuromodulation device

near the vagus nerve or directly in the brain to manage seizures with electrical stimulation. These devices reduce seizures in an estimated 25–50% of refractory epileptic individuals that pursue this intervention (Gooneratne et al., 2016). Lastly, dietary therapies are used as a clinical treatment for refractory epilepsy and are the least invasive alternatives to AEDs. The high fat, low carbohydrate ketogenic diet (KD) is the most well-known dietary therapy for refractory epilepsy. Typically, the KD has between a 22–55% success rate of at least 50% seizure reduction in adults and upwards of 60–75% success rates in infants and children (Castellano & Merlino, 2012; Henderson, Filloux, Alder, Lyon, & Caplin, 2006; Klein, Janousek, Barber, & Weissberger, 2010; Li, Zou, & Ding, 2013; Merrit & Putnam, 1938; Williams & Cervenka, 2017). While the KD can effectively reduce seizures for subsets of individuals, its restrictiveness introduces many side effects including hypoglycemia, metabolic acidosis, and weight loss. These obstacles make it difficult for epilepsy patients to maintain the diet, with about 33% dietary compliance in adults and between 42–60% compliance in children under 10years old within 6months of starting the KD (Coppola et al., 2002; Yang et al., 2022). Moreover, the mechanisms of action for the KD are poorly understood. Overall, while several AEDs are available for treating epilepsy, effective and manageable non-invasive treatments are lacking for the remaining one in three individuals with refractory epilepsy. Increased understanding of the mechanisms that enable the efficacy of diet-based therapies for subsets of individuals could uncover new therapeutic targets and approaches to improve the overall success rates, efficacy, and ease of maintenance of dietary treatments.

2. Increasing interest in the microbiome and epilepsy.

The microbiome has emerged as a mediator between diet and host, becoming a specific topic of interest to enhance the efficacy of diet-based therapies. The gut microbiota encompasses trillions of native bacteria, viruses, and fungi residing in the host gastrointestinal tract (Proctor et al., 2019). Both gut microbiome composition and function are fluid, rapidly

changing when exposed to variables such as medication, stress, and diet but simultaneously influenced by variables such as host genetics (Hasan & Yang, 2019; Turnbaugh et al., 2009). The gut microbiota significantly alters amino acid and carbohydrate metabolism, linked to microglial and astrocyte function, vagal activity, and hippocampal neurotransmitter levels (Lum, Olson, & Hsiao, 2020). Animal studies further reveal that the microbiota is linked to neurobehavior, symptoms of neurological disorders, and brain development (Vuong, Yano, Fung, & Hsiao, 2017). Additionally, alterations in the gut microbiome have been reported across several neuropsychiatric, neurodevelopmental, neurodegenerative disorders, and animal models of epilepsy (Citraro et al., 2021; Olson et al., 2018), but the connection between the microbiome and human epilepsy remains unclear (Fig. 2.1).

Only a few recent clinical studies have reported differences in baseline fecal microbiota profiles in small cohorts of epileptic individuals compared to healthy controls. One such study analyzed fecal microbiota profiles from 16S rRNA gene sequencing of stool samples collected from 14 infants and toddlers with refractory epilepsy (1–4years old) and 30 matched healthy controls who did not have any chronic illnesses, metabolic diseases, and had not taken antibiotics 1month prior to study initiation (Xie et al., 2017). Between healthy and refractory epilepsy groups, there was no significant difference observed in α-diversity measured by the Shannon index for evenness. However, principal component analysis (PCA) of the taxonomic data revealed distinct clustering of refractory epilepsy samples away from the healthy controls, suggesting differences in fecal microbiota β-diversity. Furthermore, Linear Discriminant Analysis Effect Size (LEfSe) indicated that the infant refractory epilepsy fecal microbiota had increased relative abundances of *Bacillota* and *Pseudomonadota* and decreased relative abundances of *Actinomycetota* and *Bacteroidota*. Additional differences in taxonomic membership were notable at the genus level. Refractory epilepsy infants had high levels of *Cronobacter* which was not detected in healthy infant controls. Relative abundances of Bacteroides, Bifidobacterium, and *Prevotella* were decreased in epileptic infants compared to healthy controls. Although

differences in fecal microbiota profiles were observed between epileptic and healthy infants, it remains unclear whether environmental variables, such as dietary intake and existing medications, may be responsible for the differences reported in this study.

A second human study profiled the fecal microbiota of 12 children (2–17years old) with refractory epilepsy and 11 healthy parent controls (Lindefeldt et al., 2019). Shotgun metagenomic sequencing analysis indicated that children with refractory epilepsy exhibited decreased Shannon index α-diversity compared to the healthy parent controls. PCA of fecal microbiome taxonomic profiles displayed distinct clustering of the healthy parent microbiomes away from child refractory epilepsy microbiomes. Children with refractory epilepsy also presented with increased relative abundances of *Bacillota* and *Pseudomonadota* and decreased relative abundances of *Actinomycetota* and *Bacteroidota.* Metagenomic analysis revealed differences in microbiome functional potential, highlighting decreased gene content in the acetyl-CoA pathway from β-hydroxybutyryl-CoA and crotonase in the child refractory epilepsy microbiome compared to the healthy parent control. Although the sample cohort contained household controls, the lack of age-matched controls is a major caveat in this study, considering the known age-related differences in the diversity and the function of the gut microbiome (Nagpal et al., 2018).

Another recent clinical study performed 16S rRNA gene sequencing of stool samples from a cohort of 42 refractory epilepsy individuals, 49 drug-sensitive epilepsy individuals, and 65 matched healthy family member controls, excluding individuals who took antibiotic or probiotics within the past 3 months or who had chronic illness (Peng et al., 2018). α-Diversity measured by the Chao1 diversity index for species richness was increased in the fecal microbiota from refractory epilepsy patients, specifically for those individuals experiencing more than four seizures per year. Weighted principal coordinate analysis (PCoA) revealed no distinct clustering of refractory epilepsy patient microbiomes unlike the distinct clustering seen in the Lindefeldt et al. and Xie et al. studies. Linear discriminant analysis (LDA) reported increased relative

abundances of several members of the *Bacillota* phylum including *Coprococcus, Coprobacillus, Roseburia,* and *Ruminococcus* with decreases in *Bacteroides* compared to healthy controls. Interestingly LDA revealed taxonomic differences between refractory epilepsy and drug-sensitive epilepsy groups, with refractory epilepsy individuals exhibiting increased relative abundances of *Akkermansia, Fusobacterium, Methanobrevibacter*, and *Neisseria*. Epileptic individuals were matched with a healthy family member control to decrease the impact of diet on gut microbiome composition. However, there were no dietary controls across the families that participated in this study. Notably, this study design matched age, sex, and exposure to medications across the three representative groups.

Another human study used a cohort of 30 adult patients with idiopathic focal epilepsy and 10 healthy control adults had their stool samples sequenced by 16S rRNA gene sequencing (Şafak, Altunan, Topçu, & Eren Topkaya, 2020). Weighted PCoA revealed distinct clustering between healthy and epileptic patient groups. Epileptic patients were reported to have decreased relative abundance of *Bacillota, Bacteroidota,* and *Actinomycetota*. Similar to the reports from Xie et al. and Lindefeldt et al., Şafak et al. also reports increased relative abundance of *Pseudomonadota* in epileptic individuals compared to the healthy controls. The lack of dietary control and age-related differences may explain why the findings from this study do not align with previous reported findings (Lindefeldt et al., 2019; Peng et al., 2018; Xie et al., 2017).

Another study of a cohort of 74 adults, including 24 epilepsy patients and 50 healthy adult controls, had their stool samples sequenced by 16S rRNA gene sequencing (Cui et al., 2022). α-Diversity measured by the Shannon and Simpson indexes showed the healthy controls had significantly increased α-diversity compared to epileptic individuals. Unweighted PCoA to assess β-diversity revealed distinct and separate clustering of healthy control and epileptic individuals. Epileptic individuals were shown to have significantly increased relative abundances

of *Pseudomonadota* and *Actinomycetota* and significantly decreased relative abundance of *Bacillota* compared to healthy controls.

A final human clinical study performed 16S rRNA gene sequencing on collected fecal samples from a cohort of 23 children with infantile spasms and a cohort of 21 healthy children (Wan et al., 2021). α-Diversity measured by the Shannon, Simpson, and Chao1 indexes showed no difference between epileptic and healthy individuals. Further assessment of β-diversity by PCA revealed no differences between epileptic and health individuals. LEfSe analysis revealed significantly increased abundances of *Clostridium* and significantly decreased abundances of *Lactobacillus, Roseburia,* and *Lachnospira* compared to healthy individuals.

Across these human studies, alterations in the fecal microbiota profile of refractory epilepsy individuals were observed relative to healthy controls (Cui et al., 2022; Lindefeldt et al., 2019; Peng et al., 2018; Şafak et al., 2020; Xie et al., 2017) with the exception of the Wan et al. study. Several studies reported increased relative abundance of *Bacillota* in refractory epilepsy individuals (Lindefeldt et al., 2019; Peng et al., 2018; Xie et al., 2017). However, microbial signatures differed across studies when considering more resolved taxonomic levels. α-Diversity was a point of contention across these studies with conflicting results. Differences in study design, cohort age, small cohort size, and lack of data on genetic factors and environmental controls each influence the composition and function of the gut microbiome, makes these studies difficult to compare. Differences in taxonomic analysis, by using 16S rRNA gene sequencing compared to shotgun metagenomic sequencing, also complicate study comparisons because shotgun metagenomics affords microbiome functional profiling and strain level specificity while 16S rRNA gene profiling can only capture less resolved levels of taxonomic diversity. The results from these initial studies warrant larger efforts to achieve adequately powered and matched patient and control populations, and to account for variables such as age, genetics, medications, and diet.

Notably, one initial study reported significant differences in gut microbiome composition between drug-responsive and drug-resistant epileptic individuals (Lee, Lee, Lee, & Kim, 2021). As yet, however, strong evidence is lacking for the notion that differences in the microbiota may contribute to the pathogenesis of epilepsy or be used as a biomarker for epilepsies. Several additional studies have since examined the alternative hypothesis that the microbiome could contribute to the efficacy of select interventions for epilepsy (Fig. 2.1).

3. Ketogenic diet and epilepsy

Disease and diet have shared a close relationship spanning the last 2500years. Fast-forward to the 1920s, two pivotal observations underlying the modern ketogenic diet were made. In 1921, Woodyatt observed acetone and β-hydroxybutyric acid (BHBA) buildup in a normal fasting paradigm, as well as on a diet with minimal carbohydrates and an over ingestion of fat (Woodyatt, 1921). In the same year, Wilder proposed that ketonemia could provide similar benefits to fasting but could be maintained far longer than fasting (Wilder, 1921). Upon placing patients on this high-fat, low-carbohydrate dietary intervention, he coined the term “ketogenic diet” (KD). The KD was popular as a treatment for seizures throughout the 1920s and 1930s, but with the discovery of the first AED, diphenylhydantoin, in 1938, dietary treatment of epilepsy faded from the mainstream (Merrit & Putnam, 1938). KD was thrust back into the spotlight in 1997 with an episode of NBC’s *Dateline* centered on the story of Charlie, a 2-year-old with presenting with refractory epilepsy. Dr. John M. Freeman (Johns Hopkins) treated the boy with KD therapy, and he soon became seizure free. From this, The Charlie Foundation was formed to provide physicians, dieticians, and parents with informational videos about KD therapy, and interest in the diet began to increase again.

Although the KD is not the first line treatment for epilepsy, it is an effective and non-invasive treatment for refractory epilepsy, especially for those who do not qualify for surgical treatments. However, due to the KD’s restrictiveness, patients often find it difficult to maintain on

the diet for extended periods of time. For example, a KD patient who wants to maintain ketosis with a caloric intake of 2000 calories per day needs to consume no more than 50g of carbohydrates per day. A plain bagel equates to 48g of carbohydrates. Because of this restrictiveness, adults are 50% compliant with KD therapy, seeing successful treatment rates of approximately 30% (Henderson et al., 2006; Klein et al., 2010). Children placed on KD therapy see closer to 90% compliance and successful treatment rates of approximately 40% (Ye, Li, Jiang, Sun, & Liu, 2015). The KD has taken on the role of a niche treatment for certain epilepsy syndromes, including Dravet, Rett, and Lennox-Gastaut syndromes, where it has seen marginally better success rates for reducing seizures (D'Andrea Meira et al., 2019). Exactly how the KD is effective for treating seizures and why it may be more effective for certain individuals over others remains unclear.

Several different mechanisms of action have been proposed, and there are possibly multiple pathways through which the KD works to reduce seizures. Elevated levels of ketone bodies BHBA and acetoacetate, and decreased glucose are hallmarks of the KD's global metabolic effects to increase fatty acid oxidation. As such, the theory that ketone bodies may directly mediate the anti-seizure effects of the KD has been actively pursued, with mixed results in animal models (Kim et al., 2015; Likhodii et al., 2003). Positive results have linked ketone bodies to the modulation of ATP-sensitive potassium channels and neurotransmitter release as a mechanism for seizure reduction (Gasior, Hartman, & Rogawski, 2008; Ma, Berg, & Yellen, 2007; Rho, 2017). However, these are balanced by some studies showing no striking effects of ketone bodies on seizure severity and frequency (Chang et al., 2016; Likhodii et al., 2000).

The KD has also been proposed to confer anti-seizure effects through alterations in neuronal metabolism and mitochondrial activity. Distinct from direct effects of ketone bodies, some studies propose that it is the glucose restriction that protects against seizures. Glucose is transported across the blood brain barrier (BBB) as a key neuronal precursor for neurotransmitter synthesis and ATP to fuel neuronal activity (Greene, Todorova, & Seyfried,

2003). The KD reduces global glucose levels, reducing its bioavailability for neurons in the brain. Studies in animal models have shown the anti-seizure properties of the KD can be abolished with glucose supplementation (Huttenlocher, 1976) and that administration of 2-deoxy-D-glucose, a glycolysis inhibitor, similarly decreases seizure susceptibility (Garriga-Canut et al., 2006). The KD also increases mitochondrial biogenesis and mitochondrial energy reserves in the brain (Devivo, Leckie, Ferrendelli, & McDougal, 1978). This elevates available ATP and ATP-sensitive potassium channel (KATP) function while decreasing neuronal excitability (Bough et al., 2006). As such, the KD may promote seizure protection by limiting the bioavailability of glucose and altering mitochondrial function to a state attenuates seizures.

Beyond core theories regarding ketone body production and glucose restriction, several additional hypotheses exist for the KD. For example, amino acid modulation could also play a role, as the KD is reported to increase levels of GABA in the CFS of epilepsy patients (Wang et al., 2003). KD-induced reductions in levels of aspartate may help to promote GABA synthesis by enhancing glutamate decarboxylase function and facilitating astrocyte conversion of excitatory glutamate to glutamine (Yudkoff, Daikhin, Horyn, Nissim, & Nissim, 2008). In addition, epilepsy has been linked to inflammatory cytokine production, with some evidence suggesting that the KD promotes immunosuppression, reducing IL-1β and other related pro-inflammatory cytokines (Dupuis, Curatolo, Benoist, & Auvin, 2015). Overall, several pathways have been proposed to be important for the KD but the exact mechanism how the diet protects against seizures remains unclear. More research is needed to close this gap and drive the development of more effective KD-based therapies for refractory epilepsy.

The medium-chain triglyceride KD (MCT-KD) is an alternative to the conventional KD used to treat both children and adults with refractory epilepsy with approximately the same success rate (Martin, Jackson, Levy, & Cooper, 2016; Ye et al., 2015). The MCT-KD differs from the conventional KD consisting of long-chain fats that provide 60–80% of dietary energy, are replaced with medium-chain fats, providing closer to 45% of dietary energy (Augustin et al.,

2018). This allows a more attractive option as a dietary intervention for epilepsy as it has less restrictive on carbohydrate consumption than classical KD. Ketone body biosynthesis with the MCT-KD is more metabolically efficient because of the increased number of ingested shorter-chained fatty acids. In addition to the mechanisms suggested for the conventional KD (Augustin et al., 2018; Bough et al., 2006; Devivo et al., 1978; Wang et al., 2003), MCT-KD has also been theorized to control seizures through the increase of MCTs specifically. Decanoic acid has been linked to seizure control through inhibition of glutamate receptors (Chang et al., 2016; Citraro et al., 2021). Octanoic acid is also reported to modulate seizures indirectly through adenosine receptors (Chang et al., 2016; Tan, Carrasco-Pozo, McDonald, Puchowicz, & Borges, 2017; Wlaz´ et al., 2012).

Polyunsaturated fatty acids are another subgroup of fatty acids with purported anti-seizure effects. The polyunsaturated fatty acid KD (PUFA-KD) differs from the conventional KD, switching out the traditional saturated fatty acids for PUFAs such as peanut oil, sunflower oil, butter, or cream to better control seizures. In a study done in Mumbai, India including 50 patients (ages 10months to 35years old) previously on conventional KD and not showing further seizure reduction, were switched to PUFA-KD (Nathan, Bailur, Datay, Sharma, & Khedekar Kale, 2019). It was reported that 88% of participants had a>50% reduction in seizure frequency after 12months, a significantly increased success rate compared to the conventional KD (Nathan et al., 2019). It is purported that the PUFA-KD through alterations in omega-6 and omega-3 fatty acids levels and proinflammatory cytokine production. An out of balance omega-6 to omega-3 fatty acid ratio has been linked to increased glutamate production, while increased levels of omega-3 fatty acids are linked to increased levels of GABA (Simopoulos, 2002; Taha, Burnham, & Auvin, 2010).

Lastly, the modified Atkins diet (MAD) is another common variation of the conventional KD commonly used to treat refractory epilepsy patients. MAD is a less stringent diet, which is its main advantage over the conventional KD. While on MAD, carbohydrate intake is initially

confined to 10g per day combined with unrestricted intake of calories, fats, and protein. Overall, MAD has been shown to have similar successful treatment rates to the conventional KD across several studies in both children and has been suggested to operate analogously to the conventional KD to provide its anti-seizure effects (Miranda, Mortensen, Povlsen, Nielsen, & Beniczky, 2011; Poorshiri et al., 2021; Porta et al., 2009). Like the conventional KD, several mechanisms of action have been suggested through which the MCT-KD, PUFA-KD, or MAD act to control seizures. However exactly how these alternative diets to the conventional KD provide their anti-seizure effects remains unclear.

4. Ketogenic diet impact on epilepsy via gut microbiome

The gut microbiome is shaped by diet and plays an important role in mediating the effects of diet on host physiology. Evidence is emerging for interactions between the gut microbiome and the KD in epilepsy, following the idea that KD-induced changes in the microbiome may contribute to the seizure protective effects of the diet (Lum et al., 2020). Notably, this hypothesis that the microbiome may mediate seizure protection in response to the KD is distinct from the notion that the microbiome could contribute to epileptogenesis, for which evidence is as yet limited (Fig. 2.1).

Animal studies have provided proof-of-concept that the microbiome could promote seizure protection. In the 6-Hz model of acute electrically induced seizures, mice fed the KD exhibited increased seizure resistance compared to controls fed a vitamin and mineral matched control diet (Olson et al., 2018). However, mice germ-free or treated with antibiotics to deplete the microbiota failed show increased seizure resistance, indicating that the microbiome is necessary for the anti-seizure effects of the KD. This was similarly seen in the Kcna1 -/- mouse model of sudden unexpected death in epilepsy (SUDEP), where mice treated with the KD displayed decreased frequency and duration of seizures, but treatment with antibiotics abrogated this effect. When KD was administered to mice, the gut microbiota composition was

altered, decreasing α-diversity and increasing relative abundances of *Akkermansia muciniphila* and *Parabacteroides* spp. Transplantation of the KD-associated microbiota into naïve mice fed the control diet, or treatment with select KD-associated bacterial taxa, sufficiently conferred seizure protection, further suggesting that the gut microbiota contributes to the anti-seizure effects of the KD in the 6-Hz and Kcna1 -/- mouse models for refractory epilepsy. The KD-associated microbiota was shown to reduce gamma-glutamylation of ketogenic amino acids in the intestinal lumen and serum, and to correlate with increases in bulk GABA relative to glutamate in the hippocampus.

Overall, this initial study implicates the gut microbiota in mediating the anti-seizure effects of the KD in mice. Similarly, in a mouse model with *Scn1a* gene deficiency for Dravet syndrome, increases in *Clostridium* and decreased *Romboutsia* correlated with seizure severity. KD treatment shifted the microbiome by increasing *Bacillota* and decreasing *Bacteroidota*, which was correlated with reduced frequency and duration of motor seizures (Miljanovic & Potschka, 2021). In a rat model of infantile spasms, antibiotic treatment to deplete the gut microbiota improved the effectiveness of KD. In addition, a fecal transplant from KD treated animals into mice fed a conventional diet was effective at mitigating spasms (Muet al., 2022). Additional experiments in animals and humans are warranted to investigate effects of the KD on the gut microbiota and potential roles for the gut microbiota in the KD.

4.1 Ketogenic diet on the composition of the gut microbiota in epilepsy

A few recent human studies have begun to report associations of the clinical KD with alterations in the composition of the gut microbiota in epileptic individuals (Lindefeldt et al., 2019; Peng et al., 2018; Tagliabue et al., 2017; Xie et al., 2017; Zhang et al., 2018). Several studies of children with refractory epilepsy similarly reported that the KD increased relative abundance of *Bacteroidota* and decreased relative abundance of *Bacillota* and *Actinomycetota* (Lindefeldt et al., 2019; Xie et al., 2017; Zhang et al., 2018). Interestingly, this general pattern

was seen regardless of the effectiveness of the diet against seizures or the dietary fat to carbohydrate ratio. Three months of KD treatment in epileptic children ages 2–17, where the KD ratio ranged from 3:1 to 4:1, consistently decreased the relative abundance of *Actinomycetota,* particularly of the members *Bifidobacterium longum* and *Bifidobacterium adolescentis*, in the stool microbiota (Lindefeldt et al., 2019). In another pediatric cohort, 14 patients with refractory epilepsy aged 1–4years old showed significantly increased *Bacteroides* after 1week of 2:1 KD therapy (Xie et al., 2017). Similarly, 6months of treatment with the 4:1 KD in 20 epilepsy patients, aged 1–10years, was associated with increased relative abundance of *Bacteroidota*, and decreased *Bacillota* and *Actinomycetota* (Zhang et al., 2018). In addition, 6months of KD treatment (standard protocol by Zeneca, ratio was not indicated) on children ages 2–8 with drug resistant epilepsy was correlated with decreases in *Bifidobacterium, Akkermansia, Actinomyces* and *Enterococcaceae*, though they did not reach statistical significance when compared to the pre-treatment internal controls (Gong et al., 2021).

While there are some similarities in reported results across independent studies, there are also many differences. In human studies conducted with epileptic children, there were only modest decreases in the α-diversity in KD treated individuals, but changes were not statistically significant (Gong et al., 2021; Lindefeldt et al., 2019; Xie et al., 2017; Zhang et al., 2018). This contrasts studies in mice, where the KD substantially decreased α-diversity compared to controls (Olson et al., 2018). However, in dogs with idiopathic epilepsy, the MCT-KD increased α-diversity compared to baseline diet (Pilla et al., 2020). In addition, despite the similar reports of KD-associated changes in the phylum composition of the microbiota, the specific changes varied greatly at more resolved taxonomic levels. Differential results could be due, at least in part, to heterogeneity in KD regimen, variability in initial microbiome state, differences in patient demographics and medical history, among other relevant variables. Differences in sequencing approach and analysis could also contribute. Metagenomic sequencing of the fecal microbiota revealed that 3 months of KD (3:1 to 4:1) in therapy resistant epileptic children aged 2–17 was

linked with increased *Pseudomonadota (E. coli)* and reduced *Eubacterium rectale* and *Dialister* (Lindefeldt et al., 2019). In contrast, another study of 14 epileptic infants aged 1–4 who were treated for 1 week with the clinical KD reported diminished levels of *Pseudomonadota* as measured by 16S rRNA gene sequencing (Xie et al., 2017). Increased *Prevotella* and decreased *Coronobacter, Erysipelatoclostridium, Streptococcus, Alistipes, Ruminiclostridium, Barnesiella* and *Enterococcus* were also reported. Overall, the inconsistencies in KD-associated changes in the composition gut microbiota at species and genus level point to the need to better evaluate factors that could contribute to the variability. Even in healthy individuals the diversity and the abundance of the gut microbiota vary widely, however, the metagenomic carriage stays more stable among individuals despite the variations in community structure (The Human Microbiome Project Consortium, 2012). Therefore, such dietary perturbations call for characterization of metabolic and functional output.

One consideration is whether patient responsiveness to the KD may correlate with the microbiome, and if so, whether the microbiome could serve as a biomarker to identify those subsets of epileptic individuals who would benefit from the KD. The microbiota studies to date have included cohorts of epileptic patients that varied in their responsiveness to the KD, ranging from no improvement to >50% seizure reduction or even complete absence of seizures, while many of the studies did not evaluate microbiota signatures in responders vs non-responders, a few reported some differences. Among 20 patients subjected to the 4:1 KD for 6months, compared to 10 patients with at least 50% seizure reduction, the non/low-responders (<50% reduction) had significantly increased relative abundances of *Clostridiales, Clostridia, Ruminococcaceae, Lachnospiraceae, Alistipes*, and *Rikenellaceae* (Zhang et al., 2018). In another study, epileptic patients who had positive responses to KD treatment after 6months exhibited modest elevations in the relative abundances of *Eubacterium* and *Dialister* relative to those who showed poor response to the KD (Gong et al., 2021).

KD-associated taxonomic changes in the microbiota might also be influenced by seizure etiology. Glucose transporter 1 (GLUT1) deficiency syndrome is an early-onset childhood epileptic encephalopathy caused by impaired glucose transport across the BBB. This malfunction largely originates from mutations in the *SLC2A1* gene encoding GLUT1. The KD can effectively treat seizures in individuals with this syndrome (Klepper, 2012). Six patients (three females and three males, aged 8–34years) with GLUT1 deficiency syndrome were subjected to the KD for 3months, gradually increased from 1:1 to 4:1. After 90 days of treatment, KD-treated patients showed microbiota profiles with statistically significant increases in *Desulfovibrio* (Tagliabue et al., 2017). Notably, this signature was not observed in other human epilepsy studies of the gut microbiota and KD. In another study of KD-responsive epileptic patients with mutations occurring in the genes *SCN1A, NOTCH3*, and *CDKL5*, increases in *Tannerella, Sulfurosprillum* and *Parabacteroides* were reported (Gong et al., 2021). In contrast, patients without these genetic mutations showed an increase in *Hungatella* after KD treatment (Gong et al., 2021).

Some studies are beginning to assess the hypothesis that the effectiveness of KD treatments on epilepsy could be enhanced by prebiotics and/or probiotics. In a mouse model of pentylenetetrazol (PTZ)-induced seizures, co-treatment of the KD together with the probiotic *Lactobacillus fermentum* maintained KD-associated reductions in seizure susceptibility, while also preventing some KD-associated changes in serum lipid profiles and reductions in tight junction protein expression in the BBB. Probiotic treatment was associated with reductions in relative levels of *Bacteroidota* and increases in *Bacillota* and *Defferibacteres* (Eor, Tan, Son, Kwak, & Kim, 2021). In particular, KD treatment was associated with lower abundance of *Actinomycetota* and elevated *Bacteroides* which was prevented by co-treatment with *L. fermentum*. In a separate study of the PTZ seizure model, mice were fed the 4:1 KD for 8weeks, together with *L. fermentum* MSK 408 and the prebiotic galactooligosaccharide (GOS) (Eor, Son, et al., 2021). Both the KD and co-treatment with synbiotics similarly reduced seizure

susceptibility and the diversity of the gut microbiota, with increases in *Bacillota* relative to *Bacteroidota*. This is in contrast with results observed in human studies, where KD was frequently associated with elevations in *Bacteroidota*. In the same study, *Acetatifactor, Anaerotaenia, Escherichia, Flintibacter, Oscillibacter,* and *Erysipelatoclostridium* were significantly higher in KD-treated animals. Notably, while synbiotic treatment had no added effect on KD-induced seizure protection, treatment of mice fed a control diet with the synbiotic sufficiently reduced seizure susceptibility. Together, these animal studies suggest that microbiota-targeted interventions could diminish particular side-effects of the KD and potentially confer seizure protection. More research is needed to further test these ideas in animal models and in human studies.

4.2 Ketogenic diet on the function of the gut microbiome in epilepsy

The limited consistency in microbiota composition across epileptic patients treated with the clinical KD is not surprising considering the heterogeneity of the disorder, the variability in even the healthy human microbiota, the differences in specific nutritional content of the KD, and the several additional technical, medical and lifestyle factors that shape microbial taxonomic profiles. Increasing evidence of functional redundancy across different microbial taxa suggests that common functional signatures could be identified despite variations in taxonomic composition (The Human Microbiome Project Consortium, 2012). In a study that employed metagenomic sequencing of the gut microbiota of 12 children with severe epilepsy that were treated for 3months with the KD, 29 gene clusters significantly associated with the KD (Lindefeldt et al., 2019). In particular, increases genes related to the hemin transport system and succinate dehydrogenase (which includes fumarate reductase subunits) were elevated with KD treatment, which correlated with *E. coli* and *Bacteroides*, as well as *Eggerthella lenta*, respectively (Lindefeldt et al., 2019). Notably, 26 of the 29 altered subsystems diminished with the KD treatment and seven of them were relevant to carbohydrate metabolism, which aligns

with the carbohydrate restriction in the KD. In contrast to these results from a human epilepsy cohort, 4 weeks of KD treatment in the PTZ seizure mouse model yielded microbiomes with inferred enrichment of genes related to mTOR signaling, starch and sucrose metabolism, and PPAR signaling and decreases in genes related to energy production (Eor, Tan, et al., 2021). In particular, the mTOR signaling pathway has gained attention in epilepsy due to its ability to regulate nutrient availability, cell growth, and energy metabolism in response to the KD in rats (Liu et al., 2020; McDaniel, Rensing, Thio, Yamada, & Wong, 2011). The mTOR signaling pathway is also linked to gut microbial metabolism and immunoregulation (Noureldein & Eid, 2018). Moreover, following *L. fermentum* administration in KD-treated PTZ mice, the gut microbiota exhibited inferred enrichment of genes related to the TCA cycle and ketone body synthesis, when compared to that from PTZ mice treated with KD alone and increased microbial genes related to propionate metabolism, PPAR signaling, and pyruvate metabolism compared to PTZ mice with normal diet and same probiotic control (Eor, Tan, et al., 2021). Pathways related to PPAR signaling could be relevant due to prior links to anti-inflammatory activity in the hippocampus of a mouse model for epilepsy (Noureldein & Eid, 2018).

Beyond metagenomic profiling and inferred metagenomic assessments based on taxonomic sequencing data, some microbially modulated metabolites have been implicated as the intermediates in KD-associated host-microbiome interactions (Krautkramer, Fan, & Bäckhed, 2020). SCFAs produced from microbial metabolism of carbohydrates in the gut is of interest due to their potential mediating role in gut microbiota–brain communication (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019; Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016). Children with drug resistant epilepsy prior to KD treatment showed a reduced total SCFA content compared to healthy controls (Gong et al., 2021). Although there were no significant differences between prior to KD and after KD groups, the levels of butyric acid from the KD responders were higher compared to non-responders. In contrast, 1 month of treatment with KD (4:1) in 7 epileptic patients (ages 2–46) was associated with significantly decreased total

SCFAs, including acetate, butyrate, propionate and isobutyrate, in feces (Ferraris et al., 2021). In the PTZ seizure mouse model, SCFAs were significantly lower in KD-treated mice compared to controls (Eor, Tan, et al., 2021). Concurrent treatment of KD with *L. fermentum* increased acetate and isobutyrate concentrations compared to control treated with KD alone (Eor, Tan, et al., 2021). In a follow-up study, select SCFAs also decreased with KD treatment (Eor, Son, et al., 2021).

Another approach to examine functional outcomes of the gut microbiome is to identify microbiome-dependent metabolomic alterations. While research is needed to determine effects of the KD on microbiome dependent metabolites in refractory epilepsy, some studies in animal models begin to highlight metabolites of interest. In the study of dogs with idiopathic epilepsy, LC-MS analysis of fecal metabolites revealed increases in classes of long chain triglycerides (LCT) that were associated with KD treatment, as well as 17 unidentified metabolic features that were significantly increased in both MCT and KD treatment compared to typical diet controls (Pilla et al., 2020). This suggests that the KD regardless of type of oil formulation involved may be able to induce shared metabolic responses in epileptic animals. Furthermore, in mice, random forest analysis of metabolomic profiles revealed 30 microbiome-dependent metabolites in intestinal contents and serum that were predictive of seizure protection with >90% accuracy. Notably, many metabolites were relevant to amino acid metabolism, including derivatives of lysine, tyrosine, and threonine (Olson et al., 2018). Widespread decreases in subsets of ketogenic gamma-glutamylated amino acids were observed in both colonic luminal contents and sera from seizure-protected mice compared to seizure susceptible mice. Gamma glutamylated (GG) amino acids were previously implicated in the KD as sources that modulate GABA-glutamate metabolism in the brain (Calderón, Betancourt, Hernández, & Rada, 2017). Overall, emerging studies in humans and animal models point to a potential for the gut microbiome to be modified by the KD and to in turn, contribute to alterations in host physiology and seizure susceptibility. However, the current studies are few, of limited sample size, and variable in

results. Additional well-controlled and -powered studies are required to interrogate potential interactions between the microbiome and KD in epilepsy.

4.3 Ketogenic diet on the gut microbiome in other diseases

Beyond epilepsy, the KD is increasingly being explored for its fundamental effects on host physiology and for its potential to modify risk for symptoms other metabolic, neurological, neurodevelopmental and neurodegenerative diseases. In a study of 33 obese patients enrolled in a weight loss program, treatment with a low calorie KD (2 months with very low calorie KD followed by 2 months of low calorie diet) significantly increased microbial diversity by the end of the dietary intervention (Gutiérrez-Repiso et al., 2019), with reductions in *Pseudomonadota* and increases in *Bacillota*. At the family level, *Enterobacteriaceae, Sinobacteraceae*, and *Comamonadacea* decreased, while the abundance of *Ruminococcaceae* and *Mogibacteriaceae* increased. At the genus level, a reduction in *Serratia, Erwinia*, and *Citrobacter* abundance was reported, while an increase in *Oscillospira* and *Butyricimonas* abundance was observed. In contrast, 17 obese men between the ages of 18–50 years followed a KD for 4 weeks (Ang et al., 2020). KD was associated with increases in Bacteroides and decreases in *Actinomycetota* and *Bacillota*. *Bifidobacterium* showed the greatest decrease in response to KD while no differences in SCFAs were detected in this human cohort. This is consistent with a mouse study reporting that the KD decreased the abundance of *Bifidobacterium*. Although the reduction might be concerning due to reported beneficial effects of Bifidobacterium in the human gut (O'Callaghan & van Sinderen, 2016), this shift is not surprising given the propensity for *Bifidobacteria* to rely on carbohydrates metabolism for growth (Milani et al., 2015). In addition, a recent study reported that the ketone body BHBA selectively inhibits bifidobacterial growth, which results in diminished levels of intestinal pro-inflammatory Th17 cells (Ang et al., 2020). Whether these ketone body- and microbiota-driven reductions in intestinal inflammation could play a role in the anti-seizure effects of the KD remains unclear (Agirman, Yu, & Hsiao, 2021). However, there is

some evidence that metabolites that are reduced by the KD (Heischmann et al., 2018) and correlated with lower seizure frequency (Zarnowska, 2020) could play a role in the immune response (Cervenka, Agudelo, & Ruas, 2017).

One study evaluated effects of a 6-month treatment with the 1:5:1 (protein:fat:carbs) KD on the fecal microbiota of 25 patients with multiple sclerosis (MS) compared with 14 healthy controls (Swidsinski et al., 2017). Total bacterial loads measured in the fecal microbiota of MS patients were initially decreased upon initiation of the KD, with notable restorations to levels seen in healthy controls beginning at 12 weeks and by week 23–24 of KD treatment. Notably, the KD was associated with decreases in the relative abundance of *Akkermansia*, with contrasts reported effects of the KD in healthy humans (Ang et al., 2020) and epilepsy mouse models (Olson et al., 2018). In a study of 11 individuals diagnosed with cognitive impairment (MCI) and 6 controls with normal cognitive balance 6weeks of treatment with the Mediterranean ketogenic diet (MMKD) (Nagpal, Neth, Wang, Craft, & Yadav, 2019) yielded no notable differences in α- or β-diversity between fecal microbiota from MCI compared to controls. Regardless of the cognitive status, the MMKD was associated with increased relative abundances of *Enterobacteriaceae, Akkermansia, Slackia, Christensenellaceae* and *Erysipelotriaceae* and reductions in *Bifidobacterium* and *Lachnobacterium*. Inferred metagenomic analysis of the gut microbiota revealed that MMKD treatment was correlated with decreases in microbial gene families said to be related to Alzheimer's disease, diabetes, bacterial toxins and carbohydrate metabolism and increases in gene pathways related to lipid metabolism and steroid biosynthesis.

In young healthy mice, aged 12–14weeks, 16weeks of KD treatment decreased microbial diversity and shifted community composition relative to standard diet controls (Ma et al., 2018). KD-treated mice had significant increases in the relative abundances of *A. muciniphila* and *Lactobacillus* and decreases in *Clostridium* and *Dorea*. *Desulfovibrio* and *Turicibacter* were also significantly and substantially lower in KD-treated mice. In the mouse strain BTBR, which is used to study social behavioral deficits relevant to autism spectrum

disorder, mice were fed a standard chow or KD for 10–14days (Newell et al., 2016). The KD was associated with decreases in *A. muciniphila, Methanobrevibacter*, and *Roseburia* in cecal samples and decreased *A. muciniphila, Enterobacteriaceae*, and *Lactobacillus* in fecal samples. In another study with that compared KD treatment of C57BL/6 vs BTBR (Klein et al., 2016), the KD similarly reduced α-diversity of the fecal microbiota in both mouse lines, with shared taxonomic alterations that led the authors to conclude that the KD rather than the mouse genetic background was the primary driver for microbial responses. In a study of mice exposed to hypoxia-induced cognitive impairment, the KD was associated with reductions in the relative abundance of *Clostridium cocleatum* and increases in *Bilophila* (Olson et al., 2021). These changes were only seen in mice exposed to hypoxia and on the KD. Altogether these studies reveal the potential context-specificity of microbial responses to the KD, that depend on genotype and exposure to environmental changes, among many other potential factors.

5. Potential mechanisms for microbial interactions with the ketogenic diet

5.1 Microbial effects on host lipid biology

Studies using gnotobiotic animals reveal that the gut microbiome fundamentally regulates lipid biology in the host. In comparing mice reared with a conventional microbiome or with those reared germ-free, the presence of gut microbes elevated levels of hydroxy fatty acids in plasma, small intestine, and colon (Kishino et al., 2013). Similarly, gut microbes modified lipids profiles, including phosphatidylcholines and triglycerides, in the serum, liver and adipose tissue (Velagapudi et al., 2010). Consistent with this, germ free mice transplanted with microbiota from aged mice showed elevated brain phospholipids and monounsaturated fatty acids (MUFAs), with reduced cholesterol and PUFAs, compared to mice transplanted with microbiota from young mice (Albouery et al., 2020). In a separate study, the gut microbiota promoted the synthesis of MUFAs by stearoyl-CoA desaturase 1 and the elongation of PUFAs by fatty acid elongase 5 in the liver and plasma (Kindt et al., 2018). These studies reveal

fundamental roles for the gut microbiome in regulating host lipid profiles in the blood, liver, and brain.

Microbial effects on host lipid profiles may be due to their ability to modulate the metabolism of dietary lipids (Fig. 2.2). In mice fed a high lard diet, for example, the gut microbiota was required for mediating dietary effects on hepatic triglyceride levels and the development of fatty livers (Just et al., 2018). In addition, mice treated with dietary conjugated linoleic acids exhibited increased levels of *Bacteroidota* and decreased levels of *Bacillota*, with elevated microbial production of SCFAs (Marques et al., 2015). The ability of gut microbes to regulate bile acids and SCFAs (Schoeler & Caesar, 2019) could have important implications for diets like the KD, that are based on alterations in dietary fat and carbohydrates (Fig. 2.2).

Many studies indicate that gut microbes respond to bile acids, modify levels of bile acids, and biotransform bile acids in ways that impact host lipid biology. Bile acids emulsify fat to enable lipid absorption in the small intestine. In mice fed high fat diets containing lard and palm oil, supplementation with bile acids increased weight gain, independently of the fat source (Just et al., 2018). Treatment with bile acids altered the gut microbiota, with increases in *Desulfovibrionaceae, Clostridium lactatifermentans* and *Flintibacter butyricus*, and decreases in *Lachnospiraceae*. Most bile acids are reabsorbed and recirculated to the liver, but bacterially mediated deconjugation of glycine- or taurine-conjugated bile acids reduce their reabsorption. Deconjugated bile acids can be further metabolized to secondary bile acids through dehydrogenation, dehydroxylation, and epimerization by gut bacteria (Wahlström, Sayin, Marschall, & Bäckhed, 2016). In the host, bile acids also act as signaling molecules for glucose and lipid metabolism, by interacting with host bile acid receptors, such as nuclear receptors farnesoid-X-receptor (FXR) and cell membrane receptor Takeda G protein-coupled receptor 5 (TGR5) (Li & Chiang, 2014). Thus, the ability of the gut microbiota to regulate host levels and profiles of specific bile acid species can impact not only lipid absorption, but downstream physiologies that are dependent on bile acid signaling (Yu, Raka, & Adeli, 2019).

SCFAs are the microbial end products from dietary fiber fermentation and play important roles in wide variety of metabolic processes, such as regulation of energy intake, energy harvest, glucose metabolism, lipid metabolism, and adipogenesis, as well as pathophysiology of obesity and related metabolic disorders (Koh et al., 2016). The microbial derived SCFA acetate serves as a precursor for the hepatic synthesis of C16 and C18 fatty acids in mice fed a standard chow diet containing 5% dietary fiber for 2weeks (Kindt et al., 2018). This was not observed when fiber was replaced with the unfermentable fiber cellulose, indicating a key role for microbial fiber fermentation in regulating host fatty acid synthesis. In addition, the microbial derived SCFAs butyrate and propionate have been shown to activate PPARγ in vitro (Alex et al., 2013). Consistent with this, dietary butyrate supplementation induced the activation of PPARγ and promoted lipid-mediated energy expenditure in mice fed with high fat diet (Gao et al., 2009). Notably, since many dietary fibers are unfermentable by the host and cannot be directly used as an energy source, they are often discounted as carbohydrates in clinical KD regimen (Rebecca, 2016). Given that the amount of fiber included in the KD treatment will differentially affect microbial metabolism, the production of SCFAs, and downstream consequences on host lipid metabolism, greater attention is needed to consider relative content of host non-fermentable fibers relative to host fermentable fibers and simple sugars in the diet.

5.2 Microbiome responses to variations in dietary fat content and type

Microbiome-dependent effects of dietary lipids on host physiology are influenced by both the amount and the type of the fat used in the diet (Mokkala, Houttu, Cansev, & Laitinen, 2020) (Fig. 2.2). The composition of gut microbiota differs across mice fed the high fat high sugar diet (HFD), the ketogenic diet, or the low-fat diet. In a study of mice fed the following diets for 5weeks: (i) ketogenic diet type 1 (KDR, caloric ratio of 89.5% fat, 0.1% carbohydrate, and 10.4% protein), (ii) control normal chow of KDR (NCR, caloric ratio of 10% fat, 70% carbohydrate, and 20% protein), (iii) ketogenic diet type 2 (KDH, caloric ratio of 91.3% fat, 1%

carbohydrate, and 7.7% protein), and (iv) control normal chow of KDH (NCH, caloric ratio of 15.5% fat, 64.5% carbohydrate, and 20% protein), 16S rRNA gene sequencing showed that the richness and diversity of the gut microbiota was significantly increased in KDH mice but not in KDR mice compared to their respective control groups (Li et al., 2021). Both KDs increased *Ruminococcaceae, Intestinimonas*, and *Lachnospiraceae*. However, these changes were not observed in human cohort studies (Lindefeldt et al., 2019; Peng et al., 2018; Tagliabue et al., 2017; Xie et al., 2017; Zhang et al., 2018). This highlights the need to evaluate whether there are shared functional changes in the microbiome across animal and human studies. In addition to alterations in microbial composition, the KDR but not KDH induced insulin resistance and damaged glucose homeostasis, while KDH increased fat accumulation in mice. The microbial derived tryptophan metabolites 1H-indole-3-propanoic acid (IPA) and indole acetic acid (IAA) were decreased in the KDR and KDH mice, respectively, and were negatively correlated with lipid accumulation. Furthermore, the microbial metabolite trans-2-hydroxycinnamic acid, which was elevated only in KDR mice, was positively correlated with glucose intolerance, while the SCFA isobutyrate, which was reduced in KDH mice, was negatively correlated with lipid accumulation.

Furthermore, a customized KD with the same proportions of fat as KDR and the same sources of fat as KDH was tested to evaluate the influences of sources and proportions of fat on the metabolic phenotypes in mice. 14 bacteria taxa were affected by the sources and proportions of fat in the two kinds of KD and linked to alterations in lipid metabolism (Li et al., 2021). Microbial alterations in response to the KDs also led to functional changes in the host, including decreased tryptophan metabolites and SCFAs, and increased deoxycholic acid. KDR mice had significantly higher deoxycholic acid and chenodeoxycholate levels than the KDH mice, suggesting that the differences in levels of these two bile acids may be due to the different fat sources in the KD.

Other diet studies indicate that fat type differentially affects the composition of the gut microbiome. Diets high in saturated fatty acids reduced the diversity and richness of the gut microbiota (Caesar, Tremaroli, Kovatcheva-Datchary, Cani, & Bäckhed, 2015; Devkota et al., 2012), whereas diets high in unsaturated fatty acids exerted the opposite effect (Patterson et al., 2014). In particular, consumption of a diet high in saturated (milk-derived) fat, but not polyunsaturated (safflower oil) fat, led to enrichment of a low-abundance, sulfite-reducing pathobiont, *Bilophila wadsworthia* (Devkota et al., 2012). This was associated with a pro-inflammatory T helper type 1 immune response and increased severity of colitis in genetically susceptible *Il10 -/-* mice. Milk-derived fat promoted the taurine conjugation of hepatic bile acids which increased the availability of sulfur for *B. wadsworthia*. Furthermore, mice fed high fat diets comprised of omega-3 PUFAs showed a different microbial composition than mice saturated HFD or omega-6 PUFA diet (Lam et al., 2015). Hydrogen sulfide-producing bacteria (*Bilophila*) were one of the major groups driving the diet-specific changes in the gut microbiome, with the overall microbial profile being associated with changes in body weight, HOMA-IR, and gut permeability. Besides the saturation levels, the source of the saturated fat (e.g., from lard vs palm oil) differentially alters microbial composition and lipid metabolism (Just et al., 2018). A study of mice fed with either lard or fish oil reported that fat source explained about 24% of the variability in microbiota composition (Caesar et al., 2015). TLR2 and TLR4 were activated by sera from mice fed lard, suggesting that a lard-based diet promotes the influx of microbial antigens into the systemic circulation to drives the inflammatory signatures seen in white adipose tissue (Caesar et al., 2015). Overall, these findings reveal that the specific amounts, sources, and types of fats included in the diet differentially alter the gut microbiome in ways that lead to varied host metabolic and immune outcomes. This highlights the importance of evaluating the effects of specific dietary fats on seizure protection and the gut microbiota in response to clinical KD treatments.

6. Conclusion

The gut microbiome has ability to modify brain activity, behavior, and the severity of symptoms related to a variety of neurological diseases. At the same time, diet is a major determinant of the composition and function of the gut microbiome, with the potential to shape host physiology in ways that depend upon the gut microbiome. These core principles are beginning to be supported by findings from animal studies that implicate the gut microbiome in mediating the anti-seizure effects of KD. More work is needed to extend and validate these animal studies, and to advance translational research that evaluates interactions between the human gut microbiome and clinical KD treatment in refractory epilepsy. Such studies are made challenging by the complexity and heterogeneity of the gut microbiome, refractory epilepsy, and the clinical KD regimens. Exactly what constitutes a “healthy” microbiome is poorly understood, and emerging research suggests that the definition will vary from individual to individual. As such, prospective and longitudinal studies in epileptic patients and well-matched controls before and during dietary treatment are needed to account for baseline and aging-dependent variations in microbiome composition and function. While the KD is widely implemented as a clinical treatment for refractory epilepsy, patient responsiveness to KD treatment varies greatly. This is likely due, at least in part, to the substantial heterogeneity in underlying causes and subtypes of refractory epilepsy, as well as variations in medical history, including anti-epileptic drug treatment. Large studies are needed that include clearly defined cohorts of refractory epilepsy and that account for the many confounding medical and lifestyle factors that have the potential to impact the gut microbiome. The study design, results, and interpretation may also be influenced by the specific type of clinical KD tested (e.g., classical KD, modified KD, MCT, Modified Atkins, low glycemic index), the administered dietary ratios, and the specific macro- and micro-nutritional composition of the individual meal plans. Fundamental research is needed to advance understanding of how specific gut microbes respond to, metabolize, and transform

specific dietary components, toward developing predictive tools for using diet to modify the microbiome to achieve targeted functions.

Overall, understanding the mechanistic underpinnings of microbial interactions with the clinical KD will inform the prospect of identifying targeted, personalized, and efficacious approaches for treating refractory epilepsy.

Figures and Tables:

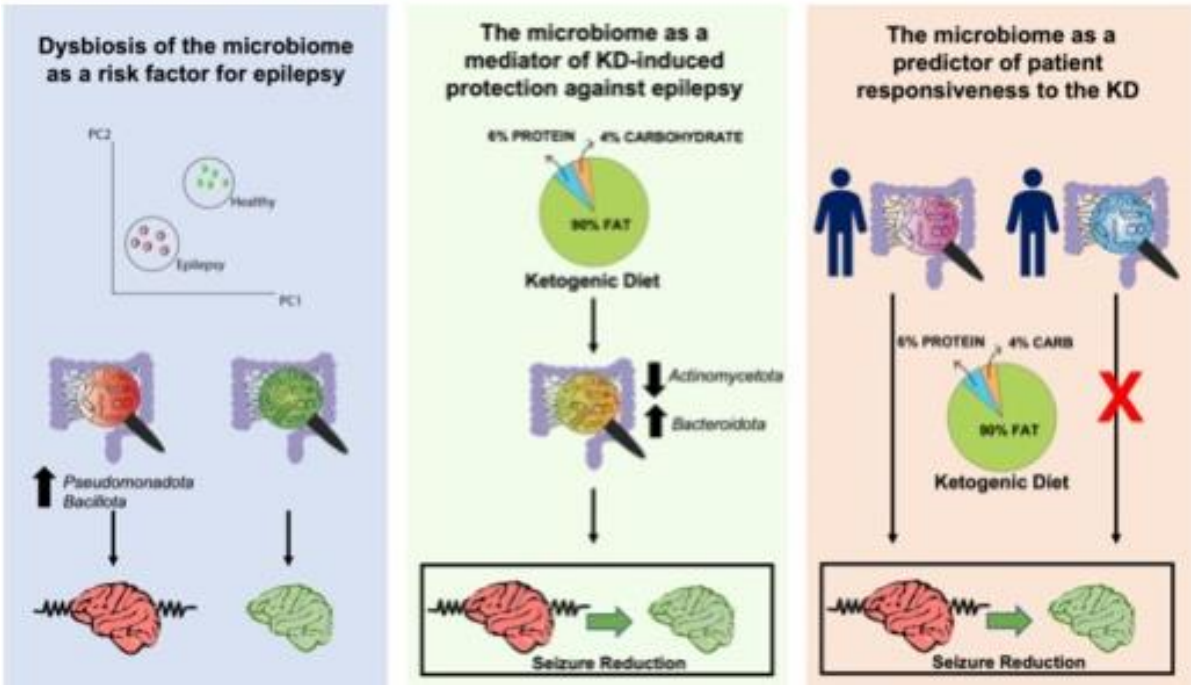

Fig. 2.1 Potential roles for the gut microbiome in epilepsy. There are many distinct and potentially overlapping ways by which the microbiome could contribute to epilepsy. Dysbiosis in the microbiome could contribute to the pathogenesis or manifestation of epilepsy. The microbiome or changes in the microbiome could contribute to the antiseizure effects of therapeutic treatment, for example with the ketogenic diet. Variations in the gut microbiome could serve as biomarkers for whether a patient responds vs fails to respond to a particular treatment, such as the ketogenic diet.

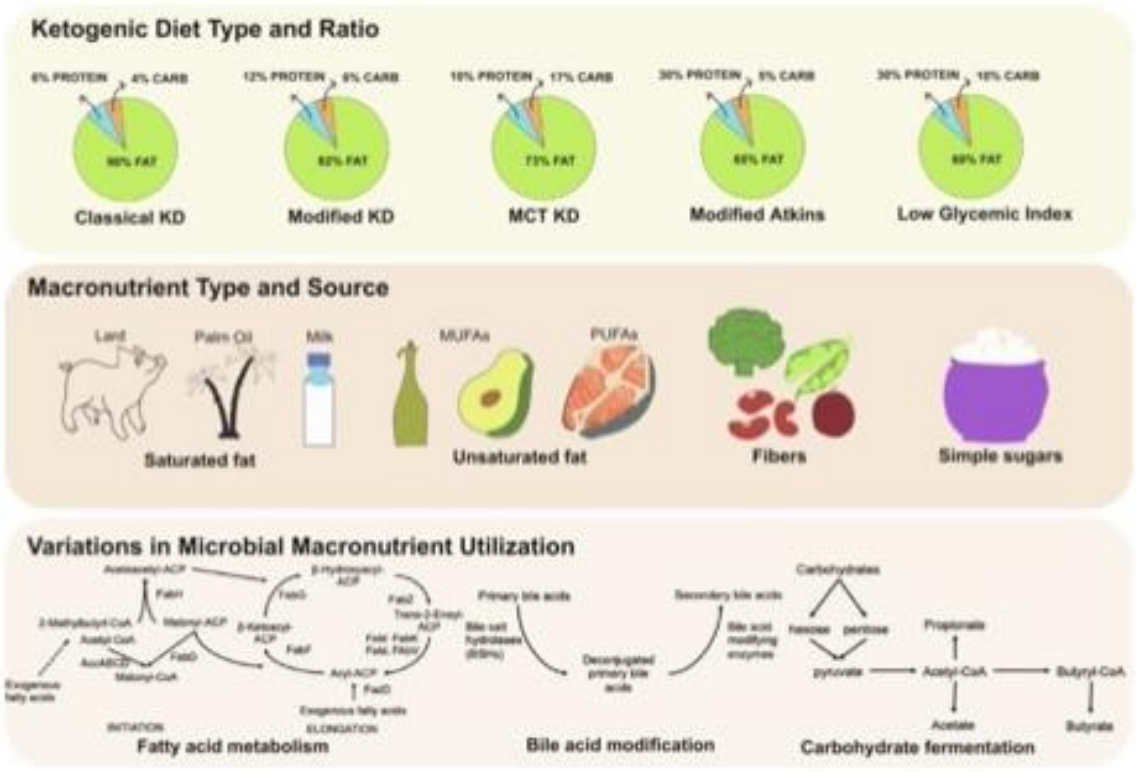

Fig. 2.2 Nuances of clinical ketogenic diet regimens and human microbiome variation could impact host responses to dietary treatment. There are various types of ketogenic diets that are used clinically and vary in the fat: carbohydrate ratio and/or the fat type and source. Within these types of clinical KDs, specific choices regarding macro- and micro-nutrient types that comprise individual meal plans could differentially impact the gut microbiome and host responses. Variations in the individual patient microbiome could consist of altered compositions and functions that impact dietary metabolism and host metabolites.

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Chapter 3: Emerging roles for the intestinal microbiome in epilepsy

Abstract

The gut microbiome is emerging as a key regulator of brain function and behavior and is associated with symptoms of several neurological disorders. There is emerging evidence that alterations in the gut microbiota are seen in epilepsy and in response to seizure interventions. In this review, we highlight recent studies reporting that individuals with refractory epilepsy exhibit altered composition of the gut microbiota. We further discuss antibiotic treatment and infection as microbiome-related factors that influence seizure susceptibility in humans and animal models. In addition, we evaluate how the microbiome may mediate effects of the ketogenic diet, probiotic treatment, and anti-epileptic drugs on reducing both seizure frequency and severity. Finally, we assess the open questions in interrogating roles for the microbiome in epilepsy and address the prospect that continued research may uncover fundamental insights for understanding risk factors for epilepsy, as well as novel approaches for treating refractory epilepsy.

1. Introduction

Epilepsy is a chronic neurological disorder affecting>50 million people worldwide and accounting for 0.5% of the global economic disease burden (WHO, 2019). It is defined as a brain pathology “characterized by an enduring predisposition to generate seizures” (Fisher et al., 2014). An estimated 2.4 million patients are diagnosed with epilepsy every year. These diagnoses include various subtypes of seizures, such as focal, generalized, combined generalized and focal, or unknown, which indicate their localization to specific brain regions or generalization across both cerebral hemispheres (Scheffer et al., 2017). Seizures occur when excitation and inhibition are imbalanced in the brain, which can be triggered by pathologies affecting synaptic connectivity, ionic channel function, and neurotransmitter reception, among several other pathways (Stafstrom and Carmant, 2015). Additionally, seizures can occur after

cerebral insult or damage, as in the case of febrile seizures, traumatic brain injury, or stroke (Herman, 2002).

While the WHO estimates that 70% of epileptic patients could be seizure-free with appropriate medication, in developing regions, less than half of the epileptic patient population has access to anti-epileptic drugs. Additionally, an estimated 15 million patients exhibit refractory epilepsy, based on their non-responsiveness to existing anti-epileptic drugs. Both genetic and environmental factors contribute to individual predisposition to epilepsy, but the exact causes of most epilepsy cases remain unclear. It is estimated that 35% of epilepsy cases can be directly attributed to genetic risk, while the remaining cases may involve both genetic risk and environmental exposures, such as head trauma or infections that lead to meningitis or encephalitis (Shorvon, 2014). Exactly how environmental factors contribute to long-term susceptibility to epilepsy remains unclear. The gut microbiota, comprising trillions of microorganisms indigenous to the gastrointestinal tract, is increasingly recognized as an important mediator of environmental risk factors on host risk for disease. The composition and function of the gut microbiota is shaped by environmental factors, such as diet, stress and medication, and also informed by human genetics. The microbiota plays a critical role in guiding brain development and neurobehavior in animal models (Vuong et al., 2017). Of particular relevance to epilepsy, the gut microbiota significantly alters carbohydrate and amino acid metabolism, microglial and astrocytic function, vagal neuronal activity, and hippocampal neurotransmitter levels (Fung, 2017 #395). In this review, we discuss current evidence for microbiome alterations in epilepsy and potential roles for the microbiome in mediating risk for epilepsy and the effects of seizure interventions (see Fig 3.1).

2. Alterations of the gut microbiota in human epilepsy

Alterations in the gut microbiome have been reported across several neurodevelopmental, neuropsychiatric and neurodegenerative disorders, but very little is known

regarding microbiome associations with human epilepsy. Only a few recent studies have highlighted differences in fecal microbiota profiles from select epileptic individuals as compared to healthy controls (Table 3.1) (Lindefeldt et al., 2019; Peng et al., 2018; Xie et al., 2017). In a cohort of 42 individuals with refractory epilepsy, 49 with drug-sensitive epilepsy, and 65 matched family members without epilepsy from West China Hospital of Sichuan University, 16S rDNA sequencing revealed distinct fecal microbiota alterations for refractory epileptic patients relative to both drug-sensitive epileptic patients and controls without epilepsy. In particular, microbiota profiles from the refractory epilepsy group exhibited elevated α- diversity, as measured by the Chao1 diversity index for species richness, which was reportedly particular to refractory epileptic patients with 4 or more seizures per year and not for those with<4 seizures per year (Peng et al., 2018). While samples from refractory epileptic patients exhibited no overt group clustering by weighted principal coordinate analysis, linear discriminant analysis (LDA) effect size analysis revealed increases in the relative abundances of select members of the phylum *Firmicutes*, including *Roseburia, Coprococcus, Ruminococcus,* and *Coprobacillus,* and decreases in *Bacteroides* relative to controls. Relative abundances of *Methanobrevibacter, Fusobacterium, Neisseria,* and *Akkermansia* were also increased in the refractory epilepsy group relative to the drug-sensitive epilepsy group. Notably, the study design matched group representation by age (mean 25.1–29.4), sex, and exposure to medication, and excluded individuals who had taken antibiotics or probiotics within the past 3 months or who had a history of another chronic disease. Primary differences between refractory and drug-sensitive groups were in seizure frequency and type (generalized, partial, or multiple), which would be expected based on the inherent biological features of the classifications. (See Table 3.1).

In another human study, fecal microbiota were profiled by 16S rDNA sequencing of stool samples collected from 14 infants with refractory epilepsy, ranging from 1 to 4 years old, and 30 matched healthy infants from Shenzhen Children's Hospital (Xie et al., 2017). In this case, there was no significant difference in α-diversity between groups when measured by the Shannon

index for evenness. However, principal component analysis showed clustering of 16S rDNA data from refractory epilepsy infants distinctly from healthy infant controls, indicating substantial differences in fecal microbial β-diversity. Similar to results from the Peng et al. study, LEfSe analysis revealed elevated relative abundance of *Firmicutes* and *Proteobacteria*, and reduced *Bacteroidetes* and *Actinobacteria*, in infants with refractory epilepsy. At the genus level, *Cronobacter* was highly enriched in epileptic infants and not detected in healthy infants, while relative levels of *Bacteroides, Prevotella* and *Bifidobacterium* were decreased in infants with refractory epilepsy relative to controls. While the study required participants to not have taken antibiotics 1 month prior to the study and excluded those with chronic illness or metabolic disease, baseline differences in infant diet which could confound the study in the absence of matched household controls were not considered.

A third human study of 12 children with refractory epilepsy, aged 2–17 years, and 11 healthy parent controls from Astrid Lindgren Children's Hospital of Karolinska Institute examined fecal microbiomes by shotgun metagenomic sequencing (Lindefeldt et al., 2019). Fecal microbiota samples of children with refractory epilepsy exhibited decreased α-diversity, as measured by Shannon index, compared to microbiota samples from the healthy control parents. Principal component analysis of taxonomic and functional profiles revealed clear clustering of microbiomes from healthy control parents, whereas those from children with refractory epilepsy exhibited larger variation and minor shifts along the first principal component. In general, taxonomic analysis indicated that microbiota from children with refractory epilepsy displayed decreased relative abundances of *Bacteroidetes* and *Proteobacteria* and increased relative abundances of *Firmicutes* and *Actinobacteria*, when compared to control parent samples. Particular differences in functional potential were reported, with refractory epilepsy microbiomes harboring decreased gene content for β-hydroxybutyryl- CoA dehydrogenase and crotonase, genes involved in the acetyl-CoA pathway, as compared to parent control microbiomes. In light

of known age-dependent changes in the gut microbiome, a key caveat of these comparisons is the lack of age-matched controls.

Overall, all three of these human studies report alterations in the fecal microbiota of individuals with refractory epilepsy relative to varied non-epileptic controls (Lindefeldt et al., 2019; Peng et al., 2018; Xie et al., 2017). While they each report increased *Firmicutes* relative to *Bacteroides* in individuals with refractory epilepsy, the reported microbial alterations varied highly across taxonomic levels more resolved than phylum. In addition, the results were conflicting with regard to whether α-diversity is altered in the epilepsy microbiota. These studies are difficult to cross-compare due to variations in study design, age differences of subjects, relatively small samples sizes, as well as a lack of data on genetic and environmental factors that could influence the structure and function of the gut microbiome. Additionally, these studies differ by sequencing methodology and analytical tools used to profile the gut microbiota, where shotgun metagenomics, as in the Lindefeldt study, delivers both strain specificity and microbiome functional profiling, while 16S rDNA taxonomic profiling captures broader, less specific levels of diversity (Poretsky et al, 2014). Larger efforts are needed to achieve adequately powered patient and control populations and to account for variables such as age, human genetics, medication, and diet.

3. Microbiome associations with Epileptogenesis

3.1. The gut microbiota and seizure susceptibility in animal models

In addition to the existing human studies reporting a correlation between refractory epilepsy and altered gut microbiota, a few animal studies highlight a causal role for the microbiome in modulating seizure susceptibility. Animal models for studying epilepsy include the use of chemoconvulsants such as kainic acid, electrical stimulation using the 6 Hz seizure model, or seizure kindling which applies repeated stimulation to increase seizure susceptibility. One in particular drew upon a wealth of literature reporting that physical and psychological

stressors alter the gut microbiota (Vuong et al., 2017) to further investigate whether stress-induced alterations in the gut microbiota impact the development of seizures (Medel-Matus et al., 2018). Sprague-Dawley rats were subjected to sham stress or chronic restraint stress for two 2-h long sessions per day for 2 weeks, and cecal contents from each group were then transplanted into naïve recipient rats that were pre-treated with antibiotics to first deplete the gut microbiome. As expected, rats exposed to chronic restraint stress required fewer number of trials of basolateral amygdalar stimulation in order to induce a full seizure response and longer seizure duration, when compared to sham stress controls. This is consistent with prior studies revealing that stress promotes epileptogenesis. Notably, transplantation of the microbiome from a stressed rat into non-stressed recipient sufficiently conferred the stress-related increases in susceptibility to kindling and duration of seizures. In contrast, transplantation of the microbiome from a nonstressed rat into a stressed rat sufficiently reduced seizure duration and increased the number of kindling trials toward levels seen in the native sham controls. These results suggest that the microbiome mediates stress-induced increases in seizure susceptibility in a rat kindling model. Limitations of the study include the small sample size of 6 rats per group, the lack of companion sequencing data to identify taxonomic and functional differences in the microbiome that underlie their proversus anti-epileptic effects, and the lack of sequencing data of donor and recipient microbiota to confirm high fidelity transplantation. Additionally, mechanisms underlying the effects of transplantation on seizure susceptibility remain unclear; it is possible that metabolites contained within the transplant material, rather than the microbiome itself, could play a role, as could any indirect effects of the procedure on the host stress response.

A separate study examined links between the microbiome and the formation of cerebral cavernous malformations (CCMs), structural abnormalities in brain capillaries that predispose to stroke and seizures (Tang et al., 2017). Initial observations in endothelial specific *Krit1*^{ECKO} and *Ccm2*^{ECKO} knockout mice, which are theoretically susceptible to CCM formation, revealed that

differences in the breeding vivarium and unexpected infections modulated resistance vs. susceptibility to CCM formation. Follow-up experiments demonstrated that intraperitoneal injection of the gram-negative bacterium *B. fragilis* or lipopolysaccharide were each sufficient to drive CCM formation through TLR4 signaling. These results suggest that infection with gram negative bacteria (GNB) or systemic injection of GNB-associated antigens accelerates CCM formation. Further supporting a role for the gut microbiome on CCM formation, *Krit1*^{ECKO} mice raised as germ-free failed to form CCM lesions, whereas those raised conventionally colonized developed CCMs by P10. Consistent with this, maternal antibiotic treatment yielded offspring that were resistant to CCM formation, a phenotype that was transmitted transgenerationally to mice in the absence of antibiotic treatment. In contrast, conventionalization of the microbiome by cross-fostering to conventionally-colonized mothers restored susceptibility to CCM formation. 16S rDNA sequencing of fecal samples from CCM susceptible versus resistant *Krit1*^{ECKO} and *Ccm2*^{ECKO} mice revealed distinct group clustering of microbiota profiles by principal coordinates analysis. Taxonomic analysis highlighted significantly increased relative abundance of *Bacteroidetes S24–7* in mice susceptible to CCM formation, as compared to resistant controls. Whether this particular taxon is sufficient to modulate CCM formation is unclear. However, the several experiments performed in the study reveal a causal relationship between the gut microbiota and formation of CCMs, a primary risk factor for seizures. Altogether, mechanistic studies in animal models have begun to highlight how the gut microbiota could modify seizure vulnerability.

3.2. Infection and risk for epilepsy

Several large epidemiological and case studies associate infections with increased risk for epilepsy. A singleton cohort study of all children born in northern Denmark from 1998 to 2008 reported increased risk for epilepsy in children born from mothers that experienced infection during pregnancy (Ahlers et al., 2019; Norgaard et al., 2012). Similarly, in a nationwide

population-based cohort study of all individuals born in Denmark from 1982 to 2012, childhood infection with hospitalization was associated with a 78% increase in risk for epilepsy (Ahlers et al., 2019). Consistent with this, infants infected with *Group B streptococcus*, a leading cause of neonatal morbidity, are more likely to be hospitalized and diagnosed with epilepsy or other neurological conditions during their childhood years (Yeo et al., 2019). A study conducted by the Norwegian Institute of Public Health reported an increase in febrile seizures characteristic of febrile infection-related epilepsy syndrome (FIRES) following the 2009 influenza A (H1N1) pandemic (Bakken et al., 2015). In addition, human herpesvirus (HHV)-6 infection has been associated with mesial temporal sclerosis (MTS), a common pathological marker in mesial temporal lobe epilepsy (MTLE), and the HHV6-B virus in particular is linked with childhood epilepsy (Leibovitch and Jacobson, 2015; Vezzani et al., 2016). In a study of 75 MTLE patients, 52 patients displaying MTS and 23 non-MTS patients, MTS patients exhibited a greater number of seizures, increased HHV-6 viral DNA load and increased markers of inflammation compared to non-MTS controls (Kawamura et al., 2015). Other studies have also suggested that HHV-6 drives MTS/MTLE pathogenesis by inducing abnormal immune or inflammatory responses (Bartolini et al., 2019; Wipfler et al., 2018). Additional human studies of bacterial and parasitic infections also suggest links between infection and seizure susceptibility. *Taenia solium*, a tapeworm with prevalence in the regions around Burkina Faso causes neurocysticercosis in infected humans, which correlates with the prevalence of epilepsy in low income countries (Sahlu et al., 2019; Vezzani et al., 2016). Overall, the diversity of infections implicated in epilepsy has led to the notion that generalized immune activation or inflammation promotes susceptibility to seizures (Pardo et al., 2014; Tan, 2018).

Animal models of various infections support a causal role for inflammation in promoting seizure vulnerability. As a model of limbic epilepsy, mice injected intracortically with Theiler's murine encephalomyelitis virus (TMEV) exhibited seizures and neuroinflammation characterized by elevated pro-inflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor

(TNF)α in the hippocampus, a focal region for seizure initiation (Cusick et al., 2017; Patel et al., 2017). Notably, blocking TNF signaling by TMEV injection into TNFα−/− or TNFR1−/- TNFR2−/− mice sufficiently reduced seizures, suggesting that TNF signaling is required for mediating the pro-epileptic effects of TMEV infection. The study provided evidence that TNFα modulates glutamate receptor trafficking via TNF receptor 1 to increase excitatory synaptic transmission, which could underlie the elevated seizure incidence seen in response to TMEV. In a separate study, Wistar rats injected systemically with the bacterial cell wall component lipopolysaccharide (Veitenhansl et al., 2004) exhibited elevated levels of pro-inflammatory cytokines TNFα, IL-6, and IL-1β in the brain and decreased thresholds for chemically- and electrically-induced seizures by pentylenetetrazole (PTZ) and corneal shock, respectively (Sewal et al., 2017). In addition, toxoplasma-infected mice displayed reduced PTZ-induced seizures as well, which were partially abrogated by blocking the dopamine receptors D1 and D2 (Babaie et al., 2017). Altogether, these animal studies corroborate human association studies by revealing that a broad range of bacteria, viruses, and parasites can similarly promote seizure propensity. Research further suggests that inflammatory responses associated with cytokines and chemokines such as TNFα (Cusick et al., 2017; Patel et al., 2017; Sewal et al., 2017) and MCP-1 (Kawamura et al., 2015) could mediate the proepileptic effects of infection.

3.3. Antibiotic treatment and seizure susceptibility

Antibiotics are commonly prescribed for treating bacterial infections (Tamma et al., 2017) but despite their widespread use, many can elicit adverse side effects, including neurological symptoms (Mattappalil and Mergenhagen, 2014). A large epidemiological study of the Danish registry reported that increased numbers of antibiotic prescriptions for a single patient correlate with increased risk for epilepsy (Norgaard et al., 2012). Another study reported increased seizure risk in hemodialysis patients that were administered cephalosporin antibiotics (Zhang et al., 2019). A meta-analysis of all randomized controlled human trials of carbapenem antibiotics

reported a significant increase in seizure risk associated with carbapenem usage (Cannon et al., 2014). Imipenem and meropenem antibiotics were also highly correlated with epileptogenesis (Leibovitch and Jacobson, 2015; Owens Jr., 2008). While the majority of studies point to neurotoxic effects of antibiotics, such as β-lactams, unsubstituted penicillins, carbapenems, and 4th generation cephalosporins (Esposito et al., 2017; Sutter et al., 2015), a few small cohort and case studies have explored antibiotics as potential treatments for epilepsy (Braakman and van Ingen, 2018; Ghanizadeh and Berk, 2015; Raposo et al., 2016; Ghanizadeh and Berk, 2015). One found that treatment with a combination of penicillin derivative and macrolide antibiotics coincided with temporary seizure-free periods in six epileptic individuals (Braakman and van Ingen, 2018). Another reported that cefixime usage correlated with seizure-free bouts in a 9 year old boy with epilepsy and comorbid autism (Ghanizadeh and Berk, 2015). A challenge to interpreting the existing human data is the inability to distinguish potential off-target effects of antibiotics from their indicated anti-bacterial effects.

Findings from laboratory models have studied potential pathways by which antibiotics regulate seizure susceptibility. Particular β-lactam antibiotics are sufficient to elicit focal seizures in mice when injected intracortically or intracerebroventricularly. For example, penicillin-inducible seizure models have been used in multiple studies to understand epileptogenesis (Arslan et al., 2017; Han et al., 2015; Marangoz et al., 2018; Tubas et al., 2017; Zhu et al., 2018). The epileptogenic potential of penicillin, among other antibiotics, has been attributed to the antagonism of gamma-aminobutyric acid (GABA) -A receptors by the β-lactam ring (Veitenhansl et al., 2004). Non-competitive inhibition in this manner and voltage-dependent alterations are thought to reduce GABAergic inhibition and thereby permit excitatory signaling to trigger epileptiform bursts. In addition to inhibiting GABA-A receptors, quinolones can also bind to benzodiazepine receptors in the GABA complex. Moreover, both quinolones and cephalosporins further display agonistic effects on glutamatergic N-methyl-D-aspartate (NMDA) receptors, which further promote seizure susceptibility. Carbapenems, which are most

frequently associated with seizures, have a higher potential to promote seizures due to their greater ability to cross the blood brain barrier and to interfere with the action of antiepileptic drugs such as valproic acid.

In contrast to these direct effects of particular antibiotics on promoting neuronal activity underling seizures, some drugs with antimicrobial properties are being pursued for their anti-epileptic effects. Rapamycin, an mTOR inhibitor and antibiotic, reduced mTOR activation, overexpression of P-glycoprotein, and seizure susceptibility in a rat Coriaria lactone kindling model of temporal lobe epilepsy (Mazumder et al., 2016; Plovier et al., 2017). Minocycline, an inhibitor of microglial activation and antibiotic, reduced sympathetic nerve activity and increased seizure thresholds in rat kainic acid and amygdalar kindling seizure models (Beheshti Nasr et al., 2013; Bhandare et al., 2017). In addition, the antibiotic gentamicin increased latency to seizure and reduced total seizure duration when injected intracerebroventricularly into rats treated with kainic acid (Zhao et al., 2018). Overall, both human and animal studies have reported opposing effects of different antibiotics on seizure susceptibility. The findings warrant increased attention to whether the particular type, dose and route of antibiotic treatment may elicit disparate influences on vulnerability to particular subtypes of seizures.

4. Microbiome implications for epilepsy treatments

4.1. The microbiome and ketogenic diet

The high-fat, low-carbohydrate ketogenic diet (KD) is used as a clinical treatment for refractory epilepsy in individuals who do not respond to existing anti-epileptic drugs. While the KD has been used for almost a century for reducing seizures, the exact mechanisms by which the diet ameliorates seizures remains unclear. A few recent studies have investigated effects of the clinical KD on the composition of the gut microbiome in epilepsy patients, drawing the attention to whether alterations in the gut microbiome may contribute to the protective effects of the KD against seizures. In a study of 12 children diagnosed with drug-resistant epilepsy, 5 out

of 12 children displayed a > 50% decrease in seizure reduction and 10 out of the 12 children exhibited improved cognition and motor functions after 3 months on classical KD (Lindefeldt et al., 2019). When comparing the gut microbiome samples collected before initiation of the KD to those taken after 3 months on KD, there was no significant difference in α-diversity. However, β-diversity analysis revealed compositional differences characterized by decreases in relative abundances of *Actinobacteria* and *Bifidobacterium* and an increase in *Proteobacteria*. Another study of 20 children with refractory epilepsy reported KD-associated reductions in epilepsy symptoms that were correlated with reduced α-diversity of the microbiome, decreases in *Actinobacteria* and *Firmicutes* relative to *Bacteroidetes* after 6 months of dietary treatment (Zhang et al., 2018). A third study of 14 epileptic infants reported reductions in *Proteobacteria* and elevations in *Bacteroides, Prevotella*, and *Bifidobacterium* after at least 1 week on the KD (Xie et al., 2017). There was little consistency across these studies in the particular microbial taxa that were affected, which could be due to variations in study design, such as the length of KD treatment, the specific KD dietary regimen implemented, and the subtypes of epilepsy and seizure semiologies represented by the patient cohorts. One study examined 6 patients diagnosed particularly with glucose transporter 1 deficiency syndrome (GLUT1 DS) (Tagliabue et al., 2017). 3 months of KD treatment correlated with alterations in the gut microbiome that were characterized by a decrease in the relative abundance of *Desulfovibrio*. Overall, these studies warrant continued efforts to examine the effects of the KD on the gut microbiome across a large cohort of epileptic individuals. In particular, profiling microbial function rather than taxonomy, and examining associations with particular dietary, seizure semiology, medical history, and demographic data may yield greater insight across studies.

Additional studies in animal models of epilepsy similarly reveal functional roles for the gut microbiome in mediating the anti-seizure effects of the diet. In a 6 Hz acute electrically-induced seizure model of refractory epilepsy, depletion of the gut microbiome by antibiotic treatment or germ-free rearing abrogated the protective effects of the KD (Olson et al., 2018).

Moreover, promoting the KD-associated microbiome in naïve mice fed a control diet sufficiently conferred seizure protection. 16S rDNA sequencing revealed that the KD decreased α- diversity of the gut microbiome within 4 days of dietary treatment and increased the relative abundance of *Akkermansia muciniphila, Parabacteroides, Sutterella*, and *Erysipelotrichaceae* relative to controls. Selective enrichment of *A. muciniphila* and *Parabacteroides* conferred protection against 6 Hz seizures. These findings were further replicated in the *Kcna1−/−* genetic mouse model for sudden unexpected death in epilepsy (SUDEP), where depletion of the gut microbiome promoted spontaneous tonic-clonic seizures whereas selective enrichment of KD-associated bacterial taxa reduced seizure frequency and duration. Metabolomic data revealed decreases in peripheral ketogenic gamma-glutamylated amino acid concentrations, which correlated with higher hippocampal GABA/glutamate ratios in seizure protected mice, suggesting a role for microbial regulation of peripheral metabolites and central neurotransmitter metabolism in regulating seizure susceptibility. These findings align with increasing interest in select microbes that regulate the biosynthesis of GABA within the gut (Strandwitz, 2018; Yunes et al., 2016), and the use of other microbiota-related metabolites to modulate seizure susceptibility. For example, ginsenoside compound K was reported to decrease seizure intensity and latency in rats challenged with pentylenetetrazole to induce seizures (Zeng et al., 2018), and GPR40, a receptor for free fatty acids, has been shown to also regulate NMDA receptor function to reduced seizure susceptibility (Yang et al., 2018). Additional studies are needed to examine mechanisms by which microbes and microbiome-dependent metabolites influence brain activity and behavior related to epilepsy.

4.2. Probiotic treatment in human epilepsy

Although there have been only a few small studies to date that report alterations in the gut microbiome in human epilepsy (Liang et al., 2017; Lindefeldt et al., 2019; Xie et al., 2017), the links between epilepsy and infection, inflammation and antibiotic treatment raise the

question of whether microbial alterations under those conditions may play a role. Two recent human studies examined the effects of probiotics on seizures. In an observational study of neonates infected with rotavirus at the Gyeongsang National University Hospital (Yeom et al., 2019), 32 out of the 78 rotavirus positive neonates and 100 out of 150 rotavirus negative neonates were treated with *Saccharomyces boulardii* or *Lactobacillus casei* as a probiotic within 24h of birth. The authors proposed that *S. boulardii* reduces seizures through inhibition of rotavirus structural protein 4, a viral enterotoxin which increases reactive oxygen species and white matter injurty, or through suppressing the inflammatory response overall. The study reported that probiotic administration within 24h of birth was associated with a 10-fold decreased risk for seizures (odds ratio of 0.09) while rotavirus infection remained a risk factor only in neonates not given probiotics (odds ratio of 4.83). Seizure reduction was also reported in a pilot open label study of 43 adults with drug-resistant epilepsy treated daily for 4 months with a cocktail of *Lactobacillus acidophilus, L. plantarum, L. casei, L. helveticus, L. brevis, Bifidobacterium lactis*, and *Streptococcus salivarius* (Gomez- Eguilaz et al., 2018). 13 out of 43 (30%) of individuals reported>50% reduction in seizure frequency in the 4 months post treatment; however, a major limitation is the small study size and lack of placebo control. In addition to probiotic treatment, one recent case study from the Second Affiliated Hospital of Nanjing Medical University performed fecal microbiota transplantation (FMT) to treat Crohn's disease (CD) in a 22-year-old individual with refractory epilepsy (He et al., 2017). After 3 treatments, there was a decrease in the patient's CD index score from 361 (pre FMT) to 131 (20 months post-FMT). In addition, the patient reportedly experienced no epileptic seizures during the 20 months after FMT during which no antiseizure medications were taken. Consistent with this potential role for select probiotics to modulate seizure susceptibility, two studies in rodent models reported that probiotic treatment with *L. rhamnosus* alone or together with *B. longum* modulated expression of select GABA receptor subunits in various brain regions (Bravo et al., 2011; Liang et al., 2017). Overall, the promising results from the limited human and animal

studies performed to date suggest that additional studies are needed to examine whether manipulation of the gut microbiome may serve as a tractable strategy for reducing seizures.

4.3. Anti-epileptic drugs and the gut microbiota

Non-antibiotic medications, including anti-epileptic drugs, can interact directly with gut microbes that modify their metabolism and thereby impact drug efficacy and toxicity. A recent study of 1197 medications reported that 27% of non-antibiotic drugs inhibited the growth of at least one of 40 bacterial isolates (Maier et al., 2018). An additional study reported that the anticonvulsant drug clonazepam is metabolized by intestinal microbes, which can contribute to drug toxicity (Zimmermann et al., 2019). Other studies report mild effects of anti-seizure treatments such as carbamazepine on select gut microbes (Gomes et al., 2018; Vasiliadou et al., 2018; Watkins et al., 2017). Moreover, in a mouse study of maternal treatment with the antiepileptic drug valproic acid (Veitenhansl et al.), offspring of valproic acid (VPA)-treated mothers exhibited fecal microbiota with decreased *Firmicutes* and increased *Bacteroidetes* when compared to vehicle-exposed control mice (Sgritta et al., 2019). Additional animal studies similarly report that maternal exposure to VPA alters offspring gut microbiota composition (de Theije et al., 2014; Lim et al., 2017; Liu et al., 2018). Notably, VPA during pregnancy has known teratogenic effects, which raises the question of whether select VPA-induced phenotypes occur via microbiome alterations as opposed to other direct effects of VPA on host physiology. These studies highlight the importance of considering drug-induced gut microbiota changes and direct xenobiotic interactions with gut microbes. Large well-controlled population studies are needed to determine whether there is a clear signature of microbiome alterations in human epilepsy, and further, whether any antiepileptic drugs may alter the microbiome in a reproducible manner.

5. Conclusion

The gut microbiota is increasingly recognized as an important factor in epilepsy. Epilepsy is a highly heterogeneous disorder requiring understanding of interactions between genetic and environment risk. The gut microbiota regulates immunity and inflammation, metabolism, and peripheral and central neuronal signaling, pathways independently linked to epileptogenesis. Continued studies are warranted to understand the gut microbiota as a mediator of environmental variables, like diet, stress, and immune challenge, on seizure outcomes. Despite human studies demonstrating that changes in the composition of the microbiota correlate with epilepsy, there is as yet little consistency in the exact microbial taxa implicated across studies. The microbiome studies in epileptic patients to date are few, underpowered, and focus largely on bacterial taxa rather than function. Future studies that evaluate functional metagenomic profiles of the microbiome in large cohorts of epileptic individuals and age-matched controls, with careful consideration of seizure semiologies, demographic, medical and dietary information, could reveal whether there are consistent functional microbial signatures for subtypes of epilepsy. Such studies have the potential to uncover whether the gut microbiome can serve as a novel biomarker of subtypes of epilepsy. Absent of consistent microbiome implications with epilepsy pathogenesis, the gut microbiome could influence efficacy of seizure treatments, such as the ketogenic diet and anti-epileptic drugs. Further study of how the microbiome is impacted by seizure interventions could identify microbial markers for treatment responsiveness or form the foundation for novel microbiome-based treatments for epilepsy. Finally, detailed studies in animal models, for how microbes impact brain metabolism, neuroimmunity and neuronal activity promise to uncover fundamental principles for host-microbiome interactions that impact brain and behavior. Overall, further investigation into roles for the microbiome in epilepsy could help to uncover mechanistic underpinnings of epilepsy pathogenesis, biomarkers for disease and therapeutic responsivity, and novel approaches for treatment of refractory epilepsy.

Figures and Tables

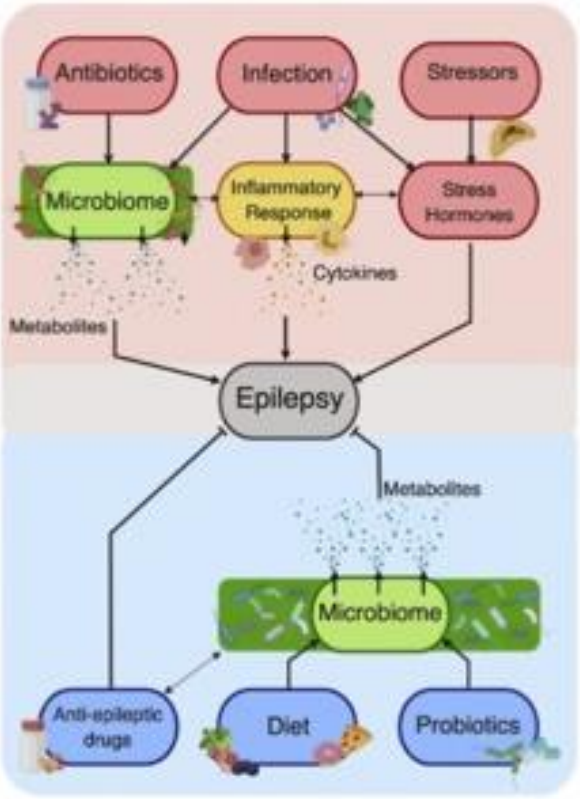

Fig. 3.1. Potential roles for the gut microbiome in mediating risk factors (red) and interventions (blue) for epilepsy. Factors associated with increased susceptibility to seizures, including antibiotics, infection, and psychological and physical stressors, also perturb the gut microbiota. Antibiotics can promote seizures directly through modulation of neuronal activity, or indirectly through modification of the microbiome. Pro-inflammatory cytokines and stress hormones that promote seizure risk can be induced by microbial antigens and can modify the gut microbiome. Particular gut microbes may alter the metabolism of antiepileptic drugs or be directly inhibited by antiepileptic drugs. The ketogenic diet is used to treat refractory epilepsy and is associated with changes in both the human and mouse gut microbiome. In two human studies, probiotic treatment was associated with reduced seizure risk. Separate animal studies report that probiotics modulate brain expression of gamma-aminobutyric acid (GABA) receptors, levels of GABA relative to glutamate, and seizure susceptibility.

Study	Location of Study	Subject	Type of Epilepsy or Disease	n	Baseline Alterations in a Refractory Epilepsy Microbiome	α-Diversity Alterations	β-Diversity	Intervention	Results after Intervention
Lindefeldt, M., Eng, A., Darban, H., Bjerkner, A., Zetterstrom, C.K., Allander, T., Andersson, B., Borenstein, E., Dahlin, M., and Prast-Nielsen, S. (2019). The ketogenic diet influences taxonomic and functional composition of the gut microbiota in children with severe epilepsy. NPJ Biofilms Microbiomes 5, 5.	Astrid Lindgren Children's Hospital of Karolinska Institute, Sweden	Children (2-17 yrs)	Refractory	12	Increase in *Firmicutes* and *Actinobacteria* Decrease in *Bacteroidetes* and *Proteobacteria*	Reduced α-diversity between refractory epilepsy group and healthy group and KD treatment did not significantly change α-diversity	PCA reported refractory epilepsy group was taxonomically different than the healthy group and pre KD microbiomes were taxonomically different than post KD microbiomes	3 months KD (3-4 g fat : 1g carbs or protein)	Increase in *Bacteroidetes* and *Proteobacteria* Decrease in *Firmicutes* and *Actinobacteria* in After 3 months KD, 5 children showed >50% decrease in the number of seizures and 83% showed improved cognition and motor functions
Peng, A., Qiu, X., Lai, W., Li, W., Zhang, L., Zhu, X., He, S., Duan, J., and Chen, L. (2018). Altered composition of the gut microbiome in patients with drug-resistant epilepsy. Epilepsy research 147, 102-107.	West China Hospital of Sichuan Univeristy, China	Children and Adults (0-52 yrs, avg age 28.4)	Refractory	42	Increase in *Firmicutes* and other rare species: *Akkermansia, Clostridium, Ruminococcus*, and *Coprobacillus* Decrease in *Bacteroidetes* and normal commensal bacteria *Lactobacillus* and *Bifidobacteria* abundance was significantly higher in children with 4 or fewer seizures when compared to children who had more than 4 seizures	Increased α-diversity in the refractory epilepsy group compared to the drug-sensitive and healthy groups	PCoA β-diversity analysis reported refractory epilepsy microbiome is different than drug-sensitive and healthy microbiomes, which were reported to be similar to each other	N/A	N/A
Tagliabue, A., Ferraris, C., Uggeri, F., Trentani, C., Bertoli, S., de Giorgis, V., Veggiotti, P., and Elli, M. (2017). Short-term impact of a classical ketogenic diet on gut microbiota in GLUT1 Deficiency Syndrome: A 3-month prospective observational study. Clin Nutr ESPEN 17, 33-37.	Department of Child Neurology at the University of Pavia, Italy	Children and Young Adults (8-34 yrs)	GLUT1 DS	6	N/A	N/A	N/A	3 months KD (2-4g fat : 1g carbs and protein)	Increase in *Disulfovibrio* No significant change in abundance of *Bacteroidetes, Firmicutes, Lactobacillus, Bifidobacterium, Enterobacteriaceae*, or *Clostridium*
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., et al. (2017). Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. World J Gastroenterol 23, 6164-6171.	Shenzhen Children's Hospital, China	Children (1-4 yrs)	Refractory	14	Increase in *Firmicutes, Cronobacter*, and *Proteobacteria* Decrease in *Bacteroidetes, Actinobacteria, Prevotella*, and *Bifidobacterium*	No significant difference in α-diversity between any groups	PCA β-diversity analysis reported differing microbiome profiles between refractory epilepsy individuals and healthy control individuals. Pre KD and post KD microbiome profiles were reported more similar to each other than to the healthy control	1 week KD (all participants given same Qitong ketogenic set meal packages)	Increase in *Bacteroidetes, Prevotella*, and *Bifidobacterium* Decrease in *Proteobacteria* and *Cronobacter* After 1 week KD, 64% of children showed >50% decrease in seizure frequency
Zhang, Y., Zhou, S., Zhou, Y., Yu, L., Zhang, L., and Wang, Y. (2018). Altered gut microbiome composition in children with refractory epilepsy after ketogenic diet. Epilepsy research 145, 163-168	Children's Hospital of Fudan University, China	Children (1-10 yrs)	Refractory	20	N/A	Reduced α-diversity between pre-KD and post-KD time points	PCoA β-diversity analysis reported differences in taxonomic diversity between the pre KD and post KD microbiome	6 months KD (4g fat : 1g carbs and protein)	Increase in *Bacteriodetes, Bacteroidia*, and *Bacteroidales* Decrease *in Firmicutes* and *Actinobacteria* During KD treatment 2 children were seizure free, 3 children had 90-99% reduction in seizure frequency, 5 children had 50-89% reduction in seizure frequency

Table 3.1. Reported alterations in the gut microbiota in human epilepsy. KD = ketogenic diet, GLUT1 DS = glucose transporter type 1 deficiency syndrome.

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Chapter 4: Ketogenic diet therapy for pediatric epilepsy is associated with alterations in the human gut microbiome that confer seizure resistance in mice

Summary

The gut microbiome modulates seizure susceptibility and the anti-seizure effects of the ketogenic diet (KD) in animal models, but whether these relationships translate to KD therapies for human drug-resistant epilepsy is unclear. Herein, we find that the clinical KD shifts the function of the gut microbiome in children with refractory epilepsy. Colonizing mice with KD-associated human gut microbes confers increased resistance to 6-Hz psychomotor seizures, as compared to colonization with gut microbes from matched pre-treatment controls. Parallel analysis of human donor and mouse recipient metagenomic and metabolomic profiles identifies subsets of shared functional features that are seen in response to KD treatment in humans and preserved upon transfer to mice fed a standard diet. These include enriched representation of microbial genes and metabolites related to anaplerosis, fatty acid beta-oxidation, and amino acid metabolism. Mice colonized with KD-associated human gut microbes further exhibit altered hippocampal and frontal cortical transcriptomic profiles relative to colonized pre-treatment controls, including differential expression of genes related to ATP synthesis, glutathione metabolism, oxidative phosphorylation, and translation. Integrative co-occurrence network analysis of the metagenomic, metabolomic, and brain transcriptomic datasets identifies features that are shared between human and mouse networks, and select microbial functional pathways and metabolites that are candidate primary drivers of hippocampal expression signatures related to epilepsy. Together, these findings reveal key microbial functions and biological pathways that are altered by clinical KD therapies for pediatric refractory epilepsy and further linked to microbiome-induced alterations in brain gene expression and seizure protection in mice.

Introduction

The low-carbohydrate, high-fat ketogenic diet (KD), is a mainstay treatment for refractory epilepsy, particularly in children who do not respond to existing anti-epileptic drugs. The efficacy of the KD is supported by multiple retrospective and prospective studies, which estimate that ~30% of pediatric patients become seizure-free and ~60% experience substantial benefit with >50% reduction in seizures (Coppola et al., 2002; Freeman et al., 1998; Hoon et al., 2005; Neal et al., 2008). However, use of the KD for treating pharmacoresistant epilepsy remains low due to difficulties with implementation, dietary compliance, and adverse side effects (Kossoff et al., 2018). Even with successful seizure reduction, retention of epileptic children on the KD is a reported 13% by the third year of dietary therapy (Hemingway et al., 2001). The primary reasons cited for discontinuation include “diet restrictiveness” and “diet side effects,” in addition to low diet responsiveness. While many pioneering studies have proposed important roles for immunosuppression, ketone bodies, anaplerosis, and gamma-aminobutyric acid (GABA) modulation in mediating the neuroprotective effects of the KD, they do not fully account for the clinical heterogeneity in patient responsiveness. Exactly how the KD confers protection against epilepsy in individuals with varied seizure semiologies remains unclear, and the biological determinants of patient responsiveness to the KD are poorly understood.

The gut microbiome plays an integral role in mediating effects of diet on multiple aspects of host physiology, including metabolism, neural activity, and behavior (Singh et al., 2017; Sonnenburg & Bäckhed, 2016). To date, a few clinical studies have reported associations between the KD regimens and alterations in the composition and/or functional potential of the gut microbiota in epileptic individuals (Lindefeldt et al., 2019; G. Xie et al., 2017; Y. Zhang et al., 2018). While promising, thus far there is little consistency across these different reports in the specific microbial taxa or gene pathways that correlate with the KD. Moreover, functional consequences of the KD-associated human epilepsy microbiome on host seizure susceptibility remain

unknown. We previously reported that KD-induced alterations in the gut microbiome were necessary and sufficient for mediating the seizure protective effects of KD chow in two mouse models for refractory epilepsy – the 6-Hz psychomotor seizure model and the *Kcna1* deficiency model for sudden unexpected death in epilepsy (SUDEP) (Olson et al., 2018). Similarly, in a rat injury model of infantile spasms, transfer of the KD-induced gut microbiota into naïve animals fed a control diet reduced spasms (Mu et al., 2022). In addition, taxonomic differences in the gut microbiome were correlated with seizure severity and seizure protection in response to KD chow in the *Scn1a* deficiency model for Dravet syndrome (Miljanovic & Potschka, 2021). Together, these findings across various seizure models provide proof-of-principle that the KD alters the gut microbiome in ways that can promote seizure protection. However, whether these results from rodent studies apply to human epilepsy, the human gut microbiome, and clinical KD regimens used to treat pediatric epilepsy is still unknown, and the core microbial functions that impact seizure susceptibility are unclear.

Herein, we perform a prospective study of KD interventions in children with refractory epilepsy and test causal effects of the human gut microbiome before and after initiating clinical KD regimens on seizure susceptibility in mice. We evaluate functional changes in the human gut microbiome that are associated with KD treatment in pediatric epilepsy patients. We further identify select features of the clinical KD-associated gut microbiome that are shared across both human donors and inoculated mouse recipients that correlate with microbiome-dependent seizure protection in mice. Finally, we identify key network interactions between the gut microbiome, metabolites, and brain transcriptome that may contribute to the ability of the clinical KD-associated human gut microbiome from pediatric epilepsy patients to promote seizure protection in mice.

Results

Clinical KD regimens elicit shared functional features of the gut microbiome in a cohort of children with refractory epilepsy

The KD is commonly prescribed for pediatric refractory epilepsy, wherein children consume commercial ketogenic infant formula and/or fat-rich, carbohydrate-restricted meals with dietary guidance from clinicians and registered dieticians (Kossoff et al., 2018). Notably, treatment regimens for the KD vary from patient to patient. KD composition depends on patient tolerability, which dictates the ratio of fat intake relative to carbohydrate and protein. Additionally, variable food sources determine the specific macro- and micro-nutrients that comprise ketogenic meals. Moreover, the KD is prescribed broadly for various forms of refractory epilepsy, the treatment population varies in genetic risk, seizure semiology, and past anti-epileptic drug exposures, among other factors. In order to assess effects of clinically-relevant KD treatments for refractory epilepsy on the gut microbiome, we therefore conducted a prospective study of 10 children with pediatric refractory epilepsy who were newly enrolled to the Ketogenic Diet Program at UCLA Mattel Children's Hospital (**Table 4.1**). From each patient, we collected a stool sample within 1 day before initiating a KD regimen (pre-KD sample) and after approximately 1 month of adherence to a clinically-guided KD (post-KD sample). 1 month was chosen as a time point at which we expected to observe stabilized microbial responses to the dietary regimen (David et al., 2014).

Data from 16S rRNA gene amplicon sequencing of fecal samples indicated no significant difference in bacterial α-diversity in the post-KD fecal microbiota from pediatric epilepsy patients relative to their matched pre-KD internal controls (**Figure 4.1A, Table S2**). Principal coordinates analysis of unweighted and weighted Unifrac distances revealed substantial variation across individuals in baseline composition of the pre-KD microbiota (**Figure 4.1B**). Additionally, the clinical KD elicited differential shifts in bacterial β-diversity and varied responses across post-KD

samples relative to their matching pre-KD controls, which were not significantly associated with demographic or clinical measures, such as age, sex, and prior anti-epileptic drug exposure (**Figure 4.1B**, **Table 4.1**, Yassour et al., 2016). Consistent with the inter-individual variation in microbial taxonomic profiles, ANCOM and ANOVA analyses (paired or unpaired) identified no significant differences in relative abundances in particular bacterial taxa when considering all post-KD sample relative to their matched pre-KD controls (**Figures 4.1C)**. These results indicate that, within this particular study cohort, there are no shared effects of the clinical KD on the microbial composition of the gut microbiota of children with refractory epilepsy.

Functional redundancy is common across different microbial species of the human gut microbiota (Tian et al., 2020). In light of the varied bacterial taxonomic profiles at baseline and in response to dietary treatment, we next asked whether the clinical KD is associated with shared alterations in the functional potential of the gut microbiota from children with pediatric epilepsy. Shotgun metagenomic profiling and pathway analysis indicated that compared to pre-KD samples, post-KD samples shared a significant decrease in relative abundance of genes belonging to the top 26 most abundant functional pathways, which together comprised >94% of the pathway diversity detected (**Figure 4.1D and 4.1E, Table S3**). This corresponded with a significant increase in the number of total observed pathways in post-KD samples compared to their respective pre-KD controls (**Figure 4.1F**). These observations suggest that the clinical KD restricts the membership of various types of microbial taxa that harbor genes related to prevalent functions and/or enriches for microbial taxa that harbor genes related to previously rare or underrepresented functions. In particular, post-KD samples exhibited significant enrichment of genes related to formaldehyde assimilation, guanosine nucleotide degradation, and L-proline biosynthesis, and decreased representation of genes related to aerobactin biosynthesis, as compared to pre-KD controls (**Figure 4.1G, Discussion**). There were also modest increases in genes related to GDP-mannose biosynthesis, 2-methylcitrate cycle, and

glycol metabolism and degradation, and decreases in genes related to polyamine biosynthesis and biotin biosynthesis, subsets of which will be discussed in greater detail in the following sections (**Figure 4.1G)**. Taken together, these data suggest that treatment with KD regimens that differ in KD ratio and specific nutritional composition elicit broad shifts in the functional potential of the gut microbiome that are shared across children with varied subtypes of refractory epilepsy.

Transferring the fecal microbiota from KD-treated pediatric epilepsy patients to mice confers seizure resistance

Causal influences of the human microbiome can be effectively studied in gnotobiotic mice, wherein transferring microbes in a clinical sample into microbiota-deficient mice is used to recapitulate the taxonomic and functional diversity of the donor human microbiota. To evaluate whether gut microbes associated with the clinical KD impact seizure susceptibility, we inoculated individual cohorts of germ-free (GF) mice with matched pre-KD and post-KD stool samples collected from children with refractory epilepsy and maintained the colonized mice on standard (non-ketogenic) mouse chow (control diet, CD). Each human donor sample (pre-KD and post-KD from 10 individuals, as biological replicates) was inoculated into 14-16 GF mice (as technical replicates) to enable cohort-level testing of susceptibility to 6-Hz psychomotor seizures (**Figure 4.2A**). The 6-Hz seizure model involves low-frequency corneal stimulation to induce acute complex partial seizures reminiscent of human limbic epilepsy (Barton et al., 2001). Consistent with refractory epilepsy, the 6-Hz model is resistant to several anti-epileptic drugs, but treatment with KD chow effectively protects against 6-Hz seizures in rodents (Hartman et al., 2008), raising the intensity of current required to elicit a seizure in 50% of the subjects tested (CC50, seizure threshold). The 4-day time point was chosen as the maximum duration of time that a KD-induced microbiota could be maintained in mice fed CD (Olson et al., 2018).

Despite the variation in bacterial diversity across patient gut microbiota (**Figure 4.1**), we observed that GF mice colonized with microbes from the post-KD microbiota required greater intensity of current to induce 6-Hz seizures (**Figure 4.2B, Table S4**) as compared to controls colonized with microbes from the pre-KD microbiota. This effect was seen when comparing post-KD vs. pre-KD microbiota transfer for individual technical replicates per patient (**Figure 4.2B)**, as well as when data were averaged across all patients (**Figures 4.2C**). There was no difference in latency to exploration at similarly tested intensities of current **(Figure 4.2D)**. In addition, compared to pre-KD controls, mice colonized with microbes from the post-KD microbiota required increased intensity of current to elicit one or more recurred seizures observed after the initial stimulus-induced seizure (**Figure 4.2E**), indicating that transfer of the post-KD human microbiota promotes resistance to both primary induced seizures and remission seizures in mice. On average, the post-KD samples raised seizure thresholds by 22.4% $\pm$ 6.4% relative to matched pre-KD controls (**Figures 4.2C**). This aligns with both our previously published data on the average effect size of KD chow on wildtype mice tested in the 6-Hz seizure assay (24.5%, Olson et al., 2018), and the observed 24.0% increase in seizure threshold seen in GF mice colonized with a conventional adult mouse microbiota (GF-conv) and fed a 6:1 KD chow, as compared to conventionalized controls fed a standard vitamin- and mineral-matched control diet (**Figure 4.2B**). Discrepancies in effect size across patient samples were largely driven by differences between responses for pre-KD controls (**Figure 4.2B**), suggesting that the comparatively low microbial diversity resulting from cross-host species transfer increases seizure susceptibility. Consistent with this, we previously observed that decreasing microbial diversity via antibiotic treatment reduced seizure threshold in the 6-Hz assay (Olson et al., 2018). Overall, these results indicate that inoculating mice with the clinical KD-associated human gut microbiota increases 6-Hz seizure threshold to levels similar to the effect sizes seen with direct consumption of the experimental 6:1 KD.

Human microbiota transplantation to mice involves oral inoculation with a human stool suspension, which is comprised of microbial biomass, as well as undigested food matter and secreted molecules from the host and microbiota. As such, effects seen in response to the transfer procedure could be due to the KD-associated gut microbiota or microbiota-independent dietary or host factors. To gain insight into whether bacteria from the gut microbiota are required for mediating the increases in seizure protection seen with inoculation of the human post-KD microbiota into mice, mice inoculated with a randomly selected post-KD donor sample were treated with broad-spectrum antibiotics (**Abx**) to deplete the microbiota, or with vehicle (**Veh**) as negative control (**Figures 4.3A and 4.3B**). Mice that were inoculated with the post-KD sample and treated with Veh displayed seizure thresholds that were comparable to that seen previously in recipient mice without the added Veh treatment (**Figures 4.3C, 4.3D, and 4.2B and Table S4**). This suggests that the post-KD sample induced increases in seizure resistance that were maintained for at least 12 days in mice fed CD. In contrast, depletion of gut bacteria in mice that were colonized with the post-KD microbiota decreased seizure thresholds to levels that were lower than previously seen in pre-KD colonized controls (**Figures 4.3C, 4.3D, and 4.2B**). These results indicate that bacterial members of the post-KD microbiota are necessary for mediating the increases in seizure threshold seen in response to transfer of the clinical-KD associated microbiota from a pediatric epilepsy patient into mice.

Administration of microbial metabolites or other microbiome-dependent molecules, in lieu of viable microbiota, has been reported to ameliorate symptoms of recurrent *Clostridiodes difficile* infection, inflammatory bowel disease, and multiple sclerosis, among other conditions (Cekanaviciute et al., 2017; Levy et al., 2015; Ott et al., 2017). To gain insight into whether administration of clinical KD-associated intestinal small molecules is sufficient to confer seizure protection in mice, a post-KD donor sample selected at random was sterile filtered and then administered to a cohort of GF recipient mice (**Figure 4.4A**), alongside controls that were

administered the unfiltered post-KD suspension, as was done previously for human microbiota inoculation (**Figures 4.4A and 4.2B**). At 4 days post inoculation, mice that were treated with the post-KD filtrate exhibited lower seizure threshold compared to controls that were treated with the corresponding unfiltered post-KD suspension (**Figures 4.4B and 4.4C and Table S4**). These data indicate that clinical KD-associated small molecules in the post-KD fecal sample from a pediatric epilepsy patient are not sufficient to confer persistent seizure protection in mice.

Orally administered microbial metabolites can be rapidly absorbed and cleared from systemic circulation within a few hours of administration (Abrams & Bishop, 1967; Williams et al., 2020). To further assess whether clinical KD-associated intestinal small molecules, including microbial metabolites, acutely modulate seizure susceptibility, mice were orally gavaged with a sterile-filtered post-KD sample and assessed 2 hours later for 6-Hz seizure threshold, rather than 4 days later as in the previous experiments (**Figure 4.5A**). Mice treated with post-KD filtrate exhibited significantly increased seizure protection compared to controls treated with pre-KD filtrate (**Figures 4.5B and 4.5C and Table S4**), with seizure thresholds that approached those seen after inoculation of the post-KD suspension (**Figures 4.5B and 1B**). These data indicate that administration of clinical KD-associated intestinal small molecules can acutely confer seizure protection in mice over short timescales (i.e., 2 hours, **Figure 4.5**), which diminishes by 4 days post treatment (**Figure 4.4**). Taken together, the results presented in these series of experiments suggest that the clinical KD for pediatric refractory epilepsy is associated with alterations in metabolic activities of the gut microbiota that promote seizure resistance in mice.

While the “humanization” of mice with microbiota from clinical stool samples is a powerful tool for translational microbiome research (Turnbaugh et al., 2009), the approach has technical and biological limitations that warrant careful consideration (Walter et al., 2020). Namely, while much of the taxonomic and functional diversity of the donor inoculum can be recapitulated in recipient

mice (Bokoliya et al., 2021), developmental influences and host-specific selection (Rawls et al., 2006), among other factors, preclude full “engraftment” of the human gut microbiota in GF mice (Walter et al., 2020). To evaluate the fidelity of fecal microbiota “transplantation” from pediatric epilepsy patients to GF mice, we subjected both the donor pre-KD and post-KD stool samples and corresponding recipient mouse fecal pellets collected at 4 days post-inoculation (the day of seizure testing) to 16S rRNA gene amplicon sequencing (**Figure 4.2A, Tables S2 and S5**). Principal coordinates analysis of bacterial taxonomic data revealed overt clustering of donor samples with matched recipient samples only for select patients, while the remaining exhibited substantial variation and no noticeable clustering (**Figure 4.6A**). There was no significant difference in α-diversity between pre-KD and post-KD fecal microbiota for either donor or recipient samples (**Figures 4.6B**). However, we observed a significant reduction in α-diversity, with an average decrease of 38% for all mouse recipient microbiota relative to all human donor microbiota (**Figure 4.6C**), indicating incomplete transfer or engraftment of the human microbiota in mice. These results align with several previous reports of reduced bacterial α-diversity in mice inoculated with human microbiota, with estimated decreases of 35%, 38%, and 50% (Blanton et al., 2016; Sharon et al., 2019; Staley et al., 2017), suggesting that we achieved levels of transfer fidelity that are consistent with those in the field. However, the inability to fully recapitulate the taxonomic diversity of the human gut microbiota from pediatric epilepsy patients in mice draws into question whether the increases in seizure resistance seen in mice inoculated with post-KD microbiota are relevant to the actual clinical condition. We therefore focused subsequent experiments on identifying and evaluating the subset of functional features of the KD-associated human gut microbiome that are recapitulated in recipient mice, and the microbiome-dependent alterations in host physiology that correspond with seizure protection in mice.

Select functional features of the clinical KD-associated human microbiome are recapitulated in colonized recipient mice and correlate with seizure protection

Given the widespread use of the clinical KD for treating epilepsy, and an increasing number of other neurodevelopmental and neurodegenerative disorders, elucidating how the activity of the gut microbiome is altered by the clinical KD could reveal important insights into its physiological effects. To identify microbiome associations with the clinical KD and further determine which of the associations, if any, may modify seizure risk, we functionally characterized the gut microbiome from pediatric epilepsy patients before and after treatment with the clinical KD, as well as from gnotobiotic mice that were inoculated with the patient samples, and tested for causal outcomes on seizure susceptibility. Metagenomic sequencing and analysis revealed microbial gene pathways that were differentially abundant in post-KD samples relative pre-KD controls, and shared between both human donor samples and mouse recipient samples (**Figure 4.7A, Tables S3 and S6**). In particular, microbial genes relevant to fatty acid β-oxidation, glycol metabolism and degradation, methylcitrate cycle I, methylcitrate cycle II, and proline biosynthesis were similarly elevated in post-KD human samples and post-KD-inoculated mice compared to their respective pre-KD controls (**Figures 4.7A and 4.7B**). These findings align with reported influences of the KD on fatty acid oxidation (A. R. Kennedy et al., 2007), of carbohydrate restriction on promoting the glyoxylate cycle (Puckett et al., 2017), and of fatty acid β-oxidation on the initiation of the methylcitrate cycle (Clark & Cronan, 2005). Proline metabolism involves reactions with glutamine, glutamate, ornithine, and arginine, which might relate to reported effects of KD on amino acid metabolism, particularly of glutamine and glutamate (Yudkoff et al., 2007). In addition, both post-KD human donor and mouse recipient samples exhibited reductions in microbial genes relevant to polyamine biosynthesis and aerobactin biosynthesis (**Figures 4.7A and 4.7B**). The main role of polyamine biosynthesis is generation of putrescine, mainly using the glucogenic amino acid L-arginine which is consumer in reduced amounts while on the KD. Aerobactin, a sidophore, biosynthesis uses the ketogenic

amino acid L-lysine, which is also essential for acetyl-CoA synthesis and energy production during ketosis. These data suggest that the consumption of a clinical KD by children with refractory epilepsy enriches for gut microbes that have the functional capacity to metabolize dietary fats and to perform anaplerotic reactions when dietary carbohydrates are restricted. The findings further indicate that these general features of the KD-associated human gut microbiome are phenocopied in recipient mice that exhibit microbiome-dependent protection against 6-Hz seizures.

The observed metagenomic signatures reveal clinical KD-associated changes in the functional potential of the gut microbiome that are preserved upon transfer to GF mice. To identify clinical KD-induced alterations in the functional activity of the gut microbiome, we performed untargeted metabolomic profiling of aliquots of the same donor fecal samples from pediatric epilepsy patients collected before and after initiating the KD regimen, and of both fecal and serum samples from recipient mice that were inoculated with the pre-KD or post-KD human fecal microbiota and fed CD (**Tables S7, S8, and S9**). Results from clinical laboratory testing of human blood samples confirmed that the month-long clinical KD regimen elevated serum β-hydroxybutyrate (**BHBA**) levels and reduced serum glucose levels, relative to pre-KD concentrations, in pediatric refractory epilepsy patients (**Figure 4.7C**). Decreases in glucose, but not BHBA, were similarly seen in human post-KD stool samples relative to matched pre-KD controls (**Figure 4.7C**), which is consistent with dietary carbohydrate restriction and KD-induced BHBA synthesis by the liver to elevate systemic, but not fecal, BHBA levels (Westman et al., 2007). Transfer of the post-KD human microbiota into mice yielded no significant differences in serum BHBA or glucose relative to pre-KD recipient controls (**Figure 4.7C**), indicating that the clinical KD-associated microbiota does not sufficiently promote key systemic features of ketosis in mice fed the standard CD. Notably, however, mice that were inoculated with post-KD human microbiota and fed CD exhibited statistically significant increases in fecal BHBA levels relative to

matched pre-KD recipient controls (**Figure 4.7C**). This could reflect alterations in intestinal synthesis of BHBA (Mierziak et al., 2021) and/or in microbial utilization of host-derived BHBA (Ang et al., 2020). Since this effect was not seen in the donor human fecal samples, we reasoned that this phenotype is likely an artifact of the "transplantation" approach and/or experimental design, and therefore not relevant to the clinical condition. These results suggest that transfer of the clinical KD-associated human gut microbiota into mice promotes resistance to 6-Hz seizures (**Figure 4.2**) through mechanisms that act independently of ketosis.

We further assessed results from untargeted metabolomic profiling to identify metabolites that were differentially abundant in post-KD samples relative to pre-KD controls and patterns that were shared across human donor and mouse recipient samples (**Figure 4.7D, Figure 4.8, Tables S7 and S8)**. Despite heterogeneity in the patient population and specific clinical KD regimens, 79 metabolites were significantly differentially abundant in fecal samples from post-KD human fecal samples relative to their matched pre-KD controls (**Figure 4.8A, Table S7**). 336 metabolites were identified in both the human fecal samples and mice fed the 6:1 KD chow vs. vitamin- and mineral-matched control chow for 2 weeks samples, previously published by our group (**Table S10,** Olson et al., 2018). 35 metabolites were differentially regulated in human fecal samples and 169 metabolites were differentially regulated in mouse fecal samples (**Figure 4.8B**). Of these significantly altered metabolites, 20 were found to be changed in the same direction across human and mouse samples (**Figure 4.8B and 4.8C**). These included KD-induced increases in levels of metabolites related to fatty acid beta-oxidation, such as palmitoleoylcarnitine (C16:1) and oleoylcarnitine (C18:1), and a decrease in kynurenine which have previously been associated with seizure susceptibility (Żarnowska et al., 2019). This statistically significant overlap suggests that there are biochemical changes that are shared across clinical KD treatments for pediatric epilepsy and mouse models of KD, and that some of the fecal metabolomic alterations observed in KD-treated epilepsy patients are a direct

consequence (rather than correlate) of dietary intervention. Of the 20 significantly differentially abundant metabolites shared in human and mouse, 14 (~70%) were further significantly altered by antibiotic treatment to deplete gut bacteria in KD-fed mice (**Figure 4.8C,** Olson et al., 2018). Taken together, these data indicate that clinical KD regimens alter fecal metabolites in children with refractory epilepsy, a subset of which have the potential to be microbiome-dependent.

Although there was substantial variability in taxonomic composition of microbiota within mouse recipients of the same experimental condition (**Figure 4.1 and 4.6**), fecal samples from mouse recipient cohorts exhibited statistically significant alterations in 45 metabolites that were shared when considering all post-KD mouse recipient fecal samples relative to their pre-KD controls (**Table S8, Figure 4.8A**). Notably, however, none of these 45 differentially abundant metabolites in mouse feces were identical to the 79 differentially abundant metabolites seen in human donor fecal samples (**Figure 4.8A**), which could reflect host specific metabolite utilization and the fact that recipient mice were fed standard chow (CD), while human donors were consuming a clinical KD at the time of sample collection. To gain insight into whether the differentially abundant metabolites relate to similar biological functions, we performed metabolite set enrichment analysis (MSEA) of the significantly altered metabolites in human vs. mouse (Pang et al., 2021). MSEA of the significantly altered metabolites identified select chemical classes (**Figure 4.7D**) and metabolic pathways (**Figure 4.7E**) that were similarly enriched in both human donor and mouse recipient post-KD samples relative to pre-KD controls. In particular, there was shared enrichment of amino acid, hydroxy fatty acid, sugar acid, phenylpropanoic acid, and monosaccharide-related metabolites across post-KD conditions for both human donors and mouse recipients (**Figure 4.7D**). Differentially abundant metabolites from human post-KD fecal samples also exhibited enrichment of bile acids and other fatty acid derivatives (**Figure 4.7D, left**), which might reflect KD- and/or microbiome-driven alterations in lipid metabolism (Joyce et al., 2014).

For metabolic pathways, post-KD samples for both human donor and mouse recipient conditions exhibited differential abundance of metabolites related to methionine metabolism, glycine and serine metabolism, and betaine metabolism (**Figure 4.7E**). While the biological relevance to KD is unclear, one possibility is that these pathways reflect known influences of the KD on one-carbon (1C) metabolism, a series of interlinking metabolic pathways that control levels of methionine, serine, and glycine, and that integrate nutrient availability with cellular nutritional status (Ducker & Rabinowitz, 2017). In addition, differentially abundant fecal metabolites from mouse post-KD recipients mapped to pathways related to alpha linolenic acid and linoleic acid metabolism, fatty acid biosynthesis, and beta-oxidation of very long chain fatty acids (**Figure 4.7E, right**), which aligns with the observed metagenomic enrichment in microbial genes related to fatty acid metabolism in response to the clinical KD (**Figure 4.7A-B**). Some of the differential metabolite chemical subclasses and metabolic pathways in mouse fecal samples were similarly seen in matched mouse serum samples (**Table S9**) – in particular, metabolites representing amino acid, hydroxy fatty acid, and unsaturated fatty acid subclasses, and related to alpha linolenic acid and linoleic acid metabolism, betaine metabolism, and beta-oxidation of fatty acids were altered in both feces and serum of mice receiving post-KD samples relative to pre-KD controls (**Figure 4.8E**). Taken together, these results suggest that the clinical KD induces alterations in the function of the gut microbiome of pediatric epilepsy patients, and that a subset of these functional characteristics may be phenocopied upon microbial transfer to mice, which develop microbiome-dependent resistance to 6-Hz seizures.

Transferring the fecal microbiota from KD-treated pediatric epilepsy patients to mice induces alterations in brain gene expression

Seizures result from atypical neural function related to discharge of electrical signals or failure to constrain the spread of these signals. To gain insight into how colonization with microbes

derived from the fecal microbiota of KD-treated individuals may alter brain function to modify seizure susceptibility, we performed transcriptomic profiling of brain tissues from cohorts of mice colonized with microbes from the post-KD human microbiota or pre-KD controls. We focused on the hippocampus and frontal cortex based on their relevance to human epilepsy, their involvement in initiating psychomotor seizures in the 6-Hz seizure assay, and evidence that the microbiome can alter gene expression and metabolites in these brain regions (Chauhan et al., 2022; Suarez et al., 2018). RNA sequencing of hippocampal tissues revealed many differentially expressed genes that were seen in post-KD samples relative to pre-KD controls (**Table S11**), including those related to core cell biological processes relating to RNA processing, translation, cellular stress response, TORC1 signaling, regulation of long-term synaptic potentiation, neuronal development, and response to nutrient levels (**Figure 4.9A**). The most drastic alterations included upregulation of *Dusp12, Bmpr1b,* and *Cmya5* and downregulation of *Abcc9*, *Ufsp1*, and *Tbx2* transcripts (**Figure 4.9B**). *Dusp12* is a dual specificity phosphatase that can dephosphorylate phosphothreonine and phosphoserine (Muda et al., 1999)*, Bmpr1b,* a member of the bone morphogenic receptor family, is a serine/threonine kinase influencing neuronal cell fate (Venugopal et al., 2012), and *Cmya5* encodes for myospyrn which is essential for structural integrity during neuritogenesis (Hsiung et al., 2019). *Abcc9* is an ATP-binding cassette transporter encoding the sulfonylurea receptor 2 subunit for potassium channels (Nelson et al., 2015), *Ufsp1* is a Ufm1 specific protease that regulates ubiquitin-like conjugation and has been linked to seizures (Millrine et al., 2022), and *Tbx2* is a transcription factor linked to neuronal cell cycle control and neuroinflammation (Reinhardt et al., 2019). STRING network analysis additionally revealed top protein interaction clusters enriched for essential biological processes including RNA processing, oxidative phosphorylation, and cell cycle regulation, consistent with results from GO enrichment analysis (**Figure 3A, 3C, 3D**), as well as endocytosis and glutathione metabolism (**Figure 3D**).

Some differentially expressed genes were also identified in frontal cortical tissues of post-KD recipients relative to pre-KD controls (**Table S12**), which similarly to hippocampus, included those related to core cell biological processes for RNA surveillance and catabolism, cellular stress responses, TORC1 signaling, and further included genes related to potassium ion transport, and core carbohydrate metabolism (**Figure 4.10A**). The most drastic alterations included upregulation of *Serpinb1a, Nqo1,* and *Slc6a12* transcripts, and downregulation of *Aldh3b1, Setmar*, and *Tfb1m* transcripts (**Figure 4.10B**). *Serpinb1a* is a serine/cysteine protease inhibitor (Huasong et al., 2015)*, Nqo1* encodes an antioxidant enzyme that primarily catalyzes the reduction of quinones (Ross & Siegel, 2021)*,* and *Slc6a12* encodes for a betaine-GABA transporter (Zhou et al., 2012)*. Aldh3b1* is an aldehyde dehydrogenase linked to oxidative stress reduction (Marchitti et al., 2007)*, Setmar* encodes a histone-lysine N-methyltransferase (Cordaux et al., 2006), and *Tfb1m* has been shown to function as methyltransferase (Metodiev et al., 2009). STRING network clustering analysis additionally revealed top protein interaction clusters enriched for transcription regulation, translation, and oxidative phosphorylation, also seen in frontal cortex GO enrichment analysis and in the hippocampal STRING network, as well as clusters enriched for calcium signaling, transcriptional regulation, and translation (**Figure 4.10C, 4.10D**). Differentially expressed gene sets from both hippocampus and frontal cortex were enriched for TORC1 signaling, cellular response to stress, and oxidate phosphorylation through GO enrichment and STRING clustering, which have all been shown to affect seizure susceptibility (Chan, 2001; Nguyen & Bordey, 2021). The similarities between transcriptomic results from hippocampus and frontal cortex suggest that colonization with post-KD microbes elicits key alterations in host metabolism that impact core biological processes that are generally consistent across different brain regions. Overall, these results indicate that mice that acquire seizure resistance in response to colonization with microbes from the post-KD human gut microbiota exhibit alterations in hippocampal and frontal cortical gene expression, relative to pre-KD recipient controls.

Multi'omics analysis reveals network connections linking microbial genomic pathways and metabolites to hippocampal transcripts related to epilepsy

To further identify key gut microbial functions that may drive particular brain gene expression signatures, we utilized microbe-metabolite vectors (MMVEC) (Morton et al., 2019) to build an integrated network of fecal metagenomic, fecal metabolomic, serum metabolomic, hippocampal transcriptomic, and frontal cortical transcriptomic datasets from mice inoculated with the human pre-KD or post-KD microbiota (**Table S13**). We generated a parallel network comprised of fecal metagenomic and fecal metabolomic datasets from human pre-KD and post-KD donor stool samples to identify features similarly underscored in both human and mouse networks, suggesting their clinical relevance. The human donor and mouse recipient networks were linked by 3 common nodes - metagenomic pathways describing branched chain amino acid (BCAA) biosynthesis (BRANCHED-CHAIN-AA-SYN-PWY), L-alanine fermentation (PROPFERM-PWY), and co-enzyme A biosynthesis (COA-PWY), as well as pathways for arginine synthesis (ARGSYN-PWY In the human network and ARGSYNSUB-PWY in the mouse network) (**Figure 4.11A,** center gray and green nodes). The shared BCAA biosynthesis, co-enzyme A biosynthesis, and arginine synthesis pathways were also identified by weighted key driver analysis as highly interconnected across the 'omics datasets and essential regulator nodes of the network (Ding et al., 2021) (**Figure 4.11A**, diamonds). The human donor network also contained an additional key driver metagenomic node for isoleucine biosynthesis (ILEUSYN-PWY), which aligns with the metagenomic node for BCAA biosynthesis. Consistent with the shared metagenomic key drivers between mouse and human networks, the human fecal metabolomic module was enriched for nodes related to valine, leucine, and isoleucine (BCAA) and CoA biosynthesis (**Figure 4.11A**, gray diamond node). In the mouse network, metabolomic modules included nodes related to glycerophospholipid metabolism for fecal metabolites and pentose and glucuronate interconversions for serum metabolites (**Figure 4.11A,** orange and

sea green nodes). Nodes for fecal 1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)*, 1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)*, 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*, 3-hydroxybutyrate (BHBA), and myo-inositol (**Figure 4.11A**, orange metabolite nodes in red font) were similarly identified as differentially abundant in individual metabolomic analyses for recipient post-KD fecal samples relative to pre-KD controls (**Table S7**). These mouse metagenomic and metabolomic modules were linked to 5 transcriptomic modules for hippocampal genes and 3 for frontal cortical genes (**Figure 4.11A,** bottom section). The hippocampal transcript modules were enriched for nodes related to regulation of telomerase RNA localization to Cajal body, glycosylphosphatidylinositol (GPI) anchor biosynthetic processes, Wnt signaling, and neuron generation and migration (**Figure 4.11A**). The frontal cortical transcript modules were enriched for nodes related to regulation of catabolic processes, lipase activity, and BCAA transmembrane transporter activity (**Figure 4.11A**). This suggests that these particular biological processes are most closely associated with the microbial functional features identified in the network. The transcript nodes included 41 hippocampal genes and 4 frontal cortical genes that were similarly identified in individual transcriptomic analyses as differentially expressed in post-KD recipient mice relative to pre-KD controls (**Figure 4.11A**, transcript nodes in red font). The higher number of connections between metabolomic modules and hippocampal transcripts suggests that the gut microbiome may exhibit a greater regulatory role for the hippocampus than for the frontal cortex in post-KD recipient mice compared to pre-KD controls. Of particular interest are the links between fecal metabolites related to glycerophospholipid metabolism, which are regulated by the microbiome (Zheng et al., 2021), and hippocampal transcript modules enriched for Wnt signaling and GPI anchor biosynthetic processes, pathways implicated in seizure susceptibility (Hodges & Lugo, 2018; Wu et al., 2020).

To gain insight into whether the hippocampal and frontal cortical transcripts that co-occur with microbial metagenomic and metabolomic features have been implicated in human epilepsy, single nucleotide polymorphisms (SNPs) identified from epilepsy genome-wide association studies (GWAS) were mapped to genes using hippocampus and frontal cortex splicing quantitative trait loci (sQTLs) and expression quantitative trait loci (eQTLs) to represent epilepsy-associated genes informed by GWAS. The mouse orthologs of these human GWAS disease genes were then compared with hippocampal and frontal cortical transcriptomic results to identify DEGs in post-KD vs pre-KD recipients that have been implicated in genetic risk for human epilepsy. There was a statistically significant enrichment of the hippocampal DEGs in the epilepsy GWAS (p=0.003), but no significant enrichment of the frontal cortical DEGs in the epilepsy GWAS (p=0.26) (**Figure 4.11B**). These results suggest that microbial alterations in hippocampal gene expression may contribute to the microbiome-dependent increases in seizure resistance seen in post-KD recipient mice compared to pre-KD controls. From the co-occurrence network, 5 hippocampal DEGs were linked to epilepsy GWAS results: *Atp5c*, which encodes mitochondrial ATP synthase; *Rprd2,* which encodes a transcriptional repressor that modulates RNA polymerase II activity; *Gnaz*, which encodes G protein alpha subunit that regulates ion equilibrium and chaperone-mediated folding; *Cfdp1*, which encodes a subunit of the chromatin remodeling complex and is important for cell division, and *Mro*, which encodes a nucleolus protein proposed to be testis-determining in the reproductive tract, but expressed in the nervous system with as yet unknown function. Overall, the multi'omics analysis of human donor and mouse recipient datasets together with epilepsy GWAS mapping to hippocampal and frontal cortical DEGs identified key microbial genomic pathways and microbially modulated metabolites that may contribute to alterations in the expression of particular hippocampal genes in mice that exhibit microbiome-induced protection against 6-Hz seizures.

Discussion

Results from this research provide evidence from a treatment study of children with refractory epilepsy, coupled with functional testing in gnotobiotic mice, that clinical KD regimens alter the function of the gut microbiome in ways that could contribute to seizure protection. We assessed microbiome composition and function in 10 children with refractory epilepsy shortly before initiating and approximately one month after adherence to classical KD regimens. Following clinical practice, the patient cohort was heterogeneous in type and underlying cause of refractory epilepsy, as well as the ratio of fat to carbohydrate and protein and specific nutritional composition of the KD they consumed (**Table 4.1**). This highlights the diversity of epilepsies that resist current antiepileptic drugs and the broad range of KD interventions that are administered to treat pediatric refractory epilepsy. Consistent with this heterogeneity, we observed that participants varied substantially in the composition of the fecal microbiota at baseline and in response to KD treatment. There was no clear KD-induced taxonomic signature of the gut microbiota that was shared across the study population, which contrasts prior studies of KD treatments for epilepsy that each reported alterations in the gut microbiota in response to a KD. Our results, however, support the finding that little to no consistency in specific microbial taxa affected exists across studies (Özcan et al., 2022).

Despite variation in microbiota composition, we observed evidence of shared functional features of the gut microbiome that were seen with KD treatment across participants in the study. This aligns with the notion of functional redundancy of gut microbes, wherein phylogenetically unrelated species can exhibit the same genetically-encoded biological activities (Tian et al., 2020). Results from metagenomic sequencing indicated that microbial genes related to fatty acid β-oxidation, 2-methylcitrate cycle, glycol metabolism, and proline biosynthesis were more highly represented in the gut microbiota of epileptic children after treatment with the KD compared to their internal pre-treatment controls. β-oxidation by select microbes in anaerobic environments enables them to utilize fatty acids from the diet as energy sources, wherein

saturated and unsaturated fatty acids are oxidized into acetyl-CoA (Yao & Rock, 2017). β-oxidation of dietary odd-chain fatty acids additionally produces propionyl-CoA, which can be toxic to cells, so the methylcitrate cycle enables microbes to further catabolize propionyl-CoA into pyruvate and succinate (Dolan et al., 2018). Glycol, including glycolate and glyoxylate, metabolism allows microbes to use products from fatty acid oxidation to fuel gluconeogenesis (Ahn et al., 2016). Proline synthesis from the central metabolite glutamate, via intermediates amino acids arginine and ornithine, is widely upregulated in bacteria to counteract growth in osmotically unfavorable conditions (Stecker et al., 2022). The elevated representation of genes related to these pathways in the post-KD samples suggests that the clinical KD shapes the gut microbiome to enrich microbial taxa that digest fat and synthesize carbohydrates under fat-rich, carbohydrate-limited conditions. These metagenomic features of the human microbiome from pediatric epilepsy patients consuming a clinical KD were preserved upon transfer to GF mice that were fed a standard diet, suggesting that the source microbes are maintained under non-ketogenic dietary conditions.

Aligning with results from metagenomic sequencing, metabolomic profiling of fecal samples from KD-treated epileptic children revealed statistically significant alterations in several metabolites, including subsets of amino acids, sugar acids, hydroxy fatty acids, bile acids, and other fatty acid derivatives, which reflect KD-, and potentially microbiome-, induced alterations in lipid and amino acid metabolism. In particular, glutamate and ornithine, both precursors of proline, were significantly decreased in post-KD human samples, relative to pre-KD controls, which may align with the observed metagenomic alterations in proline biosynthesis pathways. These metabolite alterations were induced by KD consumption in mice, and modified by microbiota depletion in mice, suggesting a causal response to the clinical KD in the human cohort that is dependent on the gut microbiome. Microbially modulated increases in palmitoleoylcarnitine (C16:1) were also seen in KD-fed mice and in post-KD human samples,

alongside several other lipid species, aligning with the high fat content of the KD and roles for the gut microbiome in lipid metabolism (Joyce et al., 2014).

The individual metabolite changes seen in human donors, including those induced by KD in a microbiome-dependent manner, were not specifically recapitulated by microbiome transfer to GF mice that were fed standard chow. This is perhaps not surprising given the important role of dietary composition in driving microbial activity (David et al., 2014). While no specific metabolite shifts were shared, a few pathway-level metabolomic changes were consistent between post-KD fecal samples from human donor (consuming the clinical KD) and mouse recipients (fed standard chow), relative to their respective pre-KD controls. Namely, differentially abundant metabolites related to metabolism of methionine, glycine, serine, and betaine were shared across post-KD conditions for human donor and microbiota-recipient mice. Methionine metabolism involves the production of homocysteine, adenosine, cysteine, and alpha-ketobutyrate, which can then be routed to glucogenic pathways by conversion to propionyl- and succinyl-CoA. Serine, synthesized via glycerate, is used to create glycine (and cysteine) via the homocysteine cycle, which can undergo microbial conversion into pyruvate or glyoxylate. Betaine (trimethylglycine), derived from diet or synthesized from choline, is metabolized by the gut microbiome (Koistinen et al., 2019) and functions as a methyl donor in transmethylation reactions, including those involved in methionine metabolism. While the relevance to KD and seizure protection is unclear, alterations in peripheral and central amino acid metabolism have been widely implicated in mediating the anti-seizure effects of the KD (Yudkoff et al., 2001). In addition, post-KD samples from both human donors and mouse recipients exhibited alterations in chemicals related to lipid metabolism, such as hydroxy fatty acids. The shared metabolite pathway- and chemical subclass-level features may reflect changes that are induced by KD in humans and generally phenocopied by gut microbes upon transfer to mice reared under non-ketogenic conditions. This suggests that transfer of clinical KD-induced gut microbes to mice

maintained under non-ketogenic conditions could result in molecular outputs that are distinct, but functionally similar, to those seen in the donor human sample.

We observed that inoculating mice with human fecal samples collected after clinical KD treatment conferred resistance to 6-Hz seizures compared to controls that received the baseline pre-treatment (pre-KD) microbiota. There was no correlation with patient responsiveness to diet, as indicated in clinician notes taken at 1 month after adherence to the clinical KD. This may be due to the unreliability of the metric, which was based on parental reporting, as well as the cross-sectional nature of the assessment, given inter-individual variation in latency to respond to KD treatments and the patient's peak KD ratio. These concerns aside, the results highlight the importance of host determinants of KD responsiveness, some of which may mask or block any beneficial influences of the KD-associated microbiota. Many patients included in this study exhibited genetic bases for refractory epilepsy, some of which could be epistatic to functional genomic changes in the KD-associated gut microbiome. Large human studies that subclassify different types of epilepsies and seizure semiologies are warranted to study potential roles for the gut microbiome in modifying or predicting responsiveness to the KD.

The microbiota-dependent increases in seizure protection were associated with brain transcriptomic alterations. In particular, both hippocampus and frontal cortex from post-KD recipient mice exhibited enrichment of differentially expressed genes related to i) RNA processing, transcriptional regulation, and translation ii) TORC1 signaling and cell cycle, and iii) oxidative phosphorylation and cellular stress response (i.e., nitric oxide, reactive oxygen species), when compared to controls colonized with pre-KD microbiota from both GO enrichment and STRING network analyses. Neuronal excitability requires protein synthesis in response to altered neuronal stimulation, and risk factors for various epilepsies include dysregulation of RNA processing, RNA stability, transcription, and translation (Malone &

Kaczmarek, 2022). TORC1 is a major nutrient- and energy-sensing serine/threonine kinase complex that controls cell growth and differentiation by coordinating core processes of transcription, translation, and autophagy. Abnormal regulation of TORC1 signaling has been implicated in a wide variety of epilepsies, and as such, is a therapeutic target of interest for treating refractory epilepsies (Nguyen & Bordey, 2021). Previous studies have reported inhibitory effects of the KD and select fatty acids on TORC1 activity (McDaniel et al., 2011; Warren et al., 2020), suggesting that it may contribute to the anti-seizure effects of the KD. Oxidative phosphorylation is a central process for cellular energy metabolism from nutrients, that generates as a byproduct reactive oxygen species (ROS) (Rowley & Patel, 2013) and is regulated by the retrograde glutamatergic neurotransmitter nitric oxide (NO). In animal epilepsy models, both ROS and NO are elevated during seizure activity due to oxidative stress-associated neuronal death (Zhu et al., 2017), which can further contribute to epileptogenesis (Chan, 2001). The KD has been previously reported to reduce oxidative stress by promoting antioxidant enzymatic activity and scavenging ROS (Greco et al., 2016). Overall, these results suggest that the KD-associated human gut microbiota alters brain transcriptional pathways that may contribute to protection against 6-Hz seizures in mice.

Integration of multi'omics datasets across human donor and mouse recipients revealed network associations between select gut microbial metagenomic pathways, fecal metabolites, serum metabolites, and hippocampal transcripts, suggesting that they may contribute to the microbiome-dependent increases in seizure protection seen in mice inoculated with human post-KD microbiota, compared to pre-KD controls. The human donor and mouse recipient co-occurrence networks were linked by shared metagenomic pathway nodes related to BCAA biosynthesis, CoA biosynthesis, L-alanine fermentation, and L-arginine biosynthesis. Key drivers for BCAA, CoA, and L-arginine biosynthesis were linked to hippocampal transcript modules enriched for genes related to neurogenesis and Wnt signaling. BCAAs modulate brain

import of precursors required for synthesis of monoamine transmitters (Larsson & Markus, 2017; Salcedo et al., 2021; Song et al., 2017). BCAAs also serve as nitrogen donors for synthesis of glutamate vs. GABA, and as such regulates synaptic balance between excitation and inhibition, a key determinant of seizure susceptibility (McKenna et al., 2019). Wnt signaling regulates calcium pathways that are important for hippocampal neurogenesis and dendrite formation, and is increasingly linked to early epileptogenesis (Hodges and Lugo, 2018). Additionally, mapping GWAS-based risk genes to the co-occurrence network identified five hippocampal nodes as linked to epilepsy. Of particular interest was *Gnaz*, which encodes G protein alpha-Z, a protein that mediates neuronal signal transduction within the hippocampus (Jang et al., 2018) and is proposed to modulate seizure susceptibility (Hultman et al., 2019). BCAA derivatives have been reported to promote phosphorylation of G-proteins, and abnormalities in GPCR mediated neuronal signaling can contribute to increased susceptibility to seizure (Shellhammer et al., 2017; Yu et al., 2019). Altogether, results from this study reveal that the clinical KD regimens used to treat pediatric refractory epilepsy are associated with alterations in the function of the child microbiome, which causally modify brain function and seizure susceptibility upon transfer to mice. Further research is warranted to define the mechanisms by which the human KD-associated microbiome signals across the gut-brain axis to modify seizure risk, and to further assess the potential for identifying microbiome-based interventions that could increase the efficacy of KD treatment, alleviate dietary side effects, and/or ease clinical implementation.

Limitations of study

A key limitation of this study design is the prioritization of experimental reproducibility, which included cohorts of 14-16 mice per patient sample, over patient sample size, which included 10 children with refractory epilepsy, each sampled before and at approximately 1 month after adherence to a clinical KD. We reasoned that by internally controlling for baseline microbiota for

each patient, we could effectively evaluate microbial alterations in response to the clinical KD within a relatively small patient group. We further posited that this study design would enable us to sample from a heterogenous patient population reflective of the etiopathological variation typically seen in refractory epilepsy. It would also us to be inclusive of the wide range of individuals with pediatric epilepsy who typically seek clinical KD treatment. This level of diversity in a small patient population may have contributed to our finding that there was no shared taxonomic response of the gut microbiome to the clinical KD, despite some shared functional genomic features when considering all post-KD microbiota relative to all pre-KD controls.

Additional constraints of the study, as discussed in the main text, are the inherent technical and biological shortcomings in “transplanting” microbiota across different mammalian hosts. In this study, we achieved levels of human-to-mouse microbiota “transplant” fidelity analogous to those reported in existing literature even when we maintained mice on a conventional rather than ketogenic diet (Bokoliya et al., 2021; Kennedy et al., 2018; Walter et al., 2020). However, the discrepancies between recipient and donor microbiota draw into question the relevance of findings in gnotobiotic mice to the human condition. To help mitigate this, we focused entirely on features of the gut microbiome that were differential between post-KD and pre-KD conditions and shared between human donors and mouse recipients. However, we acknowledge that artifacts of the microbiota transfer approach, which are not relevant to the clinical condition, may contribute to the microbiome-dependent functional differences observed in the gnotobiotic mouse experiments in this study. Nevertheless, the observed results provide important proof-of-principle that differences in the function of the gut microbiota regulate seizure susceptibility.

In assessing causal relationships between the KD-associated microbiome and host physiologies linked to seizure susceptibility, we made the major assumption that there exists a singular microbiome-dependent mechanism to increase seizure threshold that was common across all

post-KD mouse recipient cohorts relative to all pre-KD cohort controls. Our analysis does not take into account the possibility that there are multiple microbiome-dependent mechanisms that are distinct and that each result in resistance to 6-Hz seizures. Expanded studies that involve subclassification of the human participants and/or mouse recipients would aid in addressing this prospect.

Moreover, we chose to study the 6-Hz psychomotor seizure model based on its widespread use as a model of refractory epilepsy (Kehne et al., 2017), its utility for screening novel antiepileptic drugs (Barton et al., 2001), and its responsiveness to the KD (Hartman et al., 2008). We also reasoned that its measure of acutely induced seizures would preclude confounding effects of chronic genetic mutation or kindling-based models on modifying the gut microbiome (Löscher, 2017). Further research is needed to assess roles for the KD-induced gut microbiome in modifying seizures across additional epilepsy models to determine whether particular seizure semiologies or types of epilepsy are more amenable to modulation by the gut microbiome.

In light of the aforementioned heterogeneity in patient population, small sample size, variation in clinically-guided KD regimens, discrepancies introduced by the microbiota “transplantation” approach, cross-species and diet comparisons (i.e., human on KD, mouse on standard diet), and assumptions adopted for data analysis, our statistical analyses for shotgun metagenomic, untargeted metabolomic, and bulk transcriptomic data were performed with lenient thresholds for differential abundance ($p<0.05$), with a focus on pathway-level signatures that were dependent upon the clinical KD and conferred by the KD-associated microbiota. Notably, for all animal experiments, technical replicates per donor sample were averaged, and only the biological (i.e., donor) N was used for statistical analysis. Despite the expected variability, we detected consistent KD-dependent alterations in microbial genes and metabolites in epileptic children undergoing dietary treatment, and further observed KD- and microbiome-dependent

alterations in metagenomic pathways and metabolomic pathways that were shared across human donor and microbiome-recipient mice when using these parameters. Brain transcriptomic signatures were seen when comparing all mouse recipient cohorts receiving the post-KD microbiome (all of which exhibited resistance to 6-Hz seizures) relative to those receiving the pre-KD controls. Finally, results for all seizure testing experiments, which revealed shared phenotypic outcomes for post-KD groups compared to pre-KD groups, were well-powered and analyzed according to conventional statistical methods (Festing & Altman, 2002). All caveats considered, the results from this study extend existing pre-clinical research to provide initial evidence that clinical KD treatments shape the function of the gut microbiome of children with refractory epilepsy in ways that have the potential to causally modify seizure susceptibility. Continued research is warranted to elucidate the particular microbial functional activities that act together to modify signaling across the gut-brain axis to promote seizure protection and to further assess the potential to apply microbiome-based interventions to treat refractory epilepsy.

Figures and Tables

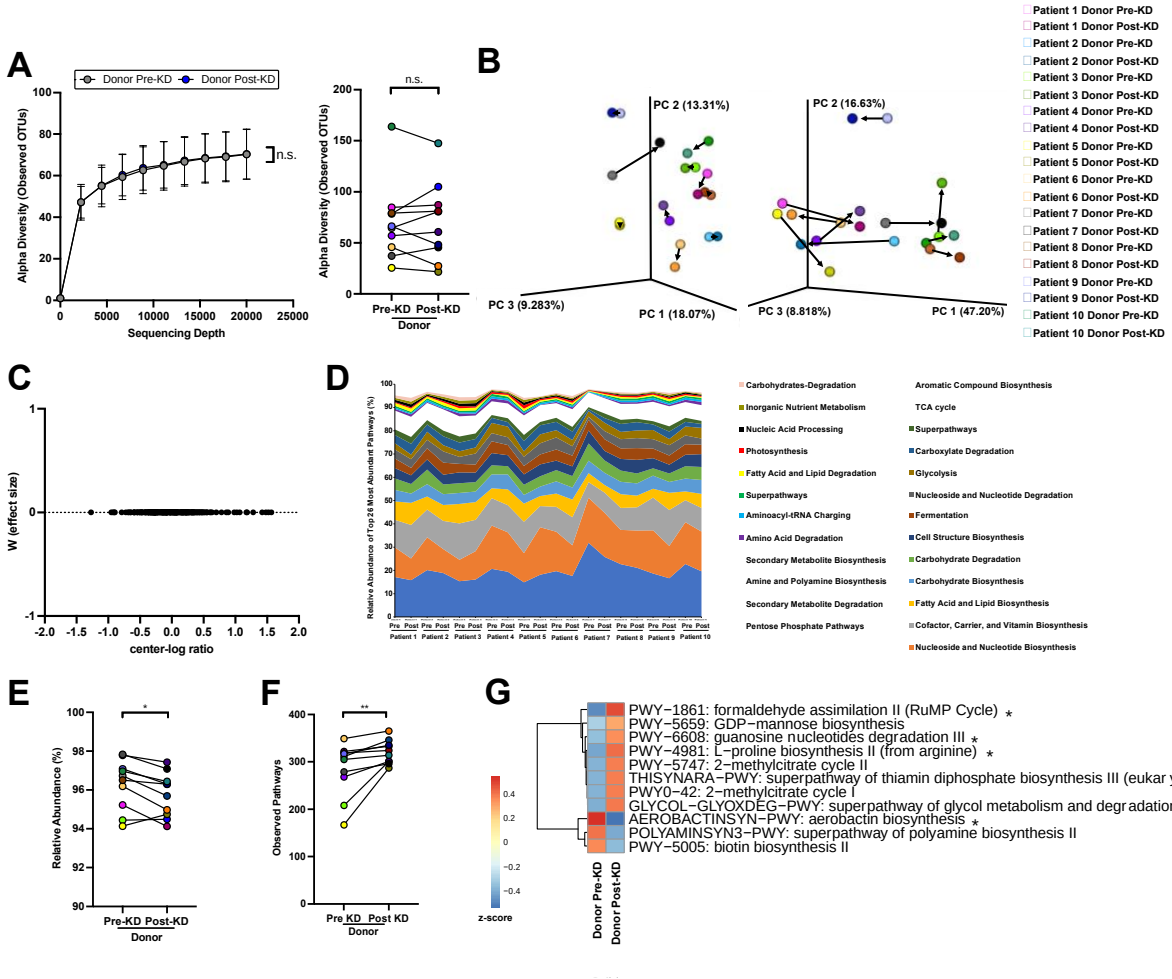


Figure 4.1. Clinical KD is associated with alterations in the functional potential, but not composition, of the gut microbiota in a cohort of children with refractory epilepsy, Related to Figure 4.2. (A) Alpha diversity as measured by rarefaction curve (left) and observed OTUs (right) of matched donor pre-KD (n=10) and post-KD (n=10) fecal microbiota samples showing no difference in alpha-diversity. (two-way ANOVA with Sidak (left); two-tailed, Wilcoxon matched-pairs signed rank test (right)). (B) Principal coordinate analysis of unweighted (left) and weighted (right) UniFrac distances from 16S rRNA gene sequencing of donor pre-KD (n=10) and post-KD (n=10) fecal microbiota samples shifting composition when introduced to the clinical KD. (C) ANCOM taxonomic differential abundance testing displaying no differentially

abundant taxa by the W score (effect size) metric when comparing donor pre-KD (n=10) and post-KD (n=10). (D) Total composition per human donor sample of the top 26 MetaCyc superclass metagenome functional pathways accounting for >94% of relative abundance, for each donor pre-KD (n=10) and post-KD (n=10) fecal microbiota samples. (E) Difference in total abundance of the 26 most abundant pathways between matched donor pre-KD (n=10) and post-KD(n=10) fecal microbiota samples (two-tailed, Wilcoxon matched-pairs signed rank test). (F) Total number of observed MetaCyc functional pathways in matched donor pre-KD (n=10) and post-KD (n=10) fecal microbiota samples (two-tailed, Wilcoxon matched-pairs signed rank test). (G) Heatmap displaying differentially abundant MetaCyc functional pathways associated with donor post-KD (n=10) relative to pre-KD (n=10) by MaAsLin2 analysis with a p-value < 0.1. (pathways with a p-value <0.05 are denoted with *). Data is displayed as mean ± SEM, unless otherwise noted. *p < 0.05, **p < 0.01; KD, ketogenic diet; n.s, no statistical significance.

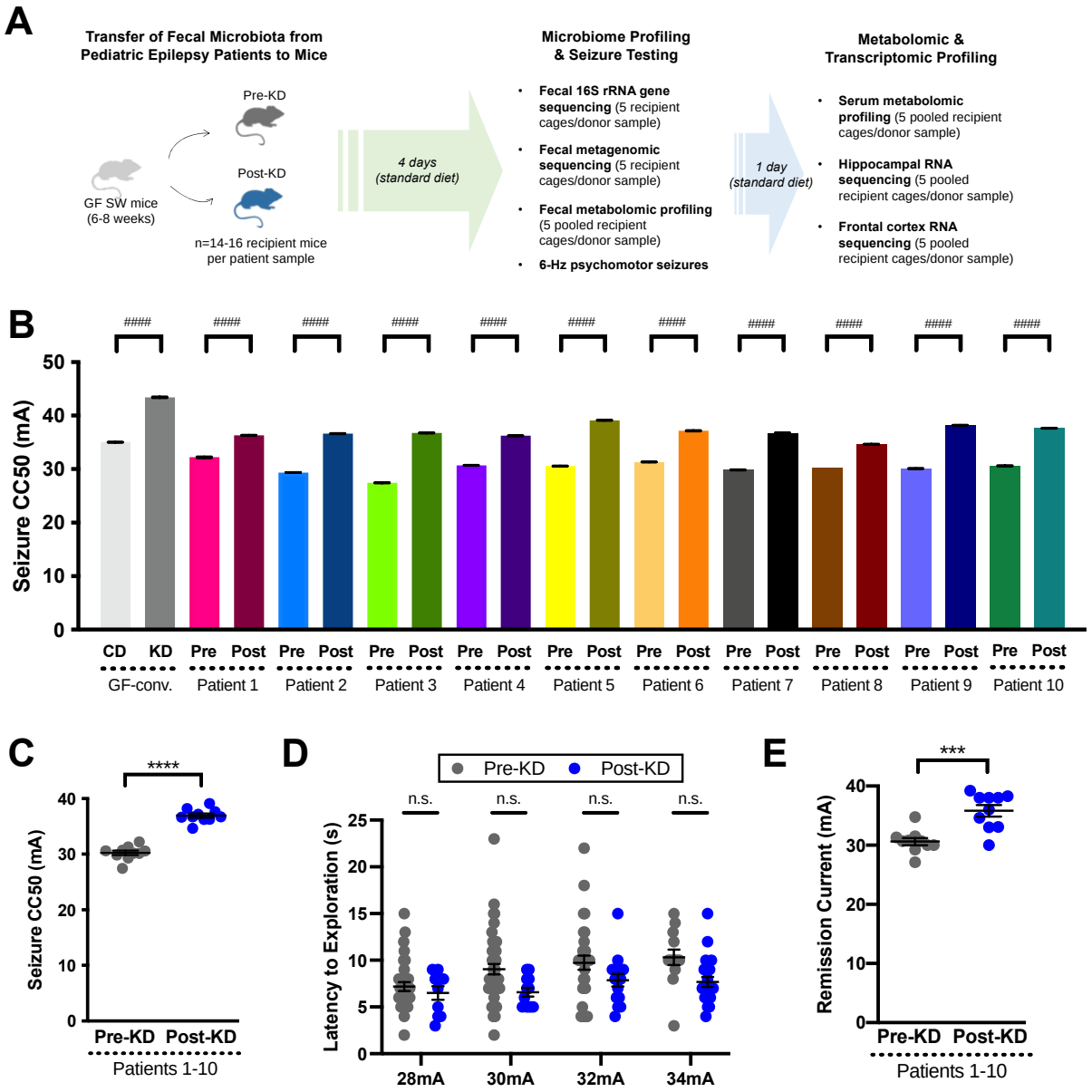


Figure 4.2: Transfer of the clinical KD-associated gut microbiota from pediatric epilepsy patients to mice confers resistance to 6-Hz seizures. (A) Experimental schematic for transplantation of human donor fecal microbiota samples into germ-free (GF) Swiss Webster mice for 6-Hz psychomotor seizure testing. (B) 6-Hz seizure thresholds for replicate mice transplanted with human microbiota from paired donor pre-KD and post-KD samples (One-way ANOVA with Tukey's, n = 13-16 mice per patient sample, with # denoting statistical differences when considering within-patient recipient mice as technical replicates). (C) Average seizure

thresholds of recipient mouse cohorts per patient donor sample. (Two-tailed, unpaired Welch's t-test. n=10 patient samples per group). (D) Comparison of latency to exploration for all transplanted pre-KD and post-KD mice that underwent 6-Hz psychomotor seizure testing at comparable currents. (Two-way ANOVA with Sidak. pre-KD mice per group at 28mA (n=33), 30mA (n=48), 32mA (n=33), 34mA (n=13); post-KD mice per group at 28mA (n=10), 30mA (n=12), 32mA (n=15), 34mA (n=22)). (E) Average current (mA) at which remission seizures were observed per patient donor sample (Two-tailed, unpaired Welch's t-test. n = 10 patient samples per group). Data is displayed as mean ± SEM, unless otherwise noted. #### $p < 0.0001$ (within-patient mouse recipients), ***$p < 0.001$, ****$p < 0.0001$

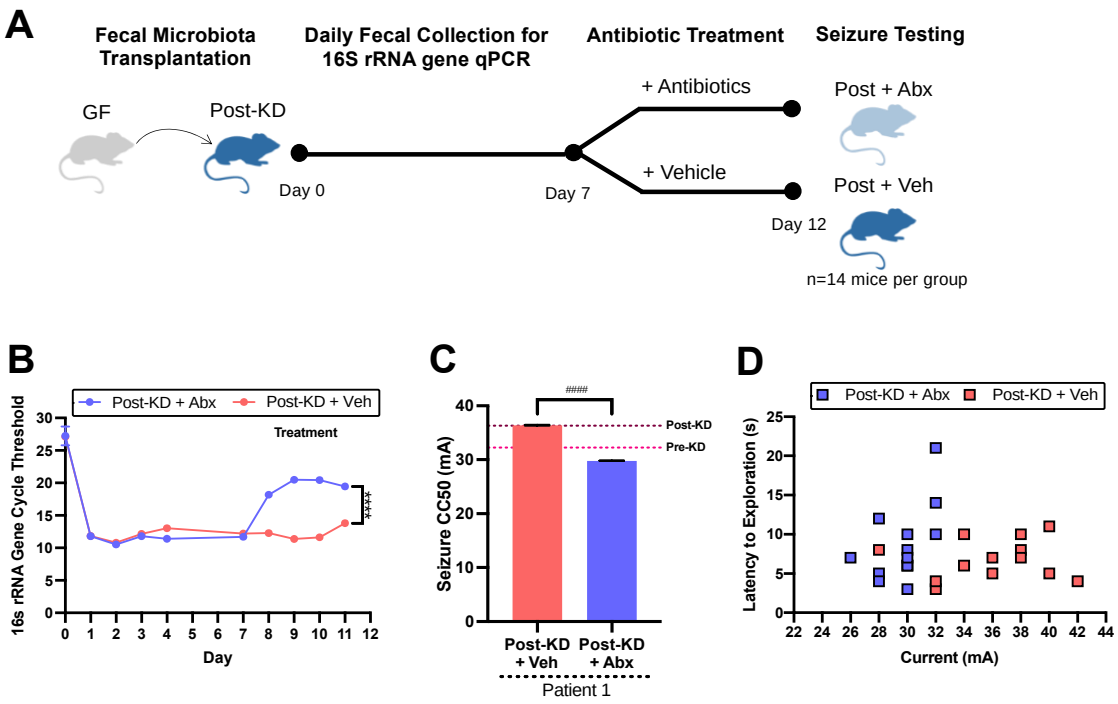


Figure 4.3: Antibiotic treatment abrogates the seizure protective effects of inoculation with the clinical KD-associated human gut microbiome, Related to Figure 4.2. (A) Experimental schematic for transfer of human donor fecal microbiota samples to germ-free (GF) mice, followed by 5 days of oral antibiotic (Abx) or vehicle (Veh) treatment, and then 6-Hz psychomotor seizure testing. (B) Bacterial loads as measured by quantitative PCR of the 16S rRNA gene from fecal pellets collected once daily before and during Abx or Veh treatment (two-way ANOVA with Sidak, n=3 cages of 3 mice each). (C) 6-Hz seizure thresholds for mice inoculated with patient 1 post-KD human microbiota treated with Abx (n=12) or Veh (n=14). Reference lines denote seizure thresholds for mice inoculated with patient 1 post-KD and pre-KD relative control fecal microbiota from Figure 1B (One-way ANOVA with Tukey's, with # denoting statistical differences when considering within-patient recipient mice as technical replicates). (D) Latency to exploration for each Abx (n=12) and Veh (n=14) mouse that underwent 6-Hz psychomotor seizure testing. Data is displayed as mean ± SEM, unless otherwise noted. ****$p < 0.0001$. $^{\#\#\#\#}p < 0.0001$ (for within-patient mouse recipients).

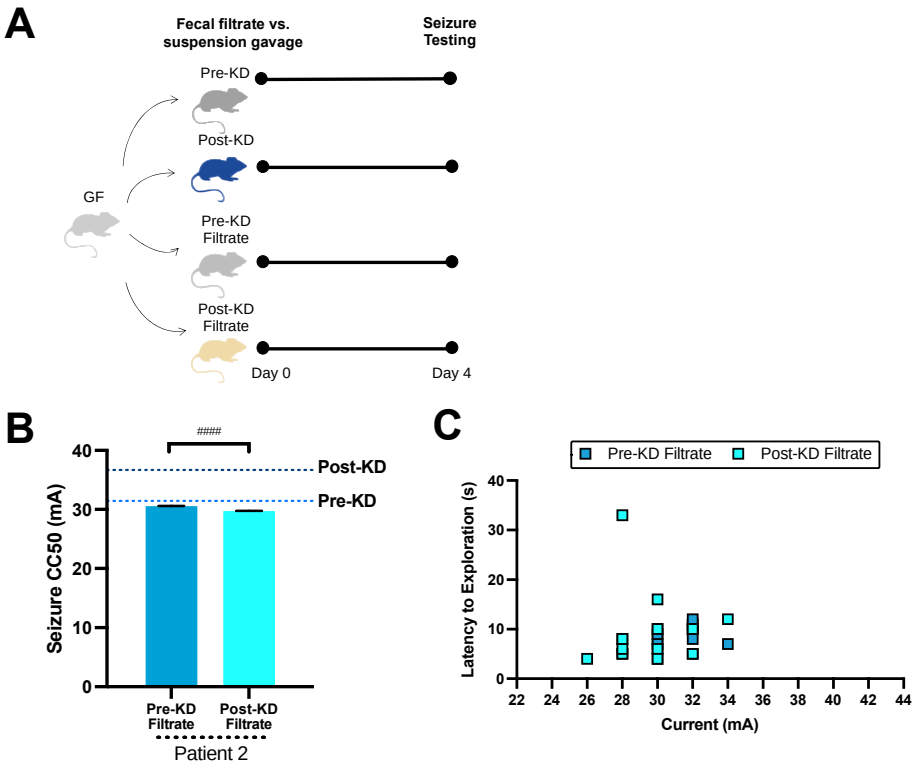


Figure 4.4: Sterile filtration prevents the seizure protective effects of transfer of the clinical KD-associated human gut microbiome, Related to Figure 4.2. (A) Experimental design for administration of human donor fecal filtrate samples to germ-free (GF) mice, followed by 6-Hz seizure testing 4 days later. (B) Seizure thresholds for mice treated with sterile filtered pre-KD (n=14) and sterile filtered post-KD (n=13) fecal samples. Reference lines denote seizure thresholds for mice transplanted with unfiltered patient 2 post-KD and pre-KD relative control fecal microbiota from Figure 1B (One-way ANOVA with Tukey's, with # denoting statistical differences when considering within-patient recipient mice as technical replicates). (C) Latency to exploration for mice treated with sterile filtered pre-KD (n=14) and sterile filtered post-KD (n=13) that underwent 6-Hz psychomotor seizure testing. Data is displayed as mean ± SEM, unless otherwise noted. #### $p < 0.0001$ (for within-patient mouse recipients).

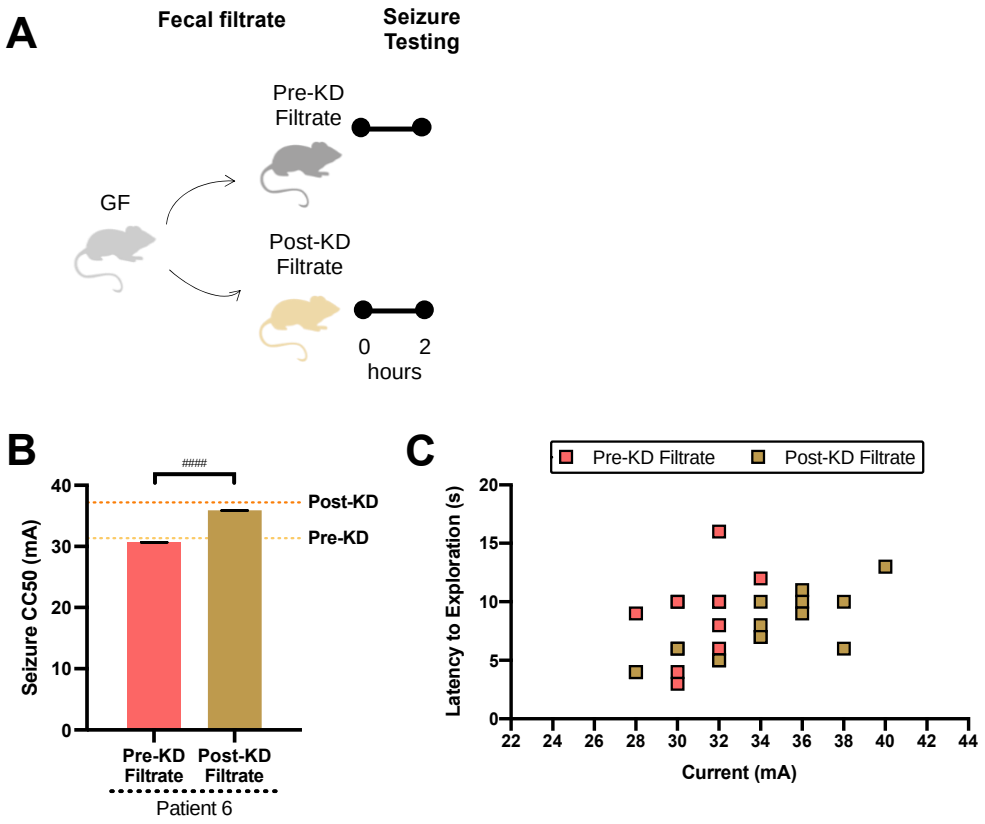


Figure 4.5: Small molecules from the clinical KD-associated human gut microbiome confer acute seizure protection, Related to Figure 4.2. (A) Experimental design for administration of human donor fecal filtrate samples to germ-free (GF) mice, followed by 6-Hz seizure testing 2 hours later. (B) Seizure thresholds for mice treated with sterile filtered pre-KD filtrate (n=13) and sterile filtered post-KD filtrate (n=14) fecal samples. Reference lines denote seizure thresholds for mice transplanted with unfiltered patient 6 post-KD and pre-KD relative control fecal microbiota from Figure 1B (One-way ANOVA with Tukey's, with # denoting statistical differences when considering within-patient recipient mice as technical replicates). (C) Latency to exploration for mice treated with sterile filtered pre-KD (n=13) and sterile filtered post-KD (n=14) that underwent 6-Hz psychomotor seizure testing. Data is displayed as mean ± SEM, unless otherwise noted. $^{\#\#\#\#}$ $p < 0.0001$ (for within-patient mouse recipients).

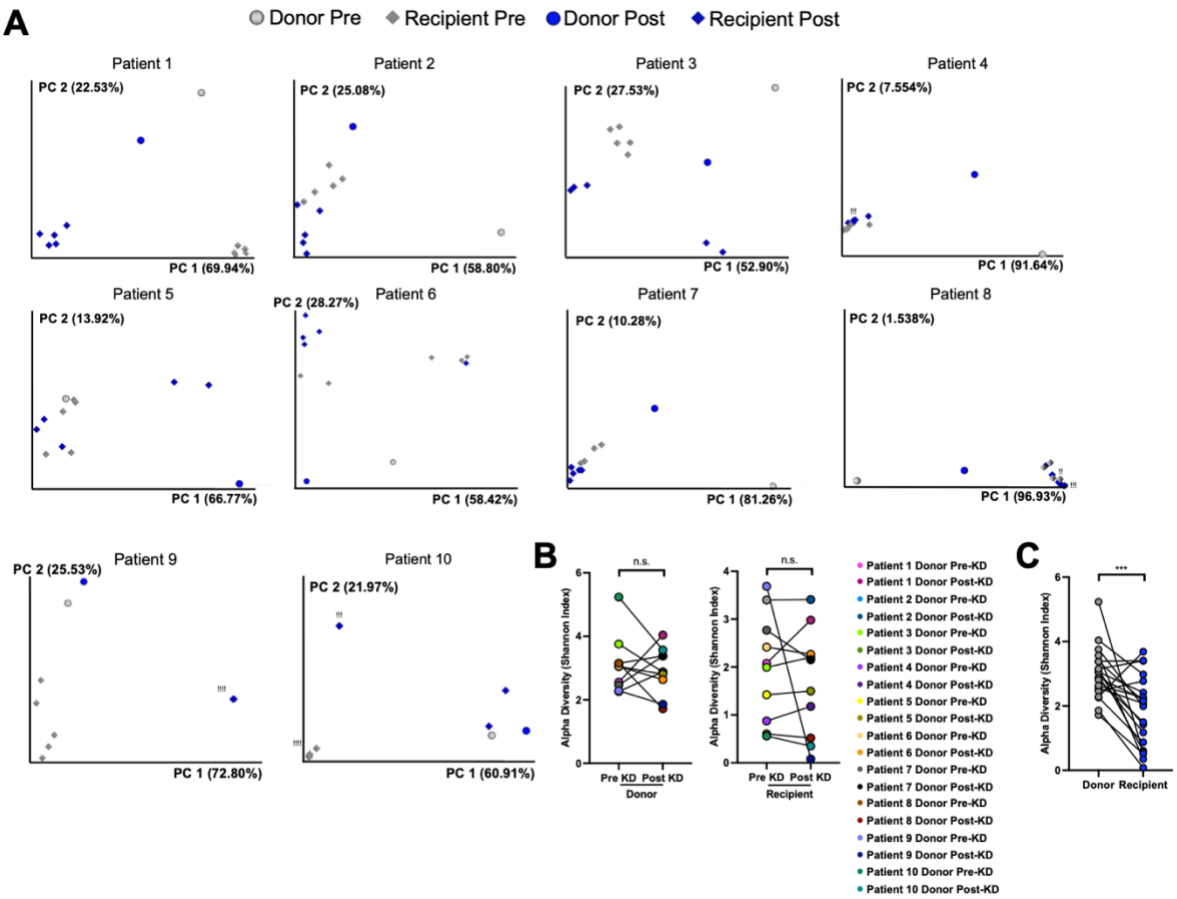


Figure 4.6: Taxonomic fidelity of human microbiota transfer to mice, Related to Figure 4.2. (A) Principal coordinates analysis of weighted UniFrac distances from 16S rRNA gene sequencing of fecal samples from matched human donors and mouse recipients (for each graph: n = 1 donor patient (10 patients total), 4-5 recipient cages of recipient mice per pre-KD vs. post-KD condition, ! = 1 overlapping data point not visible). (B) Shannon index alpha-diversity of fecal microbiota from human donor pre-KD and post-KD samples (left) and matched mouse recipient pre-KD and post-KD samples (right) (two-tailed, Wilcoxon matched-pairs signed rank test, donors: n=10 patients, recipients: n=10 per patient condition, where each n is an average from 4-5 cages per patient). (C) Shannon index alpha-diversity of fecal microbiota from all human donor samples (n=20 patients) and all matched mouse recipient samples (two-tailed, Wilcoxon matched-pairs signed rank test; n=20 patient conditions, where each n is an

average from 4-5 cages per patient). Data is displayed as mean ± SEM, unless otherwise noted. ***p < 0.001, n.s.=not statistically significant.

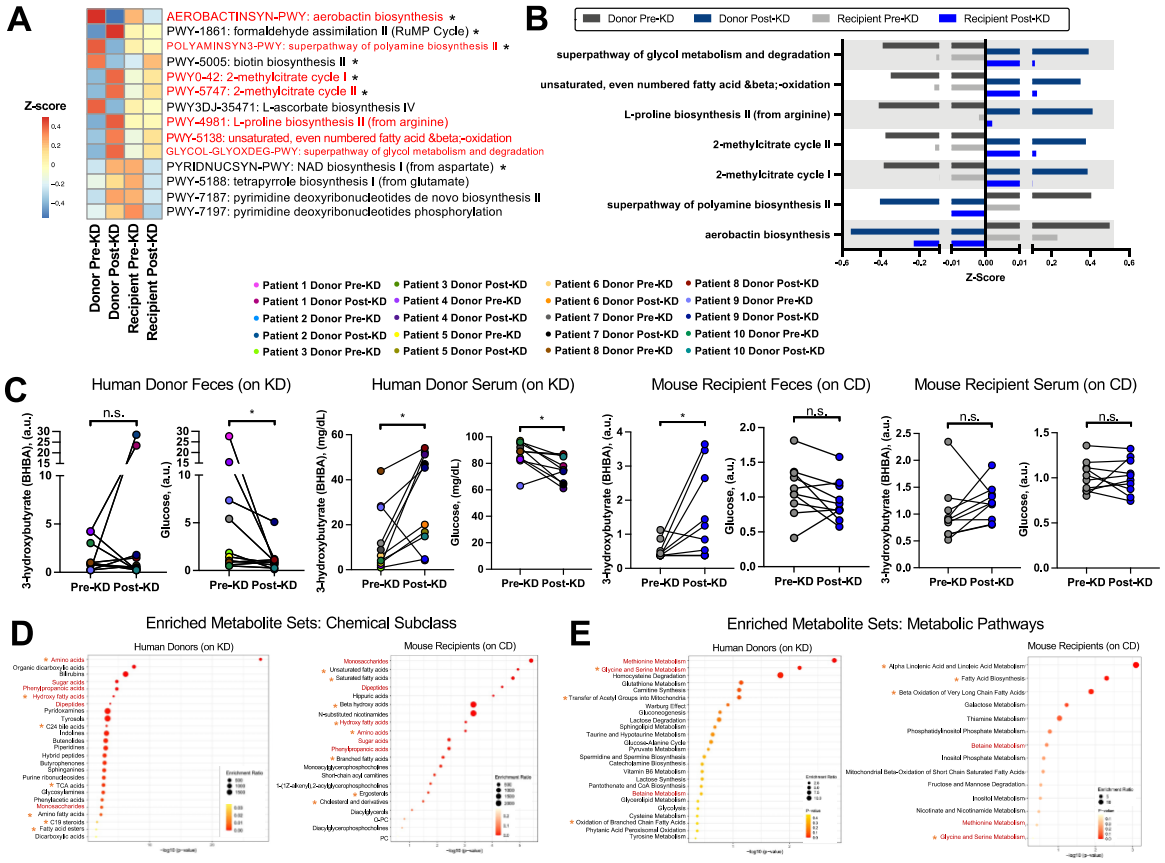


Figure 4.7: The clinical KD-associated human microbiome exhibits functional alterations that are phenocopied in seizure-protected recipient mice. (A) Microbial functional pathways differentially abundant as determined by MaAsLin2 analysis comparing post-KD (samples relative to pre-KD controls for either donor fecal samples or recipient fecal samples (donors: n=10 per diet condition; recipients: n=10 per donor diet condition [50 total, where each n reflects average of 5 technical replicate recipient mice per donor patient sample]). Red font denotes pathways that are commonly differentially abundant in the same direction in both donors fed KD and recipient mice fed CD (all pathways listed had minimum p<0.1; *denotes pathways with p<0.05). (B) Microbial functional pathways that are commonly differentially abundant by MaAsLin2 analysis in the same direction in post-KD donor and recipient controls (donors: n=10 per diet condition; recipients: n=10 per donor diet condition [50 total, where each n reflects

average of 5 technical replicate recipient mice per donor patient sample]). (C) Beta-hydroxybutyrate (BHBA) and glucose levels in human donor (left) and mouse recipient (right) pre-KD and post-KD feces and serum (two-tailed Wilcoxon matched-pairs signed rank test; donors: n=10 per diet condition; recipients: n=10 per donor diet condition [50 total, where each n reflects average of 5 technical replicate recipient mice per donor patient sample]). (D) Metabolite set enrichment analysis showing the top 25 enriched chemical subclasses ordered by p-value for the set of differentially abundant metabolites in human donor (left) post-KD vs pre-KD fecal samples ($p<0.05$, two-tailed, matched pairs Student's t-test, n=10 per diet condition). Metabolite set enrichment analysis showing enriched chemical subclasses ordered by p-value for the set of differentially abundant metabolites in recipient mouse (right) post-KD vs pre-KD fecal samples ($p<0.05$, matched pairs Student's t-test, n=10 per patient diet condition, where each sample is pooled from 5 recipient mice per donor patient sample). Red font denotes chemical subclasses altered in post-KD vs pre-KD human donor feces that are shared with those differentially regulated in post-KD vs pre-KD mouse recipient feces. Orange asterisks (*) denote additional chemical subclasses that are relevant to KD based on existing literature. (E) Metabolite set enrichment analysis showing the top 25 enriched SMPBD pathways by p-value for the set of differentially abundant metabolites in human donor (left) post-KD vs pre-KD fecal samples ($p<0.05$, matched pairs Student's t-test, n=10 per patient diet condition). Metabolite set enrichment analysis showing enriched SMPBD pathways by p-value for the set of differentially abundant metabolites in recipient mouse (right) post-KD vs pre-KD fecal samples ($p<0.05$, matched pairs Student's t-test; n=10 per patient diet condition, where each sample is pooled from 5 recipient mice per donor patient sample). Red font denotes metabolic pathways altered in post-KD vs pre-KD human donor feces that are shared with those differentially regulated in post-KD vs pre-KD mouse recipient feces. Orange asterisks (*) denote additional chemical subclasses that are relevant to KD based on existing literature. Data is displayed as mean ± SEM, unless otherwise noted. *$p < 0.05$. n.s.=not statistically significant. KD, ketogenic diet;

BHBA, beta-hydroxybutyrate; CD, control diet; SMPDB, The Small Molecule Pathway Database; PC=phosphatidylcholine

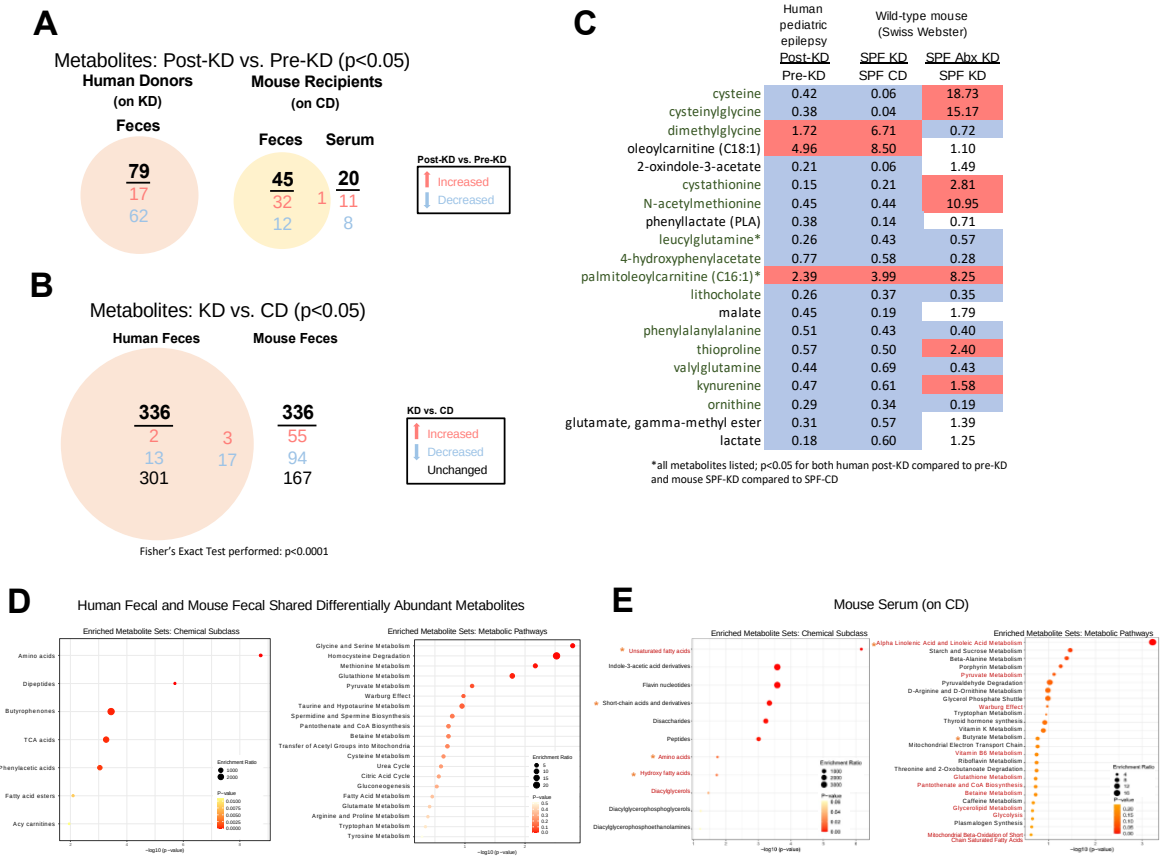


Figure 4.8: The clinical KD alters metabolomic profiles in human fecal samples and in fecal and serum samples of mice inoculated with human microbiota, Related to Figure 2.

(A) Differentially abundant metabolites (p<0.05) in post-KD compared to pre-KD samples of human donor feces, mouse recipient feces, and mouse recipient blood (Two-tailed matched pairs Student's t-test, n=10 per condition, where each recipient sample is pooled from 5 recipient mice per donor patient sample) (B) Differentially abundant metabolites (p<0.05) in post-KD compared to pre-KD samples of human donor feces, which were also significantly altered in conventional mice (SPF) fed KD chow or vitamin- and mineral- matched control diet (CD) for 14 days. Red font denotes the subset of metabolites that were further altered by pre-treating KD chow-fed mice with antibiotics (Abx) to deplete gut bacteria. (human: Two-tailed matched pairs Student's t-test, n=10 per condition; mouse: ANOVA contrasts, n=8 per

condition). (C) Differentially abundant metabolites ($p<0.05$) in human feces (post-KD compared to pre-KD) and feces of mice fed KD vs. CD chow for 14 days (Human fecal: Two-tailed matched pairs Student's t-test, n=10 per condition, where each recipient sample is pooled from 5 recipient mice per donor patient sample; Mouse fecal: two-way ANOVA with contrasts, n=8 per condition; Fisher's Exact Test). (D) Metabolite set enrichment analysis of chemical subclass for the 20 differentially abundant metabolites ($p<0.05$, matched pairs Student's t-test) found in both human post-KD vs pre-KD fecal samples and SFP mouse KD vs CD fecal samples (left) (human: n=10 per condition, where each sample is pooled from 5 recipient mice per donor patient sample; mouse: n=8 per condition). Metabolite set enrichment analysis of SMPDB pathways for the 20 differentially abundant metabolites ($p<0.05$, matched pairs Student's t-test) found in both human post-KD vs pre-KD fecal samples and SFP mouse KD vs CD fecal samples (right) (human: n=10 per condition, where each sample is pooled from 5 recipient mice per donor patient sample; mouse: n=8 per condition). (E) Metabolite set enrichment analysis of chemical subclass for differentially abundant metabolites ($p<0.05$, matched pairs Student's t-test) in recipient mouse post-KD vs pre-KD serum samples (left) (n=10 per condition, where each sample is pooled from 5 recipient mice per donor patient sample). Metabolite set enrichment analysis of SMPDB pathways for differentially abundant metabolites ($p<0.05$, matched pairs Student's t-test) in recipient mouse post-KD vs pre-KD serum samples (right) (n=10 per condition, where each sample is pooled from 5 recipient mice per donor patient sample). Red font denotes metabolic pathways altered in post-KD vs pre-KD mouse serum that are shared with those differentially regulated in post-KD vs pre-KD mouse feces and/or human feces. Orange asterisks (*) denote additional chemical subclasses that are relevant to KD based on existing literature. KD, ketogenic diet; SPF, specific pathogen free conventionalized mice; CD, control diet; Abx, antibiotic SMPDB, The Small Molecule Pathway Database

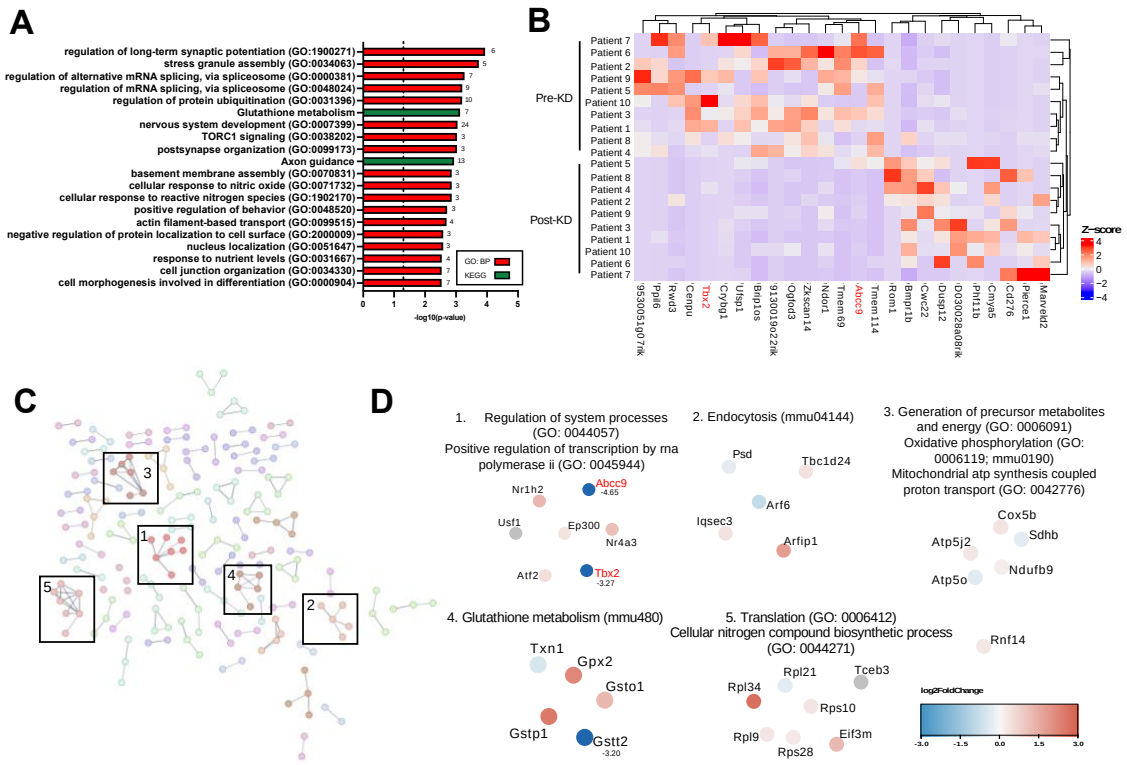


Figure 4.9: Seizure resistance in mice inoculated with the post-KD microbiota is associated with alterations in the brain transcriptome. (A) GO: Biological Process gene ontology of differentially expressed genes ($p<0.05$) in recipient mouse post-KD (n=10, where each sample is pooled from 6 recipient mice per donor patient sample) compared to pre-KD hippocampal samples, top 20 ranked by p-value (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). (B) Heatmap of euclidian row and column clustered top 25 differentially expressed genes in recipient mouse post-KD compared to pre-KD hippocampus ranked by p-value, smallest to largest, and with log2 fold-change >2 (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). (C) Protein interaction network with MCL clustering based upon mouse recipient post-KD and pre-KD hippocampal transcriptomics which appeared in both GO and STRING network enrichment analyses, STRING network enrichment score >0.7 (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient

sample). (D) Functional enrichment of top MCL sub-network clusters from hippocampal transcriptomics STRING network analysis, proteins are colored based on their overall log2FC. If log2FC >3 or <-3, the value is listed next to the node name (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). KD, ketogenic diet; GO, gene ontology; MCL, Markov Cluster Algorithm.

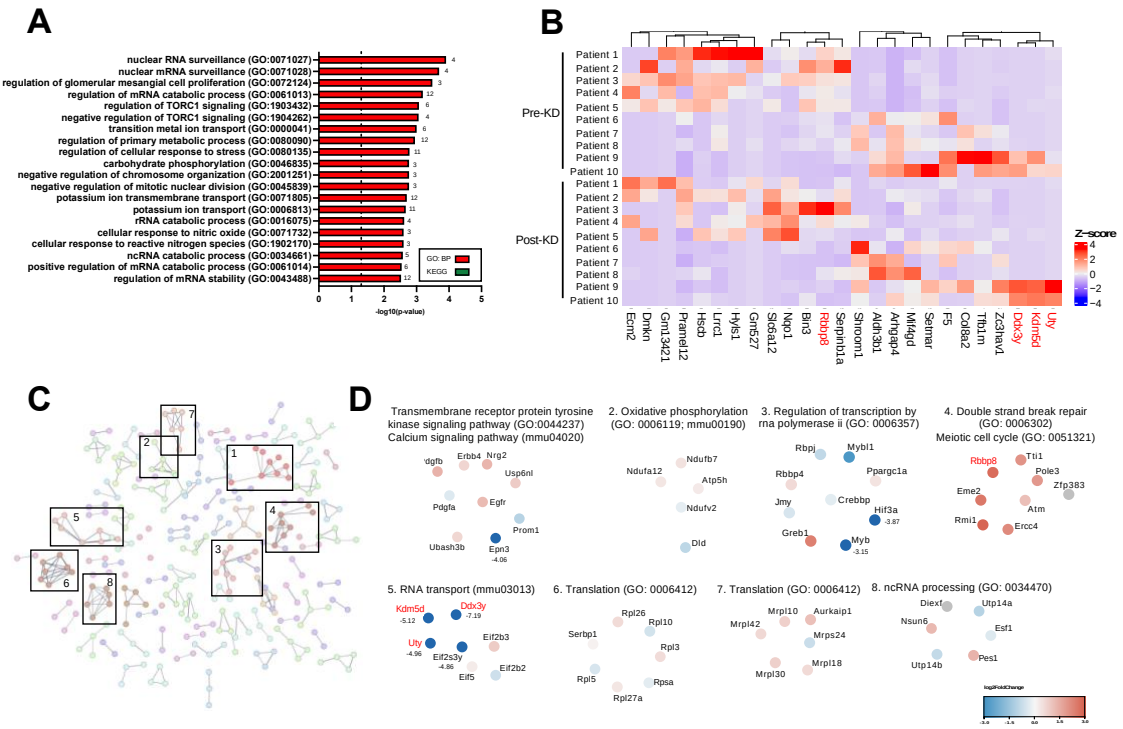
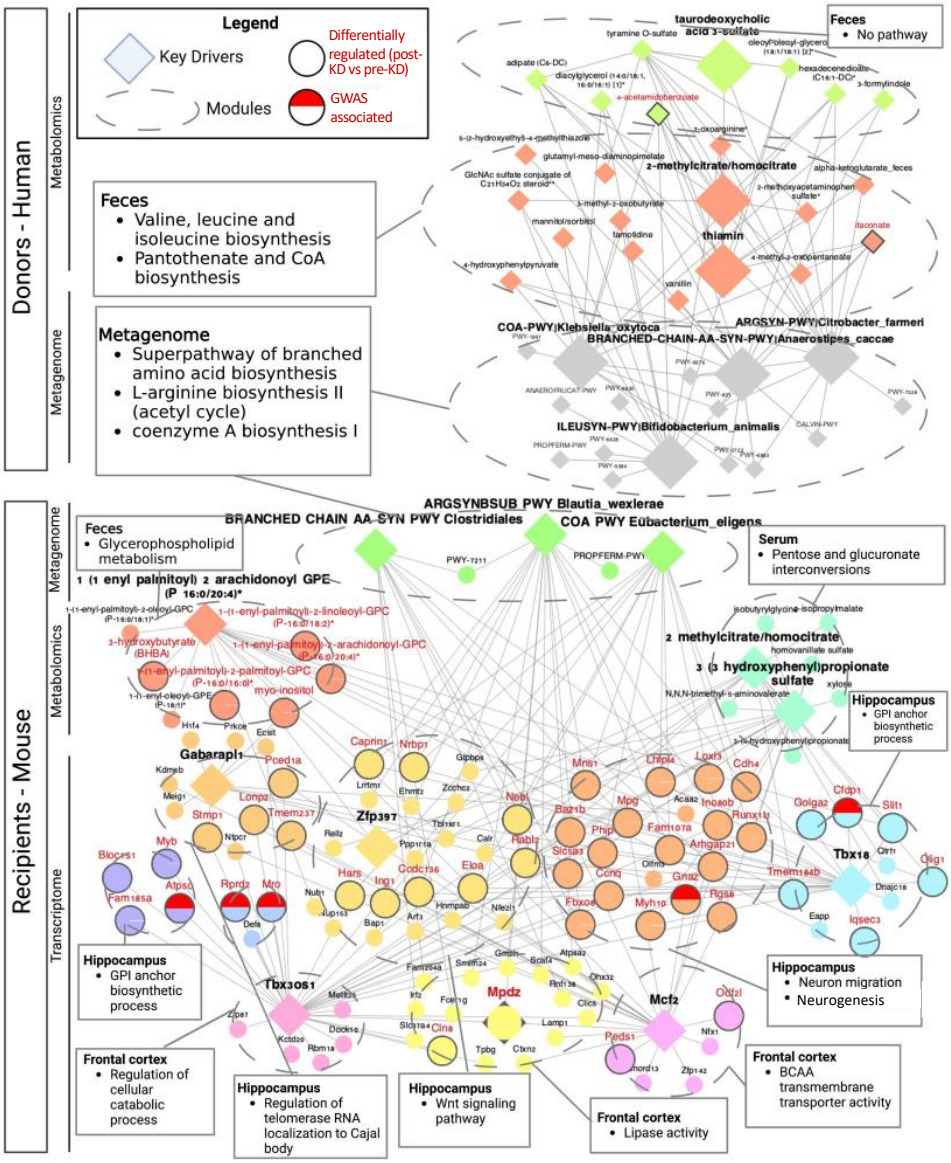


Figure 4.10: Mice inoculated with the post-KD microbiota exhibit alterations in the frontal cortical transcriptome, Related to Figure 4.9. (A) GO: Biological Process gene ontology of differentially expressed genes ($p<0.05$) in recipient mouse post-KD compared to pre-KD frontal cortex samples, top 20 ranked by p-value (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). (B) Heatmap of top 25 differentially expressed genes in recipient mouse post-KD compared to pre-KD frontal cortex ranked by p-value, smallest to largest, with log2-fold change >2 (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). (C) Protein interaction network with MCL clustering based upon mouse recipient post-KD and pre-KD frontal cortex transcriptomics which appeared in both GO and STRING network enrichment analyses, STRING network enrichment score >0.7 (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). (D) Functional enrichment of top MCL

sub-network clusters from frontal cortex transcriptomics STRING network analysis, proteins are colored based on their overall log2FC. If log2FC >3 or <-3, the value is listed next to the node name (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). KD, ketogenic diet; GO, gene ontology; MCL, Markov Cluster Algorithm.

A



B

Brain Region	p-value	GWAS - Top Associated Genes
Hippocampus	0.003	RSAD1;JAM2;GAS5;TPPP;CYB561;METTL18;AP000350.5,GSTT2B,DDT,AP000351.4, MIF-AS1,AP000350.6,GSTT2;LRRC56,RNH1;ATXN2L,SULT1A1,SULT1A2,NPIPB7, NUPR1,EIF3C,SH2B1,TUFM;ATP23
Frontal cortex	0.26	RMI1;DLD;FLOT1,VARS2,CCHCR1;DPM2;EI24;SMIM19;S100A10;RANBP10,CARMIL2; RERE;NCKIPSD,WDR6

Figure 4.11: Multi'omic network analysis identifies key microbial genomic pathways and microbially modulated metabolites associated with differential expression of hippocampal transcripts. (A) MMVEC based co-occurrence network constructed from (top)

human donor pre-KD and post-KD fecal metagenomic and fecal metabolomic datasets and (bottom) mouse recipient pre-KD and post-KD fecal metagenomic, fecal metabolomic, serum metabolomic, hippocampal transcriptomic, and frontal cortical transcriptomic datasets. wKDA analyses was performed on the network. Red text denotes pathways, metabolites, or genes that were differentially regulated ($p<0.05$) between pre-KD and post-KD in prior individual dataset analyses (donor: n=10 patients per diet condition; recipient: n=10 per patient diet condition, where each sample is pooled from 5-6 recipient mice per donor patient sample). (B) Table of top associated genes from epilepsy GWAS mapping onto mouse recipient hippocampal and frontal cortical DEGs (n=10 per patient diet condition, where each sample is pooled from 6 recipient mice per donor patient sample). MMVEC, microbe-metabolite vectors; KD, ketogenic diet; wKDA, weighted key driver analysis; GWAS genome-wide association study; DEGs, differentially expressed genes

Donor Patient	Sex	Age	KD ratio (fat:carb, in grams)	Patient Response to KD	Epilepsy Etiology	Pre-KD BHB (mg/dL)	Post-KD BHB (mg/dL)	Pre-KD Glucose (mg/dL)	Post-KD Glucose (mg/dL)	Pre-KD Period AEDs	Post-KD Period AEDs	Medications During any other visit
1	F	8	3:1	responder	Isodicentric chromosome 15q duplication syndrome	28.3	4.18	95	78	levetiracetam	levetiracetam	CBD oil, rufinamide
2	F	4	3:1	non-responder	Hypoxic-ishemic encephalopathy	11.8	52.2	82	75	levetiracetam	levetiracetam	-
3	F	3	2:1	responder	Trisomy 23 mosaicism	1.0	4.73	84	65	ACTH, CBD oil, clobozam, felbamate, levetiracetam, prednisolone, topiramate, vigabratin	-	-
4	M	1	2:1	non-responder	WWOX gene mutation	2.1	51.2	83	61	felbamate, levetiracetam, rufinamide, zonisamide	felbamate, levetiracetam, rufinamide, zonisamide	-
5	M	2	1.75:1	non-responder	Non-ketotic hyperglycinemia	3.1	16.8	97	75	CBD oil, clobozam, felbamate, levetiracetam, sodium benzoate	CBD + THCa oil, clobozam, felbamate, levetiracetam, sodium benzoate	-
6	F	1	3:1	responder	Pyruvate dehydrogenase E1-alpha deficiency + agenesis of corpus callosum	6.3	20.1	89	86	levetiracetam, vigabratin	-	levetiracetam, vigabatrin
7	F	1	3.75:1	responder	NeuroD2 mutation	8.9	47	92	64	felbamate	felbamate	-
8	M	5	2.75:1	non-responder	Bilateral perisylvian polymicrogyria	43.9	54.1	89	87	felbamate	felbamate	-
9	F	5	3:1	non-responder	Tuberous sclerosis	27.8	45.3	63	74	CBD oil, lacosamide, levetiracetam, vigabratin	CBD oil, lacosamide, levetiracetam, vigabratin	CBD oil, lacosamide, levetiracetam, vigabratin
10	F	10	2.75:1	non-responder	Neuronal ceroid lipofuscinosis type 6 (CLN6/Battens disease)	4.1	14.8	96	85	clobozam, levetiracetam, zonisamide	clobozam, levetiracetam, zonisamide	clobozam, levetiracetam, zonisamide

Table 4.1: Human Subjects. Demographic and clinical information collected from pediatric epilepsy patients (n=10) participating in this study. KD ratio denotes the ratio of grams of fat to grams of carbohydrates consumed. Patient response to KD denotes for a responder, ~50% decrease in seizures based upon observations by patients' parents and physician's notes one month after starting KD.

Materials and Methods

STAR★METHODS

Detailed methods are provided in the online version of the paper and include the following:

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    - Marker set enrichment analysis (MSEA) to connect hippocampus and frontal cortex DEGs with epilepsy GWAS
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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Stool samples from human subjects	This study	N/A

Chemicals, Peptides, and Recombinant Proteins		
Vancomycin hydrochloride	Chem-Impex International	00315
Neomycin trisulfate salt hydrate	Sigma-Aldrich	N1876
Metronidazole	Sigma-Aldrich	M1547
Ampicillin sodium salt	Sigma-Aldrich	A9518
TURBO DNase	Invitrogen	AM2238
Ultrapure water	ThermoFisher	10977015
1x PBS	ThermoFisher	10010023
Tetracaine Hydrochloride Opthalmic Solution, USP 0.5%	Oceanside Pharmaceuticals	68682-920-64
Critical Commercial Assays		
DNeasy PowerSoil Kit	Qiagen	12888-50
Qiaquick PCR purification kit	Qiagen	28104
PureLink RNA Mini Kit	Invitrogen	12183018A
QuantSeq FWD' mRNA-Seq Library Prep Kit	Lexogen	N/A
Deposited Data		
16S rRNA gene sequencing	https://qiita.ucsd.edu	14928
Metagenomic sequencing	https://qiita.ucsd.edu	14928
Untargeted metabolomics	https://data.mendeley.com/	DOI:10.17632/djzyzdbz3z.1
Hippocampal transcriptomics	Gene Expression Omnibus	GSE225682

Frontal cortex transcriptomics	Gene Expression Omnibus	GSE225682
WGCNA modules	Github	https://github.com/smha118/keto_diet_pediatric_epilepsy
Experimental Models: Organisms/Strains		
Swiss Webster	Taconic Farms	TAC-SW
Oligonucleotides		
Forward primer for digital PCR: UN00F2, 5′-CAGCMGCCGCGGTAA-3	Integrated DNA Technologies	N/A
Reverse primer for digital PCR: UN00R0, 5′-GGACTACHVGGGTWTCTAAT-3′ [1, 3])	Integrated DNA Technologies	N/A
Software and Algorithms		
Deblur	https://github.com/biocore/deblur	(Amir et al., 2017)
QIIME2-2022.2	https://qiime2.org/	(Bolyen et al., 2019)
FastQC v. 0.11.9	https://github.com/s-andrews/FastQC/releases/tag/v0.11.9	(Andrews, 2010)
ANCOM	https://github.com/FrederickHuangLin/ANCOM-Code-Archive	(Mandal et al., 2015)

Trimmomatic	https://github.com/timflutre/trimmomatic	(Bolger et al., 2014)
HISAT2	http://daehwankimlab.github.io/hisat2/	(Kim et al., 2019)
HTSeq-count	https://github.com/htseq/htseq	(Anders et al., 2015)
DESeq2	https://bioconductor.org/packages/release/bioc/html/DESeq2.html	(Love et al., 2014)
RStudio 2022.07.2	https://www.r-project.org/	(RStudio Team, 2021)
bioBakery	https://github.com/biobakery/biobakery	(McIver et al., 2018)
HUMAnN 3.0	https://github.com/biobakery/humann	(Beghini et al., 2021)
MetaPhlAn 3.0	https://github.com/biobakery/MetaPhlAn	(Beghini et al., 2021)
MaAsLin 2.0	https://github.com/biobakery/biobakery/wiki/maaslin2	(Mallick et al., 2021)
file2meco	https://github.com/ChiLiubio/file2meco	(Liu et al., 2022)

MetaboAnalyst 5.0	https://www.metaboanalyst.ca/home.xhtml	(Pang et al., 2021)
Cytoscape	https://cytoscape.org/	(Shannon et al., 2003)
EnrichR	https://maayanlab.cloud/Enrichr/	(Chen et al., 2013; Kuleshov et al., 2016; Z. Xie et al., 2021)
STRING	https://string-db.org/	(Szklarczyk et al., 2019)
WCGNA	https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/	(Langfelder & Horvath, 2008)
MMVEC	https://github.com/biocore/mmvec	(Morton et al., 2019)
wKDA	http://mergeomics.research.idre.ucla.edu/	(Ding et al., 2021)
BlueBee	Lexogen	1864011
Prism software	GraphPad	v 8.2.1
Other		
“Breeder” chow	Lab Diets	5K52

Control diet	Harlan Teklad	TD.150300
4200 Tapestation System	Agilent	G2991AA
QX200 Droplet Generator	Bio-Rad Laboratories	1864002
ECT Unit	Ugo Basile	57800

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Human Subjects

This study was approved by UCLA's Institutional Review Board (IRB protocol #15-000453). Pediatric refractory epilepsy patients were screened and enrolled in collaboration with the Ketogenic Diet Program at UCLA Mattel Children's Hospital. Prospective participants who met study criteria were provided information detailing this study by phone and email 1-2 weeks before their pre-diet initiation visit. Prior to enrollment, informed signed consent was provided by all participants and their guardians to the program clinical coordinator during the pre-diet initiation appointment. Subjects were enrolled across diverse seizure semiology and prior medical histories. Inclusion criteria: enrolled in UCLA's program for classical 4:1 KD, children aged 1-10 with refractory epilepsy, any gender, any ethnicity, any previous exposure to AEDs, any seizure semiology. Exclusion criteria: use of antibiotics or probiotics within 7 days prior to enrollment, existing diagnosis of gastrointestinal, immunological, or metabolic disorder. Human donor stool samples were collected from 10 participants, each providing 2 stool samples. The first sample was collected within 1 day before starting KD treatment (pre-KD) and the second sample was collected after maintaining on the clinical KD for 1 month (post-KD). Clinical metadata from the medical record were coded and stripped of identifiers before being shared, and included participant demographic data, medical history, AED exposure history, additional medications take during this study, laboratory blood glucose and bloody ketone body levels, seizure severity, seizure frequency, seizure semiology, and dietary regimen (Table S1).

Human stool sample collection

For in-patient fecal sample collection, once a study participant was admitted to the hospital during the pre-diet initiation visit, they were given a coded stool collection kit and sterile specimen container. Stool samples were freshly collected within 1 day prior to starting the clinical KD treatment (pre-KD). Fresh stool samples were immediately placed on dry ice for short term storage and transportation and were freshly frozen at -80°C for long-term storage. Post-KD stool samples were collected in the same manner as stated above when the study participant returned for the 1-month follow-up visit. For out-patient collection of the post-KD stool sample, which was necessitated because of hospital pandemic policies, a deidentified stool sample collection kit and sterile specimen cup was provided to the patient and guardian along with a pre-labeled return shipping box. After 1 month of the clinical KD treatment, stool samples were collected in a sterile specimen cup, immediately placed in an at home freezer, and the next day either (1) shipped back overnight to UCLA on dry ice or (2) brought with the patient to their 1 month follow-up appointment. Fresh frozen fecal samples were homogenized under liquid nitrogen and 3 ~500 mg aliquots were made per sample by sterile storage in anaerobic Balch tubes to be used for transplantation, metagenomic, and metabolomic studies.

Mice

6-8 week old wild-type germ-free Swiss Webster mice (Taconic Farms), were bred in UCLA's Center for Health Sciences Barrier Facility. Breeding animals were fed ‘‘breeder’’ chow (Lab Diets 5K52). Experimental animals were fed vitamin- and mineral-matched control diet (Harlan Teklad TD.150300). Juvenile mice were used to mimic the age range of the human donor population (<10 years old). All animal experiments were approved by the UCLA Animal Care and Use Committee.

METHOD DETAILS

16S rRNA Gene Sequencing and Analysis

Bacterial genomic DNA was extracted from human or mouse fecal samples using the Qiagen PowerSoil Kit. For human samples, the n reflects one donor sample. For mouse samples, the n reflects independent cages containing 3 mice per cage to preclude effects of co-housing on microbiota composition. The sequencing library was generated in line with (Caporaso et al., 2011). PCR amplification, run in triplicate, of the V4 region of the 16S rRNA gene was completed using individually barcoded universal primers and 30 ng of the extracted genomic DNA. The PCR product triplicates were pooled and purified using the Qiaquick PCR purification kit (Qiagen). Samples were sequenced using the Illumina MiSeq platform and 2 x 250bp reagent kit for paired-end sequencing at Laragen, Inc. Amplicon sequence variants (ASVs) were chosen by closed reference clustering based on 99% sequence similarity to the SILVA138 database. Taxonomy assignment, rarefaction, and differential abundance testing were performed using QIIME2 2022.2 (Bolyen et al., 2019; Mandal et al., 2015).

Fecal Shotgun Metagenomics

Bacterial genomic DNA was extracted from human or mouse fecal samples using the Qiagen PowerSoil Kit. 1 ng of DNA was used to prepare DNA libraries using the Nextera XT DNA Library Preparation Kit (Illumina) and genomic DNA was fragmented with Illumina Nextera XT fragmentation enzyme. IDT Unique Dual Indexes were added to each sample before 12 cycles of PCR amplification. AMpure magnetic Beads (Beckman Coulter) were used to purify DNA libraries which were eluted in QIAGEN EB buffer. Qubit 4 fluorometer and Qubit dsDNA HS Assay Kit were used for DNA library quantification. Libraries were then sequenced on Illumina HiSeq 4000 platform 2x150bp at a 6M read depth using by CosmosID. Metagenomic data was analyzed using HUMAnN 3.0 (Beghini et al., 2021) and MetaCyc database to profile gene families and pathway abundance. File2meco R package was used for MetaCyc pathway

hierarchical classification (Liu et al., 2022). MaAsLin 2.0 (Mallick et al., 2021) was used to assess significant pathway associations between pre-KD and post-KD with a p-value cutoff of 0.1, where $p < 0.05$ pathways are indicated in the figure by asterisk. Heatmaps were generated using the pheatmap v1.0.12 package for R.

Human Donor Fecal Microbiota Transfer

To prepare collected human stool samples for transplantation studies, the frozen stool sample was pulverized into a powder under liquid nitrogen stream in a sterile heavy-duty foil covered mortar and pestle, aliquoted at 500 mg per tube into 2mL screw cap tubes, and frozen at -80C. A single 500 mg aliquot of human stool sample was entered into a Coy anaerobic chamber and resuspended in pre-reduced 1x PBS + 0.05% L-cysteine. The sample was homogenized using sterile borosilicate glass beads and passed through a 100um filter. GF Swiss Webster mice were colonized by oral gavage of 200 ul fecal suspension. Excess fecal suspension was resuspended and stored at -80C in pre-reduced 1x PBS + 0.05% L-cysteine + 15% glycerol. For administration of fecal filtrates, the fecal suspension was passed through a sterile 0.2 um filter before colonization via oral gavage using 200 ul fecal filtrate.

6-Hz Psychomotor Seizure Assay

6-Hz psychomotor seizure assay testing was conducted following Samala et al., 2008. One drop (~50 ul) of 0.5% tetracaine hydrochloride ophthalmic solution was applied to the corneas of each mouse 15 min before stimulation. A thin layer of electrode gel (Parker Signagel) was applied directly to the corneal electrodes and was reapplied before each trial. A constant-current current device (ECT Unit 57800, Ugo Basile) was used to deliver current through the corneal electrodes at 3s duration, 0.2 ms pulse-width and 6 pulses/s frequency. CC50 (the milliamp intensity of current required to elicit seizures in 50% of the mouse cohort) was measured as a metric for seizure susceptibility. Pilot experiments were conducted to identify 28 mA as the

CC50 for SPF wild-type Swiss Webster mice, aged 6-8 weeks. Each mouse was seizure-tested only once, and thus at least n > 14 mice were used to adequately power each cohort. To determine CC50s for each tested cohort, 28 mA of current was administered to the first mouse per cohort, followed by stepwise fixed increases or decreases by 2 mA intervals. Mice were restrained manually during stimulation and then released into a new cage for behavioral observation. Quantitative measures for falling, tail dorsiflexion (Straub tail), forelimb clonus, eye/vibrissae twitching and behavioral remission were scored manually. For each behavioral parameter, we observed no correlation between percentage incidence during 28+ mA seizures between pre-KD or post-KD microbiota status, suggesting a primary effect of the microbiota on seizure incidence rather than presentation or form. Latency to exploration (time elapsed from when an experimental mouse is released into the observation cage (after corneal stimulation) to its first lateral movement) was scored manually with an electronic timer. Mice were blindly scored as protected from seizures if they did not show seizure behavior and resumed normal exploratory behavior within 10 s. Seizure threshold (CC50) was determined as previously described (Kimball et al., 1957), using the average log interval of current steps per experimental group, where sample n is defined as the subset of animals displaying the less frequent seizure behavior. Data used to calculate CC50 are also displayed as latency to explore for each current intensity, where n represents the total number of biological replicates per group regardless of seizure outcome.

Antibiotic Treatment

Transplanted mice were gavaged with a solution of vancomycin (50 mg/kg), neomycin (100 mg/kg) and metronidazole (100 mg/kg) every 12 hours daily for 5 days, as adapted from (Reikvam et al., 2011). Ampicillin (1 mg/ml) was provided *ad libitum* in drinking water. For mock treatment, mice were gavaged with a similar volume of 1x PBS (vehicle) water every 12 hours

daily for 7 days. Antibiotic-treated mice were maintained in sterile caging with sterile food and water and handled aseptically for the remainder of the experiments.

Fecal and Serum Metabolomics

Previously collected human donor fecal samples were aliquoted as described in section “Human Donor Fecal Microbiota Transfer”. Mouse fecal samples were collected from mice housed across independent cages, with four cages housing 3 mice and one cage housing 2 mice. Mouse serum samples were collected by cardiac puncture and separated using SST vacutainer tubes, then frozen at -80C. Samples were prepared using the automated MicroLab STAR system (Hamilton Company) and analyzed on GC/MS, LC/MS and LC/MS/MS platforms by Metabolon, Inc. Protein fractions were removed by serial extractions with organic aqueous solvents, concentrated using a TurboVap system (Zymark) and vacuum dried. For LC/MS and LC-MS/MS, samples were reconstituted in acidic or basic LC-compatible solvents containing > 11 injection standards and run on a Waters ACQUITY UPLC and Thermo-Finnigan LTQ mass spectrometer, with a linear ion-trap frontend and a Fourier transform ion cyclotron resonance mass spectrometer back-end. For GC/MS, samples were derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Chemical entities were identified by comparison to metabolomic library entries of purified standards. Following log transformation and imputation with minimum observed values for each compound, post-KD vs. pre-KD comparisons for human fecal, and mouse serum and fecal data were analyzed by paired t-test. Metabolomic data from SPF or antibiotic-treated mice fed KD vs. CD chow were acquired from (Olson et al., 2018), as log transformed and imputed with minimum observed values for each compound. Data were analyzed using two-way ANOVA to test for group effects. P and q-values were calculated based on two-way ANOVA contrasts. Principal components analysis was used to visualize variance distributions. Supervised Random Forest

analysis was conducted to identify metabolomics prediction accuracies. Metabolite set enrichment analysis (MSEA) using the Metaboanalyst 5.0 platform (Pang et al., 2021) was performed on human fecal, mouse fecal, and mouse serum metabolites statistically significantly altered in post-KD compared to pre-KD (p-val<0.05). Metabolite sets were analyzed for chemical sub-class enrichment and metabolite pathway enrichment, using The Small Molecule Pathway Database (SMPDB).

Transcriptomics

Recipient mice were sacrificed on day 4 post-colonization. Hippocampal, frontal cortex, were dissected from pre-KD and post-KD recipient mice (n=6 per cohort) and immediately placed in Trizol. RNA was extracted using the PureLink RNA Mini kit with Turbo DNAse treatment. RNA was prepared using the TruSeq RNA Library Prep kit and 2 Å~ 69-bp paired-end sequencing was performed using the Illumina HiSeq 4000 platform by the UCLA Neuroscience Genomics Core. FastQC v0.11.5, bbduk v35.92, and RSeQC v2.6.4 were used for quality filtering, trimming, and mapping. Reads were aligned to UCSC Genome Browser assembly ID: mm10 using STAR v2.5.2a, indexed using samtools v1.3, and aligned using HTSeq-count v0.6.0. Differential expression analysis was conducted using DESeq2 v1.24.041. Heatmaps were generated using the pheatmap v1.0.12 package for R. GO term enrichment analysis of differentially expressed genes with $p < 0.05$ was conducted using enrichR v3.1. Protein interaction networks were generated using STRING v10.5. Functional enrichments of network nodes were categorized by GO: biological process, molecular function, and cellular component.

Multi-omics Integration

To assess the relationships across omics layers, we first carried out dimension reduction for each data set using weighted gene co-expression network analysis (WGCNA v1.72.1) (Langfelder & Horvath, 2008). Metabolomics for human donors and mouse recipients and RNA-

seq for mouse recipients (hippocampus and frontal cortex) were used to build WGCNA modules within each dataset, where modules represent clusters of highly co-regulated/expressed molecules which are typically involved in similar biological functions. For metabolomics data, *goodSamplesGenes* function was first used with default parameters to filter out sparse metabolites across samples before constructing networks; this step was not used for RNAseq data. Standard WGCNA steps were then carried out for the filtered metabolomics and RNAseq data. Module eigengenes (MEs), or the first axis of principal component were calculated from each module. MEs were then targeted for correlation analysis with the metadata (pre-KD vs. post-KD and responder vs. non-responder). Modules that had significant correlation (p-val <0.05) with the metadata were chosen for subsequent integrative analysis.

A systematic network that combined all omics data was inferred based on the probability of co-occurrence (POC) between molecules from different omics data. To calculate POC, we leveraged a neural-net based tool called MMVEC v1.0.6 with default parameters (Morton et al., 2019). The subset of raw data that contains module components that were selected from WGCNA analysis were log normalized and combined based on sample ID. This combined data matrix was then used as input for MMVEC. For example, on donor side, modules from fecal metagenome and metabolomics were added together and, on the recipient side, the combined matrix contained the raw data from metagenome, metabolomics, and RNAseq. Due to high density of the overall network generated from MMVEC, the top 10% of POC connections were retrieved to minimize overall complexity of the network for both donors and recipients using in-house python script (https://github.com/smha118/keto_diet_pediatric_epilepsy).

The networks of modules from individual omics layers from donor (metagenome, metabolomics) and recipient (metagenome, metabolomics, RNAseq) as well as differentially expressed/abundance molecules were then seeded into Mergeomics v3.16 pipeline along with

the integrated network generated with MMVEC for weighted key driver analysis (wKDA) to identify key drivers of the networks (Ding et al., 2021). wKDA uses a χ2 -like statistic to identify molecules that are connected to significant larger module components than what would be expected by random chance. The analysis was done on the human and mouse networks separately. To further look into the network that are relevant to ketogenic diet and epilepsy, we selected key drivers (KDs) based on i) the number of modules that a key driver was invoked related to, ii) their relation to the Ketogenic diet or epilepsy. A subset of nodes in each module that were connected to the KDs were collected. These nodes were retrieved with following priority i) they are part of differentially regulated molecules ii) POC value with KDs. Finally, the network was visualized using Cytoscape (Shannon et al., 2003). To minimize overall density of the network, we chose to show the key drivers Mergeomics with the highest occurrence in their respective MEs and with > 5 degrees of connectivity.

Marker set enrichment analysis (MSEA) to connect hippocampus and frontal cortex DEGs with epilepsy GWAS

To assess the potential role of the DEGs from the hippocampus and frontal cortex in epilepsy, we collected the summary statistics of the latest epilepsy GWAS (Abou-Khalil et al., 2018). Single nucleotide polymorphisms (SNPs) that had a linkage disequilibrium of $r^2 > 0.5$ were filtered to remove redundancies. To map the epilepsy GWAS SNPs to genes, we used GTEx version 8 eQTL and sQTL data for brain hippocampus and brain frontal cortex (Aguet et al., 2020), which help us derive genes likely to be regulated by the SNPs. We next used the MSEA function of the Mergeomics package (Ding et al., 2021) to compare epilepsy disease association p-values of the SNPs representing the DEGs (hippocampus or frontal cortex) with those of the SNPs mapped to random genes to assess whether the DEGs contain SNPs that show stronger epilepsy association than random genes using a χ^2-like statistic.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted using Prism8 software v8.2.1 (GraphPad). Before statistical analysis, data was assessed for distribution to determine appropriate statistical tests to use. Data were plotted in figures as mean ± SEM. For figures: 1B, S2C, S3B, S3B, S4C, *n* = the number of technical replicates. For all other figures, *n* = the number of biological replicates. No samples or animals were excluded from data analysis. Differences between two sample conditions from parametric data sets were analyzed using two-tailed, paired Student's t-test. Differences between two sample conditions from nonparametric data sets were analyzed using two-tailed, Wilcoxon matched-pairs signed rank test. For differences among >2 groups when analyzing one variable, a one-way ANOVA with Tukey's post hoc test was used. For differences among ≥2 groups with two variables, a two-way ANOVA with Sidak's post hoc test was used. For technical replicates from within-patient analysis (Figures: 1B, S2C, S3B, S3B, S4C), differences from the above tests are denoted by: $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; $^{\#\#\#\#}p<0.0001$. For biological replicates (all other figures), differences from the above tests are denoted by: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Non-significant differences are denoted in the figures using "n.s".

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