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

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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.

42 ## INTRODUCTION

43

44 The low-carbohydrate, high-fat ketogenic diet (KD), is a mainstay treatment for refractory
45 epilepsy, particularly in children who do not respond to existing anti-epileptic drugs. The efficacy
46 of the KD is supported by multiple retrospective and prospective studies, which estimate that
47 ~30% of pediatric patients become seizure-free and ~60% experience substantial benefit with
48 >50% reduction in seizures (Coppola et al., 2002; Freeman et al., 1998; Hoon et al., 2005; Neal
49 et al., 2008). However, use of the KD for treating pharmacoresistant epilepsy remains low due to
50 difficulties with implementation, dietary compliance, and adverse side effects (Kossoff et al.,
51 2018). Even with successful seizure reduction, retention of epileptic children on the KD is a
52 reported 13% by the third year of dietary therapy (Hemingway et al., 2001). The primary reasons
53 cited for discontinuation include “diet restrictiveness” and “diet side effects,” in addition to low diet
54 responsiveness. While many pioneering studies have proposed important roles for
55 immunosuppression, ketone bodies, anaplerosis, and gamma-aminobutyric acid (GABA)
56 modulation in mediating the neuroprotective effects of the KD, they do not fully account for the
57 clinical heterogeneity in patient responsiveness. Exactly how the KD confers protection against
58 epilepsy in individuals with varied seizure semiologies remains unclear, and the biological
59 determinants of patient responsiveness to the KD are poorly understood.

60

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

68 associated human epilepsy microbiome on host seizure susceptibility remain unknown. We
69 previously reported that KD-induced alterations in the gut microbiome were necessary and
70 sufficient for mediating the seizure protective effects of KD chow in two mouse models for
71 refractory epilepsy – the 6-Hz psychomotor seizure model and the *Kcna1* deficiency model for
72 sudden unexpected death in epilepsy (SUDEP) (Olson et al., 2018). Similarly, in a rat injury model
73 of infantile spasms, transfer of the KD-induced gut microbiota into naïve animals fed a control diet
74 reduced spasms (Mu et al., 2022). In addition, taxonomic differences in the gut microbiome were
75 correlated with seizure severity and seizure protection in response to KD chow in the *Scn1a*
76 deficiency model for Dravet syndrome (Miljanovic & Potschka, 2021). Together, these findings
77 across various seizure models provide proof-of-principle that the KD alters the gut microbiome in
78 ways that can promote seizure protection. However, whether these results from rodent studies
79 apply to human epilepsy, the human gut microbiome, and clinical KD regimens used to treat
80 pediatric epilepsy is still unknown, and the core microbial functions that impact seizure
81 susceptibility are unclear.
82
83 Herein, we perform a prospective study of KD interventions in children with refractory epilepsy
84 and test causal effects of the human gut microbiome before and after initiating clinical KD
85 regimens on seizure susceptibility in mice. We evaluate functional changes in the human gut
86 microbiome that are associated with KD treatment in pediatric epilepsy patients. We further
87 identify select features of the clinical KD-associated gut microbiome that are shared across both
88 human donors and inoculated mouse recipients that correlate with microbiome-dependent seizure
89 protection in mice. Finally, we identify key network interactions between the gut microbiome,
90 metabolites, and brain transcriptome that may contribute to the ability of the clinical KD-associated
91 human gut microbiome from pediatric epilepsy patients to promote seizure protection in mice.
92
93

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 S1**). 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 S1A, 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 S1B**). Additionally, the

120 clinical KD elicited differential shifts in bacterial β-diversity and varied responses across post-KD
121 samples relative to their matching pre-KD controls, which were not significantly associated with
122 demographic or clinical measures, such as age, sex, and prior anti-epileptic drug exposure
123 (**Figure S1B**, Table S1, Yassour et al., 2016). Consistent with the inter-individual variation in
124 microbial taxonomic profiles, ANCOM and ANOVA analyses (paired or unpaired) identified no
125 significant differences in relative abundances in particular bacterial taxa when considering all
126 post-KD sample relative to their matched pre-KD controls (**Figures S1C)**. These results indicate
127 that, within this particular study cohort, there are no shared effects of the clinical KD on the
128 microbial composition of the gut microbiota of children with refractory epilepsy.
129
130 Functional redundancy is common across different microbial species of the human gut microbiota
131 (Tian et al., 2020). In light of the varied bacterial taxonomic profiles at baseline and in response
132 to dietary treatment, we next asked whether the clinical KD is associated with shared alterations
133 in the functional potential of the gut microbiota from children with pediatric epilepsy. Shotgun
134 metagenomic profiling and pathway analysis indicated that compared to pre-KD samples, post-
135 KD samples shared a significant decrease in relative abundance of genes belonging to the top 26
136 most abundant functional pathways, which together comprised >94% of the pathway diversity
137 detected (**Figure S1D and S1E, Table S3**). This corresponded with a significant increase in the
138 number of total observed pathways in post-KD samples compared to their respective pre-KD
139 controls (**Figure S1F**). These observations suggest that the clinical KD restricts the membership
140 of various types of microbial taxa that harbor genes related to prevalent functions and/or enriches
141 for microbial taxa that harbor genes related to previously rare or underrepresented functions. In
142 particular, post-KD samples exhibited significant enrichment of genes related to formaldehyde
143 assimilation, guanosine nucleotide degradation, and L-proline biosynthesis, and decreased
144 representation of genes related to aerobactin biosynthesis, as compared to pre-KD controls
145 (**Figure S1G, Discussion**). There were also modest increases in genes related to GDP-mannose

146 biosynthesis, 2-methylcitrate cycle, and glycol metabolism and degradation, and decreases in
147 genes related to polyamine biosynthesis and biotin biosynthesis, subsets of which will be
148 discussed in greater detail in the following sections (**Figure S1G)**. Taken together, these data
149 suggest that treatment with KD regimens that differ in KD ratio and specific nutritional composition
150 elicit broad shifts in the functional potential of the gut microbiome that are shared across children
151 with varied subtypes of refractory epilepsy.
152
153 **Transferring the fecal microbiota from KD-treated pediatric epilepsy patients to mice**
154 **confers seizure resistance**
155
156 Causal influences of the human microbiome can be effectively studied in gnotobiotic mice,
157 wherein transferring microbes in a clinical sample into microbiota-deficient mice is used to
158 recapitulate the taxonomic and functional diversity of the donor human microbiota. To evaluate
159 whether gut microbes associated with the clinical KD impact seizure susceptibility, we inoculated
160 individual cohorts of germ-free (GF) mice with matched pre-KD and post-KD stool samples
161 collected from children with refractory epilepsy and maintained the colonized mice on standard
162 (non-ketogenic) mouse chow (control diet, CD). Each human donor sample (pre-KD and post-KD
163 from 10 individuals, as biological replicates) was inoculated into 14-16 GF mice (as technical
164 replicates) to enable cohort-level testing of susceptibility to 6-Hz psychomotor seizures (**Figure**
165 **1A**). The 6-Hz seizure model involves low-frequency corneal stimulation to induce acute complex
166 partial seizures reminiscent of human limbic epilepsy (Barton et al., 2001). Consistent with
167 refractory epilepsy, the 6-Hz model is resistant to several anti-epileptic drugs, but treatment with
168 KD chow effectively protects against 6-Hz seizures in rodents (Hartman et al., 2008), raising the
169 intensity of current required to elicit a seizure in 50% of the subjects tested (CC50, seizure
170 threshold). The 4-day time point was chosen as the maximum duration of time that a KD-induced
171 microbiota could be maintained in mice fed CD (Olson et al., 2018).

172

173 Despite the variation in bacterial diversity across patient gut microbiota (**Figure S1**), we observed
174 that GF mice colonized with microbes from the post-KD microbiota required greater intensity of
175 current to induce 6-Hz seizures (**Figure 1B, Table S4**) as compared to controls colonized with
176 microbes from the pre-KD microbiota. This effect was seen when comparing post-KD vs. pre-KD
177 microbiota transfer for individual technical replicates per patient (**Figure 1B)**, as well as when
178 data were averaged across all patients (**Figures 1C and 1D**). In addition, compared to pre-KD
179 controls, mice colonized with microbes from the post-KD microbiota required increased intensity
180 of current to elicit one or more recurred seizures observed after the initial stimulus-induced seizure
181 (**Figure 1E**), indicating that transfer of the post-KD human microbiota promotes resistance to both
182 primary induced seizures and remission seizures in mice. On average, the post-KD samples
183 raised seizure thresholds by 22.4% $\pm$ 6.4% relative to matched pre-KD controls (**Figures 1C and**
184 **1D**). This aligns with both our previously published data on the average effect size of KD chow on
185 wildtype mice tested in the 6-Hz seizure assay (24.5%, Olson et al., 2018), and the observed
186 24.0% increase in seizure threshold seen in GF mice colonized with a conventional adult mouse
187 microbiota (GF-conv) and fed a 6:1 KD chow, as compared to conventionalized controls fed a
188 standard vitamin- and mineral-matched control diet (**Figure 1B**). Discrepancies in effect size
189 across patient samples were largely driven by differences between responses for pre-KD controls
190 (**Figure 1B**), suggesting that the comparatively low microbial diversity resulting from cross-host
191 species transfer increases seizure susceptibility. Consistent with this, we previously observed that
192 decreasing microbial diversity via antibiotic treatment reduced seizure threshold in the 6-Hz assay
193 (Olson et al., 2018). Overall, these results indicate that inoculating mice with the clinical KD-
194 associated human gut microbiota increases 6-Hz seizure threshold to levels similar to the effect
195 sizes seen with direct consumption of the experimental 6:1 KD.

196

197 Human microbiota transplantation to mice involves oral inoculation with a human stool
198 suspension, which is comprised of microbial biomass, as well as undigested food matter and
199 secreted molecules from the host and microbiota. As such, effects seen in response to the transfer
200 procedure could be due to the KD-associated gut microbiota or microbiota-independent dietary
201 or host factors. To gain insight into whether bacteria from the gut microbiota are required for
202 mediating the increases in seizure protection seen with inoculation of the human post-KD
203 microbiota into mice, mice inoculated with a randomly selected post-KD donor sample were
204 treated with broad-spectrum antibiotics (**Abx**) to deplete the microbiota, or with vehicle (**Veh**) as
205 negative control (**Figures S2A and S2B**). Mice that were inoculated with the post-KD sample and
206 treated with Veh displayed seizure thresholds that were comparable to that seen previously in
207 recipient mice without the added Veh treatment (**Figures S2C, S2D, and 1B and Table S4**). This
208 suggests that the post-KD sample induced increases in seizure resistance that were maintained
209 for at least 12 days in mice fed CD. In contrast, depletion of gut bacteria in mice that were
210 colonized with the post-KD microbiota decreased seizure thresholds to levels that were lower than
211 previously seen in pre-KD colonized controls (**Figures S2C, S2D, and 1B**). These results indicate
212 that bacterial members of the post-KD microbiota are necessary for mediating the increases in
213 seizure threshold seen in response to transfer of the clinical-KD associated microbiota from a
214 pediatric epilepsy patient into mice.
215
216 Administration of microbial metabolites or other microbiome-dependent molecules, in lieu of viable
217 microbiota, has been reported to ameliorate symptoms of recurrent *Clostridiodes difficile* infection,
218 inflammatory bowel disease, and multiple sclerosis, among other conditions (Cekanaviciute et al.,
219 2017; Levy et al., 2015; Ott et al., 2017). To gain insight into whether administration of clinical
220 KD-associated intestinal small molecules is sufficient to confer seizure protection in mice, a post-
221 KD donor sample selected at random was sterile filtered and then administered to a cohort of GF
222 recipient mice (**Figure S3A**), alongside controls that were administered the unfiltered post-KD

223 suspension, as was done previously for human microbiota inoculation (**Figures S3A and 1B**). At
224 4 days post inoculation, mice that were treated with the post-KD filtrate exhibited lower seizure
225 threshold compared to controls that were treated with the corresponding unfiltered post-KD
226 suspension (**Figures S3B and S3C and Table S4**). These data indicate that clinical KD-
227 associated small molecules in the post-KD fecal sample from a pediatric epilepsy patient are not
228 sufficient to confer persistent seizure protection in mice.
229
230 Orally administered microbial metabolites can be rapidly absorbed and cleared from systemic
231 circulation within a few hours of administration (Abrams & Bishop, 1967; Williams et al., 2020).
232 To further assess whether clinical KD-associated intestinal small molecules, including microbial
233 metabolites, acutely modulate seizure susceptibility, mice were orally gavaged with a sterile-
234 filtered post-KD sample and assessed 2 hours later for 6-Hz seizure threshold, rather than 4 days
235 later as in the previous experiments (**Figure S4A**). Mice treated with post-KD filtrate exhibited
236 significantly increased seizure protection compared to controls treated with pre-KD filtrate
237 (**Figures S4B and S4C and Table S4**), with seizure thresholds that approached those seen after
238 inoculation of the post-KD suspension (**Figures S4B and 1B**). These data indicate that
239 administration of clinical KD-associated intestinal small molecules can acutely confer seizure
240 protection in mice over short timescales (i.e., 2 hours, **Figure S4**), which diminishes by 4 days
241 post treatment (**Figure S3**). Taken together, the results presented in these series of experiments
242 suggest that the clinical KD for pediatric refractory epilepsy is associated with alterations in
243 metabolic activities of the gut microbiota that promote seizure resistance in mice.
244
245 While the “humanization” of mice with microbiota from clinical stool samples is a powerful tool for
246 translational microbiome research (Turnbaugh et al., 2009), the approach has technical and
247 biological limitations that warrant careful consideration (Walter et al., 2020). Namely, while much
248 of the taxonomic and functional diversity of the donor inoculum can be recapitulated in recipient

249 mice (Bokoliya et al., 2021), developmental influences and host-specific selection (Rawls et al.,
250 2006), among other factors, preclude full “engraftment” of the human gut microbiota in GF mice
251 (Walter et al., 2020). To evaluate the fidelity of fecal microbiota “transplantation” from pediatric
252 epilepsy patients to GF mice, we subjected both the donor pre-KD and post-KD stool samples
253 and corresponding recipient mouse fecal pellets collected at 4 days post-inoculation (the day of
254 seizure testing) to 16S rRNA gene amplicon sequencing (**Figure 1A, Tables S2 and S5**).
255 Principal coordinates analysis of bacterial taxonomic data revealed overt clustering of donor
256 samples with matched recipient samples only for select patients, while the remaining exhibited
257 substantial variation and no noticeable clustering (**Figure S5A**). There was no significant
258 difference in α-diversity between pre-KD and post-KD fecal microbiota for either donor or recipient
259 samples (**Figures S5B**). However, we observed a significant reduction in α-diversity, with an
260 average decrease of 38% for all mouse recipient microbiota relative to all human donor microbiota
261 (**Figure S5C**), indicating incomplete transfer or engraftment of the human microbiota in mice.
262 These results align with several previous reports of reduced bacterial α-diversity in mice
263 inoculated with human microbiota, with estimated decreases of 35%, 38%, and 50% (Blanton et
264 al., 2016; Sharon et al., 2019; Staley et al., 2017), suggesting that we achieved levels of transfer
265 fidelity that are consistent with those in the field. However, the inability to fully recapitulate the
266 taxonomic diversity of the human gut microbiota from pediatric epilepsy patients in mice draws
267 into question whether the increases in seizure resistance seen in mice inoculated with post-KD
268 microbiota are relevant to the actual clinical condition. We therefore focused subsequent
269 experiments on identifying and evaluating the subset of functional features of the KD-associated
270 human gut microbiome that are recapitulated in recipient mice, and the microbiome-dependent
271 alterations in host physiology that correspond with seizure protection in mice.
272

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

275

276 Given the widespread use of the clinical KD for treating epilepsy, and an increasing number of
277 other neurodevelopmental and neurodegenerative disorders, elucidating how the activity of the
278 gut microbiome is altered by the clinical KD could reveal important insights into its physiological
279 effects. To identify microbiome associations with the clinical KD and further determine which of
280 the associations, if any, may modify seizure risk, we functionally characterized the gut microbiome
281 from pediatric epilepsy patients before and after treatment with the clinical KD, as well as from
282 gnotobiotic mice that were inoculated with the patient samples, and tested for causal outcomes
283 on seizure susceptibility. Metagenomic sequencing and analysis revealed microbial gene
284 pathways that were differentially abundant in post-KD samples relative pre-KD controls, and
285 shared between both human donor samples and mouse recipient samples (**Figure 2A, Tables**
286 **S3 and S6**). In particular, microbial genes relevant to fatty acid β-oxidation, glycol metabolism
287 and degradation, methylcitrate cycle I, methylcitrate cycle II, and proline biosynthesis were
288 similarly elevated in post-KD human samples and post-KD-inoculated mice compared to their
289 respective pre-KD controls (**Figures 2A and 2B**). These findings align with reported influences of
290 the KD on fatty acid oxidation (A. R. Kennedy et al., 2007), of carbohydrate restriction on
291 promoting the glyoxylate cycle (Puckett et al., 2017), and of fatty acid β-oxidation on the initiation
292 of the methylcitrate cycle (Clark & Cronan, 2005). Proline metabolism involves reactions with
293 glutamine, glutamate, ornithine, and arginine, which might relate to reported effects of KD on
294 amino acid metabolism, particularly of glutamine and glutamate (Yudkoff et al., 2007). In addition,
295 both post-KD human donor and mouse recipient samples exhibited reductions in microbial genes
296 relevant to polyamine biosynthesis and aerobactin biosynthesis (**Figures 2A and 2B**). The main
297 role of polyamine biosynthesis is generation of putrescine, mainly using the glucogenic amino
298 acid L-arginine which is consumer in reduced amounts while on the KD. Aerobactin, a sidophore,
299 biosynthesis uses the ketogenic amino acid L-lysine, which is also essential for acetyl-CoA
300 synthesis and energy production during ketosis. These data suggest that the consumption of a

301 clinical KD by children with refractory epilepsy enriches for gut microbes that have the functional
302 capacity to metabolize dietary fats and to perform anaplerotic reactions when dietary
303 carbohydrates are restricted. The findings further indicate that these general features of the KD-
304 associated human gut microbiome are phenocopied in recipient mice that exhibit microbiome-
305 dependent protection against 6-Hz seizures.
306
307 The observed metagenomic signatures reveal clinical KD-associated changes in the functional
308 potential of the gut microbiome that are preserved upon transfer to GF mice. To identify clinical
309 KD-induced alterations in the functional activity of the gut microbiome, we performed untargeted
310 metabolomic profiling of aliquots of the same donor fecal samples from pediatric epilepsy patients
311 collected before and after initiating the KD regimen, and of both fecal and serum samples from
312 recipient mice that were inoculated with the pre-KD or post-KD human fecal microbiota and fed
313 CD (**Tables S7, S8, and S9**). Results from clinical laboratory testing of human blood samples
314 confirmed that the month-long clinical KD regimen elevated serum β-hydroxybutyrate (**BHBA**)
315 levels and reduced serum glucose levels, relative to pre-KD concentrations, in pediatric refractory
316 epilepsy patients (**Figure 2C**). Decreases in glucose, but not BHBA, were similarly seen in human
317 post-KD stool samples relative to matched pre-KD controls (**Figure 2C**), which is consistent with
318 dietary carbohydrate restriction and KD-induced BHBA synthesis by the liver to elevate systemic,
319 but not fecal, BHBA levels (Westman et al., 2007). Transfer of the post-KD human microbiota into
320 mice yielded no significant differences in serum BHBA or glucose relative to pre-KD recipient
321 controls (**Figure 2C**), indicating that the clinical KD-associated microbiota does not sufficiently
322 promote key systemic features of ketosis in mice fed the standard CD. Notably, however, mice
323 that were inoculated with post-KD human microbiota and fed CD exhibited statistically significant
324 increases in fecal BHBA levels relative to matched pre-KD recipient controls (**Figure 2C**). This
325 could reflect alterations in intestinal synthesis of BHBA (Mierziak et al., 2021) and/or in microbial
326 utilization of host-derived BHBA (Ang et al., 2020). Since this effect was not seen in the donor

327 human fecal samples, we reasoned that this phenotype is likely an artifact of the “transplantation”
328 approach and/or experimental design, and therefore not relevant to the clinical condition. These
329 results suggest that transfer of the clinical KD-associated human gut microbiota into mice
330 promotes resistance to 6-Hz seizures (**Figure 1**) through mechanisms that act independently of
331 ketosis.
332
333 We further assessed results from untargeted metabolomic profiling to identify metabolites that
334 were differentially abundant in post-KD samples relative to pre-KD controls and patterns that were
335 shared across human donor and mouse recipient samples (**Figure 2D, Figure S6, Tables S7**
336 **and S8)**. Despite heterogeneity in the patient population and specific clinical KD regimens, 79
337 metabolites were significantly differentially abundant in fecal samples from post-KD human fecal
338 samples relative to their matched pre-KD controls (**Figure S6A, Table S7**). 336 metabolites were
339 identified in both the human fecal samples and mice fed the 6:1 KD chow vs. vitamin- and mineral-
340 matched control chow for 2 weeks samples, previously published by our group (**Table S10,** Olson
341 et al., 2018). 35 metabolites were differentially regulated in human fecal samples and 169
342 metabolites were differentially regulated in mouse fecal samples (**Figure S6B**). Of these
343 significantly altered metabolites, 20 were found to be changed in the same direction across human
344 and mouse samples (**Figure S6B-D**). These included KD-induced increases in levels of
345 metabolites related to fatty acid beta-oxidation, such as palmitoleoylcarnitine (C16:1) and
346 oleoylcarnitine (C18:1), and a decrease in kynurenine which have previously been associated
347 with seizure susceptibility (Żarnowska et al., 2019). This statistically significant overlap suggests
348 that there are biochemical changes that are shared across clinical KD treatments for pediatric
349 epilepsy and mouse models of KD, and that some of the fecal metabolomic alterations observed
350 in KD-treated epilepsy patients are a direct consequence (rather than correlate) of dietary
351 intervention. Of the 20 significantly differentially abundant metabolites shared in human and
352 mouse, 14 (~70%) were further significantly altered by antibiotic treatment to deplete gut bacteria

353 in KD-fed mice (**Figure S6C,** Olson et al., 2018). Taken together, these data indicate that clinical
354 KD regimens alter fecal metabolites in children with refractory epilepsy, a subset of which have
355 the potential to be microbiome-dependent.
356
357 Although there was substantial variability in taxonomic composition of microbiota within mouse
358 recipients of the same experimental condition (**Figure S1-S2**), fecal samples from mouse
359 recipient cohorts exhibited statistically significant alterations in 45 metabolites that were shared
360 when considering all post-KD mouse recipient fecal samples relative to their pre-KD controls
361 (**Table S8, Figure S6A**). Notably, however, none of these 45 differentially abundant metabolites
362 in mouse feces were identical to the 79 differentially abundant metabolites seen in human donor
363 fecal samples (**Figure S6A**), which could reflect host specific metabolite utilization and the fact
364 that recipient mice were fed standard chow (CD), while human donors were consuming a clinical
365 KD at the time of sample collection. To gain insight into whether the differentially abundant
366 metabolites relate to similar biological functions, we performed metabolite set enrichment analysis
367 (MSEA) of the significantly altered metabolites in human vs. mouse (Pang et al., 2021). MSEA of
368 the significantly altered metabolites identified select chemical classes (**Figure 2D**) and metabolic
369 pathways (**Figure 2E**) that were similarly enriched in both human donor and mouse recipient post-
370 KD samples relative to pre-KD controls. In particular, there was shared enrichment of amino acid,
371 hydroxy fatty acid, sugar acid, phenylpropanoic acid, and monosaccharide-related metabolites
372 across post-KD conditions for both human donors and mouse recipients (**Figure 2D**). Differentially
373 abundant metabolites from human post-KD fecal samples also exhibited enrichment of bile acids
374 and other fatty acid derivatives (**Figure 2D, left**), which might reflect KD- and/or microbiome-
375 driven alterations in lipid metabolism (Joyce et al., 2014).
376
377 For metabolic pathways, post-KD samples for both human donor and mouse recipient conditions
378 exhibited differential abundance of metabolites related to methionine metabolism, glycine and

379 serine metabolism, and betaine metabolism (**Figure 2E**). While the biological relevance to KD is
380 unclear, one possibility is that these pathways reflect known influences of the KD on one-carbon
381 (1C) metabolism, a series of interlinking metabolic pathways that control levels of methionine,
382 serine, and glycine, and that integrate nutrient availability with cellular nutritional status (Ducker
383 & Rabinowitz, 2017). In addition, differentially abundant fecal metabolites from mouse post-KD
384 recipients mapped to pathways related to alpha linolenic acid and linoleic acid metabolism, fatty
385 acid biosynthesis, and beta-oxidation of very long chain fatty acids (**Figure 2E, right**), which
386 aligns with the observed metagenomic enrichment in microbial genes related to fatty acid
387 metabolism in response to the clinical KD (**Figure 2A-B**). Some of the differential metabolite
388 chemical subclasses and metabolic pathways in mouse fecal samples were similarly seen in
389 matched mouse serum samples (**Table S9**) – in particular, metabolites representing amino acid,
390 hydroxy fatty acid, and unsaturated fatty acid subclasses, and related to alpha linolenic acid and
391 linoleic acid metabolism, betaine metabolism, and beta-oxidation of fatty acids were altered in
392 both feces and serum of mice receiving post-KD samples relative to pre-KD controls (**Figure**
393 **S6E**). Taken together, these results suggest that the clinical KD induces alterations in the function
394 of the gut microbiome of pediatric epilepsy patients, and that a subset of these functional
395 characteristics may be phenocopied upon microbial transfer to mice, which develop microbiome-
396 dependent resistance to 6-Hz seizures.
397

398 **Transferring the fecal microbiota from KD-treated pediatric epilepsy patients to mice**
399 **induces alterations in brain gene expression**
400

401 Seizures result from atypical neural function related to discharge of electrical signals or failure to
402 constrain the spread of these signals. To gain insight into how colonization with microbes derived
403 from the fecal microbiota of KD-treated individuals may alter brain function to modify seizure
404 susceptibility, we performed transcriptomic profiling of brain tissues from cohorts of mice

405 colonized with microbes from the post-KD human microbiota or pre-KD controls. We focused on
406 the hippocampus and frontal cortex based on their relevance to human epilepsy, their involvement
407 in initiating psychomotor seizures in the 6-Hz seizure assay, and evidence that the microbiome
408 can alter gene expression and metabolites in these brain regions (Chauhan et al., 2022; Suarez
409 et al., 2018). RNA sequencing of hippocampal tissues revealed many differentially expressed
410 genes that were seen in post-KD samples relative to pre-KD controls (**Table S11**), including those
411 related to core cell biological processes relating to RNA processing, translation, cellular stress
412 response, TORC1 signaling, regulation of long-term synaptic potentiation, neuronal development,
413 and response to nutrient levels (**Figure 3A**). The most drastic alterations included upregulation of
414 *Dusp12, Bmpr1b,* and *Cmya5* and downregulation of *Abcc9*, *Ufsp1*, and *Tbx2* transcripts (**Figure**
415 **3B**). *Dusp12* is a dual specificity phosphatase that can dephosphorylate phosphothreonine and
416 phosphoserine (Muda et al., 1999)*, Bmpr1b,* a member of the bone morphogenic receptor family,
417 is a serine/threonine kinase influencing neuronal cell fate (Venugopal et al., 2012), and *Cmya5*
418 encodes for myospyrn which is essential for structural integrity during neuritogenesis (Hsiung et
419 al., 2019). *Abcc9* is an ATP-binding cassette transporter encoding the sulfonylurea receptor 2
420 subunit for potassium channels (Nelson et al., 2015), *Ufsp1* is a Ufm1 specific protease that
421 regulates ubiquitin-like conjugation and has been linked to seizures (Millrine et al., 2022), and
422 *Tbx2* is a transcription factor linked to neuronal cell cycle control and neuroinflammation
423 (Reinhardt et al., 2019). STRING network analysis additionally revealed top protein interaction
424 clusters enriched for essential biological processes including RNA processing, oxidative
425 phosphorylation, and cell cycle regulation, consistent with results from GO enrichment analysis
426 (**Figure 3A, 3C, 3D**), as well as endocytosis and glutathione metabolism (**Figure 3D**).
427
428 Some differentially expressed genes were also identified in frontal cortical tissues of post-KD
429 recipients relative to pre-KD controls (**Table S12**), which similarly to hippocampus, included those
430 related to core cell biological processes for RNA surveillance and catabolism, cellular stress

431 responses, TORC1 signaling, and further included genes related to potassium ion transport, and
432 core carbohydrate metabolism (**Figure S7A**). The most drastic alterations included upregulation
433 of *Serpinb1a, Nqo1,* and *Slc6a12* transcripts, and downregulation of *Aldh3b1, Setmar*, and *Tfb1m*
434 transcripts (**Figure S7B**). *Serpinb1a* is a serine/cysteine protease inhibitor (Huasong et al., 2015),
435 *Nqo1* encodes an antioxidant enzyme that primarily catalyzes the reduction of quinones (Ross &
436 Siegel, 2021), and *Slc6a12* encodes for a betaine-GABA transporter (Zhou et al., 2012). *Aldh3b1*
437 is an aldehyde dehydrogenase linked to oxidative stress reduction (Marchitti et al., 2007), *Setmar*
438 encodes a histone-lysine N-methyltransferase (Cordaux et al., 2006), and *Tfb1m* has been shown
439 to function as methyltransferase (Metodiev et al., 2009). STRING network clustering analysis
440 additionally revealed top protein interaction clusters enriched for transcription regulation,
441 translation, and oxidative phosphorylation, also seen in frontal cortex GO enrichment analysis
442 and in the hippocampal STRING network, as well as clusters enriched for calcium signaling,
443 transcriptional regulation, and translation (**Figure S7C, S7D**). Differentially expressed gene sets
444 from both hippocampus and frontal cortex were enriched for TORC1 signaling, cellular response
445 to stress, and oxidate phosphorylation through GO enrichment and STRING clustering, which
446 have all been shown to affect seizure susceptibility (Chan, 2001; Nguyen & Bordey, 2021). The
447 similarities between transcriptomic results from hippocampus and frontal cortex suggest that
448 colonization with post-KD microbes elicits key alterations in host metabolism that impact core
449 biological processes that are generally consistent across different brain regions. Overall, these
450 results indicate that mice that acquire seizure resistance in response to colonization with microbes
451 from the post-KD human gut microbiota exhibit alterations in hippocampal and frontal cortical
452 gene expression, relative to pre-KD recipient controls.
453
454 **Multi'omics analysis reveals network connections linking microbial genomic pathways**
455 **and metabolites to hippocampal transcripts related to epilepsy**
456

457 To further identify key gut microbial functions that may drive particular brain gene expression
458 signatures, we utilized microbe-metabolite vectors (MMVEC) (Morton et al., 2019) to build an
459 integrated network of fecal metagenomic, fecal metabolomic, serum metabolomic, hippocampal
460 transcriptomic, and frontal cortical transcriptomic datasets from mice inoculated with the human
461 pre-KD or post-KD microbiota (**Table S13**). We generated a parallel network comprised of fecal
462 metagenomic and fecal metabolomic datasets from human pre-KD and post-KD donor stool
463 samples to identify features similarly underscored in both human and mouse networks,
464 suggesting their clinical relevance. The human donor and mouse recipient networks were linked
465 by 3 common nodes - metagenomic pathways describing branched chain amino acid (BCAA)
466 biosynthesis (BRANCHED-CHAIN-AA-SYN-PWY), L-alanine fermentation (PROPFERM-PWY),
467 and co-enzyme A biosynthesis (COA-PWY), as well as pathways for arginine synthesis
468 (ARGSYN-PWY In the human network and ARGSYNSUB-PWY in the mouse network) (**Figure**
469 **4A,** center gray and green nodes). The shared BCAA biosynthesis, co-enzyme A biosynthesis,
470 and arginine synthesis pathways were also identified by weighted key driver analysis as highly
471 interconnected across the 'omics datasets and essential regulator nodes of the network (Ding et
472 al., 2021) (**Figure 4A**, diamonds). The human donor network also contained an additional key
473 driver metagenomic node for isoleucine biosynthesis (ILEUSYN-PWY), which aligns with the
474 metagenomic node for BCAA biosynthesis. Consistent with the shared metagenomic key drivers
475 between mouse and human networks, the human fecal metabolomic module was enriched for
476 nodes related to valine, leucine, and isoleucine (BCAA) and CoA biosynthesis (**Figure 4A**, gray
477 diamond node). In the mouse network, metabolomic modules included nodes related to
478 glycerophospholipid metabolism for fecal metabolites and pentose and glucuronate
479 interconversions for serum metabolites (**Figure 4A,** orange and sea green nodes). Nodes for fecal
480 1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)*, 1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-
481 16:0/16:0)*, 1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*, 3-hydroxybutyrate (BHBA),
482 and myo-inositol (**Figure 4A**, orange metabolite nodes in red font) were similarly identified as

483 differentially abundant in individual metabolomic analyses for recipient post-KD fecal samples
484 relative to pre-KD controls (**Table S7**). These mouse metagenomic and metabolomic modules
485 were linked to 5 transcriptomic modules for hippocampal genes and 3 for frontal cortical genes
486 (**Figure 4A,** bottom section). The hippocampal transcript modules were enriched for nodes related
487 to regulation of telomerase RNA localization to Cajal body, glycosylphosphatidylinositol (GPI)
488 anchor biosynthetic processes, Wnt signaling, and neuron generation and migration (**Figure 4A**).
489 The frontal cortical transcript modules were enriched for nodes related to regulation of catabolic
490 processes, lipase activity, and BCAA transmembrane transporter activity (**Figure 4A**). This
491 suggests that these particular biological processes are most closely associated with the microbial
492 functional features identified in the network. The transcript nodes included 41 hippocampal genes
493 and 4 frontal cortical genes that were similarly identified in individual transcriptomic analyses as
494 differentially expressed in post-KD recipient mice relative to pre-KD controls (**Figure 4A**, transcript
495 nodes in red font). The higher number of connections between metabolomic modules and
496 hippocampal transcripts suggests that the gut microbiome may exhibit a greater regulatory role
497 for the hippocampus than for the frontal cortex in post-KD recipient mice compared to pre-KD
498 controls. Of particular interest are the links between fecal metabolites related to
499 glycerophospholipid metabolism, which are regulated by the microbiome (Zheng et al., 2021), and
500 hippocampal transcript modules enriched for Wnt signaling and GPI anchor biosynthetic
501 processes, pathways implicated in seizure susceptibility (Hodges & Lugo, 2018; Wu et al., 2020).
502
503 To gain insight into whether the hippocampal and frontal cortical transcripts that co-occur with
504 microbial metagenomic and metabolomic features have been implicated in human epilepsy, single
505 nucleotide polymorphisms (SNPs) identified from epilepsy genome-wide association studies
506 (GWAS) were mapped to genes using hippocampus and frontal cortex splicing quantitative trait
507 loci (sQTLs) and expression quantitative trait loci (eQTLs) to represent epilepsy-associated genes
508 informed by GWAS. The mouse orthologs of these human GWAS disease genes were then

509 compared with hippocampal and frontal cortical transcriptomic results to identify DEGs in post-
510 KD vs pre-KD recipients that have been implicated in genetic risk for human epilepsy. There was
511 a statistically significant enrichment of the hippocampal DEGs in the epilepsy GWAS (p=0.003),
512 but no significant enrichment of the frontal cortical DEGs in the epilepsy GWAS (p=0.26) (**Figure**
513 **4B**). These results suggest that microbial alterations in hippocampal gene expression may
514 contribute to the microbiome-dependent increases in seizure resistance seen in post-KD recipient
515 mice compared to pre-KD controls. From the co-occurrence network, 5 hippocampal DEGs were
516 linked to epilepsy GWAS results: *Atp5c*, which encodes mitochondrial ATP synthase; *Rprd2,*
517 which encodes a transcriptional repressor that modulates RNA polymerase II activity; *Gnaz*, which
518 encodes G protein alpha subunit that regulates ion equilibrium and chaperone-mediated folding;
519 *Cfdp1*, which encodes a subunit of the chromatin remodeling complex and is important for cell
520 division, and *Mro*, which encodes a nucleolus protein proposed to be testis-determining in the
521 reproductive tract, but expressed in the nervous system with as yet unknown function. Overall,
522 the multi'omics analysis of human donor and mouse recipient datasets together with epilepsy
523 GWAS mapping to hippocampal and frontal cortical DEGs identified key microbial genomic
524 pathways and microbially modulated metabolites that may contribute to alterations in the
525 expression of particular hippocampal genes in mice that exhibit microbiome-induced protection
526 against 6-Hz seizures.
527
528 **DISCUSSION**
529
530 Results from this research provide evidence from a treatment study of children with refractory
531 epilepsy, coupled with functional testing in gnotobiotic mice, that clinical KD regimens alter the
532 function of the gut microbiome in ways that could contribute to seizure protection. We assessed
533 microbiome composition and function in 10 children with refractory epilepsy shortly before
534 initiating and approximately one month after adherence to classical KD regimens. Following

535 clinical practice, the patient cohort was heterogeneous in type and underlying cause of refractory
536 epilepsy, as well as the ratio of fat to carbohydrate and protein and specific nutritional composition
537 of the KD they consumed (**Table S1**). This highlights the diversity of epilepsies that resist current
538 antiepileptic drugs and the broad range of KD interventions that are administered to treat pediatric
539 refractory epilepsy. Consistent with this heterogeneity, we observed that participants varied
540 substantially in the composition of the fecal microbiota at baseline and in response to KD
541 treatment. There was no clear KD-induced taxonomic signature of the gut microbiota that was
542 shared across the study population, which contrasts prior studies of KD treatments for epilepsy
543 that each reported alterations in the gut microbiota in response to a KD. Our results, however,
544 support the finding that little to no consistency in specific microbial taxa affected exists across
545 studies (Özcan et al., 2022).
546
547 Despite variation in microbiota composition, we observed evidence of shared functional features
548 of the gut microbiome that were seen with KD treatment across participants in the study. This
549 aligns with the notion of functional redundancy of gut microbes, wherein phylogenetically
550 unrelated species can exhibit the same genetically-encoded biological activities (Tian et al.,
551 2020). Results from metagenomic sequencing indicated that microbial genes related to fatty acid
552 β-oxidation, 2-methylcitrate cycle, glycol metabolism, and proline biosynthesis were more highly
553 represented in the gut microbiota of epileptic children after treatment with the KD compared to
554 their internal pre-treatment controls. β-oxidation by select microbes in anaerobic environments
555 enables them to utilize fatty acids from the diet as energy sources, wherein saturated and
556 unsaturated fatty acids are oxidized into acetyl-CoA (Yao & Rock, 2017). β-oxidation of dietary
557 odd-chain fatty acids additionally produces propionyl-CoA, which can be toxic to cells, so the
558 methylcitrate cycle enables microbes to further catabolize propionyl-CoA into pyruvate and
559 succinate (Dolan et al., 2018). Glycol, including glycolate and glyoxylate, metabolism allows
560 microbes to use products from fatty acid oxidation to fuel gluconeogenesis (Ahn et al., 2016).

561 Proline synthesis from the central metabolite glutamate, via intermediates amino acids arginine
562 and ornithine, is widely upregulated in bacteria to counteract growth in osmotically unfavorable
563 conditions (Stecker et al., 2022). The elevated representation of genes related to these pathways
564 in the post-KD samples suggests that the clinical KD shapes the gut microbiome to enrich
565 microbial taxa that digest fat and synthesize carbohydrates under fat-rich, carbohydrate-limited
566 conditions. These metagenomic features of the human microbiome from pediatric epilepsy
567 patients consuming a clinical KD were preserved upon transfer to GF mice that were fed a
568 standard diet, suggesting that the source microbes are maintained under non-ketogenic dietary
569 conditions.
570
571 Aligning with results from metagenomic sequencing, metabolomic profiling of fecal samples from
572 KD-treated epileptic children revealed statistically significant alterations in several metabolites,
573 including subsets of amino acids, sugar acids, hydroxy fatty acids, bile acids, and other fatty acid
574 derivatives, which reflect KD-, and potentially microbiome-, induced alterations in lipid and amino
575 acid metabolism. In particular, glutamate and ornithine, both precursors of proline, were
576 significantly decreased in post-KD human samples, relative to pre-KD controls, which may align
577 with the observed metagenomic alterations in proline biosynthesis pathways. These metabolite
578 alterations were induced by KD consumption in mice, and modified by microbiota depletion in
579 mice, suggesting a causal response to the clinical KD in the human cohort that is dependent on
580 the gut microbiome. Microbially modulated increases in palmitoleoylcarnitine (C16:1) were also
581 seen in KD-fed mice and in post-KD human samples, alongside several other lipid species,
582 aligning with the high fat content of the KD and roles for the gut microbiome in lipid metabolism
583 (Joyce et al., 2014).
584
585 The individual metabolite changes seen in human donors, including those induced by KD in a
586 microbiome-dependent manner, were not specifically recapitulated by microbiome transfer to GF

587 mice that were fed standard chow. This is perhaps not surprising given the important role of
588 dietary composition in driving microbial activity (David et al., 2014). While no specific metabolite
589 shifts were shared, a few pathway-level metabolomic changes were consistent between post-KD
590 fecal samples from human donor (consuming the clinical KD) and mouse recipients (fed standard
591 chow), relative to their respective pre-KD controls. Namely, differentially abundant metabolites
592 related to metabolism of methionine, glycine, serine, and betaine were shared across post-KD
593 conditions for human donor and microbiota-recipient mice. Methionine metabolism involves the
594 production of homocysteine, adenosine, cysteine, and alpha-ketobutyrate, which can then be
595 routed to glucogenic pathways by conversion to propionyl- and succinyl-CoA. Serine, synthesized
596 via glycerate, is used to create glycine (and cysteine) via the homocysteine cycle, which can
597 undergo microbial conversion into pyruvate or glyoxylate. Betaine (trimethylglycine), derived from
598 diet or synthesized from choline, is metabolized by the gut microbiome (Koistinen et al., 2019)
599 and functions as a methyl donor in transmethylation reactions, including those involved in
600 methionine metabolism. While the relevance to KD and seizure protection is unclear, alterations
601 in peripheral and central amino acid metabolism have been widely implicated in mediating the
602 anti-seizure effects of the KD (Yudkoff et al., 2001). In addition, post-KD samples from both human
603 donors and mouse recipients exhibited alterations in chemicals related to lipid metabolism, such
604 as hydroxy fatty acids. The shared metabolite pathway- and chemical subclass-level features may
605 reflect changes that are induced by KD in humans and generally phenocopied by gut microbes
606 upon transfer to mice reared under non-ketogenic conditions. This suggests that transfer of
607 clinical KD-induced gut microbes to mice maintained under non-ketogenic conditions could result
608 in molecular outputs that are distinct, but functionally similar, to those seen in the donor human
609 sample.
610
611 We observed that inoculating mice with human fecal samples collected after clinical KD treatment
612 conferred resistance to 6-Hz seizures compared to controls that received the baseline pre-

613 treatment (pre-KD) microbiota. There was no correlation with patient responsiveness to diet, as
614 indicated in clinician notes taken at 1 month after adherence to the clinical KD. This may be due
615 to the unreliability of the metric, which was based on parental reporting, as well as the cross-
616 sectional nature of the assessment, given inter-individual variation in latency to respond to KD
617 treatments and the patient's peak KD ratio. These concerns aside, the results highlight the
618 importance of host determinants of KD responsiveness, some of which may mask or block any
619 beneficial influences of the KD-associated microbiota. Many patients included in this study
620 exhibited genetic bases for refractory epilepsy, some of which could be epistatic to functional
621 genomic changes in the KD-associated gut microbiome. Large human studies that subclassify
622 different types of epilepsies and seizure semiologies are warranted to study potential roles for the
623 gut microbiome in modifying or predicting responsiveness to the KD.
624
625 The microbiota-dependent increases in seizure protection were associated with brain
626 transcriptomic alterations. In particular, both hippocampus and frontal cortex from post-KD
627 recipient mice exhibited enrichment of differentially expressed genes related to i) RNA processing,
628 transcriptional regulation, and translation ii) TORC1 signaling and cell cycle, and iii) oxidative
629 phosphorylation and cellular stress response (i.e., nitric oxide, reactive oxygen species), when
630 compared to controls colonized with pre-KD microbiota from both GO enrichment and STRING
631 network analyses. Neuronal excitability requires protein synthesis in response to altered neuronal
632 stimulation, and risk factors for various epilepsies include dysregulation of RNA processing, RNA
633 stability, transcription, and translation (Malone & Kaczmarek, 2022). TORC1 is a major nutrient-
634 and energy-sensing serine/threonine kinase complex that controls cell growth and differentiation
635 by coordinating core processes of transcription, translation, and autophagy. Abnormal regulation
636 of TORC1 signaling has been implicated in a wide variety of epilepsies, and as such, is a
637 therapeutic target of interest for treating refractory epilepsies (Nguyen & Bordey, 2021). Previous
638 studies have reported inhibitory effects of the KD and select fatty acids on TORC1 activity

639 (McDaniel et al., 2011; Warren et al., 2020), suggesting that it may contribute to the anti-seizure
640 effects of the KD. Oxidative phosphorylation is a central process for cellular energy metabolism
641 from nutrients, that generates as a byproduct reactive oxygen species (ROS) (Rowley & Patel,
642 2013) and is regulated by the retrograde glutamatergic neurotransmitter nitric oxide (NO). In
643 animal epilepsy models, both ROS and NO are elevated during seizure activity due to oxidative
644 stress-associated neuronal death (Zhu et al., 2017), which can further contribute to
645 epileptogenesis (Chan, 2001). The KD has been previously reported to reduce oxidative stress
646 by promoting antioxidant enzymatic activity and scavenging ROS (Greco et al., 2016). Overall,
647 these results suggest that the KD-associated human gut microbiota alters brain transcriptional
648 pathways that may contribute to protection against 6-Hz seizures in mice.
649
650 Integration of multi'omics datasets across human donor and mouse recipients revealed network
651 associations between select gut microbial metagenomic pathways, fecal metabolites, serum
652 metabolites, and hippocampal transcripts, suggesting that they may contribute to the microbiome-
653 dependent increases in seizure protection seen in mice inoculated with human post-KD
654 microbiota, compared to pre-KD controls. The human donor and mouse recipient co-occurrence
655 networks were linked by shared metagenomic pathway nodes related to BCAA biosynthesis, CoA
656 biosynthesis, L-alanine fermentation, and L-arginine biosynthesis. Key drivers for BCAA, CoA,
657 and L-arginine biosynthesis were linked to hippocampal transcript modules enriched for genes
658 related to neurogenesis and Wnt signaling. BCAAs modulate brain import of precursors required
659 for synthesis of monoamine transmitters (Larsson & Markus, 2017; Salcedo et al., 2021; Song et
660 al., 2017). BCAAs also serve as nitrogen donors for synthesis of glutamate vs. GABA, and as
661 such regulates synaptic balance between excitation and inhibition, a key determinant of seizure
662 susceptibility (McKenna et al., 2019). Wnt signaling regulates calcium pathways that are important
663 for hippocampal neurogenesis and dendrite formation, and is increasingly linked to early
664 epileptogenesis (Hodges and Lugo, 2018). Additionally, mapping GWAS-based risk genes to the

665 co-occurrence network identified five hippocampal nodes as linked to epilepsy. Of particular
666 interest was *Gnaz*, which encodes G protein alpha-Z, a protein that mediates neuronal signal
667 transduction within the hippocampus (Jang et al., 2018) and is proposed to modulate seizure
668 susceptibility (Hultman et al., 2019). BCAA derivatives have been reported to promote
669 phosphorylation of G-proteins, and abnormalities in GPCR mediated neuronal signaling can
670 contribute to increased susceptibility to seizure (Shellhammer et al., 2017; Yu et al., 2019).
671 Altogether, results from this study reveal that the clinical KD regimens used to treat pediatric
672 refractory epilepsy are associated with alterations in the function of the child microbiome, which
673 causally modify brain function and seizure susceptibility upon transfer to mice. Further research
674 is warranted to define the mechanisms by which the human KD-associated microbiome signals
675 across the gut-brain axis to modify seizure risk, and to further assess the potential for identifying
676 microbiome-based interventions that could increase the efficacy of KD treatment, alleviate dietary
677 side effects, and/or ease clinical implementation.
678

679 **LIMITATIONS OF STUDY**

680

681 A key limitation of this study design is the prioritization of experimental reproducibility, which
682 included cohorts of 14-16 mice per patient sample, over patient sample size, which included 10
683 children with refractory epilepsy, each sampled before and at approximately 1 month after
684 adherence to a clinical KD. We reasoned that by internally controlling for baseline microbiota for
685 each patient, we could effectively evaluate microbial alterations in response to the clinical KD
686 within a relatively small patient group. We further posited that this study design would enable us
687 to sample from a heterogenous patient population reflective of the etiopathological variation
688 typically seen in refractory epilepsy. It would also us to be inclusive of the wide range of individuals
689 with pediatric epilepsy who typically seek clinical KD treatment. This level of diversity in a small
690 patient population may have contributed to our finding that there was no shared taxonomic

691 response of the gut microbiome to the clinical KD, despite some shared functional genomic
692 features when considering all post-KD microbiota relative to all pre-KD controls.
693
694 Additional constraints of the study, as discussed in the main text, are the inherent technical and
695 biological shortcomings in “transplanting” microbiota across different mammalian hosts. In this
696 study, we achieved levels of human-to-mouse microbiota “transplant” fidelity analogous to those
697 reported in existing literature even when we maintained mice on a conventional rather than
698 ketogenic diet (Bokoliya et al., 2021; Kennedy et al., 2018; Walter et al., 2020). However, the
699 discrepancies between recipient and donor microbiota draw into question the relevance of
700 findings in gnotobiotic mice to the human condition. To help mitigate this, we focused entirely on
701 features of the gut microbiome that were differential between post-KD and pre-KD conditions and
702 shared between human donors and mouse recipients. However, we acknowledge that artifacts of
703 the microbiota transfer approach, which are not relevant to the clinical condition, may contribute
704 to the microbiome-dependent functional differences observed in the gnotobiotic mouse
705 experiments in this study. Nevertheless, the observed results provide important proof-of-principle
706 that differences in the function of the gut microbiota regulate seizure susceptibility.
707
708 In assessing causal relationships between the KD-associated microbiome and host physiologies
709 linked to seizure susceptibility, we made the major assumption that there exists a singular
710 microbiome-dependent mechanism to increase seizure threshold that was common across all
711 post-KD mouse recipient cohorts relative to all pre-KD cohort controls. Our analysis does not take
712 into account the possibility that there are multiple microbiome-dependent mechanisms that are
713 distinct and that each result in resistance to 6-Hz seizures. Expanded studies that involve
714 subclassification of the human participants and/or mouse recipients would aid in addressing this
715 prospect.
716

717 Moreover, we chose to study the 6-Hz psychomotor seizure model based on its widespread use
718 as a model of refractory epilepsy (Kehne et al., 2017), its utility for screening novel antiepileptic
719 drugs (Barton et al., 2001), and its responsiveness to the KD (Hartman et al., 2008). We also
720 reasoned that its measure of acutely induced seizures would preclude confounding effects of
721 chronic genetic mutation or kindling-based models on modifying the gut microbiome (Löscher,
722 2017). Further research is needed to assess roles for the KD-induced gut microbiome in modifying
723 seizures across additional epilepsy models to determine whether particular seizure semiologies
724 or types of epilepsy are more amenable to modulation by the gut microbiome.
725
726 In light of the aforementioned heterogeneity in patient population, small sample size, variation in
727 clinically-guided KD regimens, discrepancies introduced by the microbiota "transplantation"
728 approach, cross-species and diet comparisons (i.e., human on KD, mouse on standard diet), and
729 assumptions adopted for data analysis, our statistical analyses for shotgun metagenomic,
730 untargeted metabolomic, and bulk transcriptomic data were performed with lenient thresholds for
731 differential abundance ($p<0.05$), with a focus on pathway-level signatures that were dependent
732 upon the clinical KD and conferred by the KD-associated microbiota. Notably, for all animal
733 experiments, technical replicates per donor sample were averaged, and only the biological (i.e.,
734 donor) N was used for statistical analysis. Despite the expected variability, we detected consistent
735 KD-dependent alterations in microbial genes and metabolites in epileptic children undergoing
736 dietary treatment, and further observed KD- and microbiome-dependent alterations in
737 metagenomic pathways and metabolomic pathways that were shared across human donor and
738 microbiome-recipient mice when using these parameters. Brain transcriptomic signatures were
739 seen when comparing all mouse recipient cohorts receiving the post-KD microbiome (all of which
740 exhibited resistance to 6-Hz seizures) relative to those receiving the pre-KD controls. Finally,
741 results for all seizure testing experiments, which revealed shared phenotypic outcomes for post-
742 KD groups compared to pre-KD groups, were well-powered and analyzed according to

743 conventional statistical methods (Festing & Altman, 2002). All caveats considered, the results
744 from this study extend existing pre-clinical research to provide initial evidence that clinical KD
745 treatments shape the function of the gut microbiome of children with refractory epilepsy in ways
746 that have the potential to causally modify seizure susceptibility. Continued research is warranted
747 to elucidate the particular microbial functional activities that act together to modify signaling across
748 the gut-brain axis to promote seizure protection and to further assess the potential to apply
749 microbiome-based interventions to treat refractory epilepsy.
750

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762

763 **AUTHOR CONTRIBUTIONS**

764 G.R.L., S.M.H, C.A.O., M.B., and J.P. performed the experiments and analyzed the data, B.R.,
765 and J.H.M. led the clinical study. G.R.L., C.A.O., J.H.M., X.Y., and E.Y.H. designed the study,
766 G.R.L. and E.Y.H. wrote the manuscript. All authors discussed the results and commented on the
767 manuscript.
768

DECLARATION OF INTERESTS

Findings reported in the manuscript are the subject of provisional patent application US 63/285,267, owned by UCLA. E.Y.H. has financial interests in Bloom Science. All other authors declare that they have no competing interests.

DIVERSITY AND INCLUSION

We worked to ensure sex balance in the selection of human subjects. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

FIGURES AND FIGURE LEGENDS

STAR★METHODS

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

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801 with epilepsy GWAS
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803

804 ## 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.re	(Ding et al.,

	search.idre.ucla.edu/	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

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elaine Hsiao (ehsiao@g.ucla.edu)

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Data from 16S rRNA gene sequencing, metagenomic profiling, and associated metadata are presented in Tables S2, S3, S5, and S6 are available online through the QIITA repository (https://qiita.ucsd.edu/) with the study accession #14928. Metabolomic data are presented in Tables S7, S8, S9, and S10 and are available online through Mendeley data with DOI:10.17632/djzyzdbz3z.1. Transcriptomic data are presented in Tables S11 and S12 and available online through Gene Expression Omnibus repository with the identification number

822 #GSE225682.

823

824 ## EXPERIMENTAL MODELS AND SUBJECT DETAILS

825 ### Human Subjects

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

844

845 ### Human stool sample collection

846 For in-patient fecal sample collection, once a study participant was admitted to the hospital during
847 the pre-diet initiation visit, they were given a coded stool collection kit and sterile specimen

848 container. Stool samples were freshly collected within 1 day prior to starting the clinical KD
849 treatment (pre-KD). Fresh stool samples were immediately placed on dry ice for short term
850 storage and transportation and were freshly frozen at -80°C for long-term storage. Post-KD stool
851 samples were collected in the same manner as stated above when the study participant returned
852 for the 1-month follow-up visit. For out-patient collection of the post-KD stool sample, which was
853 necessitated because of hospital pandemic policies, a deidentified stool sample collection kit and
854 sterile specimen cup was provided to the patient and guardian along with a pre-labeled return
855 shipping box. After 1 month of the clinical KD treatment, stool samples were collected in a sterile
856 specimen cup, immediately placed in an at home freezer, and the next day either (1) shipped
857 back overnight to UCLA on dry ice or (2) brought with the patient to their 1-month follow-up
858 appointment. Fresh frozen fecal samples were homogenized under liquid nitrogen and 3 ~500 mg
859 aliquots were made per sample by sterile storage in anaerobic Balch tubes to be used for
860 transplantation, metagenomic, and metabolomic studies.
861
862 **Mice**
863 6-8 week old wild-type germ-free Swiss Webster mice (Taconic Farms), were bred in UCLA's
864 Center for Health Sciences Barrier Facility. Breeding animals were fed ''breeder'' chow (Lab Diets
865 5K52). Experimental animals were fed vitamin- and mineral-matched control diet (Harlan Teklad
866 TD.150300). Juvenile mice were used to mimic the age range of the human donor population
867 (<10 years old). All animal experiments were approved by the UCLA Animal Care and Use
868 Committee.
869
870 **METHOD DETAILS**
871
872 **16S rRNA Gene Sequencing and Analysis**

873 Bacterial genomic DNA was extracted from human or mouse fecal samples using the Qiagen
874 PowerSoil Kit. For human samples, the n reflects one donor sample. For mouse samples, the n
875 reflects independent cages containing 3 mice per cage to preclude effects of co-housing on
876 microbiota composition. The sequencing library was generated in line with (Caporaso et al.,
877 2011). PCR amplification, run in triplicate, of the V4 region of the 16S rRNA gene was completed
878 using individually barcoded universal primers and 30 ng of the extracted genomic DNA. The PCR
879 product triplicates were pooled and purified using the Qiaquick PCR purification kit (Qiagen).
880 Samples were sequenced using the Illumina MiSeq platform and 2 x 250bp reagent kit for paired-
881 end sequencing at Laragen, Inc. Amplicon sequence variants (ASVs) were chosen by closed
882 reference clustering based on 99% sequence similarity to the SILVA138 database. Taxonomy
883 assignment, rarefaction, and differential abundance testing were performed using QIIME2 2022.2
884 (Bolyen et al., 2019; Mandal et al., 2015).
885
886 **Fecal Shotgun Metagenomics**
887 Bacterial genomic DNA was extracted from human or mouse fecal samples using the Qiagen
888 PowerSoil Kit. 1 ng of DNA was used to prepare DNA libraries using the Nextera XT DNA Library
889 Preparation Kit (Illumina) and genomic DNA was fragmented with Illumina Nextera XT
890 fragmentation enzyme. IDT Unique Dual Indexes were added to each sample before 12 cycles of
891 PCR amplification. AMpure magnetic Beads (Beckman Coulter) were used to purify DNA libraries
892 which were eluted in QIAGEN EB buffer. Qubit 4 fluorometer and Qubit dsDNA HS Assay Kit
893 were used for DNA library quantification. Libraries were then sequenced on Illumina HiSeq 4000
894 platform 2x150bp at a 6M read depth using by CosmosID. Metagenomic data was analyzed using
895 HUMAnN 3.0 (Beghini et al., 2021) and MetaCyc database to profile gene families and pathway
896 abundance. File2meco R package was used for MetaCyc pathway hierarchical classification (Liu
897 et al., 2022). MaAsLin 2.0 (Mallick et al., 2021) was used to assess significant pathway
898 associations between pre-KD and post-KD with a p-value cutoff of 0.1, where $p < 0.05$ pathways

899 are indicated in the figure by asterisk. Heatmaps were generated using the pheatmap v1.0.12
900 package for R.
901
902 **Human Donor Fecal Microbiota Transfer**
903 To prepare collected human stool samples for transplantation studies, the frozen stool sample
904 was pulverized into a powder under liquid nitrogen stream in a sterile heavy-duty foil covered
905 mortar and pestle, aliquoted at 500 mg per tube into 2mL screw cap tubes, and frozen at -80C.
906 A single 500 mg aliquot of human stool sample was entered into a Coy anaerobic chamber and
907 resuspended in pre-reduced 1x PBS + 0.05% L-cysteine. The sample was homogenized using
908 sterile borosilicate glass beads and passed through a 100um filter. GF Swiss Webster mice were
909 colonized by oral gavage of 200 ul fecal suspension. Excess fecal suspension was resuspended
910 and stored at -80C in pre-reduced 1x PBS + 0.05% L-cysteine + 15% glycerol. For administration
911 of fecal filtrates, the fecal suspension was passed through a sterile 0.2 um filter before colonization
912 via oral gavage using 200 ul fecal filtrate.
913
914 **6-Hz Psychomotor Seizure Assay**
915 6-Hz psychomotor seizure assay testing was conducted following Samala et al., 2008. One drop
916 (~50 ul) of 0.5% tetracaine hydrochloride ophthalmic solution was applied to the corneas of each
917 mouse 15 min before stimulation. A thin layer of electrode gel (Parker Signagel) was applied
918 directly to the corneal electrodes and was reapplied before each trial. A constant-current current
919 device (ECT Unit 57800, Ugo Basile) was used to deliver current through the corneal electrodes
920 at 3s duration, 0.2 ms pulse-width and 6 pulses/s frequency. CC50 (the milliamp intensity of
921 current required to elicit seizures in 50% of the mouse cohort) was measured as a metric for
922 seizure susceptibility. Pilot experiments were conducted to identify 28 mA as the CC50 for SPF
923 wild-type Swiss Webster mice, aged 6-8 weeks. Each mouse was seizure-tested only once, and
924 thus at least n > 14 mice were used to adequately power each cohort. To determine CC50s for

925 each tested cohort, 28 mA of current was administered to the first mouse per cohort, followed by
926 stepwise fixed increases or decreases by 2 mA intervals. Mice were restrained manually during
927 stimulation and then released into a new cage for behavioral observation. Quantitative measures
928 for falling, tail dorsiflexion (Straub tail), forelimb clonus, eye/vibrissae twitching and behavioral
929 remission were scored manually. For each behavioral parameter, we observed no correlation
930 between percentage incidence during 28+ mA seizures between pre-KD or post-KD microbiota
931 status, suggesting a primary effect of the microbiota on seizure incidence rather than presentation
932 or form. Latency to exploration (time elapsed from when an experimental mouse is released into
933 the observation cage (after corneal stimulation) to its first lateral movement) was scored manually
934 with an electronic timer. Mice were blindly scored as protected from seizures if they did not show
935 seizure behavior and resumed normal exploratory behavior within 10 s. Seizure threshold (CC50)
936 was determined as previously described (Kimball et al., 1957), using the average log interval of
937 current steps per experimental group, where sample n is defined as the subset of animals
938 displaying the less frequent seizure behavior. Data used to calculate CC50 are also displayed as
939 latency to explore for each current intensity, where n represents the total number of biological
940 replicates per group regardless of seizure outcome.
941

942 **Antibiotic Treatment**

943 Transplanted mice were gavaged with a solution of vancomycin (50 mg/kg), neomycin (100
944 mg/kg) and metronidazole (100 mg/kg) every 12 hours daily for 5 days, as adapted from (Reikvam
945 et al., 2011). Ampicillin (1 mg/ml) was provided *ad libitum* in drinking water. For mock treatment,
946 mice were gavaged with a similar volume of 1x PBS (vehicle) water every 12 hours daily for 7
947 days. Antibiotic-treated mice were maintained in sterile caging with sterile food and water and
948 handled aseptically for the remainder of the experiments.
949

950 **Fecal and Serum Metabolomics**

951 Previously collected human donor fecal samples were aliquoted as described in section “Human
952 Donor Fecal Microbiota Transfer”. Mouse fecal samples were collected from mice housed across
953 independent cages, with four cages housing 3 mice and one cage housing 2 mice. Mouse serum
954 samples were collected by cardiac puncture and separated using SST vacutainer tubes, then
955 frozen at -80C. Samples were prepared using the automated MicroLab STAR system (Hamilton
956 Company) and analyzed on GC/MS, LC/MS and LC/MS/MS platforms by Metabolon, Inc. Protein
957 fractions were removed by serial extractions with organic aqueous solvents, concentrated using
958 a TurboVap system (Zymark) and vacuum dried. For LC/MS and LC-MS/MS, samples were
959 reconstituted in acidic or basic LC-compatible solvents containing > 11 injection standards and
960 run on a Waters ACQUITY UPLC and Thermo-Finnigan LTQ mass spectrometer, with a linear
961 ion-trap frontend and a Fourier transform ion cyclotron resonance mass spectrometer back-end.
962 For GC/MS, samples were derivatized under dried nitrogen using bistrimethyl-silyl-
963 trifluoroacetamide and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-
964 quadrupole mass spectrometer using electron impact ionization. Chemical entities were identified
965 by comparison to metabolomic library entries of purified standards. Following log transformation
966 and imputation with minimum observed values for each compound, post-KD vs. pre-KD
967 comparisons for human fecal, and mouse serum and fecal data were analyzed by paired t-test.
968 Metabolomic data from SPF or antibiotic-treated mice fed KD vs. CD chow were acquired from
969 (Olson et al., 2018), as log transformed and imputed with minimum observed values for each
970 compound. Data were analyzed using two-way ANOVA to test for group effects. P and q-values
971 were calculated based on two-way ANOVA contrasts. Principal components analysis was used
972 to visualize variance distributions. Supervised Random Forest analysis was conducted to identify
973 metabolomics prediction accuracies. Metabolite set enrichment analysis (MSEA) using the
974 Metaboanalyst 5.0 platform (Pang et al., 2021) was performed on human fecal, mouse fecal, and
975 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

1002 before constructing networks; this step was not used for RNAseq data. Standard WGCNA steps
1003 were then carried out for the filtered metabolomics and RNAseq data. Module eigengenes (MEs),
1004 or the first axis of principal component were calculated from each module. MEs were then targeted
1005 for correlation analysis with the metadata (pre-KD vs. post-KD and responder vs. non-responder).
1006 Modules that had significant correlation (p-val <0.05) with the metadata were chosen for
1007 subsequent integrative analysis.
1008
1009 A systematic network that combined all omics data was inferred based on the probability of co-
1010 occurrence (POC) between molecules from different omics data. To calculate POC, we leveraged
1011 a neural-net based tool called MMVEC v1.0.6 with default parameters (Morton et al., 2019). The
1012 subset of raw data that contains module components that were selected from WGCNA analysis
1013 were log normalized and combined based on sample ID. This combined data matrix was then
1014 used as input for MMVEC. For example, on donor side, modules from fecal metagenome and
1015 metabolomics were added together and, on the recipient side, the combined matrix contained the
1016 raw data from metagenome, metabolomics, and RNAseq. Due to high density of the overall
1017 network generated from MMVEC, the top 10% of POC connections were retrieved to minimize
1018 overall complexity of the network for both donors and recipients using in-house python script
1019 (https://github.com/smha118/keto_diet_pediatric_epilepsy).
1020
1021 The networks of modules from individual omics layers from donor (metagenome, metabolomics)
1022 and recipient (metagenome, metabolomics, RNAseq) as well as differentially
1023 expressed/abundance molecules were then seeded into Mergeomics v3.16 pipeline along with
1024 the integrated network generated with MMVEC for weighted key driver analysis (wKDA) to identify
1025 key drivers of the networks (Ding et al., 2021). wKDA uses a $\chi 2$ -like statistic to identify molecules
1026 that are connected to significant larger module components than what would be expected by
1027 random chance. The analysis was done on the human and mouse networks separately. To further

1028 look into the network that are relevant to ketogenic diet and epilepsy, we selected key drivers
1029 (KDs) based on i) the number of modules that a key driver was invoked related to, ii) their relation
1030 to the Ketogenic diet or epilepsy. A subset of nodes in each module that were connected to the
1031 KDs were collected. These nodes were retrieved with following priority i) they are part of
1032 differentially regulated molecules ii) POC value with KDs. Finally, the network was visualized
1033 using Cytoscape (Shannon et al., 2003). To minimize overall density of the network, we chose to
1034 show the key drivers Mergeomics with the highest occurrence in their respective MEs and with >
1035 5 degrees of connectivity.
1036

1037 **Marker set enrichment analysis (MSEA) to connect hippocampus and frontal cortex**
1038 **DEGs with epilepsy GWAS**

1039 To assess the potential role of the DEGs from the hippocampus and frontal cortex in epilepsy, we
1040 collected the summary statistics of the latest epilepsy GWAS (Abou-Khalil et al., 2018). Single
1041 nucleotide polymorphisms (SNPs) that had a linkage disequilibrium of $r^2 > 0.5$ were filtered to
1042 remove redundancies. To map the epilepsy GWAS SNPs to genes, we used GTEx version 8
1043 eQTL and sQTL data for brain hippocampus and brain frontal cortex (Aguet et al., 2020), which
1044 help us derive genes likely to be regulated by the SNPs. We next used the MSEA function of the
1045 Mergeomics package (Ding et al., 2021) to compare epilepsy disease association p-values of the
1046 SNPs representing the DEGs (hippocampus or frontal cortex) with those of the SNPs mapped to
1047 random genes to assess whether the DEGs contain SNPs that show stronger epilepsy association
1048 than random genes using a χ^2-like statistic.
1049

1050 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1051 Statistical analyses were conducted using Prism8 software v8.2.1 (GraphPad). Before statistical
1052 analysis, data was assessed for distribution to determine appropriate statistical tests to use. Data
1053 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".

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and fourteen tables that contain source data and can be found with this article.

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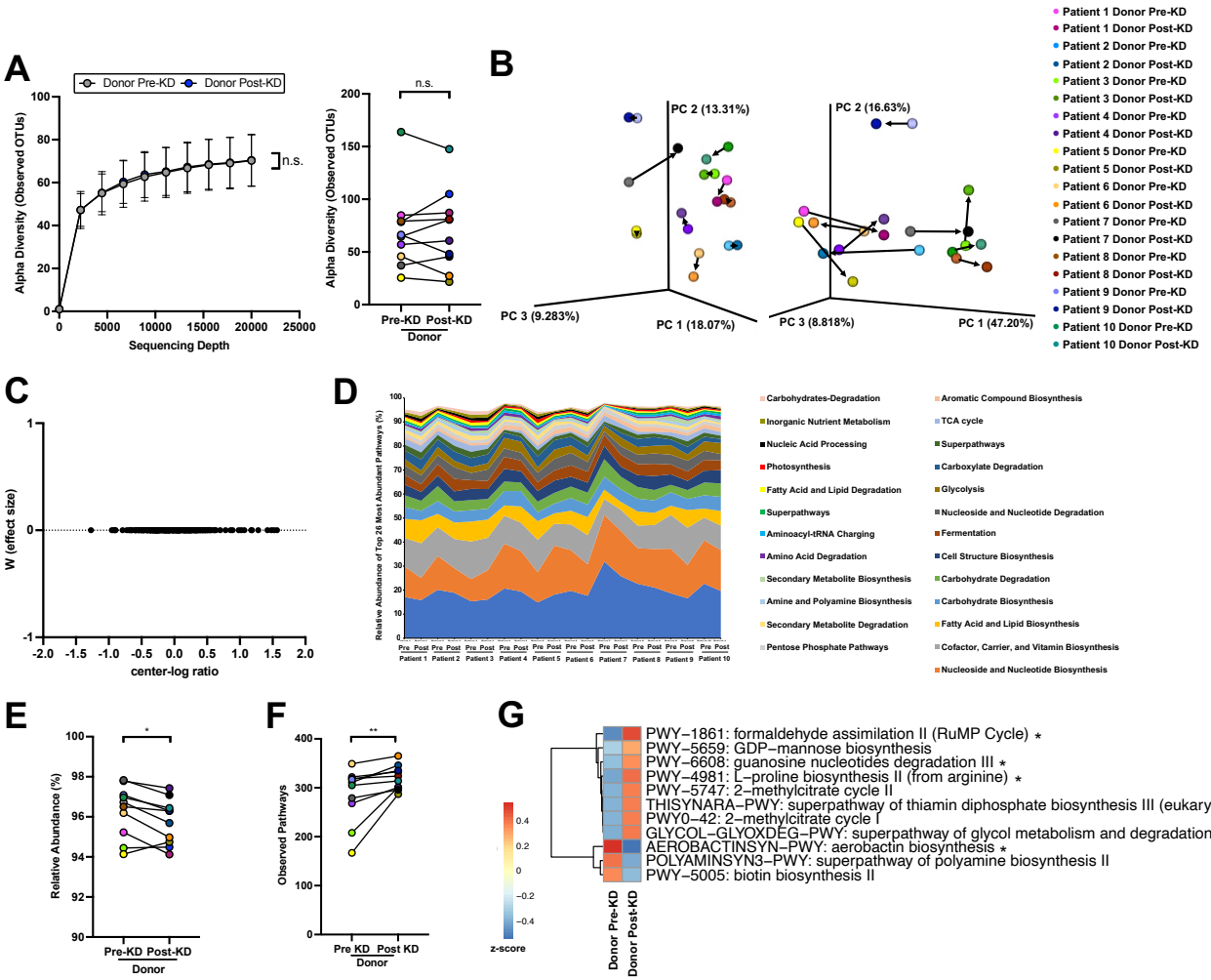


Figure S1: Clinical KD is associated with alterations in the functional potential, but not composition, of the gut microbiota in a cohort of children with refractory epilepsy, Related to Figure 1. **(A)** Alpha diversity as measured by rarefaction curve (left) and observed OTUs (right) of matched donor 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.

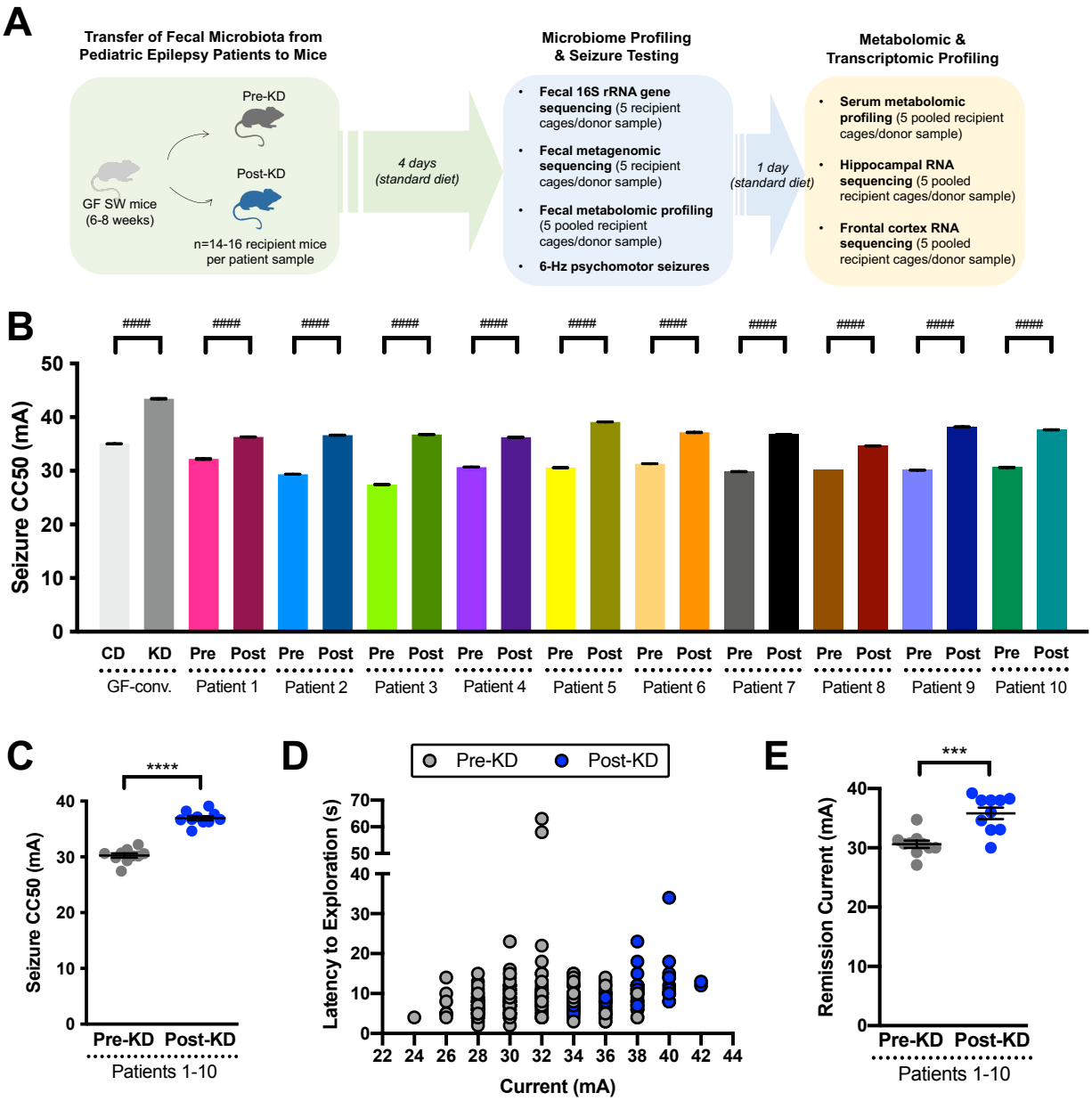


Figure 1: Transfer of the clinical KD-associated gut microbiota from pediatric epilepsy patients to mice confers resistance to 6-Hz seizures. **(A)** Experimental schematic for transplantation of human donor fecal microbiota samples into germ-free (GF) Swiss Webster mice for 6-Hz psychomotor seizure 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)** Latency to exploration for all individual pre-KD (n=140) and post-KD (n=141) recipient mice. **(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), ***$p < 0.001$, ****$p < 0.0001$

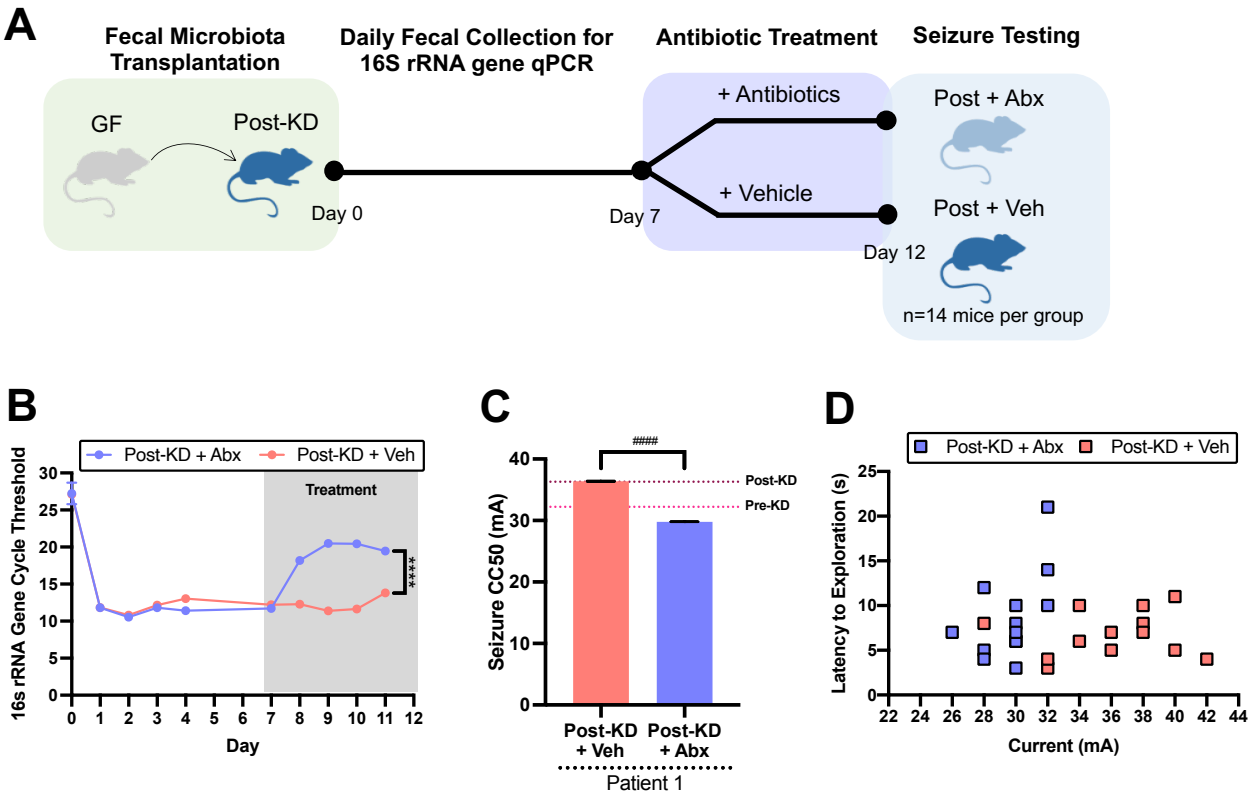


Figure S2: Antibiotic treatment abrogates the seizure protective effects of inoculation with the clinical KD-associated human gut microbiome, Related to Figure 1. (A) Experimental schematic for transfer of human donor fecal microbiota samples to germ-free (GF) mice, followed by 5 days of oral antibiotic (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).

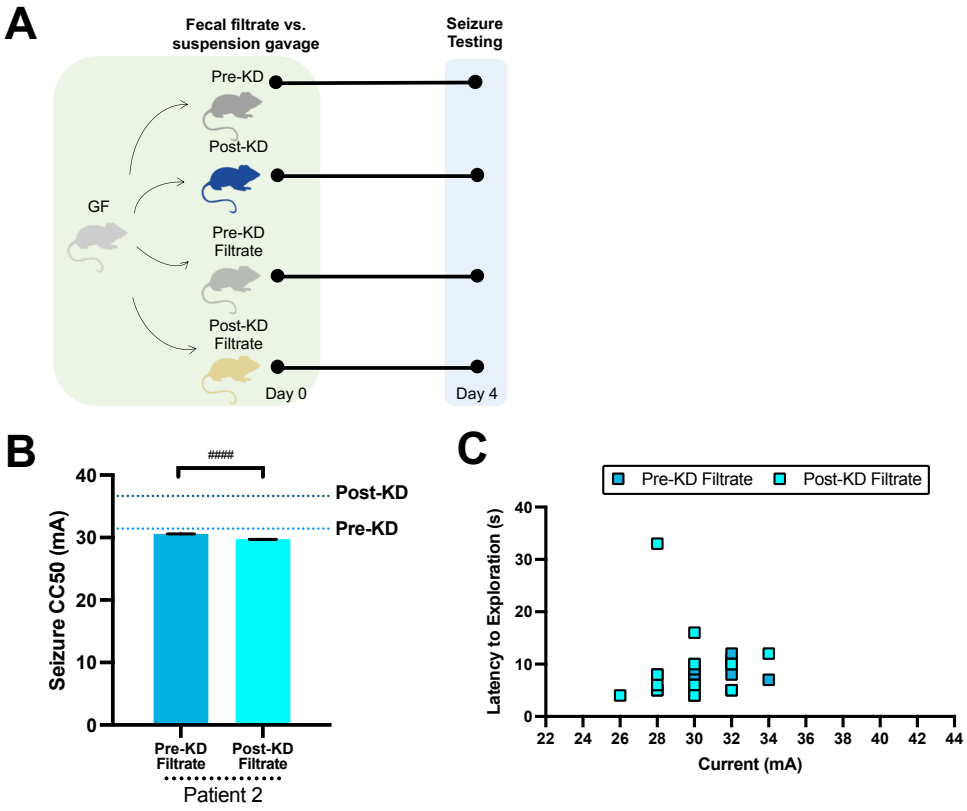


Figure S3: Sterile filtration prevents the seizure protective effects of transfer of the clinical KD-associated human gut microbiome, Related to Figure 1. **(A)** Experimental design for administration of human donor fecal filtrate samples to germ-free (GF) mice, followed by 6-Hz seizure testing 4 days 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).

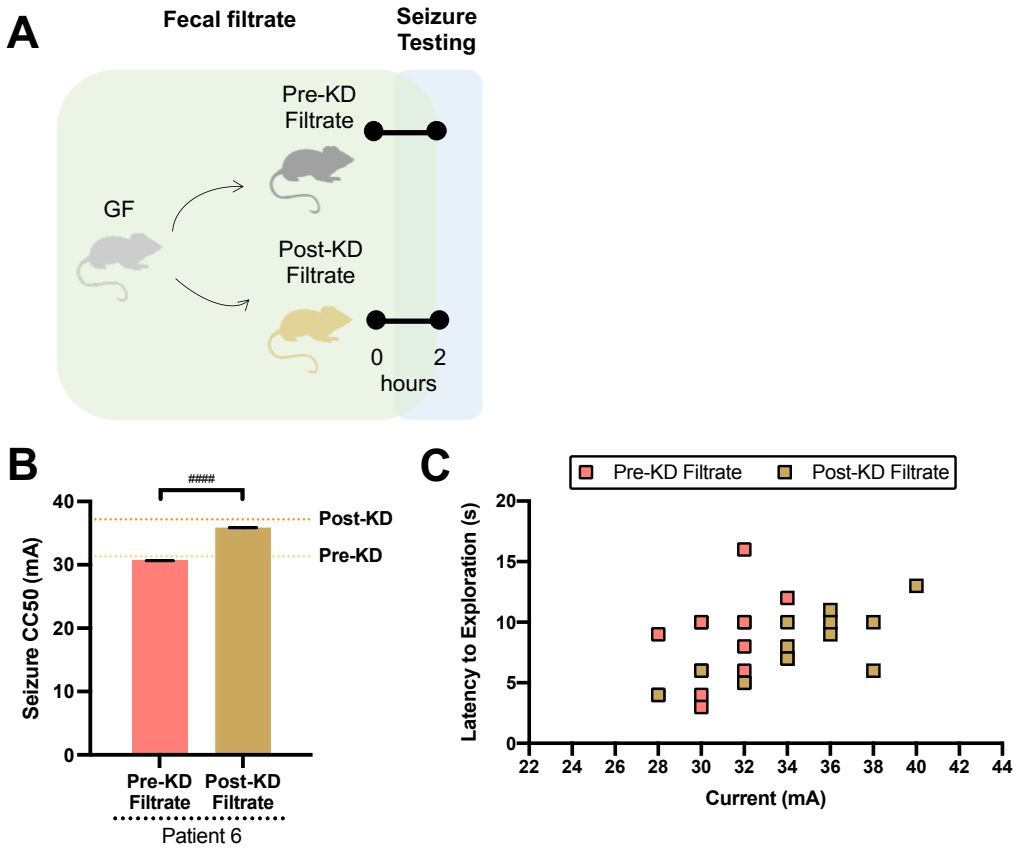


Figure S4: Small molecules from the clinical KD-associated human gut microbiome confer acute seizure protection, Related to Figure 1. **(A)** Experimental design for administration of human donor fecal filtrate samples to germ-free (GF) mice, followed by 6-Hz seizure testing 2 hours later. **(B)** Seizure 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).

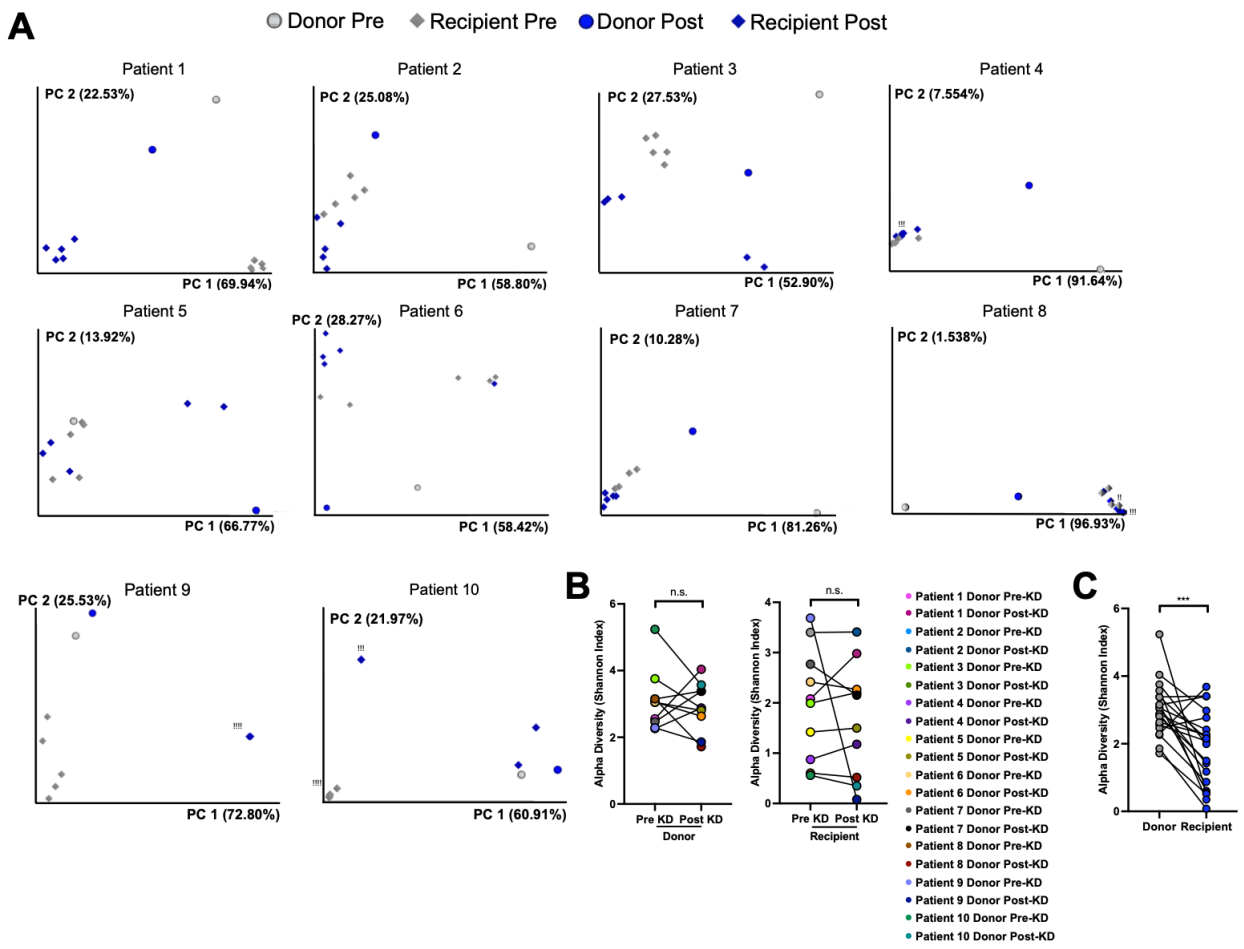


Figure S5: Taxonomic fidelity of human microbiota transfer to mice, Related to Figure 1. **(A)** Principal coordinates analysis of weighted UniFrac distances from 16S rRNA gene sequencing of fecal samples from matched human donors and mouse recipients (for each graph: n = 1 donor patient (10 patients 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.

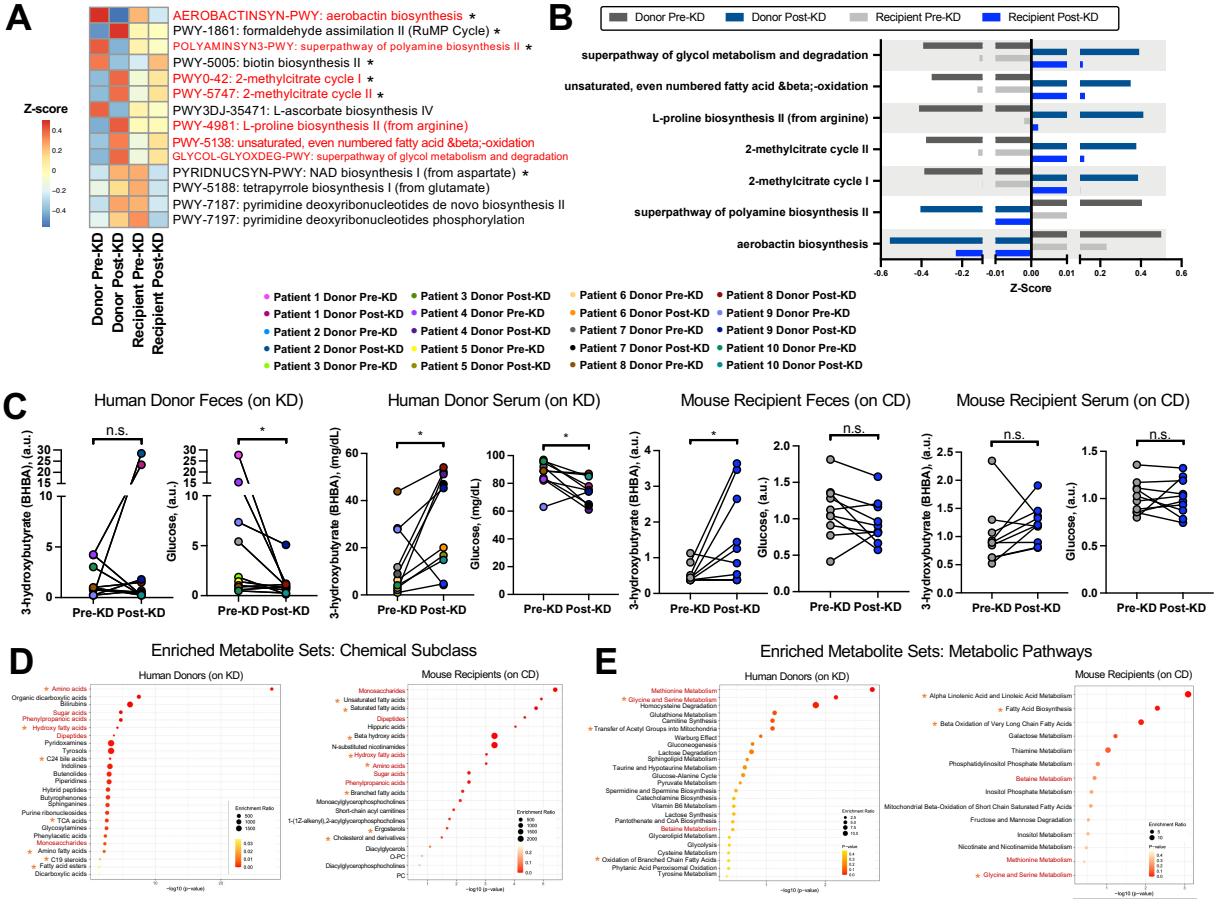
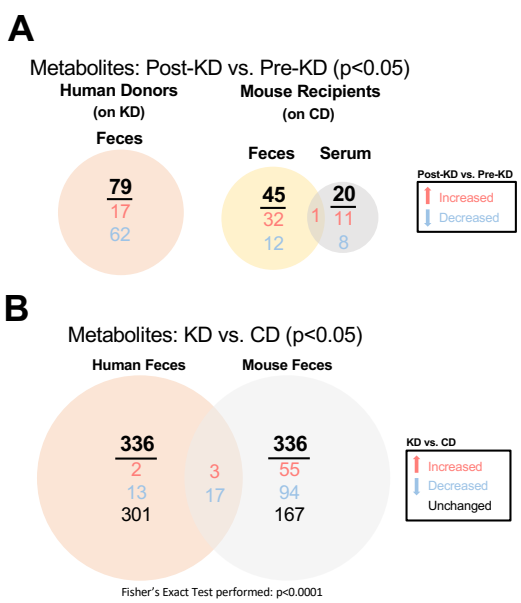


Figure 2: The clinical KD-associated human microbiome exhibits functional alterations that are phenocopied in seizure-protected recipient mice. **(A)** Microbial functional pathways differentially abundant as determined by MaAsLin2 analysis comparing post-KD (samples relative to pre-KD controls for either 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



C

	Human pediatric epilepsy	Wild-type mouse (Swiss Webster)	
	Post-KD / Pre-KD	SPF KD / SPF CD	SPF Abx KD / SPF KD
cysteine	0.42	0.06	18.73
cysteinylglycine	0.38	0.04	15.17
dimethylglycine	1.72	6.71	0.72
oleoylcarnitine (C18:1)	4.96	8.50	1.10
2-oxindole-3-acetate	0.21	0.06	1.49
cystathionine	0.15	0.21	2.81
N-acetylmethionine	0.45	0.44	10.95
phenyllactate (PLA)	0.38	0.14	0.71
leucylglutamine*	0.26	0.43	0.57
4-hydroxyphenylacetate	0.77	0.58	0.28
palmitoleoylcarnitine (C16:1)*	2.39	3.99	8.25
lithocholate	0.26	0.37	0.35
malate	0.45	0.19	1.79
phenylalanylalanine	0.51	0.43	0.40
thioproline	0.57	0.50	2.40
valylglutamine	0.44	0.69	0.43
kynurenine	0.47	0.61	1.58
ornithine	0.29	0.34	0.19
glutamate, gamma-methyl ester	0.31	0.57	1.39
lactate	0.18	0.60	1.25

*all metabolites listed; p<0.05 for both human post-KD compared to pre-KD and mouse SPF-KD compared to SPF-CD

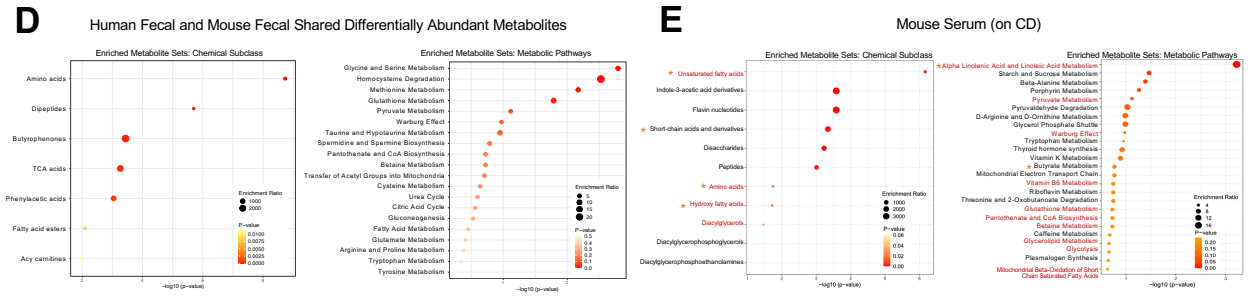


Figure S6: The clinical KD alters metabolomic profiles in human fecal samples and in fecal and serum samples of mice inoculated with human microbiota, Related to Figure 2. (A) Differentially abundant metabolites (p<0.05) in post-KD compared to pre-KD samples of human donor feces, mouse recipient feces, 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

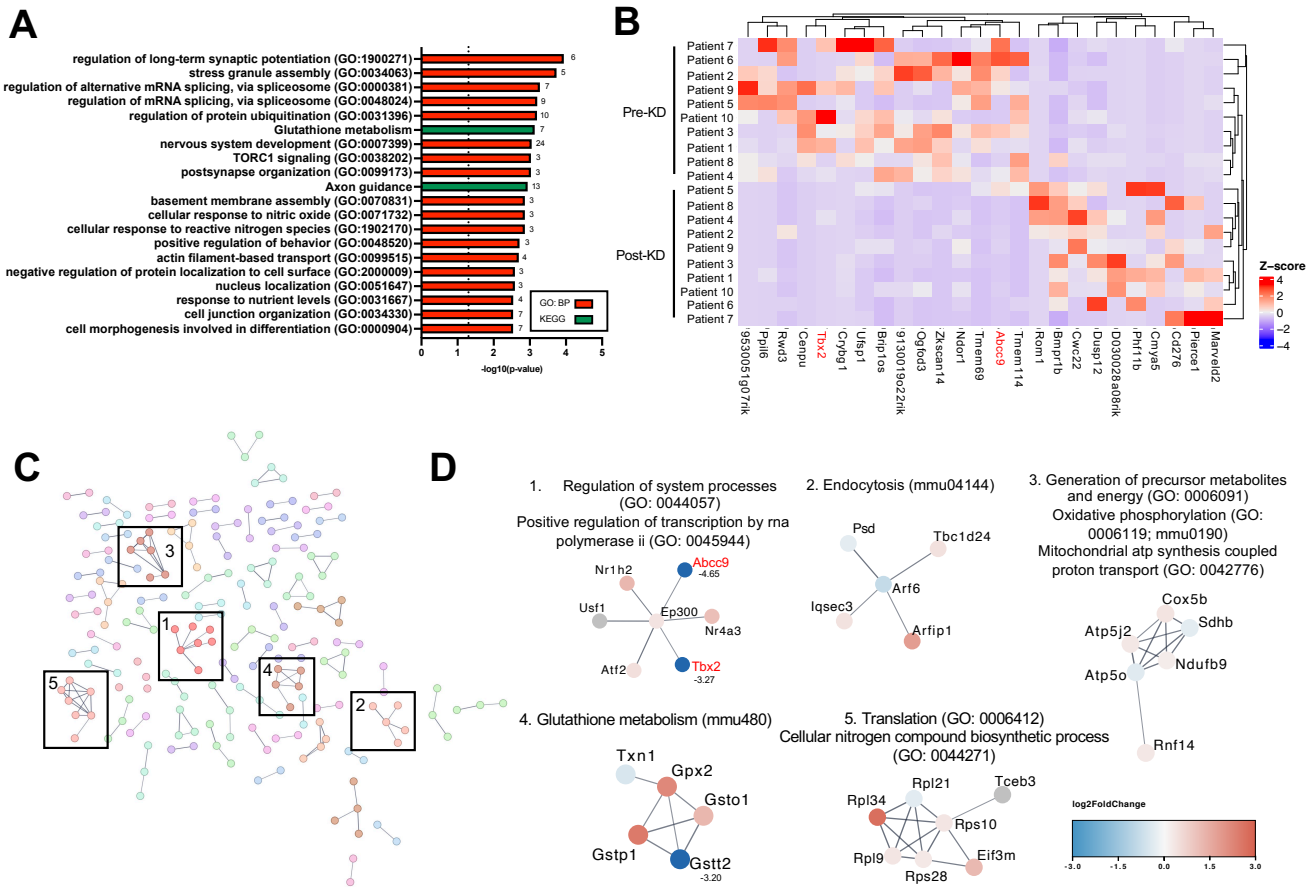


Figure 3: Seizure resistance in mice inoculated with the post-KD microbiota is associated with alterations in the brain transcriptome. **(A)** GO: Biological Process gene ontology of differentially expressed genes ($p<0.05$) in recipient mouse post-KD (n=10, where each sample is pooled from 6 recipient 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.

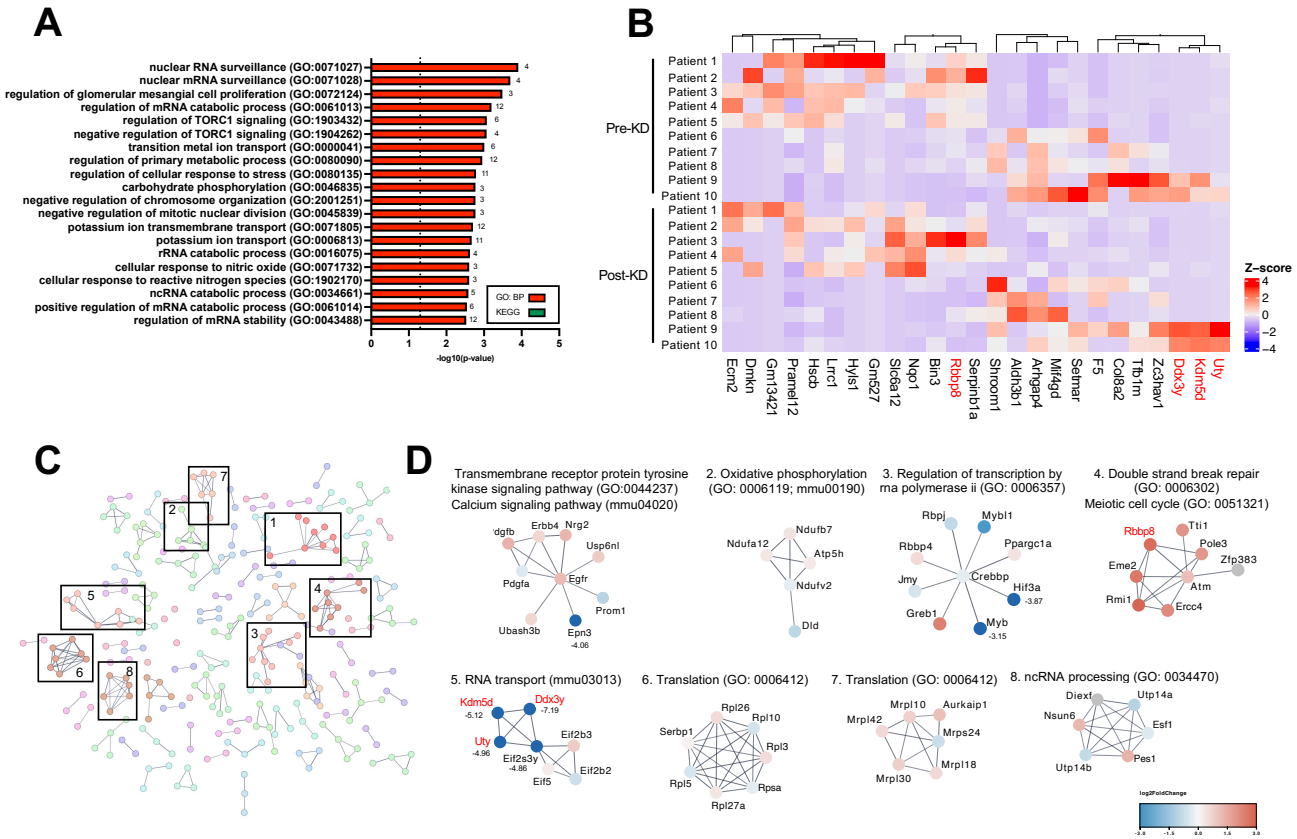
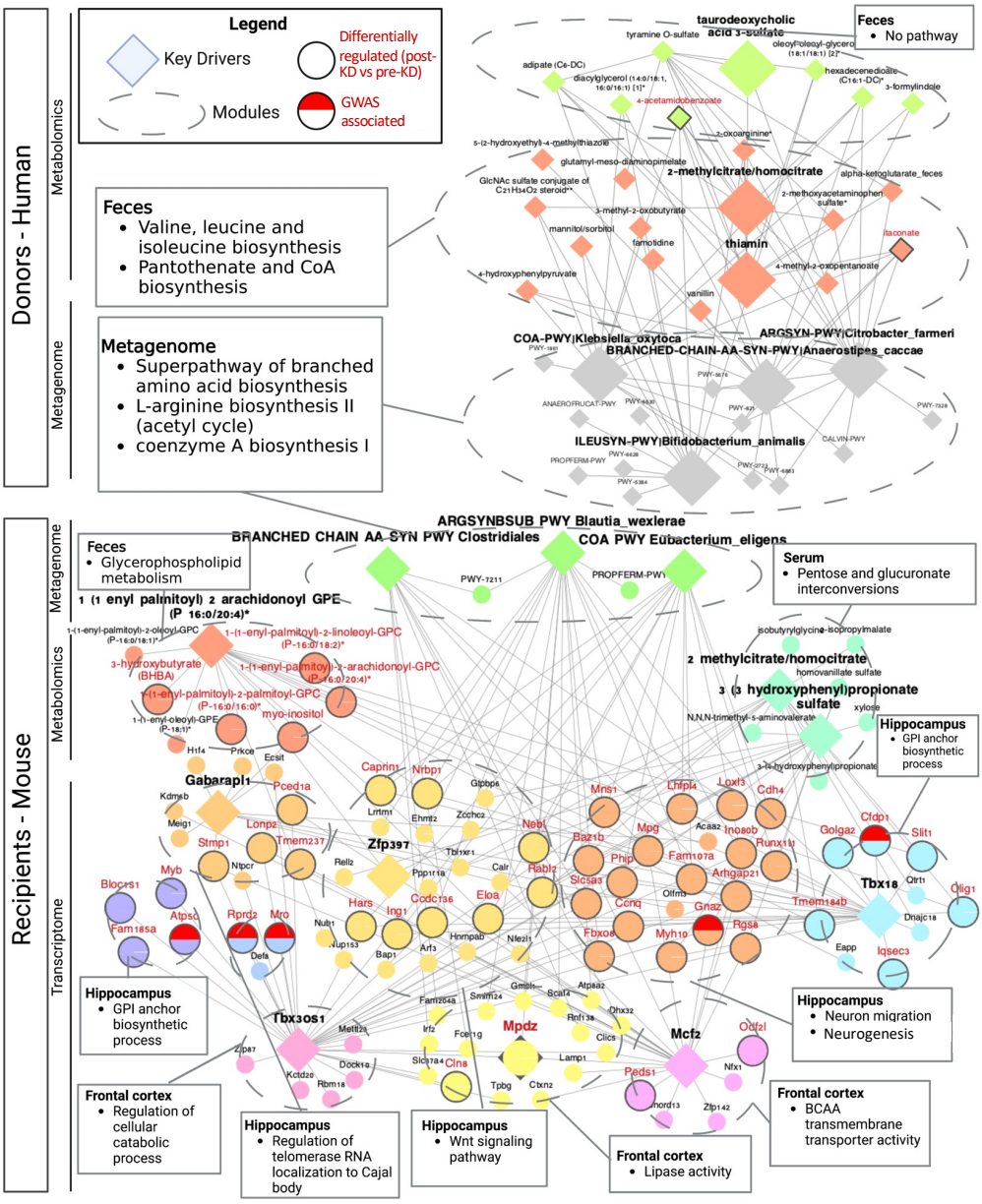


Figure S7: Mice inoculated with the post-KD microbiota exhibit alterations in the frontal cortical transcriptome, Related to Figure 3. **(A)** GO: Biological Process gene ontology of differentially expressed genes ($p<0.05$) in recipient mouse post-KD compared to pre-KD frontal cortex samples, top 20 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: 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