



The impact of *Scn1a* deficiency and ketogenic diet on the intestinal microbiome: A study in a genetic Dravet mouse model

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ARTICLE INFO

Keywords:

Gut microbiome
Epileptic encephalopathy
Ketogenic diet
Scn1a
Mice

ABSTRACT

Purpose: The gut-brain axis has been discussed as a possible factor contributing to ictogenesis and epilepsy. While recent preclinical studies have proposed a link between the antiseizure effect of a ketogenic diet (KD) and alterations to the gut microbiota, there is a knowledge gap about microbial composition as a result of *Scn1a* genetic deficiency and how this is affected by KD in Dravet syndrome.

Methods: A large-scale microbiome analysis using 16S rRNA gene sequencing was performed in fecal samples collected from wildtype and Dravet mice fed either control diet (CD) or KD. Microbial alterations associated with the Dravet phenotype or triggered by KD exposure were identified.

Results: The comprehensive microbial analysis revealed pronounced alterations in gut microbiota between wildtype and Dravet mice. The regulation of Chao index indicated a reduced species richness in Dravet mice when compared to wildtype controls. The ratio between Firmicutes and Bacteroidetes phyla was increased in mice with the Dravet genotype, therefore implying a microbial dysbiosis in these animals. Following the switch to CD or KD, several bacteria phyla and genera were regulated in Dravet mice. Interestingly, an increased abundance of the Clostridium genus and a decreased abundance of the Romboutsia genus showed a significant correlation with the severity of the phenotype in Dravet mice. KD increased the abundance of Firmicutes and reduced the abundance of Bacteroidetes phyla in Dravet mice. The degree of these microbial alterations correlated with the reduction in the frequency and duration of motor seizures in these animals.

Conclusion: In conclusion, the comprehensive microbial analysis demonstrated pronounced alterations in the gut microbiota with evidence of a gut dysbiosis as a consequence of the *Scn1a* genetic deficiency. Exposure to KD affected the gut microbiome in Dravet mice. Interestingly, abundance of selected genera correlated with the seizure phenotype of Dravet mice. Future studies investigating the functional relevance of disease-associated and KD-triggered changes would be essential to confirm the relevance of these findings.

1. Introduction

Recent experimental and clinical findings suggest a bidirectional relationship between the gut microbiome and the epileptic brain (De Caro et al., 2019). Comparisons between the gut microbiome of patients with drug-refractory epilepsy and of healthy control patients suggested an impact of epilepsy and non-controlled seizures on alpha diversity and the abundance of different bacterial species (Lindfeldt et al., 2019; Peng et al., 2018). In line with cumulating evidence for a regulatory influence of gut microbiota on brain function and their direct or indirect

impact on gut-to-brain signaling, experimental data pointed to a functional relevance of the gut microbiome for seizure susceptibility in a mouse model (Olson et al., 2018). These findings seem to be in line with effects of the ketogenic diet (KD) on the diversity of the gut microbiome and the abundance of different bacterial species in patients and mice (Lindfeldt et al., 2019; Olson et al., 2018; Tagliabue et al., 2017; Xie et al., 2017; Zhang et al., 2018). However, the findings from different clinical studies did not reveal consistent effects of epilepsy and of KD-associated changes in alpha diversity and abundance of specific bacteria species (Lum et al., 2020). In view of the heterogeneity of

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<https://doi.org/10.1016/j.epilepsyres.2021.106826>

Received 29 July 2021; Received in revised form 30 September 2021; Accepted 18 November 2021

Available online 24 November 2021

0920-1211/© 2021 The Authors

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numerous influencing factors such as the individual diet composition the variance in the outcome of respective clinical studies does not come as a surprise. Based on a highly standardized environment in animal facilities, animal models offer obvious advantages to address the question to what extent a disorder or dietary approaches affect the gut microbiome. Although species differences in the microbiome composition need to be considered for translational conclusions, respective studies can provide valuable fundamental information about the respective consequences of a disease and a therapeutic dietary approach.

For patients with Dravet syndrome suffering from drug-refractory seizures not adequately responding to different antiseizure drugs, the administration of a KD is recommended by neuropediatric experts (Cross et al., 2019). The recommendation builds on clinical studies, which provided proof for a relevant efficacy of KD in this patient population. In view of a possible contribution of gut microbiota to the efficacy of KD, it is of particular interest to assess the impact of the Dravet syndrome and of a dietary treatment approach on the composition of the gut microbiome. We recently provided first data from the phenotypic and Omics characterization of a genetic Dravet mouse model with a *Scn1a* deficiency (Miljanovic et al., 2021a). The findings confirmed a good face validity of the new genetic model with lowered thresholds for hyperthermia-associated seizures, spontaneous recurrent seizures, hyperactivity, and gait disturbances. Thus, this model provides a basis for the analysis of disease- and KD-associated alterations in the gut microbiome.

2. Methods

2.1. Animals

Experimental animals were generated by crossing two parental breeding lines, B6(Cg)-*Scn1a*^{tm1.1Dsf}/J (#026133 (Kuo et al., 2019; Ricobaraza et al., 2019)) and 129S1/Sv-*Hprt*^{tm1(CAG-cre)Mnn}/J (#004302 (Tang et al., 2002)), both purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The offspring resulted in heterozygous A1783V-*Scn1a* (Dravet) and wildtype mice. Animals' breeding and genotyping was done as previously described (Miljanovic et al., 2021a).

In-vivo experiments were approved by the government of Upper Bavaria (license number 55.2-1-54-2532-168-2016) and conducted in agreement with the German Animal Welfare act and the EU directive 2010/63/EU for animal experiments. Experiments were conducted in line with ARRIVE guidelines and Basel declaration (<http://www.baseldeclaration.org>) including the 3R concept.

In total, 35 Dravet and 28 wildtype mice were generated for this study. Nine Dravet mice died as a result of probable SUDEP. From the remaining 26 Dravet and 28 wildtype mice, 22 Dravet mice (11 males; 11 females) and 20 wildtype mice (10 males; 10 females) were selected for the experiment and were divided in two cohorts based on their birth date (block randomization). The cohorts were two weeks apart from each other.

Please note that metabolomics, seizure and behavioral data have previously been presented in the context of studies providing proteomic and metabolomic characterization of the line (Miljanovic et al., 2021a, 2021b). One Dravet female mouse had a skin wound during the second video-EEG monitoring phase and was therefore sacrificed and excluded from analyses.

2.2. Housing of animals

Each litter was housed in individually ventilated cages (Tecniplast, Hohenpeißenberg, Germany) until the time of weaning (P19–21). After weaning mice were kept in standard Makrolon type III cages (Ehret, Emmendingen, Germany) in groups of 3–5 mice per cage. Following surgery, mice were single-housed.

Each cage was once per week supplied with fresh sawdust as a bedding material (Lignocel, Rosenberg, Germany), an animal house

(Tecniplast, Hohenpeißenberg, Germany; Zoonlab GmbH, Castrop-Rauxel, Germany), and 7 g of Enviro-dri® nest material (Claus GmbH, Neuwied Germany).

Environmental conditions in the animal facility were kept at standard settings (temperature 20–24 °C, humidity 40–60 %, regular 12 h light/dark cycle).

All mice received tap water and food (ssniff® R/M-H, Sniff, Soest, Germany) ad libitum. From P14–26 all animals were offered a Dietgel76A as a supplement (Sniff, Soest, Germany). Following group allocation, mice were supplied ad libitum either with ketogenic diet (KD; fat:protein=6:1; #TD07797, Envigo, Italy), or a vitamin and mineral balanced control diet (CD; #TD150300, Envigo, Italy). Mice were offered CD or KD over a period of 41–42 days (subject to the day of sacrifice). Since KD has a consistency of paste, mice were offered wooden popsicles as an additional enrichment (Pura Sticks, Labodia AG, Niederglatt, Switzerland).

2.3. The experimental timeline

Wildtype and Dravet mice (12 weeks old) underwent a survival surgery for the implantation of telemetric transmitters and hippocampal depth electrode (CA1 region; ap: -2.00, lat: +1.3, dv: -1.6). Dravet mice were prepared for EEG-ECG recording, while wildtype mice received a dummy transmitter, with no possibility for recording, as previously described (Miljanovic et al., 2021b). Briefly, meloxicam (1 mg/kg s.c.; Metacam®, Boehringer Ingelheim, Germany) was used for analgesia 30 min before and 24 h after anesthesia induction. Isoflurane (4 % for induction, 1.5 % for maintenance of anesthesia; Isoflurane CP®, Henry Schein Vet, Hamburg, Germany) was used as a general anesthetic. For local anesthesia bupivacaine (0.25 % s.c.; Jenapharm®, Mibe GmbH, Brehna, Germany) was applied to surgical areas affected by telemetric transmitter and placement of leads, while bupivacaine with epinephrine (0.5 % + 0.0005 % s.c.; Jenapharm®, Mibe GmbH, Brehna, Germany) was applied for intracranial electrode placement.

The skin was opened dorsocaudal from the scapula region and a telemetric transmitter (HD-X02, DSI, St. Paul, USA) was placed subcutaneously. ECG leads were fixed intramuscularly and the skin was closed with absorbable sutures (Smi AG, St. Vith, Belgium). Mice were then fixed in the stereotactic surgical frame and three screws were fixed to the skull. The positive and negative EEG leads were connected to a bipolar teflon-isolated stainless-steel electrode and the screw placed over the cerebellum, respectively.

Electrode and screws were fixed with Paladur (Heraeus®, Hanau, Germany) and the skin around the skull was closed with absorbable sutures and a tissue adhesive (Surgibond®, Henry Schein Vet, Hamburg, Germany). Mice were supplied with oxygen (Oxyboy oxygen generator, Hugo Sacks Electronic, March-Hugstetten, Germany) until waking up. Following two weeks for post-surgical recovery, information about seizure activity was obtained by a one-week continuous video-EEG recording session (baseline) acquired with Ponemah software (Ponemah R, v. 5.2.0, DSI, St. Paul, USA). Motor seizures were firstly automatically detected (NeuroscoreTM v. 3.0, DSI, St. Paul, USA) and then confirmed and analyzed using video recordings (Axis communications, Lund, Sweden).

Following the baseline video-EEG recording, baseline fecal samples were collected from all animals for microbial analysis. Mice were then randomly (R software) allocated to four groups based on their genotype and consumed diet: wildtype control diet (n = 10, WT CD), wildtype ketogenic diet (n = 10, WT KD), Dravet control diet (n = 11, Dra CD) and Dravet ketogenic diet (n = 11, Dra KD) group. The number of motor seizures in Dravet mice was considered, when distributing mice to groups (R software, stratified randomization).

Mice were switched to either CD or KD over the next six weeks (Fig. 1). Seizure activity was analyzed during the fourth week of the diet phase based on continuous video-EEG recordings. Two female and one male Dravet mice with no motor seizures during the baseline recording,

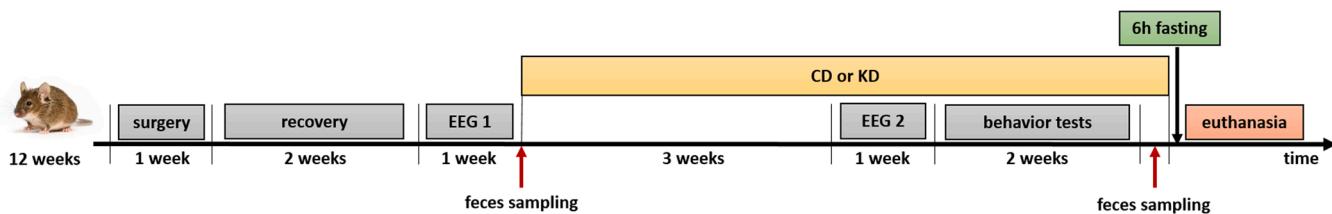


Fig. 1. Experimental timeline. CD=control diet, KD=ketogenic diet.

were excluded from this second recording session. Over the next two weeks, i.e. the fifth and sixth week of diet exposure, behavioral patterns were analyzed in the morning hours starting from 8:00 a.m. During the fifth week, nest building activity and saccharin preference were assessed in the home cage. In the following week, the open field test, the novel object recognition test, and gait assessment were conducted and video recorded in a test room (standard conditions: temperature 20–24 °C, humidity 40–60 %, lighting 15–20 lx). Ten-minute-long sessions in the open field and novel object recognition test were simultaneously performed in two white cylinders ($\varnothing=61$ cm, height=40 cm). Relevant parameters (distance moved; velocity; time spent in wall, middle, and center zone; immobility time; time sniffing objects) were detected automatically with Ethovision 8.5 software (EthoVision XT, Noldus, Wageningen, The Netherlands). Rearing behavior was manually scored by an observer blinded to animals' genotype and treatment. For gait analysis, animals were recorded from below (Bastler acA1300–60gm, Noldus, Wageningen, The Netherlands) while walking on a transparent plexiglas runway (length=100 cm, width=10 cm). Parameters of interest (stride length; forelimb and hindlimb base of support; the angle between forelimb paw and direction of body) were measured from two consecutive strides. A detailed description of the surgical procedure and all behavioral paradigms is provided in Miljanovic et al. (Miljanovic et al., 2021b).

Following the behavioral testing phase, fecal samples were collected from all animals for microbial analysis and animals were sacrificed during the next two days. The experimental timeline is presented in Fig. 1.

2.4. Microbial sampling

Fresh fecal samples were collected from mice in morning hours (9:30–11:00 a.m.) at two time points: prior to diet onset (baseline) and one day prior to sacrifice (diet). Each mouse was placed into a clean cage, cleaned with DNA-ExitusPlus spray (KISKER BIOTECH GMBH & CO. KG Steinfurt, Germany). Feces was collected into 2 ml PCR clean safe-lock tubes (Eppendorf, Wesseling-Berzdorf, Germany) with 800 µl of DNA and RNA stabilization solution for nucleic acids (Cat# R1100–250, ZYMO RESEARCH EUROPE GMBH, Freiburg, Germany). Samples were stored on 4 °C and shipped on ice for DNA extraction and microbial profiling to BaseClear (Leiden, The Netherlands). The samples were double-labeled, and the person analyzing samples was blinded to their genotype and treatment. Briefly, genomic DNA was isolated from feces, bacterial 16S rRNA gene (V3-V4) was PCR amplified, barcoded and the library was prepared for 10,000 Illumina MiSeq sequencing. All samples passed the quality check (PhiX and adaptor sequences removal), were quantified and FASTQ reads were generated. Relative microbial abundance was calculated and used for statistical analysis.

2.5. Euthanasia

Prior to sacrificing the animals, all mice were fasted for six hours during the light cycle phase to limit the variance related to individual differences in recent food intake. Mice were euthanized with 600 mg/kg Pentobarbital in 10 ml/kg injection volume (i.p.) between 12:00 and 3:30 p.m. Animals were randomly distributed into groups, which were

euthanized at two consecutive days (R software). Hippocampal tissue and blood were sampled for metabolomics profiling as previously described (Miljanovic et al., 2021b).

2.6. Statistics

Statistical analysis was performed using R software (version 3.6.1.). Graphs illustrating alpha diversity and principal component analysis were prepared with GraphPad Prism (Version 5.04, GraphPad, USA), and graphs illustrating microbial diversity were prepared with Genome Explorer (BaseClear). Spearman correlation matrix was calculated and visualized using R software (R package "gplots" (Warnes et al., 2016)). The significance level was set at $-0.5 < R < 0.5$ and missing data from individual animals were not considered for the particular correlation analysis.

A two-tailed unpaired *t*-test was used for the comparison of microbiome between wildtype and Dravet mice fed standard chow (baseline). The genotype and diet effect on microbiome in mice fed CD or KD (diet) were tested by two-way ANOVAs and a Bonferroni post-hoc test. The significance level was set at $p < 0.05$ for all tests.

3. Results

3.1. Alpha diversity

Microbial samples were taken before diet exposure (baseline, standard chow) and following the complete experiment (diet, CD or KD). Firstly, alpha diversity was assessed at both time points. Chao index is frequently used as an abundance-based estimator of species richness in a sample (Chao, 1984). The index proved to be lower in Dravet mice as compared to wildtype mice (baseline, CD, KD) (Fig. 2A). Moreover, the implementation of CD or KD reduced the Chao index in both wildtype and Dravet mice, when compared to baseline data (Fig. 2B-C).

Shannon and Simpson indices evaluate species diversity based on operational taxonomic units (OTUs), with more focus on species richness and evenness, respectively (Kim et al., 2017). Regardless of the genotype, species diversity indicated by the Shannon index in mice with KD exceeded that in mice with CD (Fig. 2D). Interestingly, the Shannon index was reduced in wildtype and Dravet mice fed CD, when compared to baseline data (Fig. 2E). This was in apparent contrast to animals with KD, in which the Shannon index remained at baseline levels (Fig. 2F). For the Simpson index differences between animals with CD and KD exposure were only evident in wildtype mice, indicating a lower level of species richness and evenness in mice with KD exposure (Fig. 2G). The switch from standard chow to the specific diet affected the Simpson index in wildtype mice with CD. The increase of the Simpson index indicated that CD reduced bacterial diversity only in wildtype mice (Fig. 2H). No effect was observed in mice fed KD (Fig. 2I).

3.2. Principal component analysis (PCA)

PCA performed for fecal microbiome data in baseline samples, revealed that the first principal components explain 46.91 % of total data variance (PC1: 29.99 %, PC2: 16.92 %; Fig. 3A). An unpaired *t*-test revealed a significant effect of genotype along PC1 ($p = 0.03$). For the

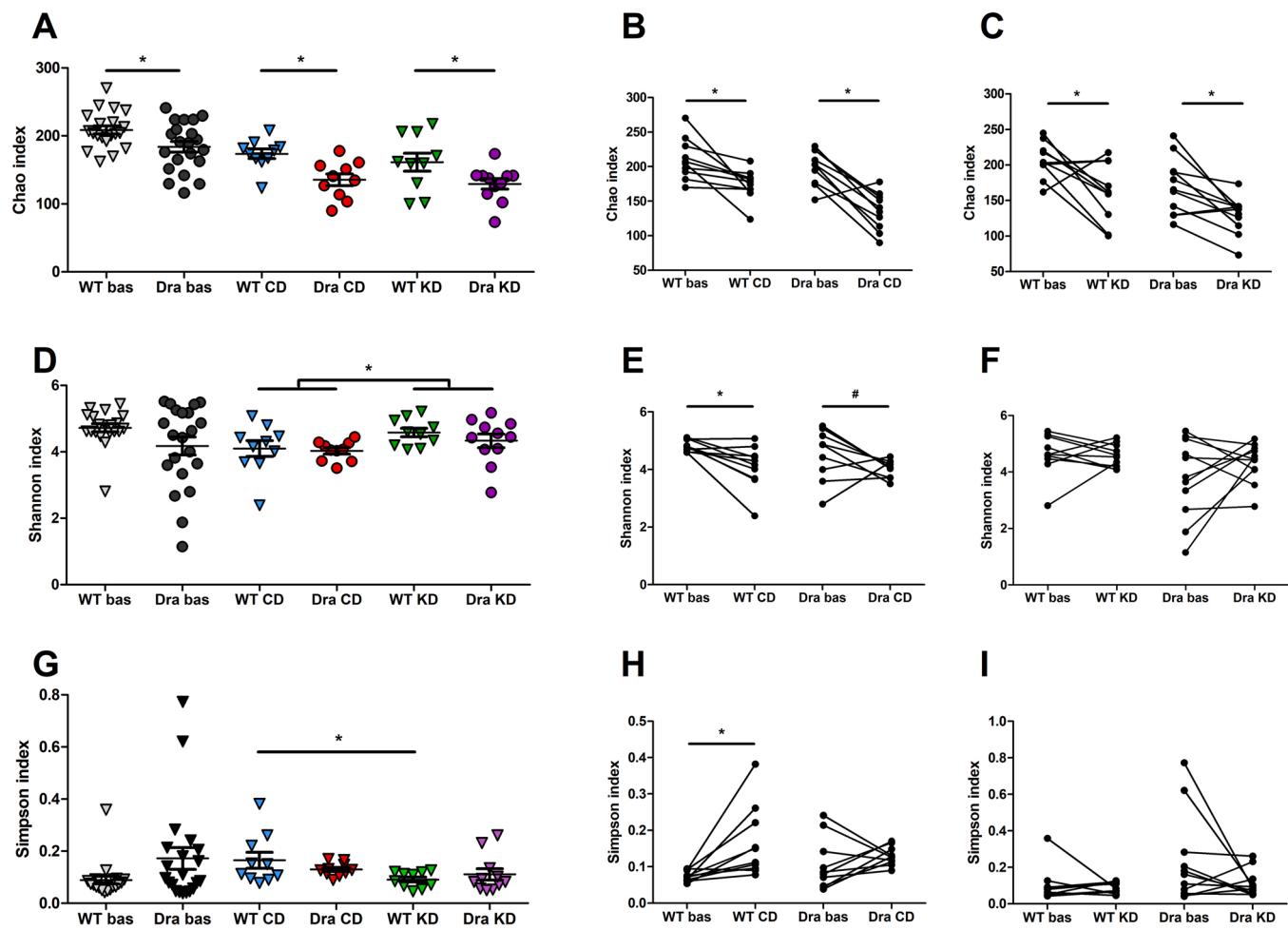


Fig. 2. Alpha diversity index. A-C Chao index of species diversity. Chao index was reduced in Dravet mice when compared to wildtype mice, regardless of the diet. In addition, wildtype and Dravet mice fed CD or KD exhibited a reduced Chao index in comparison to their baseline data. D-F Shannon index. CD reduced Shannon index in both wildtype and Dravet mice when compared to baseline data. KD fed wildtype and Dravet mice had an increased Shannon index in comparison to CD fed mice. G-I Simpson index. Wildtype mice fed KD had a lower Simpson index than wildtype mice fed CD. In addition, CD increased the index value in wildtype mice, when compared to their baseline data. Data shown are from 20 wildtype mice (20 baseline, 10 CD, 10 KD) and 21 Dravet mice (21 baseline, 10 CD, 11 KD) (baseline comparison: unpaired *t*-test; post diet comparison: Two-way ANOVA; comparison between baseline and post diet: paired *t*-test; significance level $p < 0.05$).

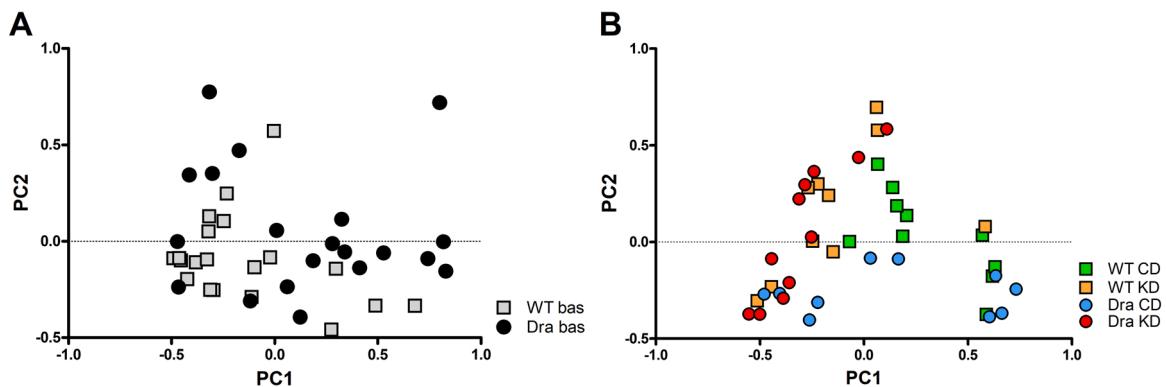


Fig. 3. Principal component analysis (PCA) of microbial samples for baseline (A) and post diet (B) time points. Scatter plot of individual animals and their microbial composition along PC1 and PC2. A PCA revealed that the microbiome of wildtype ($n = 20$) and Dravet mice ($n = 21$) significantly differed (unpaired *t*-test; significance level $p < 0.05$). B PCA revealed that both, genotype and diet, affect the intestinal microbiome of mice. Data shown are from 20 wildtype mice (10 CD, 10 KD) and 21 Dravet mice (10 CD, 11 KD) (Two-way ANOVA; significance level $p < 0.05$).

PCA performed for fecal microbiome data following exposure to CD or KD, the first principal components explain 41.41 % of total variance in the data (PC1: 26.01 %, PC2: 15.4 %; Fig. 3B). Two-way ANOVA revealed a significant impact of the diet along PC1 ($F(2,38) = 19.45$,

$p < 0.0001$) and the impact of diet ($F(2,38) = 6.36$, $p = 0.02$) and genotype ($F(2,38) = 5.52$, $p = 0.02$) along PC2.

3.3. Microbial alterations

The baseline microbiome was dominated by two bacteria phyla, Bacteroidetes and Firmicutes in both wildtype and mutants. When compared to wildtype animals, analysis in Dravet mice demonstrated a reduced abundance of Bacteroidetes (WT: $49.36 \pm 4.19\%$; Dravet: $32.99 \pm 5.75\%$) and an enhanced abundance of Firmicutes (WT: $46.80 \pm 4.36\%$, Dravet: $64.17 \pm 5.87\%$) (mean \pm SEM; Fig. 4A). Assessment of the ratio between bacterial phyla revealed a higher prevalence of Firmicutes over Bacteroidetes indicating microbial dysbiosis in Dravet mice. A trend towards an increase of Deferribacteres has been noted in Dravet mice (Fig. 4A). Moreover, its abundance in microbiome showed a strong positive correlation with seizure frequency and duration in Dravet mice (Fig. 5B).

Following CD or KD introduction, an increase of Firmicutes and a decrease of Bacteroidetes was observed in groups receiving KD resulting in an increase of the Firmicutes to Bacteroidetes ratio (WT: $1.04 \pm 0.28\%$ vs. $1.48 \pm 0.25\%$, Dravet: $1 \pm 0.18\%$ vs. $4.18 \pm 1.4\%$; mean \pm SEM) (Fig. 4B). While the abundance of the phylum Bacteroidetes showed a positive correlation with the frequency and duration of motor seizures in Dravet mice, a negative correlation with these seizure parameters was identified for the phylum Firmicutes (Fig. 6).

On a genus taxonomic level, a lower abundance of Barnesiella and a higher abundance of Bacteroides became evident in Dravet mice during baseline analysis. Moreover, we observed a trend towards a higher abundance of Lachnoclostridium and Mucispirillum in Dravet mice (Fig. 5A). Interestingly, the abundance of Mucispirillum positively correlated with the frequency and duration of motor seizures in Dravet mice (Fig. 5B).

Following CD or KD introduction, an increase in Clostridium, Acetatifactor, Oscillospira, and Enterohabitus abundance was noted in Dravet mice. On the contrary, abundance of Romboutsia was reduced (Fig. 7). For wildtype and Dravet mice fed CD, a strong correlation was confirmed between the abundance of Clostridium, Romboutsia, and Alloprevotella genera and behavioral parameters characterizing the phenotype of Dravet mice (Fig. 6). Interestingly, Romboutsia abundance also showed a pronounced correlation with hippocampal levels of glucose, glycolysis intermediates, and several amino acids (Fig. 6).

Regardless of the genotype, KD exerted an impact on the fecal

microbiome by increasing Acetatifactor, Alloprevotella, and Enterohabitus and reducing relative abundance of Barnesiella, Lactobacillus, and Bacteroides. A trend towards an increase of Lachnoclostridium and Eisenbergiella was observed (Fig. 7). Moreover, the abundance of Lachnoclostridium showed a negative correlation with the frequency and duration of motor seizures ($R=-0.65, -0.55$, respectively). Interestingly, the level of abundance changes of Lactobacillus, Bacteroides, and Enterohabitus genera correlated with the level of ketosis (β -hydroxybutyrate) in the hippocampus of Dravet mice ($R=-0.58, -0.58, 0.64$, respectively). We have previously reported that KD improved one of the gait parameters (BOS_hindlimb) in Dravet mice (Miljanovic et al., 2021b). There was no correlation between this effect and KD-induced microbial changes.

4. Discussion

Taxonomic analysis of the gut microbiome revealed a significant influence of *Scn1a*-deficiency in a genetic Dravet mouse model. Manifestation of epilepsy along with behavioral alterations and motor dysfunction was accompanied by changes in alpha diversity and changes in the abundance of different bacterial phyla and genera. While alterations in relative abundance were evident following the switch from the standard chow to both KD and the respective control diet, selected changes in the gut microbiome became only evident in mice on KD suggesting that these alterations are specific for the exposure to a high fat diet.

Analysis of alpha diversity revealed a negative impact of the genetic deficiency on species richness in the samples. A reduced Chao index was evident in Dravet mice regardless of the diet exposure.

This finding confirms our hypothesis that manifestation of the Dravet syndrome can be associated with alterations in the gut microbiome. To our knowledge, there is a lack of respective data from patients with Dravet syndrome. Different studies have assessed disease-associated alterations in gut microbiota in children and adults with epilepsy related to different etiologies (Lindefeldt et al., 2019; Lum et al., 2020; Peng et al., 2018; Xie et al., 2017). Concerning the analysis of alpha diversity these studies revealed inconsistent findings with either no change, an increase or a decrease in alpha diversity. However, only one of these studies analyzed the Chao index, which pointed to an increase in

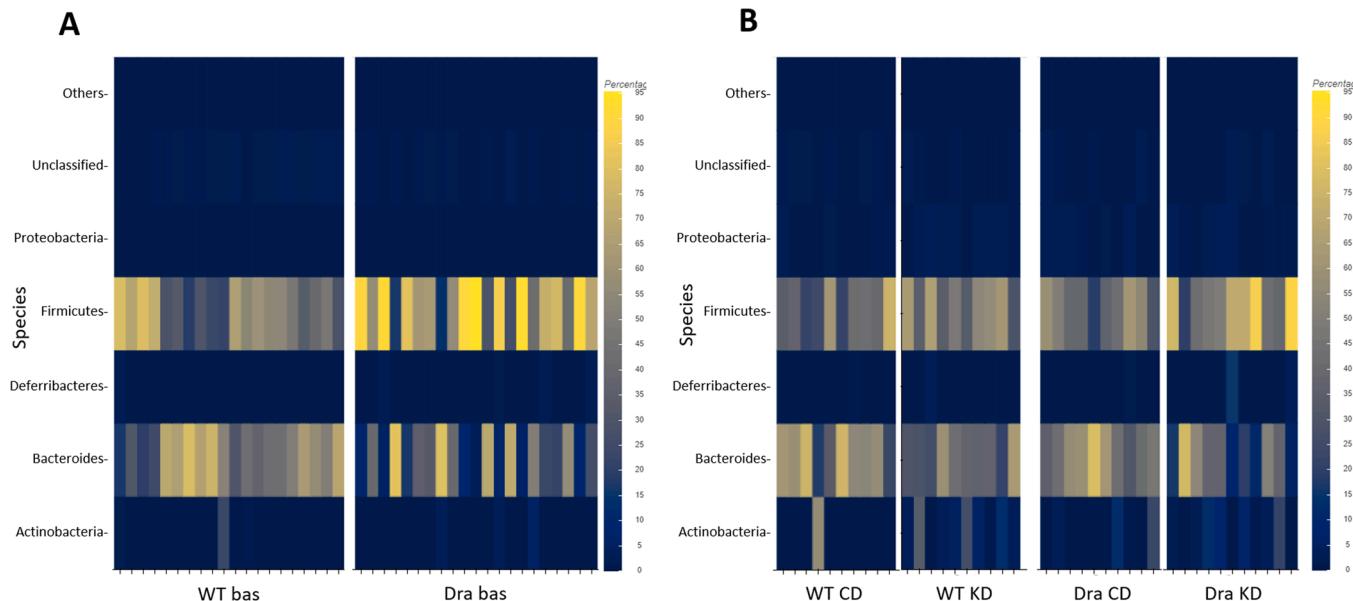


Fig. 4. Microbial diversity on a phylum taxonomic level during baseline (A) and diet (B) time points. A A reduced abundance of Bacteroidetes and an increased abundance of Firmicutes bacteria phyla were observed in Dravet mice during baseline. B Following the switch to CD or KD, an increased abundance of Firmicutes and a decreased abundance of Bacteroidetes phyla were noted in groups receiving KD. Data shown are from 20 wildtype mice (20 baseline, 10 CD, 10 KD) and 21 Dravet mice (21 baseline, 10 CD, 11 KD) (baseline comparison: unpaired t-test; post diet comparison: Two-way ANOVA; significance level $p < 0.05$).

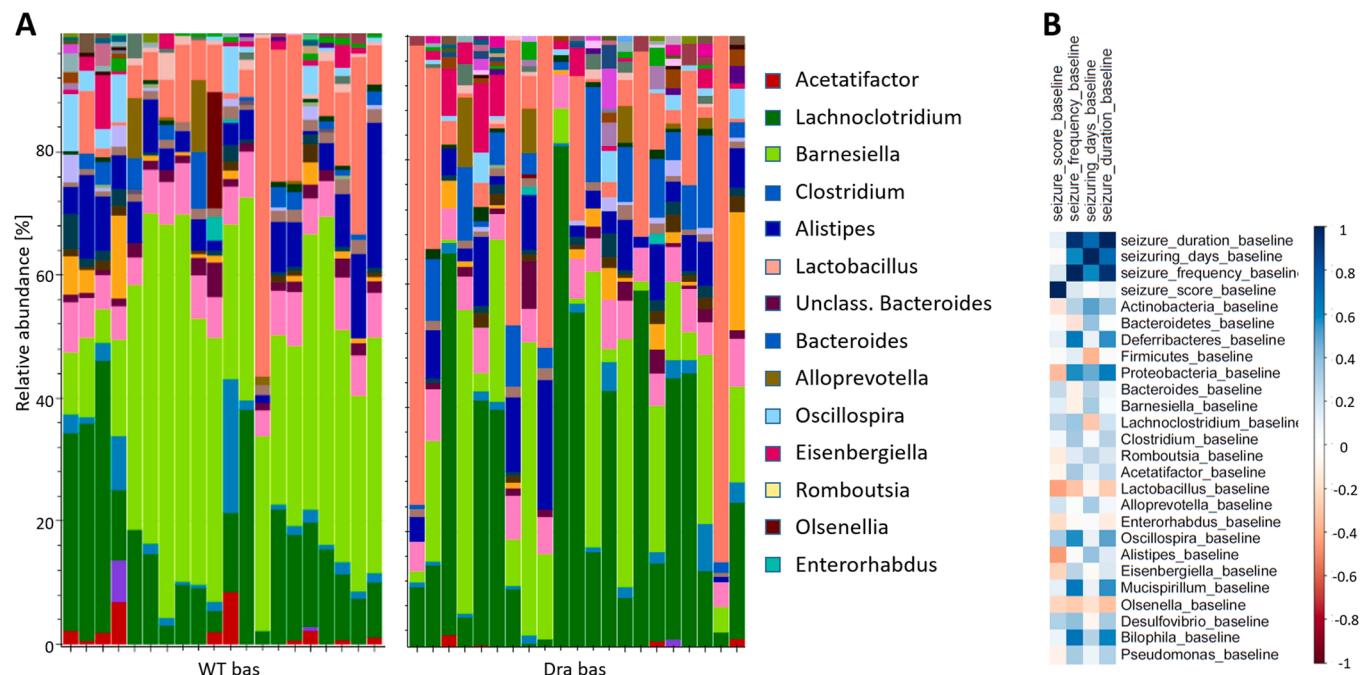


Fig. 5. Microbial diversity on a genus taxonomic level during baseline. A Microbial relative abundance in WT and Dravet mice fed standard chow diet (baseline). Data shown are from 20 wildtype mice and 21 Dravet mice (unpaired *t*-test; significance level $p < 0.05$). B Spearman correlation matrix between assessed motor seizure parameters and gut microbiota during baseline. The heat map represents individual Spearman correlations between selected parameters in WT and Dravet mice. Color scale is shown below the matrix, with blue color indicating a positive and red color indicating a negative correlation. Seizure severity (total seizure frequency and duration) showed a strong positive correlation with abundances of Deferribacteres and Proteobacteria phyla, and with Oscillospira, Mucispirillum, and Bilophila genera. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

alpha diversity in patients with refractory epilepsy compared to patients with drug-sensitive epilepsy or healthy persons (Peng et al., 2018). In this context, it needs to be considered that assessment of alpha diversity only provides very general information.

Thus, we have explored the impact of Dravet syndrome on the abundance of bacterial taxa. Interestingly, our findings demonstrated a shift in the Firmicutes to Bacteroidetes ratio with an increase of Firmicutes and a decrease of Bacteroidetes. Corresponding changes are generally considered as a marker of gut dysbiosis (Magne et al., 2020), and have for instance been associated with obesity in patients and experimental animals (de Wit et al., 2012; Hildebrandt et al., 2009; Krajmalnik-Brown et al., 2012; Ley et al., 2006; Turnbaugh and Gordon, 2009). Moreover, evidence exists from experimental studies that the shift in the ratio of Firmicutes and Bacteroidetes can contribute to enhanced neuroinflammation, impaired neurovascular function, a decline in cognitive function, and an increase in anxiety levels occurring with increasing age (Hoffman et al., 2017).

In this context, it is of particular interest that an increase in Firmicutes and a decrease in Bacteroidetes represented a consistent finding throughout different clinical studies with comparison between patients with refractory epilepsy and different control groups (Lindfeldt et al., 2019; Peng et al., 2018; Xie et al., 2017). Thus, our present findings indicate that the impact of epilepsy and uncontrolled seizures on these bacterial phyla in the gut microbiome is a reproducible consequence, which seems to be preserved across different species. This conclusion was further confirmed by the fact that we identified significant correlations between alterations in the two bacteria phyla and duration and the frequency of motor seizures in Dravet mice.

Gut microbiome-brain signaling has been linked with different signaling factors (Carabotti et al., 2015). Some of these factors including short chain fatty acids seem to be associated with beneficial effects such as a limitation of inflammatory signaling in microglia mediated by butyrate (Huuskonen et al., 2004). In contrast, other gut microbiota metabolites can exert detrimental effects (Ma and Ma, 2019).

Trimethylamine N-oxide can affect glia function, trigger neuroinflammation, and can cause an impairment of learning and memory (Brunt et al., 2021). In consideration of the fact that a high abundance of Firmicutes has been linked with enhanced formation of trimethylamine and its cometabolite trimethylamine N-oxide (Martinez-del Campo et al., 2015), microbiome alterations associated with Dravet syndrome may aggravate cognitive dysfunction in patients. Future studies would be of particular interest to compare the experimental taxonomic findings of the present study with clinical data from patient cohorts and to additionally assess possible alterations in trimethylamine levels.

KD is recommended as an adjunctive therapeutic approach in patients with Dravet syndrome and failure to achieve an adequate seizure control despite trials with three to four antiseizure drugs (Cross et al., 2019). While the mechanisms of KD and alternate dietary approaches are not yet fully understood, various mechanisms have been discussed and explored generating a different level of evidence (Rogawski et al., 2016; Youngson et al., 2017). Among others the list of putative mechanisms comprises possible direct effects of ketone bodies, effects of polyunsaturated fatty acids, increased GABA synthesis, increased adenosine levels, improved mitochondrial function and reduced production of reactive oxygen species (Rogawski et al., 2016; Youngson et al., 2017). More recent experimental evidence suggested that an impact of KD on the gut microbiome might also play a role for the antiseizure effect of the diet (Olson et al., 2018). Upon exposure to a KD, we observed an increase in abundance of Acetatifactor, Alloprevotella, and Enterorhabdus and a decrease in abundance of Barnesiella, Lactobacillus, and Bacteroides, thereby confirming a general influence of KD on the gut microbiome composition. The diet did not impact the seizure or behavioral phenotype of Dravet mice but managed to improve their gait disturbance (Miljanovic et al., 2021b). Yet, this effect of KD seems to be independent of its impact on microbiome of Dravet mice. KD-induced changes in the microbiome pattern have already been reported from studies in patients with epilepsy (Gong et al., 2021; Lindfeldt et al., 2019; Xie et al., 2017; Zhang et al., 2018). While the studies consistently

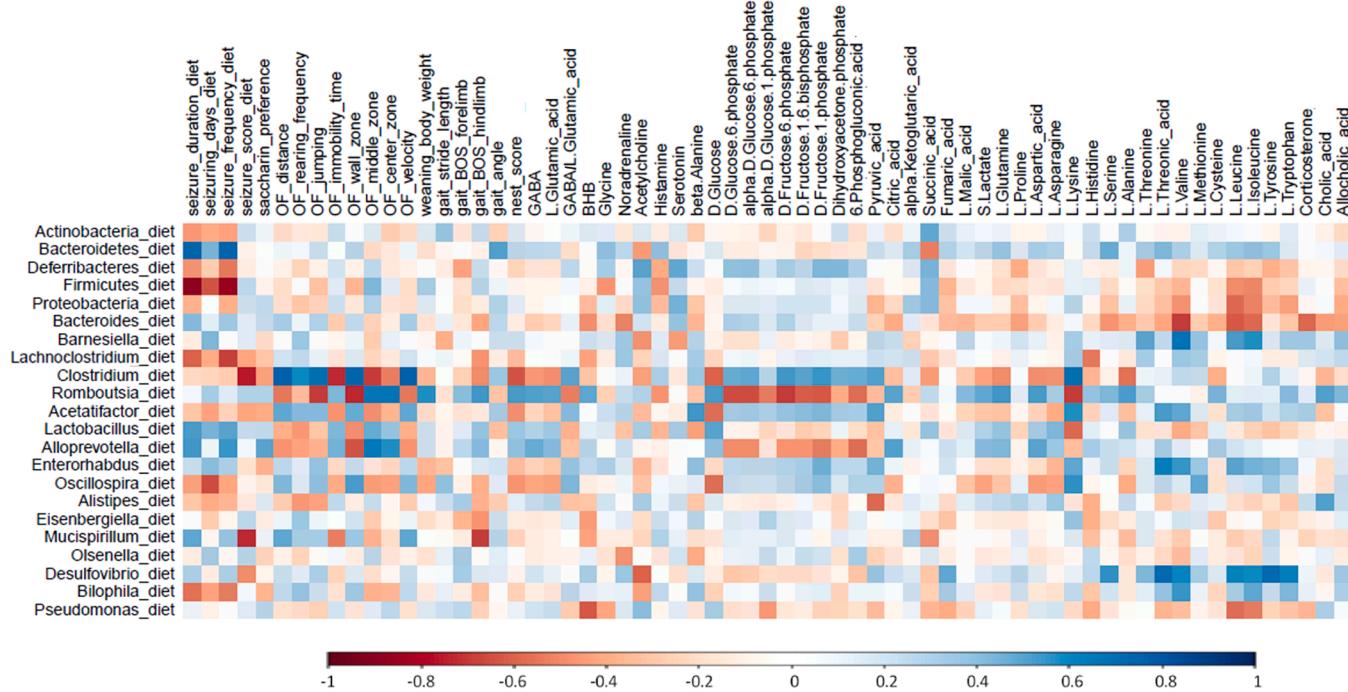
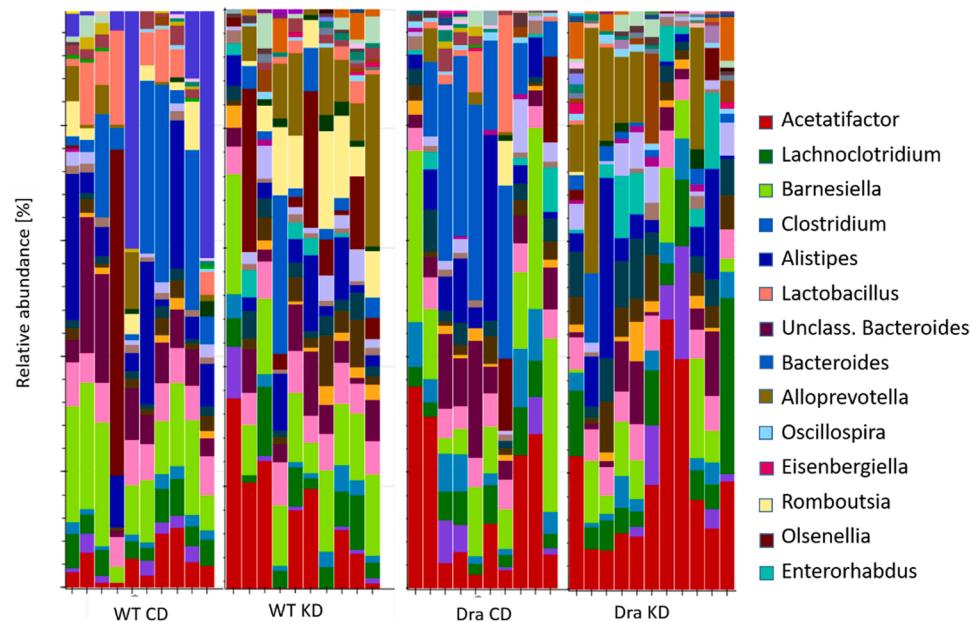


Fig. 6. Microbial diversity in WT and Dravet mice. Spearman correlation matrix for abundance of bacteria phyla and genera, and seizure parameters, behavioral parameters, and hippocampal metabolites (except for corticosterone and bile acids measured in plasma). The heat map represents individual Spearman correlations between selected parameters in WT and Dravet mice fed CD. The color scale is shown below the matrix, with blue color indicating a positive and red color indicating a negative correlation. Romboutsia genus abundance showed a strong negative correlation with hyperlocomotion and thigmotaxic behavior in the open field test and with gait disturbance (BOS_hindlimb), as well as a positive correlation with the nest complexity score. The bacteria abundance showed a positive correlation with hippocampal levels of glucose, glutamine, aspartic acid, and alanine, as well as a negative correlation with the hippocampal GABA:glutamic acid ratio, glycolysis intermediates, and lysine levels. Clostridium genus abundance showed a strong positive correlation with hyperlocomotion and thigmotaxic behavior in the open field test, and a negative correlation with nest complexity score. The bacteria abundance showed a positive correlation with fructose-6-phosphate, fructose-1-phosphate, and lysine, as well as a negative correlation with glucose and alanine levels in the hippocampus. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)



confirmed an impact, the specific alterations in specific microbial taxa showed relevant differences between studies (Lum et al., 2020). Interestingly, some of the altered bacteria in the aforementioned studies were also affected in our experimental setup. For instance, an increase in

abundance of Alloprevotella genus in KD-fed Dravet mice was in line with results from the study in patients with drug-refractory epilepsy (DRE). Moreover, genus Bacteroides proved to be reduced in Dravet mice after KD, as well as in KD-responsive when compared to

Fig. 7. Microbial diversity on a genus taxonomic level in wildtype and Dravet mice. Following CD or KD introduction, Dravet mice showed an increased abundance of Clostridium, Acetatifactor, Oscillospira, and Enterohabclus, and a reduced abundance of Romboutsia. KD altered the fecal microbiome by increasing relative abundance of Acetatifactor, Alloprevotella, and Enterohabclus and reducing relative abundance of Barnesiella, Lactobacillus, and Bacteroides. Additionally, a trend towards an increase of Lachnoclostridium and Eisenbergiella was observed in Dravet mice. Data shown are from 20 wildtype mice (10 CD, 10 KD) and 21 Dravet mice (10 CD, 11 KD) (Two-way ANOVA; significance level $p < 0.05$).

KD-nonresponsive patients with DRE (Gong et al., 2021). On the other hand, some of these studies reported contrasting results to our findings, with reduced alpha diversity, an increase of Bacteroidetes (Xie et al., 2017; Zhang et al., 2018), and a decrease of Firmicutes phyla (Zhang et al., 2018) as a result of exposure to KD. These opposing microbial alterations may explain why KD failed to improve the seizure phenotype in Dravet mice. However, as discussed by Lum and colleagues (2020), the limited consistency in the specific changes may be related to several factors including differences in species, the duration of KD exposure, the specific KD composition, subtype of epilepsy and seizures as well as technological differences in the assessment of the gut microbiome.

Functional evidence for a relevant role of the gut microbiome for the antiseizure effects of KD came from an experimental study that demonstrated that the efficacy of KD is lost in germ-free mice and in mice with antibiotic pre-treatment and microbiome depletion, and that the efficacy can be restored by fecal gavage in germ-free mice (Olson et al., 2018). Based on an analysis of the abundance of different microbiota, the authors were able to trace the KD effects back to changes in Akkermansia muciniphila and Parabacteroides. Their key role was confirmed in experiments, in which selective enrichment of these bacterial taxa mediated protection in an acute seizure model and a genetic epilepsy model with a potassium channel deficiency (Olson et al., 2018).

These findings triggered an interest to explore respective gut microbiome changes in models of epilepsies, which are considered important clinical indications for KD such as the Dravet syndrome. While we also observed a relevant influence of KD, we did not observe the regulation of aforementioned two bacteria taxa and could not reproduce the findings reported by Olson and colleagues (Olson et al., 2018). The differences may reflect a specific impact of the manifestation of Dravet syndrome with a high seizure frequency in the mouse model used in the present study. However, they may also be related to methodological differences and environmental factors in the animal facilities. Nevertheless, our data provide evidence that it would be of interest to further study the functional relevance of KD-associated gut microbiome alterations in models of Dravet syndrome and patients. In respective follow-up studies, profiling of the gut metabolome can provide further information about microbial function and its relevance for seizure susceptibility in Dravet syndrome.

Correlation between microbiome data and in-vivo data points to a possible link between microbiome composition and *Scn1a* genetic deficiency. Dravet mice fed standard chow with higher abundance of Deferribacteres and Proteobacteria phyla and their genera Desulfovibrio and Mucispirillum, were prone to higher seizure severity. Interestingly, previous studies reported an increased or reduced abundance of Proteobacteria phylum in patients with epilepsy (Gong et al., 2020; Safak et al., 2020; Xie et al., 2017). Furthermore, its genus Desulfovibrio is a proinflammatory gut bacteria involved in production of short chain fatty acids including propionic acid, which can trigger seizure activity in rats (Macfabe, 2012).

An increase in Deferribacteres and its genus Mucispirillum observed in Dravet mice was previously associated with an increase in inflammatory cytokines including interleukin-6 (Diling et al., 2020, 2019; Sauer et al., 2019). This is of particular interest considering that excessive inflammatory signaling represents one of the hallmarks of epileptogenesis and epilepsy manifestation and may contribute to development and progression of Dravet syndrome.

Mucispirillum genus was found increased in microbial samples of Shank3 $\alpha\beta$ knock-out mice with autistic features. Besides neuronal inflammation, these mice also showed signs of astrogliosis (Sauer et al., 2019) previously reported in our model (Miljanovic et al., 2021a). Albeit our data demonstrate correlation and not causation, alterations in abundances of these bacteria might trigger an immune response by increasing levels of cytokines contributing to an excessive inflammatory response and astrogliosis, which in turn can compromise normal brain function and affect neuronal excitability, behavioral patterns, and the emotional state, and thus exacerbate symptoms of Dravet syndrome

(Devinsky et al., 2013).

Following the switch to CD or KD, lower levels of Romboutsia genus and higher levels of Clostridium genus correlated with the severity of the Dravet mice phenotype as well as with the concentrations of GABA/Glutamate-glutamine cycle components and glucose levels in the hippocampus. Respective metabolic alterations have previously been characterized in detail in a metabolomic study (Miljanovic et al., 2021b). Considering the gap in knowledge about Romboutsia as a short chain fatty acid producing bacteria (Xie et al., 2017) and its role in epilepsy, it may be of interest to further explore its potential as a disease marker in Dravet syndrome and other epilepsies. On the other hand, Clostridium is a proinflammatory, short chain fatty acid producing gut bacteria, linked to increased epileptiform activity and autism-spectrum disorders in rats (Macfabe, 2012) and epilepsy in patients (Huang et al., 2019; Zhang et al., 2018). Our data thus implies that it may also play a role in progression of pathophysiology of Dravet syndrome characterized by pronounced seizure activity and autistic features (Dravet, 2011).

Concerning the potential links between *Scn1a* genetic deficiency and alterations in gut microbiota one can only speculate. Previously, we demonstrated that mice with a Dravet genotype have lower levels of corticosterone and two bile acids in their plasma (Miljanovic et al., 2021c). Bile acids can alter the gut microbiome and show antimicrobial properties by destroying bacterial membranes directly or indirectly through FXR and induction of antimicrobial inflammatory agents including IL-18 and iNOS (Inagaki et al., 2006). These events may further impact the gut-brain axis (Carabotti et al., 2015) and thus further deteriorate the phenotype of Dravet mice. However, future studies need to further address the mechanistic links.

5. Conclusions

In conclusion, the comprehensive microbial analysis revealed pronounced alterations in the gut microbiota in mice with the *Scn1a* genetic deficiency. The findings point towards a gut dysbiosis as a consequence of Dravet syndrome in mice. Exposure to KD further altered the gut microbiome in Dravet mice. Interestingly, abundance of selected genera correlated with the seizure phenotype of Dravet mice. Future studies investigating the functional relevance of disease-associated and KD-triggered changes would be essential to confirm a relevance of these findings.

Declaration of Interest

None.

Acknowledgements

The authors thank Dr. R. Maarten van Dijk, Verena Buchecker, Sarah Glisic, Sieglinde Fischlein, Katharina Gabriel, Sabine Vican, and Uwe Roßberg for their excellent technical assistance.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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