Shared microbial community changes in female rats and humans with Rett syndrome

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Research

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Abstract

Background

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder predominantly caused by alterations of the methyl-CpG-binding protein 2 (MECP2) gene. The gut microbiome has been implicated in neurodevelopmental disorders such as Autism Spectrum Disorder (ASD) as a regulator of disease severity. Although the gut microbiome has been previously characterized in humans with RTT, the impact of MECP2 mutation on the composition of the gut microbiome in animal models where the host and diet can be experimentally controlled remains to be elucidated.

Methods

We evaluated the microbial community through 16S sequencing of fecal samples collected across postnatal development as behavioral symptoms appear and progress in a novel zinc-finger nuclease rat model of RTT. Additionally, we profiled fecal levels of fatty acids in MecP2 deficient rats. Lastly, we compared our results to predicted functional shifts in the microbiota of females with RTT compared to their mothers to further examine the translational potential of the current RTT rat model.

Results

We have identified microbial taxa that are differentially abundant across key timepoints in a zinc-finger nuclease rat model of RTT compared to WT. Furthermore, we have characterized functional categories of gut microbes that are similarly affected in females with RTT and female RTT rats, including similar alterations in pathways related to short chain fatty acid (SCFA) activity. Lastly, we have demonstrated that SCFA levels are decreased in the feces of RTT rats compared to WT.Limitations The current study is potentially limited by age related differences in the microbiome of RTT participants and controls as well as medication effects on the microbiome. Additionally, the current study did not assess male MeCP2-deficient rats, and it may be relevant in future studies to address potentially disparate microbial changes in male and female rats and humans with RTT.

Conclusions

The results of our studies establish distinct microbial community shifts that occur in RTT across developmental time points independently of diet or environmental factors. We identify p105 as a key translational timepoint at which microbial shifts most closely mirror reported microbiota communities in RTT patients. Overall, these results represent an important step in translational RTT research.

Background

RTT is an X-linked progressive neurodevelopmental disorder that affects up to 1 in 10,000 girls annually in the United States (1). RTT is characterized by seemingly typical development until 6–18 months of age followed by sometimes rapid neurodevelopmental regression. Greater than 95% of all cases of RTT harbor alterations in the methyl-CpG-binding protein 2 (MECP2) gene (2). However, severity of RTT symptoms can vary wildly across individuals due to differences in X-chromosome inactivation and specific gene mutation (2,3). While the neurologic sequelae, loss of motor, cognitive, and social skills, are defining features in all Rett individuals,

chronic issues related to other organ systems also characterize disease progression. A 2012 survey of nearly 1,000 families of individuals with RTT revealed that comorbid gastrointestinal (GI) symptoms are present in a large majority (92%) of people with RTT (4) and represent a significant quality of life issue for long-term care. Symptoms such as constipation and oropharyngeal dysmotility predominate, however gastroparesis, gastroesophageal reflux, and biliary dysmotility may also occur. Importantly, severe sequelae of abnormal nutritional status such as low bone mineral density and fractures increase with age, suggesting an insidious disease course with respect to GI symptoms (4). Inadequate oral mechanical function surely affects nutritional intake and contributes to these sequelae. However, given that MeCP2 is expressed throughout the enteric nervous system in all gut regions (5), it is not unreasonable to posit that changes in gut MeCP2 expression may directly contribute to GI symptoms. In fact, mechanisms whereby this may occur have recently been proposed (5).

The trillions of bacteria that inhabit the gut are termed the gut microbiota and have long been known to impact GI disorders such as Crohn's disease and irritable bowel syndrome (IBD) (6,7). More recently, gut microbiota have been shown to be altered in a variety of neurological disorders, including Autism Spectrum Disorder (ASD) (8), Parkinson's Disease (9), and epilepsy (10). The contribution of the gut microbiome not only to comorbid GI distress in these conditions but also disease pathogenesis itself are now being heavily investigated. For instance, a variety of animal and human studies of ASD and neuropsychiatric disorders describe a spectrum of alterations in gut microbiota as well as improvements in behavior phenotypes following treatments targeting the gut microbiome (11). The microbiome has also been shown to be critical for appropriate brain development with respect to microglial cells (12), which function as central nervous system (CNS) immune mediators and appear to be aberrantly distributed and activated in the brains of individuals with ASD (13). Thus, we hypothesize that mechanisms of microbiome-mediated changes in gut/brain axis may be observed more broadly across other neurological disorders. In RTT, MECP2 mutation may directly alter GI motility, precipitating changes in the gut environment that favor certain bacterial taxonomies. Changes in microbiota ultimately affect nutrient metabolism and absorption, immune mediators, and neuroendocrine signaling (14), which may act in a feed-forward fashion to contribute to overall disease pathogenesis.

Previous studies have demonstrated that RTT diagnosis is related to changes in microbial diversity (15) as well as changes in production of microbial metabolites such as short chain fatty acids (SCFAs) (15,16), which are known to affect blood-brain barrier (BBB) integrity (17) and have been implicated in ASD (18). However, the relationship between MeCP2-deficiency and the gut microbiome has remained unexplored in animal models of RTT. Given the emerging importance of the gut microbiome in neurologic disease, characterizing and understanding changes in the microbiome of animal models relative to humans with RTT is an appropriate next step to explore the role of the gut microbiome in disease pathogenesis. Importantly, previous RTT human studies have utilized age-matched control designs, which appropriately account for known age-dependent alterations in the microbiome (19). However, other genetic influences, household or environmental exposures, geographic location, and diet also significantly influence the microbiome (19,20,21). Thus, our study approaches this issue from a novel angle, utilizing each patient’s mother as a control, potentially minimizing the effects of environmental and genetic covariates in our study.

RTT has historically been modeled in mice through a variety of genetic manipulations (22,23). However, studying cognitive and social deficits such as those in RTT can represent a significant challenge in many animal models. Rats have been shown to participate in complex social interactions (24) and have the ability to

complete higher level cognitive tasks (25) relative to mice, offering another approach to this challenge. Recently, a novel zinc-finger nuclease model of RTT in rats was developed by Sage laboratories. In this model, the MeCP2 protein is absent due to a 71 base pair deletion in exon 4 of Mecp2. The motor, behavioral, and social deficits across development in this model have been previously described (26,27). Importantly, development of abnormalities in MeCP2 Mecp2ZFN/+ heterozygous females begins as early as the third to fourth postnatal week, comparable to the developmental age of symptom onset in females with RTT. Severe weight, motor, and behavioral symptoms progress most profoundly in Mecp2ZFN/+ heterozygous female rats between 4 and 12 months of age, at which comparable developmental stage symptoms have generally also progressed and begun to stabilize in girls with RTT. As the microbiome changes rapidly throughout development (28), an animal model that recapitulates developmental disease timepoints and can be easily studied at early phases of disease course is essential to characterize the impact of the microbiome on pathogenesis. The aim of the current study was to characterize the gut microbiome in a MeCP2 rat model of Rett syndrome across development in comparison the human gut microbiome in individuals with RTT.

Methods

Participants

Participants were recruited through the Rare Disease Clinical Research Network US Natural History Study of Rett syndrome and related disorders (HD-061222). This protocol is listed on ClinicalTrials.gov (NCT00299312 and NCT02738281). The protocol was approved by UAB IRB. The human fecal samples were processed and stored at -80C in Cary/Blair supplemented with 10% glycerol as a cryopreservative as described (PMID 25042718)

Animals

All experiments were conducted in accordance with NIH guidelines and were carried out with approval from the Animal Care and Use Committee of the University of Alabama at Birmingham. All animals in the present study were bred as previously described (26). Briefly, Sprague Dawley females lacking one copy of MeCP2 (Mecp2ZFN/+) were crossed to wildtype (WT) S100b eGFP (enhanced green fluorescent protein) males obtained from the National Bioresource Project Rat (Japan). Originally Wistar, WT S100b eGFP males were back crossed over 10 generations onto a Sprague Dawley background prior to crossing with Mecp2ZFN/+. For the current study only Mecp2ZFN/+ female rats were used as experimental animals. Mecp2ZFN/+ rats were weaned according to genotype at postnatal day 21 (PND 21). WT and Mecp2ZFN/+ experimental animals were not co-housed for this study. Animals were provided with food and water ad libitum, and kept under standard 12-h light-dark cycles.

Fecal Collection

Animals were individually removed from the home cage and placed into a clean, open, plastic container. Fresh fecal pellets were collected using clean tweezers and stored at -80C at time points indicated in the text.

DNA isolation

Human and rat fecal DNA was isolated using the DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research) according to manufacturer's instructions. For rats fecal isolation, one fecal pellet was used for each isolation.

For human fecal samples 25 mg of human fecal sample was utilized for each isolation. DNA was quantified and samples stored at -80C until sequenced.

Sequencing

Isolated DNA was quantitated prior to PCR and barcoded PCR amplification of the V4 region of the 16S rRNA gene (51) was accomplished using degenerate primers originally taken from Caporaso et al. 2011 (52). We used primers as described by Kumar et al. 2014 (53) for use on the Illumina MiSeq sequencer. PCR was carried out under conditions described by Kumar et al. PCR products were resolved on agarose gels; DNA isolated and purified using Qiagen kits; and then quantitated. The products were sequenced on the MiSeq platform, a single flowcell, single lane instrument that can generate approximately 9Gb of sequence data from our paired end 250bp run.

16S analysis

Sequencing data was analyzed with QIIME (52). Forward and reverse reads were joined, and data was quality filtered and trimmed using BBMAP. Operational Taxonomic Units (OTUs) were picked using closed reference OTU picking with 97% sequence similarity. OTUs were assigned to taxonomies using a 97% similarity threshold with the greengenes database. Observed OTUs alpha diversity metrics, Bray Curtis beta diversity metrics, and individual taxonomy differences were assessed using QIIME. Rarefaction curves were generated for alpha diversity metrics (Fig. S1). OTU tables from QIIME were utilized to predict abundances of KEGG orthologs (KOs) and collapse KOs into KEGG pathways for functional analysis. KEGG pathways were analyzed and graphed using STAMP (32). Linear Discriminant effect size analysis (LEfSe) was performed with default parameters (39). OTU tables generated in QIIME were assigned LDA scores and graphed utilizing the Galaxy web application.

UPLC-MS/MS Analysis of Short Chain Fatty Acids

Standards and samples were prepared using the SOP for SCFA analysis developed in the DPMSR laboratory based on the published method of Han, et al., 2015 (60). A 12-point calibration curve from 10 mM to 0.975 uM for acetic acid, and 1 mM to 0.098 uM for the C3-C8 short chain fatty acids was prepared for quantitation of short chain fatty acids in study samples. The rat fecal samples were homogenized at 10 uL/mg volume to weight ratio in 50/50 v/v EtOH/water. The sample extracts were then derivatized using 1:1 200 mM 3-nitrophenyl hydrazine (3-NPH) in 50% ethanol with 6% pyridine: 120 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) in 50% ethanol in a 1:1 ratio of sample: derivatization reagent. At the end of the incubation, the reaction was quenched with the addition of 20x excess cold 10% ethanol in water with 1% formic acid. A 25 ul aliquot of the reaction solution from each sample was transferred and 25 ul aliquot of the stable isotope standards (SIS) solution was added. 5 uL injections were used for UPLC separation of the SCFAs, performed using a Exion AD liquid chromatograph (Sciex, Framingham, MA) with a Waters Acquity 2.1 mm x 50 mm 1.7 um BEH C18 column fitted with a Waters Acquity C18 1.7 um Vanguard guard column. Analytes were separated using a gradient from 15% solvent B to 100% solvent B in 9.5 minutes. Solvents A & B were 0.1% formic acid in water and acetonitrile, respectively. The total UPLC analysis time was approximately 13.5 minutes. The method uses electrospray ionization in negative mode introduced into a 6500+ QTrap mass spectrometer (Sciex) operating in the Multiple Reaction Monitoring (MRM) mode. MRM transitions (compound-

specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time window and the data were analyzed using Skyline v19.1 (https://skyline.ms).

Statistics

Human alpha diversity values were calculated with observed OTU metrics. Human beta diversity analyses were calculated with bray-curtis distance. LDA score for LEfSe analysis was performed on differential abundance tables generated with QIIME. All taxonomy differences in human participants as well as differential KEGG pathways were analyzed using relative abundance difference scores between each daughter and her paired mother (mother-daughter). Differences between RTT and controls for both human participants and rat models were assessed using Kruskal-Wallis H tests. All p-values for taxonomy differences and KEGG pathways are FDR adjusted. SCFA log2 values were analyzed using Mann-Whitney U tests.

Results

MeCP2-deficient rats have altered microbiota across development in parallel with behavioral symptoms of RTT

We first sought to characterize changes in microbiota at different developmental time points selected to parallel previously identified behavioral symptoms present in the female rat model, which range in appearance from p21 (time of weaning) to 4-12 months of age. Alpha diversity measured by observed operational taxonomic units (OTUs) was not significantly different between the MeCP2-deficient rats at any of the selected timepoints (Fig. 1 a). Beta diversity measured by bray-curtis difference was also similar across groups at p21 (Fig. 1 b). However, Mecp2ZFN/+ rats begin to diverge from WT rats in beta diversity at p49, and continue to be significantly distinct through p196 (Fig. 1 c-i). These clustering changes in beta diversity despite lack of changes in alpha diversity indicate that although the Mecp2ZFN/+ and WT rats have similar numbers of bacterial taxonomies in their guts throughout development, the makeup of bacterial taxonomies is altered across development at multiple timepoints associated with the development of behavioral symptoms in Mecp2ZFN/+ rats.

MeCP2-deficient rats show developmental shifts in the microbiome

The human RTT microbiome has previously been characterized by two groups (15,16). In a large cohort of humans with RTT, Strati et al. found decreases in the relative abundance of Bacteroidetes in RTT compared to healthy controls, as well as increased Firmicutes/Bacteroidetes ratio in RTT. Strati et al. also characterized RTT through genus level changes in the relative abundance of Actinomyces, Bifidobacterium, Clostridium XIVa Eggerthella, Enterococcus, Erysipelotrichaceae incertae sedis, Escherichia/Shigella, and Megasphaera. Borghi et al. found that although a cohort of humans with RTT had similar phylum level microbial communities to controls, RTT participants differed in family Bacteroidaceae as well as Clostridium and Suterella species, and that differences in Bacteroidaceae correlate with disease severity.

In the current study, we also sought to characterize specific microbial shifts of RTT rats compared to WT rats across development as shown in Table 1. (For full taxonomies see Table S1). At p21, the gut microbiota of both Mecp2ZFN/+ and WT rats is characterized by dominance of Bacteroidetes and Firmicutes phyla, as is also apparent in the aforementioned human studies. As expected, there are no significant differences in relative abundance across any level in p21 RTT rats and WT rats, possibly due to cohousing of RTT and WT rats until weaning at p21. At p35, the gut microbiota of both RTT and WT rats is still characterized by dominance of

Bacteroidetes and Firmicutes phyla. At this age, however, the RTT rat microbiome begins to diverge from WT rats with the inclusion of the family Barnesiellaceae, the abundance of which is increased in sedentary women and is predicted by increased body fat percentage (29). This is of interest given that previous studies show that RTT rats are significantly heavier than WT rats by p60-90, despite being housed under the same dietary conditions.

When the animals reach p49, divergence appears at the class Epsilonproteobacteria and order Campylobacterales in the Proteobacterium phylum, and class RF39 in the phylum Tenericutes. These broad category changes in relative abundance likely drive the separation in beta diversity that also begins at this time point. By p77, however, RTT rats and WT rats no longer show changes in relative abundance of Proteobacteria, but differences in the Phylum Tenericutes remain significant. With regard to previously reported differences in RTT body weight, it should be noted that changes in Mollicutes, a class of Tenericutes, has been associated with rodent obesity related to the western diet (30).

Of note, the gut microbiomes of RTT and WT rats significantly diverge at p105 across multiple diverse taxonomies. Changes in order RF39 in phylum Tenericutes still persist. Additionally, we note shifts in multiple orders in classes Bacilli and Clostridia in phylum Firmicutes, and class Bacteroidia in phylum Bacteroidetes. By p133, though relative abundances of order RF39 and class Bacilli still trend toward significant differences, the microbiomes of WT and RTT rats do not significantly diverge at any level of taxonomy. This indicates that the microbial changes in RTT exist in a specific developmental window independent of age or weight related microbial shifts previously documented in rats (31). At p196, changes in classes Bacilli and Clostridia in phylum Firmicutes re-appear, and changes in the order Bacteroidales in phylum Bacteroidetes appear. This may indicate that the microbiomes of RTT rats are broadly characterized by shifts in Firmicutes and Bacteroides bacteria. Another possibility is that the reemergence of microbiota changes are related to disease progression, as we have previously described the appearance of significant motor abnormalities in female RTT rats at ~6 months of age (26).

Specific microbial shifts flag p105 as an important developmental stage in MeCP2-deficient rats

As higher level analyses indicated large taxonomy shifts at p105 in RTT rats, we examined differences in abundance of specific bacterial species at this time point. At this age, abundance of B. *acidifaciens*, which has been shown to promote IgA production in the large intestine (33), is significantly increased in RTT rats compared to WT (Table 1). Serum IgA counts are associated with gastrointestinal inflammation in individuals with RTT (15) and gut IgA content is increased in children with autism relative to typically developing children (34), suggesting a potential role for IgA-mediated gut inflammation in these neurologic conditions. C. *perfringens* is also significantly increased in p105 RTT rats compared to WT (Table 1). This species is known to produce epsilon toxin (35), which has deleterious effects on neurons, among other cell types (36). Increases in Clostridium genera abundance have also been previously identified in children with autism (37). Additionally, A. *muciniphila* trend lower in abundance in Mecp2ZFN/+ rats compared to WT (FDR adj. $p = 0.07$). Notably, A. *muciniphila* has been described as protective against epilepsy in rodents (10), and its reduction in abundance has also been reported in children with autism (38).

Previous studies have additionally utilized LEfSe scores to identify statistically significant differences in microbiota classifications (39) (Fig. 2). We performed LEfSe analysis on p105 rats to supplement our abundance data. The epsilon toxin-producing family Clostridiaceae has a significantly large linear discriminant analysis (LDA) score in Mecp2ZFN/+ rats compared to WT, with biological relevance of the sub classification

genera Clostridium noted as above. A variety of clostridium-related species, which have been broadly described as associated with RTT (15,16) also demonstrate large LDA scores between genotypes. The SCFA-producing genus Lachnobacterium and family Lachnospiracaea in turn are similarly reduced in Mecp2ZFN/+ rats, again paralleling findings in Strati et al. and Borghi et al. Other taxonomies of note with large LDA score differences indicating relative reduction in Mecp2ZFN/+ vs WT rats include A. *muciniphila*, noted above, and *Ruminococcus gnavus*, which is involved in tryptophan metabolism in the gut (40). The broader Ruminococcus genus was also noted to be depleted in RTT participants in Borghi et al. and elevated in IBD patients (41). As the changes in abundance of specific bacterial species at p105 in RTT rats most closely mimics reported changes noted in humans with RTT, this suggests that p105 is a key translational time point in the presented RTT model.

Gut microbiota changes in p105 MeCP2-deficient rats are reflected by changes in predicted microbiome function

It is not unexpected that an animal model of a complex condition such as RTT would demonstrate differences in significant disease-associated bacterial species relative to human studies. Despite these differences, the general functions that significantly altered bacteria perform in the body may be similar between rats and humans and thus identify common biologically relevant pathways in RTT. We utilized PICRUSt (42) to predict functional differences between RTT and WT rat microbiota at p105. KEGG level 1 pathway analysis predicted that RTT rats have significant decreases in pathways related to cellular processes and environmental information processing, and inversely showed increases in pathways related to metabolism and genetic information processing (Fig. 3a).

Strati et al. previously showed enrichment in KEGG pathways related to SCFA metabolism, including carbohydrate metabolism, in humans with RTT. Similarly, RTT rats demonstrate enriched carbohydrate metabolism as well as differences in KEGG pathways that are related to SCFA production. Other altered pathways of note include purine metabolism and fatty acid elongation in mitochondria (Fig. 3 b-d).

Humans with RTT and Mecp2ZFN/+ rats share common alterations in microbiota and resulting predicted functional pathways

To further assess the relationship between the Mecp2ZFN/+ model of RTT and human RTT, we recruited 6 individuals with RTT and their mothers to examine gut microbiome changes. All RTT participants were females with various MECP2 mutations. Most experienced constipation as a gastrointestinal comorbidity, as well as neurological comorbidities including seizures and sleep problems. All RTT participants were taking a variety of medications for various comorbidities, and one patient was taking probiotics in an attempt to ease constipation (Table 2).

To determine changes in microbiome function in our patient samples relative to control samples, we examined differences in LDA score. Generally, anaerobic bacteria had higher LDA scores in mothers compared to RTT participants. Of note, the SCFA producing taxonomies Lachnobacterium and Faecalibacterium are decreased in RTT participants compared to their mothers. In contrast, the family Clostridiaceae, which can produce epsilon toxin, is increased in those with RTT (Fig. 4).

Given these significant LEfSe findings, we next utilized QIIME (43) to map taxonomies in each group to KEGG Orthologs (KOs), and mapped KOs to KEGG pathways with PICRUSt. Consistent with a decrease in SCFA producing bacteria, RTT participants show a decrease compared to their mothers in KEGG pathways related to

microbial SCFA production including the pentose phosphate pathway and purine metabolism. (Fig. 5). The data reveal clear functional similarities between RTT patient microbiomes and the microbiomes of MeCP2-deficient rats, inviting the potential for increased therapeutic relevance from RTT rat microbiome studies.

Impact of MecP2 mutation on fecal SCFA levels at p105

As previous studies in humans with RTT showed alterations in fecal SCFA content (15,16) and our sequencing results indicate alterations at p105 in SCFA-related microbial pathways, we next examined the content of 12 SCFAs in fecal samples from p105 RTT and WT rats. Unbiased hierarchical clustering of total SCFA profiles in RTT rats and WT shows clear clustering of RTT samples separately from WT (Fig. 6A). There were no measurable levels of 3-methyl valeric acid or octanoic acid in our samples. Additionally, there were no differences between RTT and WT feces in levels of propionic acid, butyric acid, iso-burtyic acid, 2-methyl butyric acid, iso-caproic acid, valeric acid, or iso-valeric acid (Fig. 6A-G). However, RTT samples do contain significantly lower levels of acetic acid ($p = 0.0062$), caproic acid ($p= 0.0044$), and heptanoic acid ($p=0.0186$) (Fig 6 H-J). Our findings suggest that RTT rats have a distinct fecal SCFA profile.

Discussion

Previous studies indicated that the microbiomes of humans with RTT differ significantly in alpha and/or beta diversity compared to those of healthy controls (15,16). In the current study, we found that Mecp2ZFN/+ differed from WT rats only in beta diversity measures. This indicates that Mecp2ZFN/+ rats experience changes in the diversity, but not number, of the taxonomies present compared to WT rats, suggesting an obvious potential effect on the gut/brain axis through changes in the microenvironment. Indeed, we observed broad taxonomy shifts in the microbiomes of Mecp2ZFN/+ rats compared to WT rats beginning at p49, persisting through p105, and re-emerging at p196. Specifically, at p105, we observed significant changes in the abundance of B. acidifaciens, C. perfringens, and trends in the abundance of A. muciniphila in Mecp2ZFN/+ rats compared to WT. LefSe analysis also revealed significant differences in the family Clostridiaceae, genus Lachnobacterium, family Lachnospiracaea, and R. gnavus between Mecp2ZFN/+ and WT rats at this age. We also report significant changes in family Clostridiaceae and genus Lachnobacterium in RTT participants relative to controls, paralleling findings from the rat model.

SCFAs are important metabolic products of carbohydrate and protein breakdown. Based on LDA effect size (LEfSe) in this study, several SCFA-producing bacterial classifications are reduced in RTT rats (family Lachnospiracaea, genus Lachnobacterium, and R. gnavus) and RTT participants (genus Lachnobacterium and genus Faecalibacterium). These changes in microbial diversity are reflected in reductions in some KEGG pathways related to microbial SCFA production, including the pentose phosphate pathway (humans with RTT), purine metabolism (humans with RTT and RTT rats), and fatty acid elongation in mitochondria (RTT rats). Strati et al. and Borghi et al. also identified similar changes in LefSe score and abundance, respectively, in family Lachnospiraceae. Lachnospiraceae and Faecalibacterium are implicated in gut production of butyrate (44), which was detected at similar levels in WT and RTT rat fecal samples in this study. Among other SCFAs, butyrate specifically appears to improve intestinal epithelial integrity via anti-inflammatory pathways (45), and has also been shown to improve BBB integrity (17). Data is conflicting on butyrate's role in neurological disorders; humans with RTT reportedly have increased levels (per Borghi et al.) while levels are decreased in some ASD children (46). Notably, Faecalibacterium and Lachnospiraceae are also increased in healthy controls

relative to patients with Parkinson disease (47), another neurological disorder in which the role of the gut/brain axis appears increasingly important.

Family Clostridiaceae LDA scores are elevated in RTT rats and humans with RTT, with C. perfringens specifically being enriched in RTT rats. Clostridium species are known producers of SCFAs via fermentation of carbohydrates. Increases in stool SCFA concentration in humans with RTT has been previously noted by Strati et al. and Borghi et al., and more broadly been implicated in ASD (18). In particular, propionic acid, a known metabolic product of Clostridia species, is elevated in RTT participants in other studies (15,16). This SCFA is proposed to have a negative impact on mitochondrial metabolic pathways and has been used to develop an animal model recapitulating a variety of ASD symptoms (18,48). Interestingly, one mechanism by which the ketogenic diet is hypothesized to improve ASD symptoms is via a decrease in propionic acid transport across the BBB (48). Although carbohydrate metabolism is noted to be increased in RTT rats paralleling increases in C. perfringens, there does not appear to be an associated increase in propionic acid relative to WT at the examined time point. Whether or not differences might be identified at other time points corresponding to behavioral changes in the rat model remains to be elucidated.

In the current study, we demonstrated decreases in fecal content of acetic acid in RTT rats compared to WT. Acetic acid is detectable in the cerebrospinal fluid of humans (54) and radiolabeled colonic acetic acid can be found in biologically relevant amounts in the rodent brain soon after injection (55), indicating that acetic acid could be an important target in the gut-brain axis in neurological disorders. Indeed, humans with ASD have decreased fecal levels of acetic acid (56). Strati et al. show a modest decrease in acetic acid levels in RTT females compared to healthy controls; however, Borghi et al. show a slight increase in acetate (15,16). These disparate findings may be due to the effects of diet on acetic acid content, as our rats were not fed a humanized diet in any way. It may be important for future translation of SCFA-related pathways in rodent RTT research to consider dietary effects outside of disease effects.

We also show decreases in fecal content of caproic acid and heptanoic acid in RTT rats compared to WT. Caproic acid levels have been shown to be increased after chronic unpredictable stress in rats (57). Additionally, heptanoic acid levels were found to be increased in a cohort of ASD children compared to children with pervasive developmental disorders not otherwise specified (59). The unique fecal fatty acid profile in RTT rats may provide insight into the role of fatty acids in neurodevelopmental disease.

Additional metabolic pathways involved in amino acid metabolism are also predicted to be altered in RTT rats and participants in this study. A decrease in tryptophan metabolism may be mediated by a reduction in Ruminococcus gnavus (40) in the RTT rat. Tryptophan metabolites have been noted to affect gut immunity, motility, and permeability (40). Alterations in metabolic pathways involving free amino acids may also have direct effects on neurotransmitter homeostasis (e.g. serotonin). A reduction in free amino acid metabolism could lead to elevations in free amino acids, a phenomenon observed children with pervasive neurodevelopmental disorders and autism (46).

Limitations

The current study is potentially limited by age related differences in the microbiome of RTT participants and controls as well as medication effects on the microbiome. Additionally, the current study did not assess male

MeCP2-deficient rats, although the abundance of past RTT studies have focused on behavioral traits of male models of MeCP2 deficiency, and previous studies have indicated vast differences in the male and female rodent microbiome (49,50). Thus, it may be relevant to characterize additionally the microbiomes of male MeCP2-deficient rats to assess the translational relevance of this model to human RTT more broadly.

Conclusions

The current study represents an important step in translational RTT research. We have directly identified microbiome changes in an animal model that also are reflected in humans with RTT. Additionally, the rat model may provide unique advantages in future studies, as several of the RTT-associated microbial shifts identified herein and elsewhere consist of species that are found at abundances similar to humans in rats but not in mice (e.g. Akkermansia and some Clostridium and Lachnospiraceae clusters) (31). Finally, the described rat model provides for experimental control of diet and environment, as well as the ability for experimental manipulation, opening up potential for future translational studies on the gut-brain axis in RTT.

Abbreviations

RTT, Rett syndrome; MECP2, methyl-CpG-binding protein 2; ASD, Autism Spectrum Disorder; SCFA, short chain fatty acid; IBD, Irritable bowel syndrome; GI, gastrointestinal; CNS, Central Nervous System; BBB, blood brain barrier; WT, wild type; OUT, operational taxonomic unit; QIIME, Quantitative Insights Into Microbial Ecology; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; LefSe, Linear discriminant analysis effect size.

Declarations

Ethics approval and consent to participate

All experiments were conducted in accordance with NIH guidelines and were carried out with approval from the Animal Care and Use Committee of the University of Alabama at Birmingham. Consent for publication

All participants have consented to data publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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ML Olsen- Conceptualization, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing, Funding Acquisition, Project Administration, Supervision

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References

1. Percy AK, Lane JB. Rett Syndrome: Model of Neurodevelopmental Disorders. J Child Neurol. 2005 Sep 1;20(9):718–21.
2. Amir RE, Veyver IBV den, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2 , encoding methyl-CpG-binding protein 2. Nat Genet. 1999 Oct;23(2):185–8.
3. Cuddapah VA, Pillai RB, Shekar KV, Lane JB, Motil KJ, Skinner SA, et al. Methyl-CpG-binding protein 2 (MEPC2) mutation type is associated with disease severity in Rett Syndrome. J Med Genet. 2014 Mar;51(3):152–8.
4. Motil KJ, Caeg E, Barrish JO, Geerts S, Lane JB, Percy AK, et al. Gastrointestinal and Nutritional Problems Occur Frequently Throughout Life in Girls and Women with Rett Syndrome. J Pediatr Gastroenterol Nutr. 2012 Sep;55(3):292–8.

5. Wahba G, Schock SC, Claridge E, Bettolli M, Grynspan D, Humphreys P, et al. MeCP2 in the enteric nervous system. Neurogastroenterol Motil. 2015;27(8):1156–61.
6. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006 Feb;55(2):205–11.
7. Balsari A, Ceccarelli A, Dubini F, Fesce E, Poli G. The fecal microbial population in the irritable bowel syndrome. Microbiologica. 1982 Jul;5(3):185–94.
8. de Theije CGM, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, et al. Altered gut microbiota and activity in a murine model of autism spectrum disorders. Brain Behav Immun. 2014 Mar;37:197–206.
9. Scheperjans F, Aho V, Pereira PAB, Koskinen K, Paulin L, Pekkonen E, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord Off J Mov Disord Soc. 2015 Mar;30(3):350–8.
10. Olson CA, Vuong HE, Yano JM, Liang QY, Nusbaum DJ, Hsiao EY. The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet. Cell. 2018 14;173(7):1728-1741.e13.
11. Vuong HE, Yano JM, Fung TC, Hsiao EY. The Microbiome and Host Behavior. Annu Rev Neurosci. 2017;40(1):21–49.
12. Erny D, Hrabě de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci. 2015 Jul;18(7):965–77.
13. Takano T. Role of Microglia in Autism: Recent Advances. Dev Neurosci. 2015;37(3):195–202.
14. Sampson TR, Mazmanian SK. Control of Brain Development, Function, and Behavior by the Microbiome. Cell Host Microbe. 2015 May 13;17(5):565–76.
15. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. Altered gut microbiota in Rett syndrome. Microbiome. 2016 Jul 30;4(1):41.
16. Borghi E, Borgo F, Severgnini M, Savini MN, Casiraghi MC, Vignoli A. Rett Syndrome: A Focus on Gut Microbiota. Int J Mol Sci [Internet]. 2017 Feb 7 [cited 2019 Aug 9];18(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5343879/
17. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Tóth M, et al. The gut microbiota influences blood-brain barrier permeability in mice. Sci Transl Med. 2014 Nov 19;6(263):263ra158.
18. MacFabe DF. Enteric short-chain fatty acids: microbial messengers of metabolism, mitochondria, and mind: implications in autism spectrum disorders. Microb Ecol Health Dis. 2015;26:28177.
19. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012 Jun;486(7402):222–7.
20. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan 23;505(7484):559–63.
21. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. Nature. 2018;562(7728):583–8.
22. Lombardi LM, Baker SA, Zoghbi HY. MECP2 disorders: from the clinic to mice and back. J Clin Invest. 2015 Aug 3;125(8):2914–23.
23. Li W, Pozzo-Miller L. Beyond Widespread Mecp2 Deletions to Model Rett Syndrome: Conditional Spatio-Temporal Knockout, Single-Point Mutations and Transgenic Rescue Mice. Autism-Open Access. 2012;2012(Suppl 1):005.

24. Thor DH, Holloway WR. Social play in juvenile rats: a decade of methodological and experimental research. Neurosci Biobehav Rev. 1984;8(4):455–64.
25. Urcelay GP, Miller RR. On the generality and limits of abstraction in rats and humans. Anim Cogn. 2010 Jan;13(1):21–32.
26. Patterson KC, Hawkins VE, Arps KM, Mulkey DK, Olsen ML. MeCP2 deficiency results in robust Rett-like behavioural and motor deficits in male and female rats. Hum Mol Genet. 2016 Aug 1;25(15):3303–20.
27. Veeraragavan S, Wan Y-W, Connolly DR, Hamilton SM, Ward CS, Soriano S, et al. Loss of MeCP2 in the rat models regression, impaired sociability and transcriptional deficits of Rett syndrome. Hum Mol Genet. 2016 01;25(15):3284–302.
28. Voreades N, Kozil A, Weir TL. Diet and the development of the human intestinal microbiome. Front Microbiol [Internet]. 2014 [cited 2019 Aug 9];5. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2014.00494/full
29. Bressa C, Bailén-Andrino M, Pérez-Santiago J, González-Soltero R, Pérez M, Montalvo-Lominchar MG, et al. Differences in gut microbiota profile between women with active lifestyle and sedentary women. PLOS ONE. 2017 Feb 10;12(2):e0171352.
30. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe. 2008 Apr 17;3(4):213–23.
31. Flemer B, Gaci N, Borrel G, Sanderson IR, Chaudhary PP, Tottey W, et al. Fecal microbiota variation across the lifespan of the healthy laboratory rat. Gut Microbes. 2017 Jun 6;8(5):428–39.
32. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. (2014). STAMP: Statistical analysis of taxonomic and functional profiles. Bioinformatics, 30, 3123-3124.
33. Yanagibashi T, Hosono A, Oyama A, Tsuda M, Suzuki A, Hachimura S, et al. IgA production in the large intestine is modulated by a different mechanism than in the small intestine: Bacteroides acidifaciens promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells. Immunobiology. 2013 Apr;218(4):645–51.
34. Wang M, Zhou J, He F, Cai C, Wang H, Wang Y, et al. Alteration of gut microbiota-associated epitopes in children with autism spectrum disorders. Brain Behav Immun. 2019 Jan 1;75:192–9.
35. Yang NJ, Chiu IM. Bacterial Signaling to the Nervous System via Toxins and Metabolites. J Mol Biol. 2017 Mar 10;429(5):587–605.
36. Wioland L, Dupont J-L, Bossu J-L, Popoff MR, Poulain B. Attack of the nervous system by Clostridium perfringens Epsilon toxin: from disease to mode of action on neural cells. Toxicon Off J Int Soc Toxinology. 2013 Dec 1;75:122–35.
37. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, et al. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. PloS One. 2013;8(10):e76993.
38. Wang LW, Tancredi DJ, Thomas DW. The prevalence of gastrointestinal problems in children across the United States with autism spectrum disorders from families with multiple affected members. J Dev Behav Pediatr JDBP. 2011 Jun;32(5):351–60.
39. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011 Jun 24;12(6):R60.

40. Gao J, Xu K, Liu H, Liu G, Bai M, Peng C, et al. Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism. Front Cell Infect Microbiol. 2018;8:13.
41. Pozuelo M, Panda S, Santiago A, Mendez S, Accarino A, Santos J, et al. Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome. Sci Rep. 2015 Aug 4;5:12693.
42. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013 Sep;31(9):814–21.
43. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335–6.
44. Vital M, Karch A, Pieper DH. Colonic Butyrate-Producing Communities in Humans: an Overview Using Omics Data. mSystems. 2017 Dec;2(6).
45. Zheng L, Kelly CJ, Battista KD, Schaefer R, Lanis JM, Alexeev EE, et al. Microbial-Derived Butyrate Promotes Epithelial Barrier Function through IL-10 Receptor–Dependent Repression of Claudin-2. J Immunol. 2017 Sep 11;ji1700105.
46. Liu F, Li J, Wu F, Zheng H, Peng Q, Zhou H. Altered composition and function of intestinal microbiota in autism spectrum disorders: a systematic review. Transl Psychiatry. 2019 Jan 29;9(1):1–13.
47. Gerhardt S, Mohajeri MH. Changes of Colonic Bacterial Composition in Parkinson's Disease and Other Neurodegenerative Diseases. Nutrients. 2018 Jun 1;10(6).
48. Frye RE, Rose S, Chacko J, Wynne R, Bennuri SC, Slattery JC, et al. Modulation of mitochondrial function by the microbiome metabolite propionic acid in autism and control cell lines. Transl Psychiatry. 2016 Oct;6(10):e927.
49. Elderman M, Hugenholtz F, Belzer C, Boekschoten M, van Beek A, de Haan B, et al. Sex and strain dependent differences in mucosal immunology and microbiota composition in mice. Biol Sex Differ [Internet]. 2018 Jun 18 [cited 2019 Aug 9];9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6006852/
50. Org E, Mehrabian M, Parks BW, Shipkova P, Liu X, Drake TA, et al. Sex differences and hormonal effects on gut microbiota composition in mice. Gut Microbes. 2016 03;7(4):313–22.
51. Woese CR, Gutell RR. Evidence for several higher order structural elements in ribosomal RNA. Proc Natl Acad Sci U S A. 1989 May;86(9):3119–22.
52. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. PNAS. 2011 Mar 15;108(Supplement 1):4516–22.
53. Kumar A, Grover S, Batish VK. A multiplex PCR assay based on 16S rRNA and hly for rapid detection of L. monocytogenes in Milk. Food Measure. 2014 Sep 1;8(3):155–63.
54. Nagashima H, Morio Y, Meshitsuka S, Yamane K, Nanjo Y, Teshima R. High-resolution nuclear magnetic resonance spectroscopic study of metabolites in the cerebrospinal fluid of patients with cervical myelopathy and lumbar radiculopathy. Eur Spine J. 2010 Aug;19(8):1363–8.
55. Frost G, Sleeth ML, Sahuri-Arisoylu M, Lizarbe B, Cerdan S, Brody L, et al. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nat Commun [Internet]. 2014 Apr 29 [cited 2019 Sep 22];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4015327/

56. Liu S, Li E, Sun Z, Fu D, Duan G, Jiang M, et al. Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder. Sci Rep [Internet]. 2019 Jan 22 [cited 2019 Sep 22];9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6342986/
57. Li Y, Peng Y, Ma P, Yang H, Xiong H, Wang M, et al. Antidepressant-Like Effects of Cistanche tubulosa Extract on Chronic Unpredictable Stress Rats Through Restoration of Gut Microbiota Homeostasis. Front Pharmacol [Internet]. 2018 Aug 21 [cited 2019 Sep 22];9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6112285/
58. Skonieczna-Żydecka K, Grochans E, Maciejewska D, Szkup M, Schneider-Matyka D, Jurczak A, et al. Faecal Short Chain Fatty Acids Profile is Changed in Polish Depressive Women. Nutrients [Internet]. 2018 Dec 7 [cited 2019 Sep 22];10(12). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6316414/
59. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, et al. Fecal Microbiota and Metabolome of Children with Autism and Pervasive Developmental Disorder Not Otherwise Specified. PLoS One [Internet]. 2013 Oct 9 [cited 2019 Sep 22];8(10). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3793965/
60. Han, Jun, Karen Lin, Carita Sequeira, and Christoph H. Borchers. “An Isotope-Labeled Chemical Derivatization Method for the Quantitation of Short-Chain Fatty Acids in Human Feces by Liquid Chromatography-Tandem Mass Spectrometry.” Analytica Chimica Acta 854 (January 7, 2015): 86–94. https://doi.org/10.1016/j.aca.2014.11.015.

Tables

Table 1

	OTU	RTT Mean	WT Mean	Test Statistic	p-value	FDR p-value
	n.s	n.s	n.s	n.s	n.s	n.s
	f__Barnesiellaceae	0.0003	0	11.6667	0.0006	0.0318
	o__Campylobacterales	0.0124	0.0032	9.0536	0.0026	0.0498
	o__RF39	0.0002	0.0016	8.4308	0.0037	0.0498
	o__RF39	0.0006	0.0047	8.3705	0.0038	0.0515
5	o__Turicibacterales	0.0002	0.0089	10.5188	0.0012	0.0236
	o__RF39	0.0007	0.0052	9.2182	0.0024	0.0236
	o__Lactobacillales	0.2157	0.0675	9.0536	0.0026	0.0236
	f__Turicibacteraceae	0.0002	0.0089	10.5188	0.0012	0.0298
	o__Clostridiales	0.0148	0.0694	10.5000	0.0012	0.0298
	f__Odoribacteraceae]	0.0004	0.0015	9.0536	0.0026	0.0328
	f__Ruminococcaceae	0.0727	0.1806	7.7143	0.0055	0.0391
	g__Turicibacter	0.0002	0.0089	10.5188	0.0012	0.0446
	g__Lactobacillus	0.0846	0.0064	10.5000	0.0012	0.0446
	s__acidifaciens	0.0079	0.0007	9.7634	0.0018	0.0462
	g__Blautia	0.0637	0.0004	9.0536	0.0026	0.0462
	g__Odoribacter	0.0002	0.0011	8.3705	0.0038	0.0462
	g__Oscillospira	0.0278	0.0922	8.3705	0.0038	0.0462
	s__animalis	0.0002	0.0000	8.2286	0.0041	0.0462
	s__perfringens	0.0022	0.0000	7.8893	0.0050	0.0464
	s__uniformis	0.0023	0.0006	7.7143	0.0055	0.0472
	f__Lactobacillaceae	0.2011	0.0623	7.0848	0.0078	0.0486
3	n.s	n.s	n.s	n.s	n.s	n.s
5	o__Turicibacterales	0.0001	0.0571	10.6909	0.0011	0.0255
	f__Turicibacteraceae	0.0001	0.0571	10.6909	0.0011	0.0263
	f__Lachnospiraceae	0.1832	0.0579	9.7634	0.0018	0.0263
	f__Barnesiellaceae	0.0001	0.0000	9.4577	0.0021	0.0263
	g__Turicibacter	0.0001	0.0571	10.6909	0.0011	0.0385
	g__SMB53	0.0001	0.0018	10.5000	0.0012	0.0385
	f__Clostridiaceae	0.0001	0.0068	9.0698	0.0026	0.0385
	f__Lachnospiraceae	0.0712	0.0246	9.0536	0.0026	0.0385
3	n.s	n.s	n.s	n.s	n.s	n.s

2

ent	MecP2 mutation	Age	Mother Age	Weight	Feeding tube	Constipation	Seizures	Medication
	R255X	31	55	44.7 kg	Y	Y	Y	carbamazepine for seizures; multiple meds for pulmonary function: albuterol, Advair, ipratropium bromide, nasone; omeprazole (GE reflux); symethicone for gas; probiotic (constipation); bethanecol (stomach emptying); ciproflaxin antibiotic; glycopyrrolate (drooling); depo-provera (birth control)
	R294X	26	56	69.8 kg	N	Y	Y	Topamax, Vimpat, Lamotrigine, Diastat rescue (seizures); Miralax (constipation); Klonopin for sleep or agitation; trazodone (sleep); Namenda and Latuda (mood); Prilosec (GE reflux)
	3' truncation	16	46	34.6 kg	N	Y	Y	Miralax and senna (constipation); baclofen (tone); norethindrone (birth control); Lamictal, Tranxene, Valium (seizures); CBD oil
	splice site	14	47	54.0 kg	N	N	N	trazodone (sleep)
	806delG	10	32	39.6 kg	N	N	Y	trazodone and melatonin (sleep); Miralax; Prevacid (GE reflux); glycopyrrolate (drooling); Keppre, phenobarb (seizures); carnitine
	789dupC	9	46	NA	Y	N	Y	Compleat Pediatric; glycopyrrolate (drooling); Miralax; Erythromycin (stomach emptying); trazodone (sleep); Lamictal (seizures)

Additional Files

Additional File 1, .pdf, Figure S1. Alpha Diversity rarefaction curves for RTT and WT rats p21-p548.

Additional File 2, .pdf, Figure S2. Beta Diversity metrics for RTT and WT rats p35-548.

Additional File 3, .pdf, Figure S3. Complete KEGG pathway analysis for RTT and WT rats at p105.

Additional File 4, .pdf, Figure S4. Short Chain Fatty Acid (SCFA) analysis by LC-MS/MS. Overlaid chromatogram traces for each of the SCFA species, from Acetate (C2) to Octanoate (C8).

Additional File 5, .pdf, Table S1. Complete taxonomy information for significant OTU differences in RTT rats and WT rats p21-548.

Figures

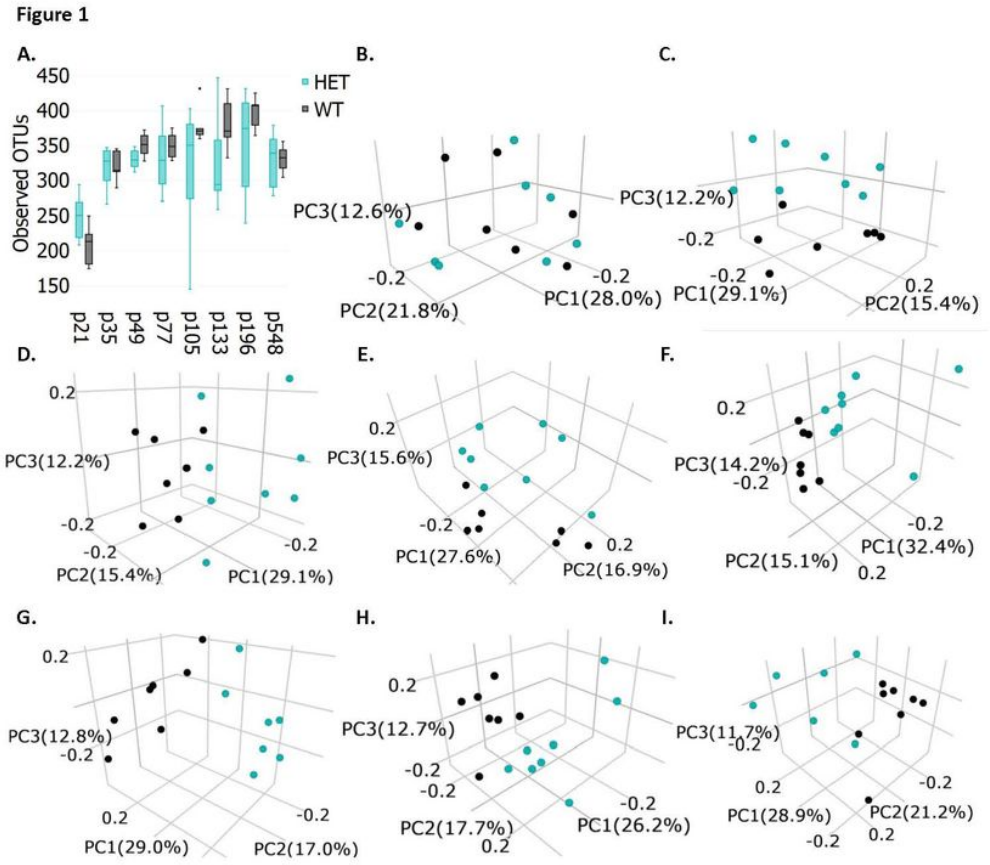

Figure 1. (A) Alpha diversity of HET and WT rats as measured by observed OTUs n.s. using Mann-Whitney U tests. (B) Beta diversity of p21 HET and WT rats. n.s. (C) Beta diversity of p35 HET and WT rats. n.s (D) Beta diversity of p49 HET and WT rats. p = 0.007. (E) Beta diversity of p77 HET and WT rats. p = 0.019. (F) Beta diversity of p105 HET and WT rats. p = 0.001. (G) Beta diversity of p133 HET and WT rats. p = 0.001. (H) Beta diversity of p196 HET and WT rats. p = 0.003. (H) Beta diversity of p548 HET and WT rats. p = 0.003. All beta diversity graphs depict bray curtis distance, p values calculated using Kruskal Wallis metrics.

Figure 1

(A) Alpha diversity of HET and WT rats as measured by observed OTUs n.s. using Mann-Whitney U tests. (B) Beta diversity of p21 HET and WT rats. n.s. (C) Beta diversity of p35 HET and WT rats. n.s (D) Beta diversity of p49 HET and WT rats. p = 0.007. (E) Beta diversity of p77 HET and WT rats. p = 0.019. (F) Beta diversity of p105 HET and WT rats. p = 0.001. (G) Beta diversity of p133 HET and WT rats. p = 0.001. (H) Beta diversity of p196 HET and WT rats. p = 0.003. (H) Beta diversity of p548 HET and WT rats. p = 0.003. All beta diversity graphs depict bray curtis distance, p values calculated using Kruskal Wallis metrics.

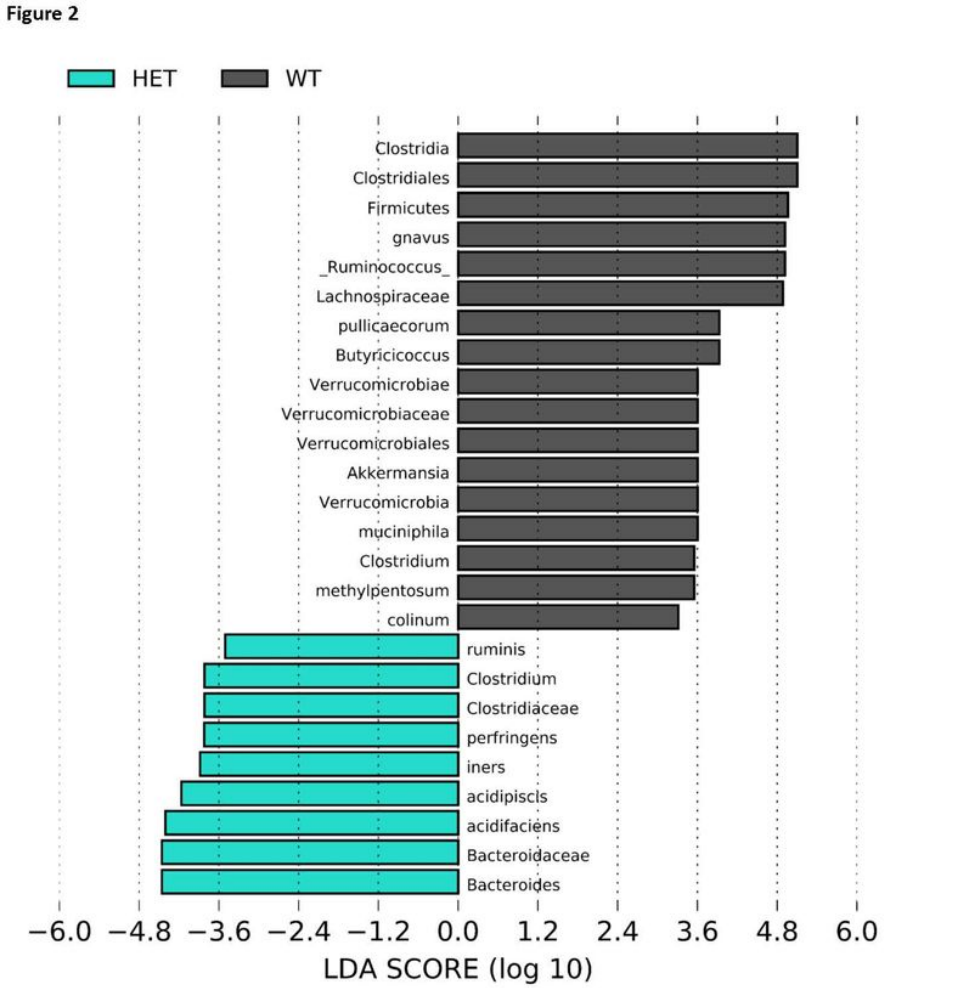

Figure 2. Functional characterization of OTUs represented in the gut microbiota of p105 RTT rats and p105 WT rats. Significant OTUs have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA > 2. Teal histograms: OTUs enriched in RTT rats. Gray histograms: OTUs enriched in WT rats.

Figure 2

Functional characterization of OTUs represented in the gut microbiota of p105 RTT rats and p105 WT rats. Significant OTUs have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA > 2. Teal histograms: OTUs enriched in RTT rats. Gray histograms: OTUs enriched in WT rats.

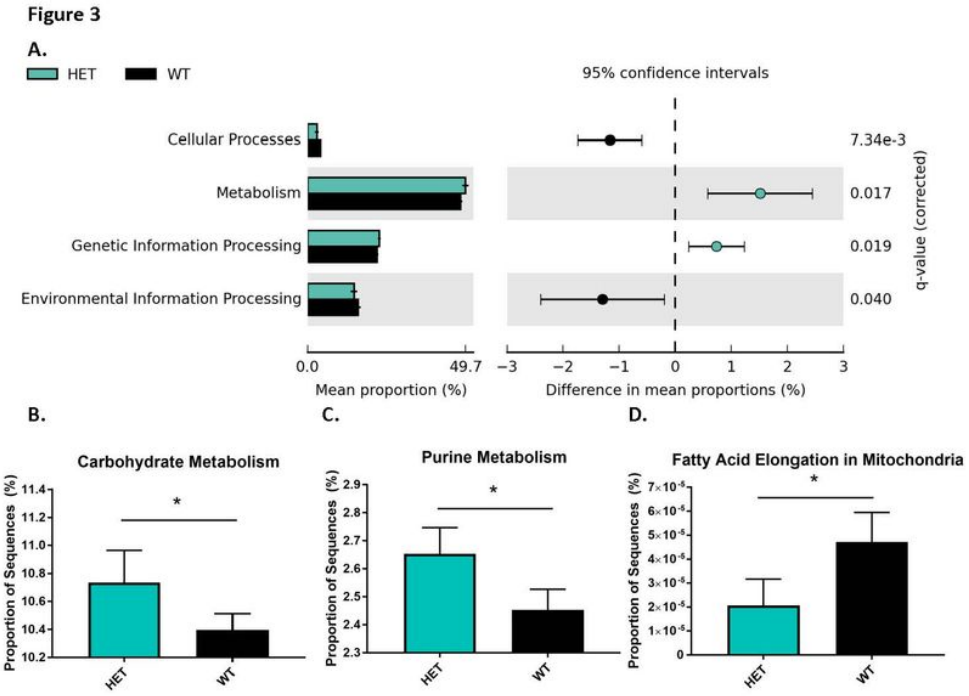

Figure 3. (A) Level 1 KEGG pathway predicted difference for p105 RTT rats vs. WT controls. All p values are FDR adjusted. (B) Carbohydrate Metabolism predicted difference p105 in RTT rats vs. WT controls (p = 0.042). (C) Purine Metabolism predicted difference at p105 in RTT rats vs. WT controls (p = 0.028). (D) Fatty Acid Elongation in Mitochondria predicted difference at p105 in RTT rats vs. WT controls (p = 0.036). All p values are FDR adjusted. * represents FDR adjusted p > 0.05.

Figure 3

(A) Level 1 KEGG pathway predicted difference for p105 RTT rats vs. WT controls. All p values are FDR adjusted. (B) Carbohydrate Metabolism predicted difference p105 in RTT rats vs. WT controls (p = 0.042). (C) Purine Metabolism predicted difference at p105 in RTT rats vs. WT controls (p = 0.028). (D) Fatty Acid Elongation in

Mitochondria predicted difference at p105 in RTT rats vs. WT controls (p = 0.036). All p values are FDR adjusted. * represents FDR adjusted p > 0.05.

Figure 4

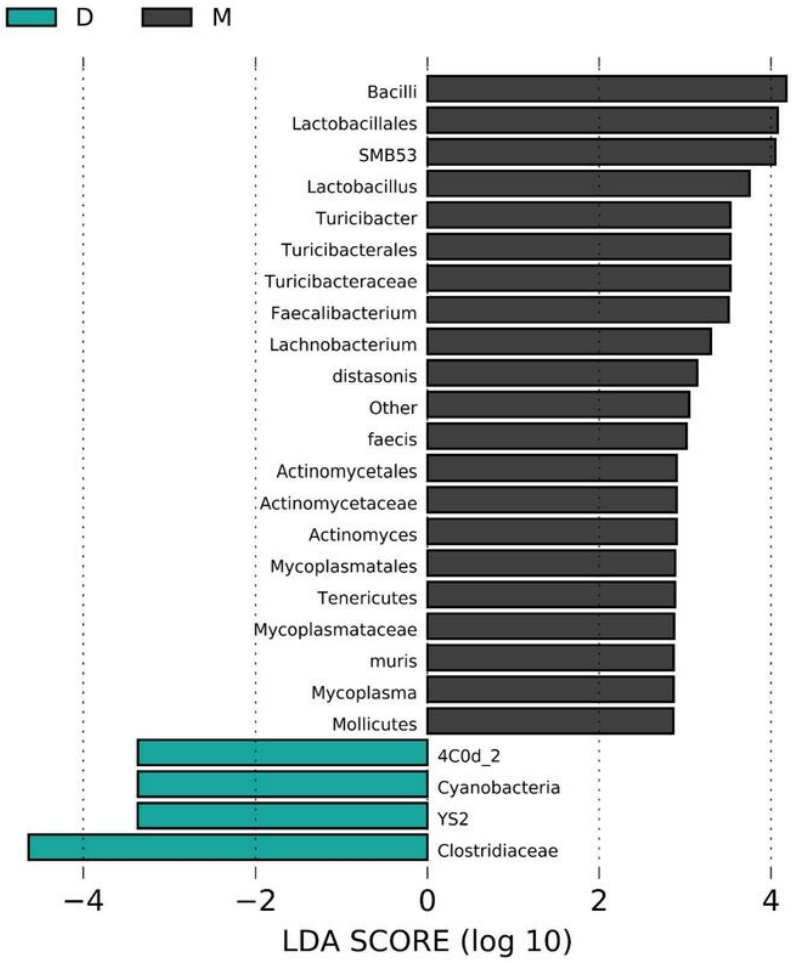

Figure 4. Functional characterization of OTUs represented in the gut microbiota of patients with RTT (D) compared with their mothers (M). Significant OTUs have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA > 2. Teal histograms: OTUs enriched in RTT patients (D). Gray histograms: OTUs enriched in mothers of RTT patients (M).

Figure 4

Functional characterization of OTUs represented in the gut microbiota of patients with RTT (D) compared with their mothers (M). Significant OTUs have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA > 2. Teal histograms: OTUs enriched in RTT patients (D). Gray histograms: OTUs enriched in mothers of RTT patients (M).

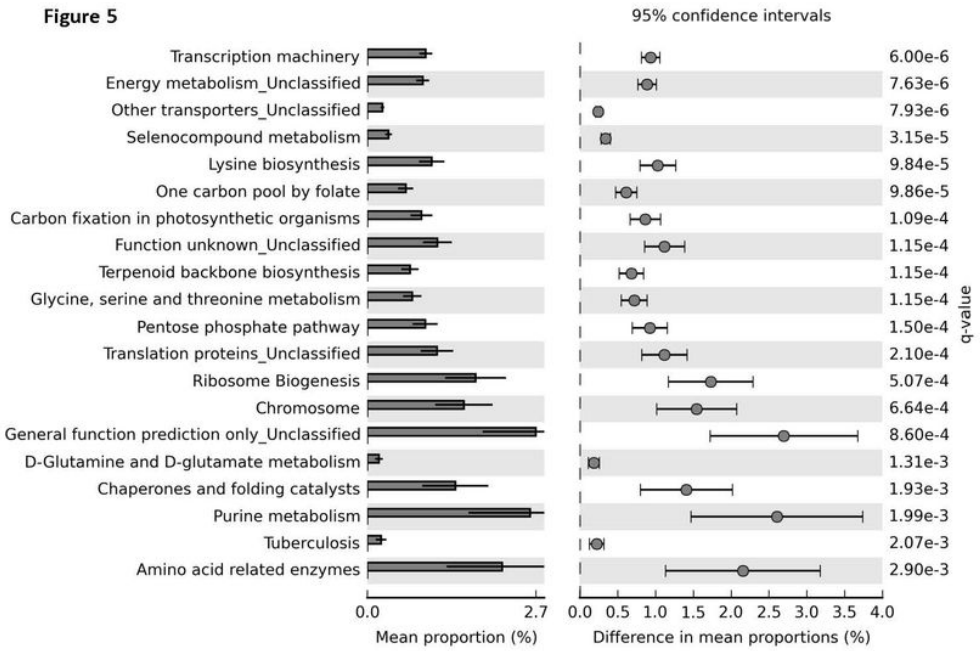

Figure 5. Predicted KEGG pathway differences in RTT patients and their mothers (controls). All significant pathways were predicted to be enriched in mothers compared to their daughters with RTT. All p values are FDR adjusted.

Figure 5

Predicted KEGG pathway differences in RTT patients and their mothers (controls). All significant pathways were predicted to be enriched in mothers compared to their daughters with RTT. All p values are FDR adjusted.

Figure 6

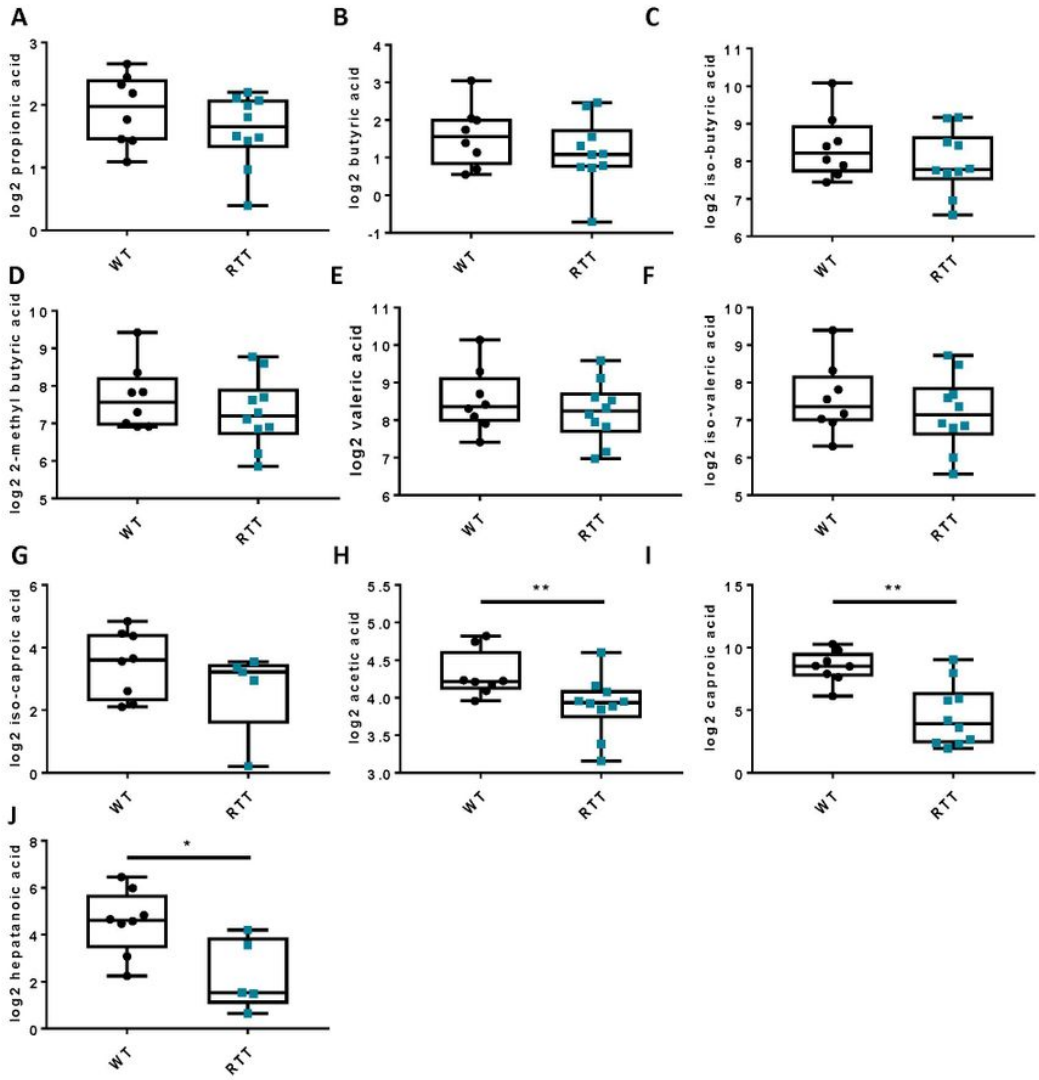

Figure 6. Fecal concentrations of short chain fatty acids (SCFAs) in RTT vs. WT p105 rats. Log2 fecal levels of (a) propionic acid n.s. (b) butyric acid n.s. (c) iso-butyric acid n.s. (d) 2-methyl butyric acid n.s. (e) valeric acid n.s. (f) iso-valeric acid n.s. (g) iso-caproic acid n.s. (h) acetic acid p = 0.0062 (i) caproic acid p = 0.0044, (i) heptanoic acid p = 0.0186. Note: for some SCFAs, multiple RTT samples were excluded from analysis because samples were below measurable levels.

Figure 6

Fecal concentrations of short chain fatty acids (SCFAs) in RTT vs. WT p105 rats. Log2 fecal levels of (a) propionic acid n.s. (b) butyric acid n.s. (c) iso-butyric acid n.s. (d) 2-methyl butyric acid n.s. (e) valeric acid n.s. (f) iso-valeric acid n.s. (g) iso-caproic acid n.s. (h) acetic acid p = 0.0062 (i) caproic acid p = 0.0044, (i) heptanoic acid p = 0.0186. Note: for some SCFAs, multiple RTT samples were excluded from analysis because samples were below measurable levels.

Supplementary Files

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- TableS1.pdf
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