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

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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 Lachnospiraceae 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 Lachnobotrium 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
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 Lachnospiraceae, 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 Lachnospiraceae, 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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**Author contributions**

AF Gallucci – Conceptualization, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing
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Tables

Table 1
| Age | OTU | RTT Mean | WT Mean | Test Statistic | p-value |
|-----|-----|----------|---------|----------------|---------|
| p21 | n.s | n.s      | n.s     | n.s            | n.s     |
| p35 | f__Barnesiellaceae | 0.0003 | 0       | 11.6667        | 0.0003  |
| p49 | o__Campylobacterales | 0.0124 | 0.0032  | 9.0536         | 0.0003  |
|     | o__RF39         | 0.0002 | 0.0016  | 8.4308         | 0.0003  |
| p77 | o__RF39         | 0.0006 | 0.0047  | 8.3705         | 0.0003  |
| p105| o__Turicibacterales | 0.0002 | 0.0089  | 10.5188        | 0.0003  |
|     | o__RF39         | 0.0007 | 0.0052  | 9.2182         | 0.0003  |
|     | o__Lactobacillales | 0.2157 | 0.0675  | 9.0536         | 0.0003  |
|     | f__Turicibacterales | 0.0002 | 0.0089  | 10.5188        | 0.0003  |
|     | o__Clostridiales | 0.0148 | 0.0694  | 10.5000        | 0.0003  |
|     | f__Odoribacteraceae | 0.0004 | 0.0015  | 9.0536         | 0.0003  |
|     | f__Ruminococcaceae | 0.0727 | 0.1806  | 7.7143         | 0.0003  |
|     | g__Turicibacter | 0.0002 | 0.0089  | 10.5188        | 0.0003  |
|     | g__Lactobacillus | 0.0846 | 0.0064  | 10.5000        | 0.0003  |
|     | s__acidifaciens | 0.0079 | 0.0007  | 9.7634         | 0.0003  |
|     | g__Blautia | 0.0637 | 0.0004  | 9.0536         | 0.0003  |
|     | g__Odoribacter | 0.0002 | 0.0011  | 8.3705         | 0.0003  |
|     | g__Oscillospira | 0.0278 | 0.0922  | 8.3705         | 0.0003  |
|     | s__animalis | 0.0002 | 0.0000  | 8.2286         | 0.0003  |
|     | s__perfringens | 0.0022 | 0.0000  | 7.8893         | 0.0003  |
|     | s__uniformis | 0.0023 | 0.0006  | 7.7143         | 0.0003  |
|     | f__Lactobacillaceae | 0.2011 | 0.0623  | 7.0848         | 0.0003  |
| p133| n.s | n.s      | n.s     | n.s            | n.s     |
| p196| o__Turicibacterales | 0.0001 | 0.0571  | 10.6909        | 0.0003  |
|     | f__Turicibacterales | 0.0001 | 0.0571  | 10.6909        | 0.0003  |
|     | f__Lachnospiraceae | 0.1832 | 0.0579  | 9.7634         | 0.0003  |
|     | f__Barnesiellaceae | 0.0001 | 0.0000  | 9.4577         | 0.0003  |
|     | g__Turicibacter | 0.0001 | 0.0571  | 10.6909        | 0.0003  |
|     | g__SMB53 | 0.0001 | 0.0018  | 10.5000        | 0.0003  |
|     | f__Clostridiaceae | 0.0001 | 0.0068  | 9.0698         | 0.0003  |
|     | f__Lachnospiraceae | 0.0712 | 0.0246  | 9.0536         | 0.0003  |
| p548| n.s | n.s      | n.s     | n.s            | n.s     |
## Table 2

| Patient | MecP2 mutation | Age | Mother Age | Weight | Feeding tube | Constipation | Seizures |
|---------|----------------|-----|------------|--------|--------------|--------------|----------|
| D1      | R255X          | 31  | 55         | 44.7 kg| Y            | Y            | Y        |
| D2      | R294X          | 26  | 56         | 69.8 kg| N            | Y            | Y        |
| D3      | 3' truncation  | 16  | 46         | 34.6 kg| N            | Y            | Y        |
| D7      | splice site    | 14  | 47         | 54.0 kg| N            | N            | N        |
| D5      | 806delG        | 10  | 32         | 39.6 kg| N            | N            | Y        |
| D6      | 789dupC        | 9   | 46         | NA     | Y            | N            | Y        |

### 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.

(A) Alpha diversity of HET and WT rats as measured by observed OTUs n.s. using
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.

(A) Level 1 KEGG pathway predicted difference for p105 RTT rats vs. WT controls.
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).

Functional characterization of OTUs represented in the gut microbiota of patients
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. 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, (j) heptanoic acid p = 0.0186. Note: for some SCFAs, multiple RTT samples were excluded from analysis because samples were below measurable levels.
Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

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Table S1.pdf
Fig S1.pdf
Fig S2.pdf
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