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Microbial community changes in a female rat model of Rett syndrome

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Abstract

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that is predominantly caused by alterations of the methyl-CpG-binding protein 2 (MECP2) gene. Disease severity and the presence of comorbidities such as gastrointestinal distress vary widely across affected individuals. The gut microbiome has been implicated in neurodevelopmental disorders such as Autism Spectrum Disorder (ASD) as a regulator of disease severity and gastrointestinal comorbidities. Although the gut microbiome has been previously characterized in humans with RTT compared to healthy controls, 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.

By evaluating the microbial community across postnatal development as behavioral symptoms appear and progress, we have identified microbial taxa that are differentially abundant across developmental timepoints in a zinc-finger nuclease rat model of RTT compared to WT. We have

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Ethical Statement

All experiments were conducted in accordance with NIH guidelines, the U.K. Animals (Scientific Procedures) Act 1986, and were carried out with approval from the Animal Care and Use Committee of the University of Alabama at Birmingham.

additionally identified p105 as a key translational timepoint. Lastly, we have demonstrated that fecal SCFA levels are not altered in RTT rats compared to WT rats across development. Overall, these results represent an important step in translational RTT research.

1. Introduction

RTT is an X-linked progressive neurodevelopmental disorder that affects up to 1 in 10,000 girls annually in the United States (Percy and Lane, 2005). 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 (Amir et al., 1999). However, severity of RTT symptoms can vary wildly across individuals due to differences in X-chromosome inactivation and specific gene mutation (Amir et al., 1999; Cuddapah et al., 2014). 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 1000 families of individuals with RTT revealed that comorbid gastrointestinal (GI) symptoms are present in a large majority (92%) of people with RTT (Motil et al., 2012) 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 (Motil et al., 2012). 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 (Wahba et al., 2015), 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 (Wahba et al., 2015).

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) (Manichanh et al., 2006; Balsari et al., 1982). More recently, gut microbiota have been shown to be altered in a variety of neurological disorders, including Autism Spectrum Disorder (ASD) (de Theije et al., 2014), Parkinson's Disease (Scheperjans et al., 2015), and epilepsy (Olson et al., 2018). 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 (Vuong et al., 2017). The microbiome has also been shown to be critical for appropriate brain development with respect to microglial cells (Erny et al., 2015), which function as central nervous system (CNS) immune mediators and appear to be aberrantly distributed and activated in the brains of individuals with ASD (Takano, 2015). 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 (Sampson and Mazmanian, 2015), which may act in a feed-forward fashion to contribute to overall disease pathogenesis.

Previous studies have reported changes in the microbiome in patients with RTT compared to typically developing females (Strati et al., 2016) as well as changes in production of microbial metabolites such as short chain fatty acids (SCFAs) (Strati et al., 2016; Borghi et al., 2017), which are known to affect blood-brain barrier (BBB) integrity (Braniste et al., 2014) and have been implicated in ASD (MacFabe, 2015). 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.

RTT has historically been modeled in mice through a variety of genetic manipulations (Lombardi et al., 2015; Li and Pozzo-Miller, 2012). 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 (Thor and Holloway, 1984) and have the ability to complete higher level cognitive tasks (Urcelay and Miller, 2010) 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 (Patterson et al., 2016; Veeraragavan et al., 2016). Importantly, development of abnormalities in MeCP2 *Mecp2*ZFN/+ 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 *Mecp2*ZFN/+ 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 (Voreades et al., 2014), 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.

2. Methods

2.1 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 (Patterson et al., 2016). Briefly, Sprague Dawley females lacking one copy of MeCP2 (*Mecp2*ZFN/+) 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, however, care was taken to balance number of cages across groups to prevent cage-effects. Animals were provided with food and water ad libitum, and kept under standard 12-h light-dark cycles.

2.2 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 -80°C at time points indicated in the text.

2.3 DNA isolation

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. DNA was quantified and samples stored at -80°C until sequenced.

2.4 Sequencing

Isolated DNA was quantitated prior to PCR and barcoded PCR amplification of the V4 region of the 16S rRNA gene (Woese and Gutell, 1989) was accomplished using degenerate primers originally taken from Caporaso et al., 2011 (Caporaso et al., 2011). We used primers as described by Kumar et al., 2014 (Kumar et al., 2014) 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 250 bp run. *2.5. 16S analysis* Sequencing data was analyzed with QIIME (Caporaso et al., 2011). 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 (Parks et al., 2014). Linear Discriminant effect size analysis (LEfSe) was performed with default parameters (Segata et al., 2011). OTU tables generated in QIIME were assigned LDA scores and graphed utilizing the Galaxy web application.

2.5 16S analysis

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2.6 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 (Han et al., 2015). A 12-point calibration curve from 10 mM to 0.975 μ M for acetic acid, and 1 mM to 0.098 μ M 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 μ L/mg volume to weight ratio in 50/50 v/v EtOH/water. The sample extracts were then derivatized using 1:1200 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 20 \times excess cold 10% ethanol in water with 1% formic acid. A 25 μ L aliquot of the reaction solution from each sample was transferred and 25 μ L aliquot of the stable isotope standards (SIS) solution was added. 5 μ L 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 \times 50 mm 1.7 μ m BEH C18 column fitted with a Waters Acquity C18 1.7 μ m Vanguard guard column. Analytes were separated using a gradient from 15% solvent B to 100% solvent B in 9.5 min. Solvents A & B were 0.1% formic acid in water and acetonitrile, respectively. The total UPLC analysis time was approximately 13.5 min. 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>).

2.7 Statistics

Alpha diversity values were calculated with observed OUT, Chao1, Shannon, and Simpson metrics. Beta diversity analyses were calculated with bray-curtis distance. LDA score for LEfSe analysis was performed on differential abundance tables generated with QIIME. Differences between RTT and controls were assessed using Mann-Whitney *U* tests. All *p*-values for taxonomy differences and KEGG pathways are FDR adjusted. SCFA log₂ values were analyzed using Mann-Whitney *U* tests.

3. Results

3.1 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 p35 to p196. 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-d). Beta diversity measured by bray-curtis difference was also similar across groups at p35 (Fig. 2 a). However, Mecp2ZFN/+ rats begin to diverge from WT rats in beta diversity at p49, and continue to be significantly distinct through p196 (Fig. 2 b-f). Although beta diversity values separate at these timepoints, Firmicutes/Bacteroides ratio does not differ between RTT and WT rodents at any of the selected timepoints (Supp. Fig. 1). 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.

3.2 MeCP2-deficient rats show developmental shifts in the microbiome

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 p35, 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. Although behavior and metabolic symptoms begin to develop following p35, there are no specific shifts in microbial taxonomies at p35, p49, or p77 timepoints, suggesting a stable microbiome across early development.

Of note, the gut microbiomes of RTT and WT rats significantly diverge at p105 across multiple diverse taxonomies. Importantly, we note shifts in *Bacteroides* spp., *Lactobacillus* spp., and family Ruminococcaceae that mirror similar microbiome alterations in patients with RTT (Strati et al., 2016; Borghi et al., 2017) (Fig. 3 b-d). Additionally, *A. muciniphila* are lower in abundance in Mecp2ZFN/+ rats compared to WT (Fig. 3 a). Notably, *A. muciniphila* has been described as protective against epilepsy in rodents (Olson et al., 2018), and its reduction in abundance has also been reported in children with autism (Wang et al., 2011). Additionally, bacteria in genus *Turicibacter* were reduced in p105 RTT rats. Interestingly, reduction in *Turicibacter* is also reported in patients with ASD (Liu et al., 2019), and reduction in *Turicibacter* correlates with reduced microbiome stability in ASD patients (Berding and Donovan, 2020). Other taxonomies reduced in p105 RTT rodents included *Desulfovibrio*, *Oscillospira*, *Odoribacter*, and *Clostridiales*. Alternatively, *Mogibacteriaceae*, *Blautia*, and *Pediococcus* taxonomies were increased in p105 RTT rodents compared to WT.

By p133, although beta diversity values still show separation, 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 (Flemer et al., 2017). This lack of specific taxonomy alterations persists at all of the later timepoints examined in this study. Taken together, these findings highlight p105 as an important translational timepoint as microbial community shifts at this timepoint display the most similarity to published studies in RTT patients.

3.3 Linear Discriminant Analysis reveals additional developmental shifts in the microbiome of MeCP2-deficient rats

Previous studies have additionally utilized LEfSe scores to identify statistically significant differences in microbiota classifications (Segata et al., 2011) (Fig. 4, Table 2). We performed LEfSe analysis on RTT and WT rats to supplement our abundance data and further compare our findings to data on RTT patients. We found no significant differences between genotypes at the p35 timepoint, confirming that microbiota alterations due to genotype do not occur immediately following weaning. In accordance with significant alterations in beta diversity, RTT rats at p49 display enrichment in *Streptococcus agalactiae*, *Bifidobacterium bifidum*, and *Lactobacillus acidipiscis*. By p77 however, RTT samples begin to display reductions in *Campylobacter rectus* and *Bifidobacterium pseudolongum* compared to WT, while maintaining an enrichment in *Streptococcus agalactiae* and introducing an enrichment in genus *Pediococcus*. These enrichments are maintained through p105, and enrichments in *Lactobacillus ruminis*, genus *Bilophila*, and genus *Holdemania* appear at this timepoint. Importantly, these enrichments in *Lactobacillus* species mirror enrichments found in LEfSe analyses in human RTT patients (Strati et al., 2016). Additionally, WT samples display significant enrichment in *Enterococcus casseliflavus*, understudied WAL 1855D, genus *Anaerofustis*, as well as genus *Lachnobacterium*. At p133, WT enrichment in *Anaerofustis* is maintained and WT samples are additionally enriched in *Bifidobacterium longum*, *Bifidobacterium pseudolongum*, and genus *Turcibacter*. RTT samples at p133 maintain enrichment in *Pediococcus*, but additionally are enriched in understudied bacteria SHA_98 and AF12, as well as genus *Mannheimia*. At p196 RTT samples are only enriched in *Pediococcus*, but WT samples are still enriched in *Bifidobacterium longum*, *Bifidobacterium pseudolongum*, genus *Anaerofustis*, and genus *Facklamia*.

In summary, beginning at p77 RTT samples display sustained enrichment in *Pediococcus* as well as sustained deficiency in *Bifidobacterium* species. These results suggest that RTT rats may benefit from *Bifidobacterium* probiotic therapy in adulthood. Additionally, these results again highlight p105 as a translational timepoint at which the RTT rat microbiome most closely resembles changes that occur in the microbiomes of humans with RTT.

3.4 Impact of MecP2 mutation on fecal SCFA levels across development

As previous studies in humans with RTT showed alterations in fecal SCFA content (Strati et al., 2016; Borghi et al., 2017) and our sequencing results indicate alterations at p105 in SCFA-related microbes, we next examined the content of 12 SCFAs across development in fecal samples from p35, p105, and p196 RTT and WT rats. There were no measurable levels of 3-methyl valeric acid or octanoic acid in our samples. As expected, we found no differences in fecal SCFA levels in p35 compared to WT rodents (Table 3). However,

although we report marked decreases in bacteria that produce SCFAs, such as lactic acid bacteria and Ruminococcaceae at both p105 and p196, we additionally found no difference in any of the measured SCFAs in our WT vs our RTT samples at p105 and at p196 (Table 3). As SCFAs exhibit distinct biological gradients throughout the digestive tract (reviewed in Morrison and Preston, 2016), it is possible that fecal SCFA measurements may not be precise enough to catch site-specific alterations in SCFA content. Additionally, some SCFAs such as acetate are produced by a variety of microbiota (Miller and Wolin, 1996), indicating that the loss of specific microbes is not enough to alter overall SCFA production.

3.5 Timeline of microbial, behavioral, and motor shifts in female RTT rats

Rat models of RTT have previously been utilized to assess the longitudinal effects of MECP2 mutations on neural, motor, and metabolic symptoms. Fig. 5 (Supplementary table 2) depicts the timeline of gut microbiome shifts in female RTT rats in the current study compared to previously published longitudinal data on other RTT behavioral characteristics. Published work from our lab shows changes in brain weight in female RTT rats beginning at p21 and persisting through p540 (Patterson et al., 2016). Previous studies have additionally reported social abnormalities in the three chamber test in female RTT rat models as early as p35 (Wu et al., 2016). Although microbiome alteration has been reported to drive behavioral symptoms in rodent models of ASD (Sharon et al., 2019), and a healthy gut microbiome is essential for typical social development in rodents (Arentsen et al., 2015; Desbonnet et al., 2014), it is clear that neural correlates of RTT appear before significant microbiome alterations in female rat models of RTT.

Changes in body weight are known to affect the gut microbiome in both humans and rodents (Palleja et al., 2016; Ussar et al., 2015). Previous work from our lab and others shows weight gain in female RTT rats compared to WT beginning at p77 and persisting through late adulthood, although WT rodents do also experience considerable weight gain by p360 (Patterson et al., 2016). It is likely that weight gain drives later timepoint microbiome shifts, as body weight difference from WT is most robust at p196, and microbial changes also fluctuate at p196. However, it is possible that microbiome alterations at earlier timepoints actually drive some of these metabolic abnormalities in RTT rodents, as studies in germ free rodents indicate that the gut microbiome regulates weight gain (Bäckhed et al., 2004).

Difficulty walking is another common symptom of RTT in humans. Previous studies have reported rotor rod task deficiencies in female RTT et al., 2016). As rotor rod tasks do not account for complex gait changes, Patterson et al. also reported Catwalk™ gait analysis. Fig. 5 depicts an averaged score of female Catwalk gait analysis in comparison to microbial shifts identified in the current study. Most gait changes in female RTT rats were observed to onset at p180 and persist into late adulthood. These motor abnormalities largely appear after changes in the gut microbiome, suggesting a possible role for microbial community changes in affecting severity of motor symptoms in RTT rats.

4. Discussion

The human RTT microbiome has previously been characterized by two groups (Strati et al., 2016; Borghi et al., 2017). Previous studies indicated that the microbiomes of humans

with RTT differ significantly in alpha and/or beta diversity compared to those of healthy controls (Strati et al., 2016; Borghi et al., 2017). 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. 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 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. ovatus*, *B. uniformis*, *L. ruminis*, and *A. muciniphila* in Mecp2ZFN/+ rats compared to WT. Excitingly, these findings mirror alterations in similar bacterial taxonomies found in published studies of humans with RTT, humans with ASD, and rodent models of epilepsy. LefSe analysis also revealed significant differences in species in the genus Bifidobacterium as well as species in the genus Lactobacillus, further mirroring published findings in humans (Borghi et al., 2017). In contrast, we show decreases in Desulfovibrio, Odoribacter, Clostridiales, and Oscillospira, and increases in Blautia in p105 RTT rats compared to WT, while studies in humans have indicated that these species are altered in the opposite direction in patients with ASD (Tomova et al., 2015; Zhai et al., 2019; Coretti et al., 2017; Luna et al., 2017). This may point to differential microbiome profiles in RTT compared to ASD. Specifically, some of these microbiome differences may be due to sex-specific effects, as the current study examines a female model of RTT while many published studies on ASD describe male patients, and Oscillospira has been noted to change in abundance in male versus female ASD rodent models (Coretti et al., 2017).

LEfSe analysis revealed additional taxonomic differences in RTT rodents compared to WT. Specifically, RTT p49 samples begin to show an enrichment in *L. acidipiscis*, *B. bifidum*, and *S. agalactiae*. Interestingly, *Streptococcus agalactiae* is classified as a group B streptococcus (GBS) pathogen, and GBS exposure during gestation can lead to ASD-like behavioral symptoms in male rats (Allard et al., 2017) although it is unclear if adulthood GBS infection has effects on behavior. Over-abundance of *S. agalactiae* continues through p77, and a persistent overabundance of Pediococcus appears at this timepoints. Additionally, a reduction in Bifidobacterium and Campylobacter appear at p77, highlighting the possible benefits on Bifidobacterium-based probiotics in RTT rats beginning at this timepoint. P105 still represents the most severe microbiome alterations in the RTT rats, highlighted by additional alterations in Lactobacillus species which mirror alterations in humans with RTT (Strati et al., 2016). Additionally, a reduction in Anaerofustis begins at p105 and persists through all measured timepoints.

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. Strati et al. and Borghi et al. also identified similar changes in LefSe score and abundance in *Lactobacillus*. Although 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 (MacFabe, 2015) we found no alterations in fecal SCFA levels across development in female RTT rats. SCFAs exhibit distinct biological gradients across various mucosal surfaces (reviewed in Morrison and Preston 2016), bringing to light the possibility that fecal SCFA measurements may not be precise enough to catch site-specific alterations in SCFA content. Additionally, genes encoding SCFA production are spread out across a wide variety of bacteria (Miller and Wolin, 1996), indicating that the loss of specific microbes may not be enough to alter fecal SCFA levels. Lastly, diet has been shown to have a large impact on fecal SCFA concentration (Flint et al., 2015), and our rodents were not fed a humanized diet, which could explain differences in human and rodent fecal SCFA levels. Whether or not differences might be identified at other time points corresponding to behavioral changes in the rat model or in rodents on humanized diets remains to be elucidated.

Finally, we compared the timeline of disease progression in previously published work on female RTT rats to our findings on developmental microbiome shifts. Social abnormalities in female RTT rodents compared to WT appear before the onset on microbiome changes (Miller and Wolin, 1996). The current study did not examine pre-weaning developmental timepoints, nor did we examine the maternal microbiome, so there is a possibility that microbial changes occur outside of the measured timepoints. The majority of microbiome alterations occur before the peaks of metabolic and motor symptoms, indicating the possible role of host-microbiota interaction on disease progression in RTT rats. Experimental manipulation of the gut microbiota at the key timepoints described in the current study is necessary to further elucidate the role of the gut microbiome in the severity and progression of RTT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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

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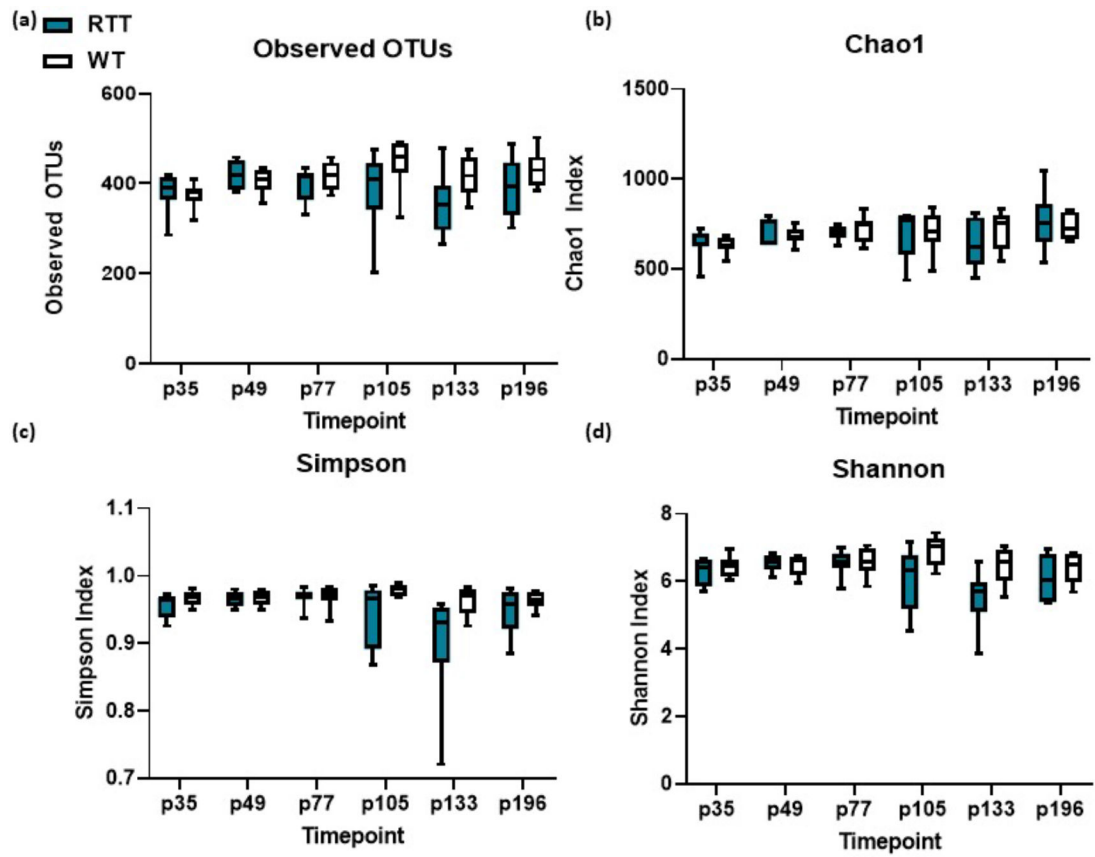


Fig. 1. (a-d) Alpha diversity of HET and WT rats as measured by observed OTUs, Chao1, Simpson, and Shannon indices. n.s. using Mann-Whitney U tests.

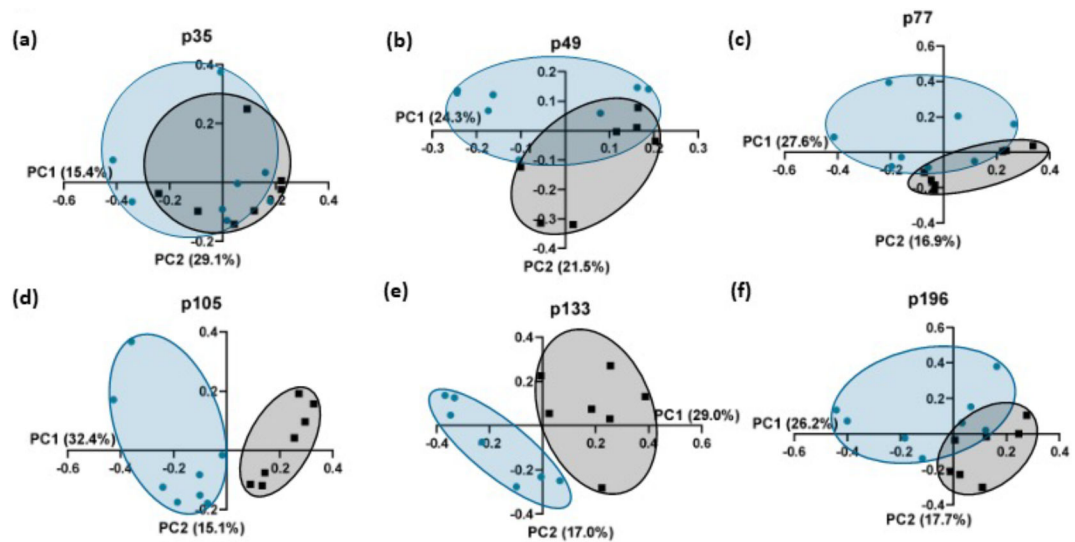


Fig. 2.

(a) Beta diversity of p35 RTT ($n = 8$) and WT ($n = 7$) rats. n.s. (b) Beta diversity of p49 RTT ($n = 8$) and WT ($n = 7$) rats. $p = 0.007$. (c) Beta diversity of p77 RTT ($n = 8$) and WT ($n = 7$) rats. $p = 0.019$. (d) Beta diversity of p105 HET and WT rats. $p = 0.001$. (e) Beta diversity of p133 HET and WT rats. $p = 0.001$. (f) Beta diversity of p196 HET and WT rats. $p = 0.003$. All beta diversity graphs depict bray curtis distance, p values calculated using Kruskal Wallis metrics.

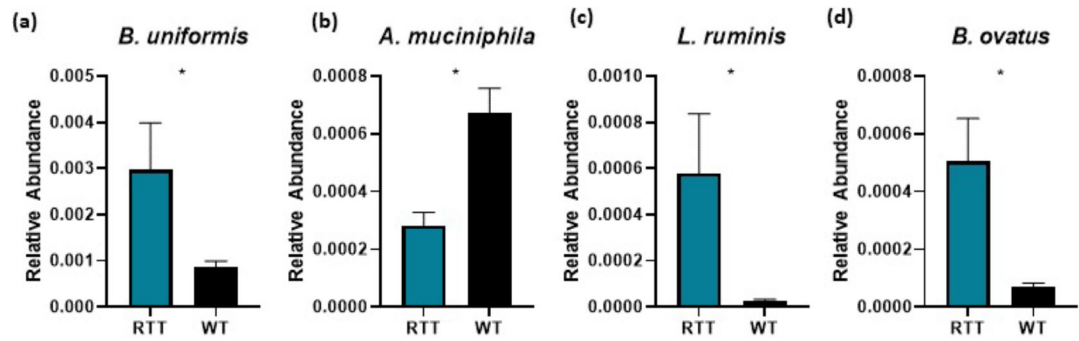


Fig. 3.

Relative abundance of selected L7 OTUs (a) Relative abundance of *B. uniformis*. $p = 0.034$

(b) *A. muciniphila*. $p = 0.034$ (c) *L. ruminis*. $p = 0.034$. (d) *B. ovatus*. $p = 0.034$. All p values

represent FDR-adjusted bootstrapped Mann-Whitney U tests of L7 relative abundances.

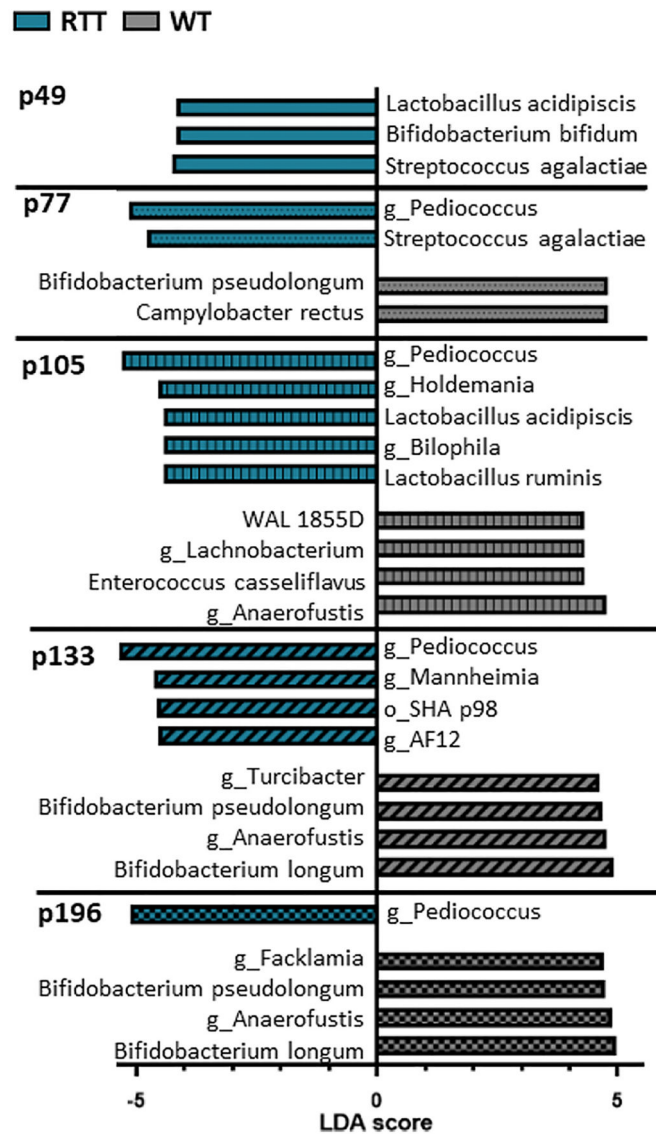


Fig. 4. Functional characterization of OTUs represented in the gut microbiota of RTT rats and WT rats across development. Significant OTUs have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA >4. The p35 timepoint showed no significant OTUS. Teal histograms: OTUs enriched in RTT rats. Gray histograms: OTUs enriched in WT rats.

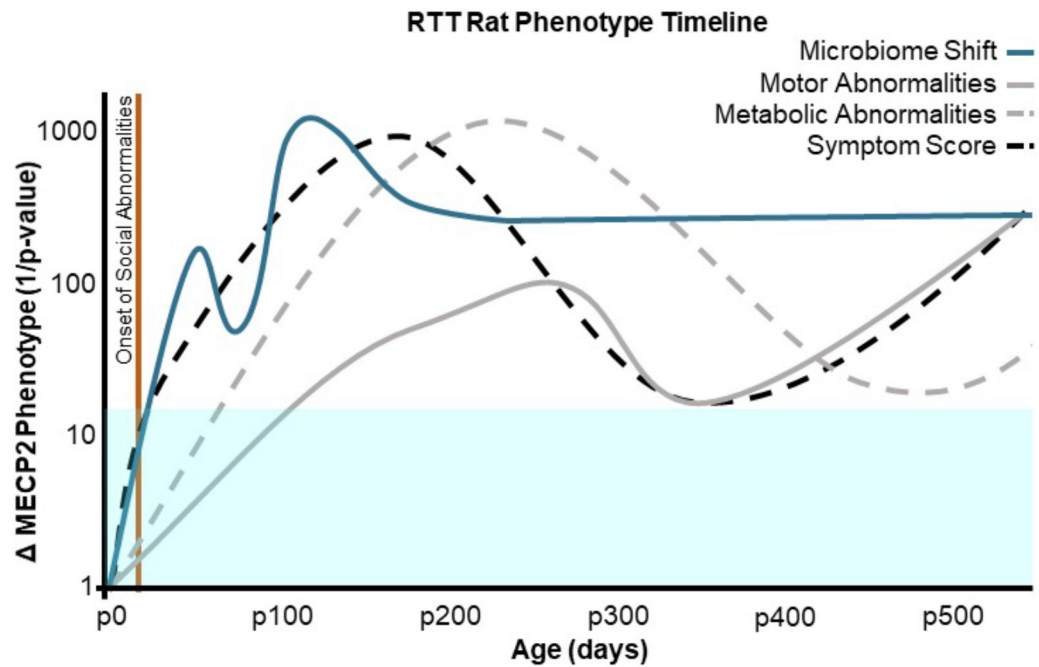


Fig. 5.

Timeline of microbiome shifts in the current study compared to previously published longitudinal data on neural, motor, and metabolic abnormalities in female RTT rats. Microbiome shifts here are defined by Bray-Curtis beta diversity. Metabolic abnormalities describe severity of weight difference from WT and were taken from Patterson et al., 2016. Motor abnormalities here depict an average score on all catwalk categories measured in Patterson et al., 2016. Symptom score describes overall wellness described by Patterson et al., 2016. Onset of social abnormalities describes the age in Wu et al., 2016 performed the three chamber task on RTT and WT female rats and found significant differences in socialization in RTT rats. All values are inverse p -value scores indicating statistical significance. The blue box indicates a significance cutoff value of $p = 0.05$, all lines within this box indicate no statistically significant differences from WT rats.

Table 1

Bold taxonomies were identified via literature search, and indicate taxonomies altered in the same direction in published studies in RTT patients. Table 1 depicts OTUs significantly altered in RTT rats compared to WT across developmental timepoints (Bootstrap Mann-Whitney U test, FDR adjusted). Bold taxonomies depict bacteria that were also found to be altered in the same direction in published work on the microbiome in human RTT patients.

Age	OTU	RTT Mean	WT Mean	FDR p-value	Human RTT studies
p21	n.s.	n.s.	n.s.	n.s.	
p35	n.s.	n.s.	n.s.	n.s.	
p49	n.s.	n.s.	n.s.	n.s.	
	n.s.	n.s.	n.s.	n.s.	
p77	n.s.	n.s.	n.s.	n.s.	
p105	<i>g_Turicibacter</i>	0.000001	0.000128	0.034	
	<i>B. ovatus</i>	0.000506	0.000068	0.034	genus in Borghi et al., 2017
	<i>L. ruminis</i>	0.000578	0.000024	0.034	genus in Strati et al., 2016
	<i>A. municipihila</i>	0.000282	0.000676	0.034	
	<i>f_Mogibacteriaceae</i>	0.000587	0.003411	0.034	
	<i>B. uniformis</i>	0.002976	0.000874	0.034	genus in Borghi et al., 2017
	<i>g_Desulfovibrio</i>	0.001522	0.004543	0.034	
	<i>g_Blautia</i>	0.068587	0.000649	0.034	
	<i>g_Oscillospira</i>	0.028362	0.094498	0.034	
	<i>g_Pediococcus</i>	0.000628	0.000047	0.044	
	<i>g_Odoribacter</i>	0.000311	0.001384	0.044	
	<i>g_Bacteroides</i>	0.066382	0.005045	0.044	Borghi et al., 2017
	<i>f_Ruminococcaceae</i>	0.042262	0.096028	0.044	Borghi et al., 2017
	<i>o_Clostridiales</i>	0.123888	0.292810	0.044	
133	n.s.	n.s.	n.s.	n.s.	
196	n.s.	n.s.	n.s.	n.s.	
548	n.s.	n.s.	n.s.	n.s.	

Table 2

Bold taxonomies were identified via literature search, and indicate taxonomies altered in the same direction in published studies in RTT patients. Table 2 depicts OTUs significantly altered in RTT rats compared to WT across developmental timepoints (Lefse analysis, LDA cutoff of 4.0). Bold taxonomies depict bacteria that were also found to be altered in the same direction in LEfSe analyses in published work on the microbiome in human RTT patients.

Age	OTU	RTT enriched or WT enriched	LDA score	Human RTT Studies
p35	n.s.	n.s	n.s	
p49	<i>Streptococcus agalactiae</i>	RTT	4.26	
	<i>Bifidobacterium bifidum</i>	RTT	4.17	genus in Strati et al., 2016
	<i>Lactobacillus acidipiscis</i>	RTT	4.17	genus in Strati et al., 2016
p77	<i>Campylobacter rectus</i>	WT	4.81	
	<i>Bifidobacterium pseudolongum</i>	WT	4.79	
	<i>Streptococcus agalactiae</i>	RTT	4.77	
	<i>g_Pediococcus</i>	RTT	5.17	
p105	<i>g_Anaerofustis</i>	WT	4.77	
	<i>Enterococcus casseliflavus</i>	WT	4.32	
	<i>g_Lachnobacterium</i>	WT	4.32	
	WAL 1855D	WT	4.30	
	<i>Lactobacillus ruminis</i>	RTT	4.42	genus in Strati et al., 2016
	<i>g_Bilophila</i>	RTT	4.43	
	<i>Lactobacillus acidipiscis</i>	RTT	4.42	genus in Strati et al., 2016
	<i>g_Holdemania</i>	RTT	4.56	
<i>g_Pediococcus</i>	RTT	5.31		
p133	<i>Bifidobacterium longum</i>	WT	4.92	
	<i>g_Anaerofustis</i>	WT	4.76	
	<i>Bifidobacterium pseudolongum</i>	WT	4.68	
	<i>g_Turcibacter</i>	WT	4.62	
	<i>g_AF12</i>	RTT	4.54	
	<i>o_SHA_98</i>	RTT	4.58	
	<i>g_Mannheimia</i>	RTT	4.62	
	<i>g_Pediococcus</i>	RTT	5.37	
p196	<i>Bifidobacterium longum</i>	WT	4.97	
	<i>Anaerofustis</i>	WT	4.88	
	<i>Bifidobacterium pseudolongum</i>	WT	4.73	
	<i>Facklamia</i>	WT	4.71	
	<i>Pediococcus</i>	RTT	5.12	

Table 3

depicts fecal levels of acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methyl butyric acid, isovaleric acid, valeric acid, isocaproic acid, and caproic acid in RTT and WT rats at p35, p105, and p196. There were no significant differences in RTT and WT samples at any developmental timepoint measured. Values depicted are log₂ transformed data values.

SCFA	Age	RTT Average pmol/mg	RTT STE pmol/mg	WT Average pmol/mg	WT STE pmol/mg	p-value
acetic acid	p35	14.75	0.27	14.68	0.02	n.s.
	p105	14.95	0.20	14.41	0.21	n.s.
	p196	14.40	0.28	14.88	0.18	n.s.
propionic acid	p35	12.55	0.22	12.49	0.07	n.s.
	p105	12.78	0.19	12.13	0.32	n.s.
	p196	12.12	0.23	12.66	0.15	n.s.
iso-butyric acid	p35	7.54	0.43	7.96	0.33	n.s.
	p105	8.15	0.40	7.50	0.54	n.s.
	p196	7.55	0.40	8.38	0.23	n.s.
butyric acid	p35	11.30	0.49	11.73	0.13	n.s.
	p105	11.92	0.46	11.02	0.39	n.s.
	p196	11.72	0.39	12.24	0.29	n.s.
2-methyl butyric acid	p35	6.10	0.62	6.70	0.48	n.s.
	p105	6.65	0.56	6.31	0.59	n.s.
	p196	6.24	0.53	7.29	0.23	n.s.
iso-valeric acid	p35	6.46	0.47	7.04	0.40	n.s.
	p105	7.09	0.50	6.60	0.58	n.s.
	p196	6.39	0.40	7.42	0.24	n.s.
valeric acid	p35	7.77	0.66	8.67	0.19	n.s.
	p105	8.71	0.48	7.79	0.70	n.s.
	p196	8.19	0.37	9.18	0.25	n.s.
iso-caproic acid	p35	2.59	0.38	1.68	0.22	n.s.
	p105	2.42	0.51	2.03	0.31	n.s.
	p196	2.29	0.40	1.91	0.36	n.s.
caproic acid	p35	5.59	1.37	5.47	1.09	n.s.
	p105	5.28	1.42	3.29	0.70	n.s.
	p196	3.00	0.53	3.74	0.70	n.s.