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Sex Influences Gut Microbial Composition in Mice with Familial Dysautonomia but is not the Primary Determinant of Microbial Functional Diversity

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SUMMARY Familial dysautonomia is a genetic neurological disease characterized by impaired nervous system functions due to a mutation of Elongator acetyltransferase Complex subunit 1. Previous studies have demonstrated that the severity of familial dysautonomia is associated with variations in the gut microbiota and metabolite profiles in both human patients and mice. However, the mouse model for familial dysautonomia has yet to be characterized in terms of demographic factors (i.e. sex and age) associated with their gut microbial dysbiosis and metabolic composition. Hence, we aim to investigate the variables associated with gut microbial dysbiosis and how they impact gut microbial dynamics and metabolic functions in mice with familial dysautonomia. First, through univariate regression analysis with Bray-Curtis distance and principle coordinate analysis, we determined that sex was the strongest indicator correlated with compositional variations. Next, our taxonomic composition analysis revealed a decrease in the abundance of Bacteroidota and increased Firmicutes in male familial dysautonomia mice compared to females. Furthermore, indicator species analysis identified signature genera of class Clostridia, that were representative in male familial dysautonomia mice. Despite no significant sex differences across metabolic pathways from a predictive functional analysis, correlation analysis identified a strong association between genera *Romboutsia* and creatinine degradation II pathways that were shown to be upregulated in males compared to female FD mice. Ultimately, our findings presented that microbial dysbiosis is intricately linked with sex in the familial dysautonomia mouse model. Further characterization of the mouse model allowed insight into its representation of FD and applicability to human patients.

INTRODUCTION

Neurodegenerative diseases are chronic conditions that progressively impair different parts of the nervous system. While most neurodegenerative diseases are genetically-based, progressive, late-onset, and will shorten life expectancy, Familial Dysautonomia (FD) stands out showing symptoms during infancy (1–3). Familial dysautonomia (FD) is an autosomal recessive disease that affects the autonomic nervous system (4). FD patients experience involuntary organ dysfunctions, such as breathing difficulty, blood pressure instability, pain and temperature perception impairment, and movement coordination difficulties (5). They are homozygous for a deleterious allele of Elongator Acetyltransferase Complex subunit 1 (*ELP1*) (6, 7). ELP1 is a scaffolding protein subunit that binds to tRNA to facilitate mRNA translation within the central nervous system and peripheral nervous system (8, 9). Reduced ELP1 production decreases the association of the Elongator complex to tRNA, and therefore, gene translation (8). This impairs neuronal development and degeneration of sensory and autonomic neurons crucial to organ innervation (3). The

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bidirectional communication between the gut microbiota and parts of the nervous system is often referred to as the Gut-Brain Axis (GBA) (10). It suggests that impaired neurons can alter the gut microbiome and metabolomes (11). Consequently, neural symptoms experienced by FD patients are often correlated with gut microbiome dysregulations (12–14). Additionally, FD patients exhibit lower fat content and body mass index, along with heightened metabolic rates, which also suggests that gut metabolism and function are affected due to imbalanced or altered gut microbiota, often referred to as gut microbial dysbiosis (15).

Previously, Cheney et al. studied the microbiota and metabolite profiles in healthy and ELP1-deficient individuals to investigate whether the GBA is involved in FD pathology (12). They demonstrated disparities between healthy and FD-affected individuals, which suggested a correlation between ELP1-deficiency, and gut microbiome and metabolic disruptions (12). Further analysis of these individuals revealed that variables, such as age, diet, antibiotic history, stomach acid conditions, stool choline levels, and fundoplication, were potentially associated with microbial dysbiosis in FD patients (12). Interestingly, studies on other neurodegenerative diseases revealed that host factors are strongly associated with disease onset and progression (16–19). In the FD mouse model, Khatra et al. observed a significant increase in microbial richness as disease severity increased, as well as altered metabolic functions (20). Additionally, several microbial species have been identified to be indicative of different FD severities (20). However, other host variables in mouse models, including age, sex, body weight, and genotype, remain unexplored in terms of their impact on gut microbial and metabolomic composition.

Based on these observations, we hypothesize that we will discover additional significant variables, other than FD severity, which are associated with gut microbial dysbiosis in FD mice. We also hypothesize that FD mice will exhibit different microbial and metabolic characteristics among different levels of the identified significant variable(s).

While FD is a human disease, it is challenging to understand disease mechanisms and maximize research findings from human models while balancing ethical considerations. Mouse models provide advantages in studying human diseases as they are biologically similar to humans (21). They also provide convenience in result interpretation and studying biological changes over the lifespan with fewer ethical constraints (21). Despite the metabolic differences between mice and humans, an improved understanding of the mouse model would lead to useful findings that are more likely to be applicable to FD in humans (21).

METHODS AND MATERIALS

Familial Dysautonomia dataset. The mouse FD 16s rRNA amplicon sequencing datasets were generated by Cheney et al. (9). Stool samples were collected from Specific Pathogen Free C57BL/6 mice carrying *Tuba1a-Cre*$^{+}$*; Elp1*$^{loxp/loxp}$ mutation (FD mice) with FD scores ranging from 0 (no disease) to 12 (severe disease) and *Tuba1a-Cre*$^{-}$*; Elp1*$^{+/loxp}$ littermates (control) (9). This study included three different experimental groups (cohoused, succinate, and general) and two different treatment types (separate or cohoused). No analyses were done on the succinate experiment group and the cohoused treatment type in our current study. Both male and female mice were included in this study with ages ranging from 21 days to 485 days of age.

Data processing using the QIIME2 pipeline. Data processing steps were conducted using the Quantitative Insights into Microbial Ecology version 2 (QIIME2) (v2023.7) (22). The raw pair-ended 16s rRNA sequences from the mouse Familial Dysautonomia dataset were imported and demultiplexed. No trimming was applied to the reads since all sequences in the dataset were 251 nucleotides long with a median Phred quality score of 30. Next, denoising and clustering were performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (23). Taxonomic classification of the ASVs was conducted using the SILVA 138-99 database (24) for the V4 region of the 16s rRNA gene, amplified with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (25). Any detected chloroplast and mitochondrial sequences were filtered out.

Data processing in R. The metadata file, along with files generated through QIIME2 were imported into R using the tidyverse package (version 2.0.0) (26). The information from these

files was formatted and then combined into a single phyloseq object using the phyloseq package (version 1.38.0) (27). Subsequently, ASVs with less than five counts total and samples with less than 100 reads were filtered out. The phyloseq object was then further refined to include only general and cohoused experimental groups under the "experimental group" variable within the separate treatment groups under the "treatment group" variable. Samples were then rarefied to a sampling depth of 9069. The rarefied phyloseq object had 33 samples and the unrarefied phyloseq object had 59 samples.

Univariate regression analysis. The rarefied phyloseq object was filtered to retain as many variables that may be associated with gut microbiome dysbiosis. This included age, cage ID, experiment group, mouse ID, phenotype score, FD severity, sex, and weight in grams. Missing data was removed, NA values were filtered out, and a dissimilarity matrix based on microbial community composition using the Bray-Curtis distance was calculated using the vegan (version 2.6-4) package (28). Finally, a permutational multivariate analysis of variance (PERMANOVA) statistical test was performed while controlling for cage ID, a confounding variable. A result matrix was populated with R-squared and p-values obtained from the PERMANOVA analysis for each variable and then filtered to retain only statistically significant (p-adjusted<0.05) biological variables which included age, sex, genotype, and weight (g).

Taxonomic composition analysis. Taxonomic analysis was performed on the unrarefied phyloseq object. Relative abundance (%) at the phylum level was calculated by the dplyr (version 2.0.0) package (29) and visualized by ggplot2 (version 3.5.0) package (30) stratified by sex and genotype.

Alpha and beta diversity analyses. Alpha diversity was calculated in Shannon, Chao1, and Pielou's evenness from the phyloseq (1.38.0) package (27), and Faith's phylogenetic diversity indices from the picante (version 1.8.2) package (31). The significant differences in alpha diversity between sexes were determined by a generalized linear model with mouse age as a covariable, following Gaussian distribution ($p<0.05$) from the stats (version 4.1.1) package (32). Beta diversity was calculated in Bray-Curtis, Jaccard, and weighted UniFrac distances. The significance of different microbial compositions of mouse sex and age were analyzed by PERMANOVA ($p<0.05$). Kruskal-Wallis test and Wilcoxon rank-sum test ($p<0.05$) were performed.

Indicator species analysis (ISA). ISA was performed on the unrarefied phyloseq object. The phyloseq object was subsetted to remove data from control mice samples. Data was analyzed at all taxonomic levels from Phylum to ASV level to reveal patterns of the indicator species. The loaded phyloseq object was grouped by each taxonomy level, using the phyloseq (version 1.46.0) package (27). The sample counts were transformed into relative abundances. For all ASVs, indicator values and p-values were calculated using the indicspecies (version 1.7.14) package (33). All indicator values and p-values were combined with the phyloseq object's taxonomy table into a table. This table was then filtered to remove non-significant p-values ($p>0.05$) and indicator values ($stat<0.80$).

Differential sequence expression analysis (DESeq). DESeq was performed on the unrarefied phyloseq object. The phyloseq object was filtered to remove data from control mice samples, subsetted into FD male and female mice, and converted into a DESeq object using the DESeq2 (version 1.42.0) package (34). The sample ASVs were transformed into relative abundances to create a matrix of differential abundance, with female FD mice assigned as the reference group. A volcano plot was created using the ggplot2 (version 3.5.0) package (30) under two conditions to assess significance — adjusted p-values lower than 0.05 and |log2FoldChange| values greater than 1.5 — to show effect size and significance of the results. Significant and non-significant ASVs were differentiated by different plot colours. Finally, a results table was created with the list of significant ASVs, which was pruned using the phyloseq (version 1.46.0) package (27). To visualize the specific abundances of the

identified significant ASVs in the results table, a bar plot was created using the ggplot2 (version 3.5.0) package (30) and RColorBrewer (version 1.1-3) package (35).

Predictive functional analysis. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) analysis was performed using QIIME2. The input file was created from the QIIME2 denoising and clustering step with sequences grouped into respective ASVs and chloroplast and mitochondrial sequence filtered out. Next, the file was filtered to keep only ASVs with a frequency greater than 5. To compare the metabolic pathways between male and female FD mice, the predicted gene family abundances were mapped to known metabolic pathways, and the relative counts per sample were analyzed to derive pathway abundance information using the qiime2-picrust2 (version 2023.2) plugin (36). The output of the pathway abundance table was converted to a human-readable file. Downstream analysis in R was done using ggpicrust2 (version 1.7.3) package (37) and with the metadata of the rarified phyloseq object that was used for taxonomic analysis. The abundance table was filtered to contain samples that existed in the filtered metadata. The pathway identifiers were annotated using MetaCyc pathway descriptions. A Principal Component Analysis (PCA) plot was created using all the pathways with mutant genotypes of the filtered samples, between sexes. A log2FoldChange bar graph was created using only significant pathways with p-adjusted<0.05 and |log2FoldChange|>2, using female FD mice as the reference group. A heatmap was created using pathways of both genotypes and with p-adjusted<0.05 and |log2FoldChange|>1.5.

Correlation analysis between taxa composition and metabolic pathways. Correlation analysis was performed by calculating relative abundance using the taxa table from taxonomic composition analysis, and relative abundance of significant metabolic pathways in FD mice. Using Spearman's rank correlation test, only genera in FD samples with p-adjusted<0.05 were kept. A scatter plot was created using the relative abundance of each FD sample of both sexes in the genera and the four significant pathways from predictive functional analysis.

RESULTS

Sex was strongly associated with changes to the gut microbial composition in mouse models of FD. A univariate regression analysis using the Bray-Curtis dissimilarity matrix was performed to determine variables associated with variation in gut microbial composition in mouse models of FD (Fig. 1A). Four biological factors were found to influence gut microbial composition: age, sex, weight, and genotype (Fig. 1A). Among these factors, age and sex emerged as the primary contributors to variations in gut microbial composition (Fig. 1A). Further investigation into these two biological factors through beta diversity analysis measured by Bray-Curtis revealed gut microbial composition variance due to mouse age and sex (Fig. 1B, 1C). Although both factors were shown to be significant to an equivalent level, the F value for sex is approximately twice that of age (Fig. 1B, 1C). This suggests that the variation between male and female mice exceeds the variation observed among young, middle-aged, and old mice by approximately two-fold. Moreover, distinct groupings were observed in the Principal Coordinate Analysis (PCoA) plots for sex but not age (Fig. 1B, 1C). In terms of age, although old and middle-aged mice clustered into two distinct groups along axis one, the young mice were dispersed across both groups, whereas in terms of sex, along axis one, male and female mice formed two clearly separated clusters. The same clustering pattern is observed across Jaccard and Weighted UniFrac beta diversity analyses of the sexes (Supplementary Fig. S2A and S2B). In terms of alpha diversity, there was no difference observed between male and female mice when sex was taken into consideration (Supplementary Fig. S1). Overall, these findings suggest that sex exerts a stronger influence on gut microbial composition changes compared to age.

Microbial taxonomic composition and abundance vary between male and female FD mice. To further investigate the microbial structures between female and male mice, we compared taxonomic composition within sex groups. The taxonomic composition varied at a phylum level between sex and also between genotypes for each sex (Fig. 2A). Interestingly, there was an overall increase in Bacteroidota abundance in female mice and Firmicutes in

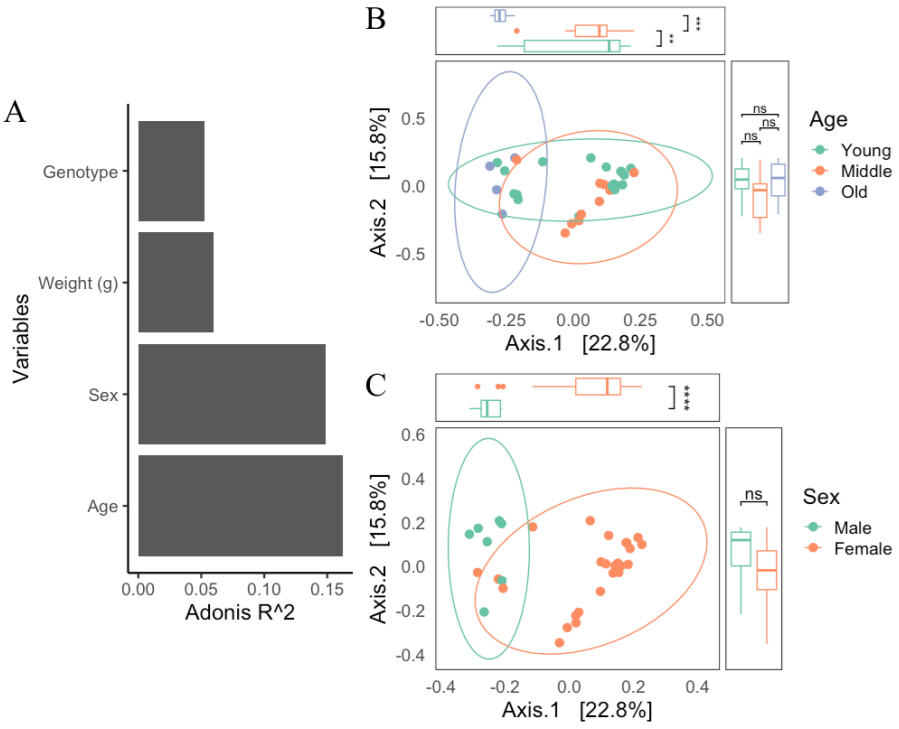

FIG. 1 Sex and age were the strongest variables associated with changes to gut microbial diversity of FD mice. (A) A univariate regression analysis using Bray-Curtis was conducted on the mouse metadata to identify significant (p-adjusted<0.05, PERMANOVA) biological factors influencing gut microbial composition (age, sex, weight, and genotype). Principal coordinates analysis plots illustrating the gut microbial composition of mouse (B) sex (p-adjusted = 0.002, R^2 = 0.14868, F = 5.4139, PERMANOVA) and (C) age (p-adjusted = 0.002, R^2 = 0.16221, F = 2.9042, PERMANOVA), with a 95% eclipse for each group in the variables. The boxplots show PCoA1 and PCoA2 scores distribution within each group, showing quartiles and outliers. (B-C) The sample sizes for sex (female, male) and age (young, middle, and old) were 26, 7, 18, 11, and 4, respectively. Wilcoxon rank-sum tests were performed. ns, $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

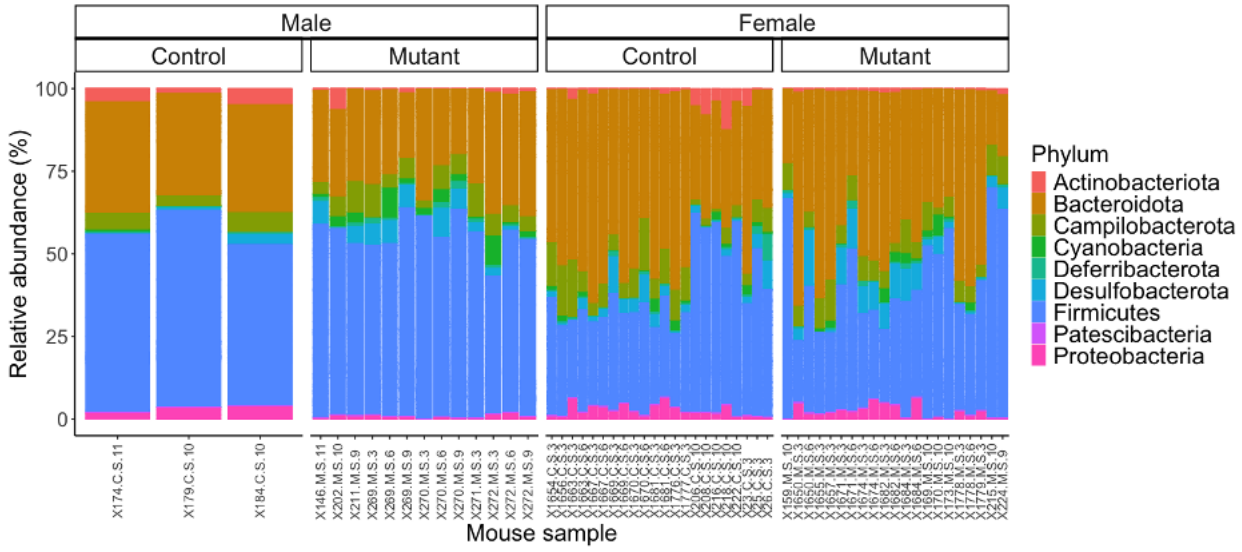

FIG. 2 Female FD mice have higher levels of Bacteroidota but lower levels of Firmicutes in their gut microbiota than male FD mice. Comparison of relative abundance (%) between female and male mice at a phylum level. The mouse sample size was 59, where n = 43 for females (22 controls and 21 mutants) and n = 16 for males (3 controls and 13 mutants).

male mice. DESeq was performed to assess differences in microbial composition at the ASV level in terms of relative abundances. Ten ASVs were different between male and female FD mice (Supplementary Fig. S3). Overall, male FD mice showed increased abundance of ASVs in phyla Firmicutes and Proteobacteria (Fig. 3). The pattern of Firmicutes abundances aligns with our beta diversity analysis. Contrarily, female FD mice showed increased abundance in phylum Bacteroidota. One genus of Firmicutes, *Anaeroplasma*, was shown to be particularly more abundant in female FD mice (23 |log2FoldChange|).

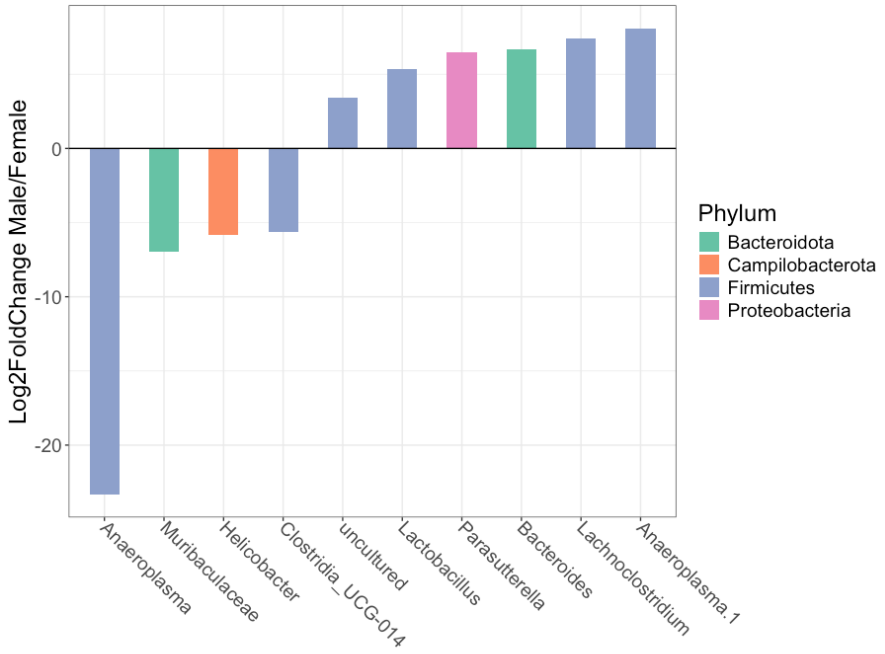

FIG. 3 Male FD mice showed four decreased and six increased ASVs compared to female FD mice. DESeq was performed to compare the ASVs abundance at the genus level between male and female FD mice. Female FD mice data was set as reference. Identified ASVs were filtered for unidentified taxa and statistical significance (p-adjusted<0.05, |log2FoldChange|>1.5). Each ASV is annotated in their respective colour-coded phylum, and in order of increasing log2FoldChange. n = 21 for females and n = 13 for males.

Indicator species analysis identified species exclusive to male FD mice, but none was specific to female FD mice. ISA was performed to characterize unique species in each sex and revealed eight ASVs that are strongly associated with gut microbiome dysbiosis in male FD mice. Contrarily, female FD mice did not exhibit any signature species. These ASVs showed both a strong indicator value and statistical significance as outlined in Table 1. Notably, of these eight indicator species in male FD mice, three genera are from class Clostridia.

TABLE. 1 Eight ASVs identified as strongly associated with gut microbiome dysbiosis in male FD mice, resolved to genus level. Indicator Species Analysis was performed on FD affected mice samples at the ASV level. Illustrated are ASVs from phylum to genus level after filtering for unidentified taxa, significant indicator value (stat > 0.85), and statistical significance ($p < 0.05$). n = 21 for females and n = 13 for males.

Phylum	*Class:Order:Family:Genus*	Sex	Stat	p-value
Bacteroidota	*Bacteroidia:Bacteroidales:Muribaculaceae:Muribaculaceae*	Male	0.90	0.005
	Bacteroidia:Bacteroidales:Prevotellaceae:Prevotellaceae_UCG-001	Male	0.89	0.005
Campilobacterota	*Campylobacteria:Campylobacterales:Helicobacteraceae:Helicobacter*	Male	0.88	0.005
Cyanobacteria	*Vampirivibrionia:Gastranaerophilales: Gastranaerophilales Gastranaerophiales*	Male	0.87	0.005
Firmicutes	*Clostridia:Oscillospirales:Oscillospiraceae:uncultured*	Male	0.90	0.005
	Clostridia:Oscillospirales:Ruminococcaceae:Anaerotruncus1	Male	0.88	0.005
	Clostridia:Oscillospirales:Ruminococcaceae:Anaerotruncus2	Male	0.88	0.005
Proteobacteria	*Gammaproteobacteria:Burkholderiales:Sutterellaceae:Parasutterella*	Male	0.91	0.005

Sex was not a main factor influencing functional diversity in FD mice. After investigation into sex differences in gut microbial composition of FD mice, functional analysis using PICRUSt was performed in order to determine whether metabolic pathways differ between sexes. There was a more diverse metabolic pathway abundance across samples in females compared to male FD mice, as observed by the widespread distribution in marginal density plots (Fig. 4A). There was a significant overlap between the sexes, suggesting that there was no clear separation in the abundance of metabolic pathways between male and

female FD mice. Fig. 4B displayed four upregulated pathways in male FD mice than in female FD mice: catechol degradation to 2-oxopent-4-enoate II, catechol degradation II (meta-cleavage pathway), methyl ketone biosynthesis, and creatinine degradation II. Correlation experiment analysis was conducted to assess the relative abundance between the genus *Romboutsia* of FD mice and the four significant pathways of FD mice mentioned. The creatinine degradation II pathway had a significant positive correlation with the genus *Rombutsia* belonging to the phylum Firmicutes while the other pathways exhibited non-significant and less robust correlations with *Rombutsia* (Fig. 4C).

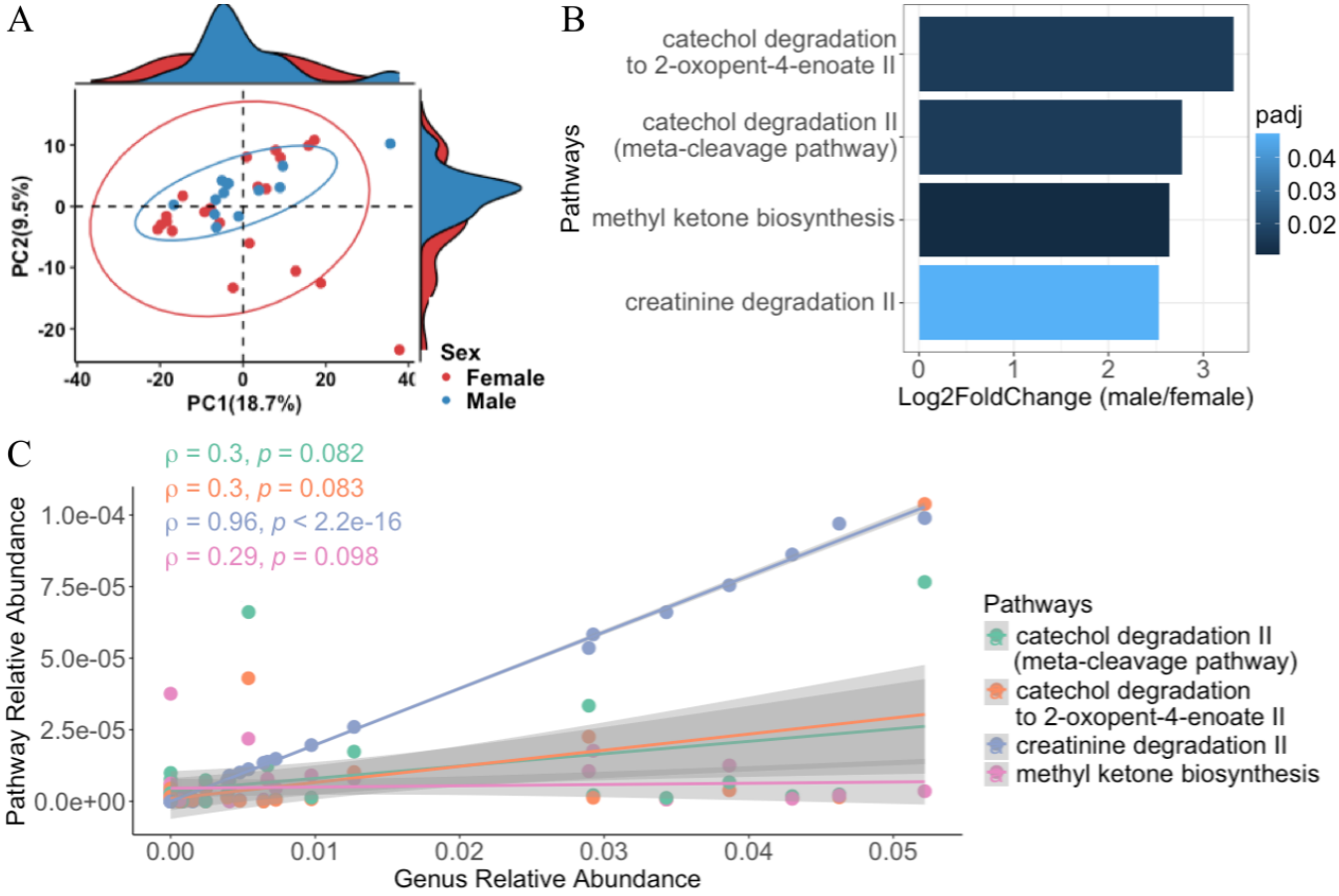

FIG. 4 Sex was not a main factor that influenced functional diversity and the genus *Romboutsia* positively correlated with creatinine degradation II pathway in male FD mice. (A) PCA plot of pathways predicted in total of 34 FD genotype samples (n = 13 for males and n = 21 for females) based on metabolic expression profiles, with females in red and males in blue. (B) Bar plot of log2FoldChange for pathways predicted in FD genotype using female as reference, with p-adjusted<0.05 and |log2FoldChange|>2, colour-coded by p-value significance. (C) Scatter plot of correlation between the significant pathways (B) relative abundance vs. genus *Romboutsia* relative abundance in a total of 34 FD mice samples (n = 34). Spearman's rank correlation coefficient (ρ) was calculated and visualized with best-fit line and 95% confidence interval (significant level, p<0.05). Pathways were annotated by colour.

DISCUSSION

In this study, we aimed to identify variables associated with gut microbiome dysbiosis, a key driver of metabolic dysfunction in individuals with FD, and to investigate their effects on the dynamics and metabolic functions of the gut microbial community. By characterizing the mouse model, we gain insight into its accuracy as a representation of FD and the extent to which findings can be extrapolated to human FD patients.

Through univariate regression analysis using Bray-Curtis and representational PCoA plots, we found that sex is the biological variable most strongly associated with gut microbiome dysbiosis in FD mouse models (Fig. 1). Previous research on FD in human

patients has suggested age as the most significant factor associated with FD, akin to many other neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (12, 18, 19). Given the prominence of age as a factor in various human neurodegenerative diseases, including FD, we initially predicted that age would similarly be the most significant factor associated with FD in mice. While Cheney et al. did not find a significant relationship between microbial diversity and sex in FD humans, the experiment conditions were quite different between human FD and the mouse model FD studies (12). Variables associated with FD in mouse models may more readily align with variables associated with FD in humans, if the experimental conditions were similar. Regardless of alignment, such an endeavor would yield a more conclusive understanding of the factors contributing to FD in mouse models and whether they parallel those in humans.

Through Bray-Curtis, Jaccard, and weighted UniFrac beta diversity analyses, we found that gut microbial diversity is significantly different between FD male and female mice (Fig. 1B, Supplementary Fig. 2A, 2B). Cage effect is one of the main drivers of divergent gut microbial compositions (38). However, data points of different cages overlap in the beta-diversity analysis of Cage ID and Sex (Supplementary Fig. 2C), indicating that other factors, such as sex, are also contributing to the divergent microbial composition across samples. While the different-cage effects could have skewed the results, we decided to only work with mice housed in different cages as there were limited cohoused mice available for comparison in the dataset. Collectively, these observation supports our initial hypothesis that the microbial distributions between male and female FD mice would be different. These results are also consistent with studies that investigated microbial diversities between sexes in other genetically-caused neurodegenerative diseases, such as autism spectrum disorder, where the two sexes harbour different gut microbiome structures (39). Hence, the correlation between gut microbiome dysbiosis and sexes of FD mice indicates a potential role for the GBA in mediating disease progression differently between sexes.

Previous studies have suggested that FD severity and environmental factors, such as housing style (cohoused vs. non-cohoused), influence the homogeneity of gut microbial composition in FD mice (12, 20). Through characterizing the microbial taxonomy between sexes, we observed an increase in Bacteriodota abundance and a decrease in Firmicutes abundance in female mice compared to male mice (Fig. 2). The taxonomic variations were expected and supported our hypothesis. However, previous studies have shown that female patients have higher phylum-level Firmicutes/Bacteriodota (F/B) ratios compared to male patients (40). The discrepancy shown in FD patients and mice studies suggests that the taxonomic composition trends in the mouse FD disease model are not yet translatable to represent that of the gut microbial dysbiosis patterns in humans. Moreover, the F/B ratio was greater between sexes than between genotypes (i.e. mutants and controls) of each sex (Fig. 2). Previous studies have also demonstrated that higher F/B ratios were associated with several pathological conditions in humans, including obesity and amyotrophic lateral sclerosis (41, 42).

Distinct microbial compositions between sexes were further characterized by ISA and DESeq. While ISA identified species that were signatures in male FD mice, female FD mice did not exhibit any indicator species (Table 1). The difference between the sexes in our ISA results supports our initial hypothesis that FD mice would exhibit different microbial characteristics among the levels of host variables that contribute to significant microbiome diversities between FD and healthy mice models. Furthermore, our results align with recent research which highlights those neurological diseases may be influenced by the sex-specific microbiota beyond the interindividual variation within each sex (43). As sex differences have been known to confer microbial diversity, particularly in the gut, the signature species of male FD mice may be reflective of the unique microbiota shaped by sex-specific factors such as hormones and gene activation (44). Furthermore, identified signature species may influence disease progression and response to treatment.

Notably, three of eight species that were identified to be strongly associated with gut microbiome dysbiosis in male FD mice are of class Clostridia, making them an interesting target for further examination. As prevalent regulators of intestinal homeostasis, some species of Clostridia are known to be beneficial for gut health while others are known to contribute to microbial dysbiosis (45). Clostridia are involved in the regulation of amino acid

metabolism. Additionally, all identified Clostridia species from this analysis belong in the order *Oscillospirales* and subdivide into families *Oscillospiraceae* and *Ruminococcaceae.* Many genera of *Oscillospirales* are associated with the production of short-chain fatty acids (SCFA), as well as the reduction of chronic inflammation (46). However, increased abundance of *Oscillospirales* was found with dysbiotic microbiota of anti-N-methyl-D-aspartate receptor encephalitis (NMDARE) microbiota recipient mice, along with compromised blood-brain barrier and higher susceptibility to inflammation (47). Bacteria of family *Ruminococcaceae* are involved in the promotion of biosynthesis of serotonin in the intestine to aid in gastrointestinal motility (48). In another study, *Ruminococcaceae* has been shown to affect the speed of disease progression in PD, specifically on global cognitive functions (49). In the case of irritable bowel syndrome (IBS), the changes in abundance of *Ruminococcaeae* species have demonstrated their influence on the brain regions of patients (48). The interaction between gut microbiota and the central nervous system depicted in IBS reflects the gut-brain axis dynamics seen in FD. The presence of *Oscillospiraceae* and *Ruminococcaeae* as indicator species for male FD mice could suggest that their disproportion is linked to gut microbiome dysbiosis, akin to the patterns observed in NMDARE, PD, and IBS.

Through DESeq analysis, we identified ten genera of microbes in four major phyla that displayed significantly different abundances between male and female FD mice (Fig. 3). With the reference group as female FD mice, male mice presented four genera with decreased abundance and six genera with increased abundance. Among the identified major phyla, the increase of phylum Proteobacteria in male FD mice is consistent with our beta diversity analysis. As an important constituent of the core gut microbiota, the genus *Parasutterella* of Proteobacteria contributes to the regulation of amino acid, bilirubin, purine, bile acid, and cholesterol metabolisms (50). Interestingly, a study using murine infection models revealed that decreased abundance of *Parasutterella* is correlated to positive health outcomes after infection, suggesting its potential role in homeostatic regulations (51). Notably, we also observed an increased abundance of Campylobacteria in female FD mice, specifically *Helicobacter*. This opportunistic bacterium modulates the gut microbiota by protecting the host against some allergic diseases, autoimmune disorders, and IBS (52).

Additionally, *Anaeroplasma,* a genus from phylum Firmicutes, has over twenty |log2FoldChange| (Fig. 3). The significant change in abundance between the sexes underscores its potential role in the sex-specific microbiota. Bacterial species of genus *Anaeroplasma* are involved in gut homeostasis regulation and inflammation mediation by promoting the expression of immune-regulatory transforming growth factor beta and mucosal immunoglobulin A (53).

Variations in microbial abundances, particularly *Parasutterella*, *Helicobacter,* and *Anaeroplasma* between the sexes indicate the interplay between host sex and gut microbiota composition, which could significantly influence the pathophysiology of FD. Extended analyses on the sex-specific microbial profiles may have implications for understanding how sex hormones influence gut microbiota composition and function.

Predictive functional analysis using PICRUSt provided a comprehensive overview of the metabolic pathways within the gut microbiomes of FD mice. Despite the clear taxonomic and diversity distinctions identified between male and female mice, our analysis indicates that these differences do not necessarily translate into significant variations in metabolic or functional pathways. These findings did not support our hypothesis, which was that levels of host variables significantly associated with microbiota diversities will display different predicted metabolic pathways. In defiance of the pronounced sex-based distinctions observed in beta diversity and in the taxonomic compositions of the microbiomes, predictive functional analysis revealed considerable overlap in the metabolic pathways present in both male and female FD mice. This overlap suggests that while the microbial communities are distinct, the functional outcomes are similar between sexes. Despite no clear distinction between sexes (Fig. 4A), we observed four significant pathways that were upregulated in males than females (Fig. 4B). Among these pathways, catechol degradation II is a superpathway of catechol degradation 2-oxopent-4-enoate II (54), and this meta-cleavage pathway is a key process involved in breaking down aromatic compounds (55). Degradation of aromatic compounds such as catechol can be linked to energy production and the formation

of intermediary metabolites like SCFAs (56). Alterations in SCFA levels are linked to systemic inflammation which may affect neuroinflammation and gut-brain axis signaling, and potentially influence neurodegenerative disorders (57, 58). Catechols and their metabolites play a significant role in neurological function, and dysregulation of catecholic neurotransmitters is associated with SCFA production and PD (59–61), but there is no direct evidence suggesting that catechol degradation 2-oxopent-4-enoate II or catechol degradation II pathway directly influence neurological function or neurodegenerative diseases. There are no implications that the engineered methyl ketone biosynthesis pathway is associated with neurodegenerative pathways or FD. Creatinine degradation II is involved in the degradation of creatinine, which is derived from creatine phosphate in muscle (54, 62). Creatinine levels in the blood are linked to the regulation of glucose in humans (63) and impairment in glucose is recognized as one of the significant characteristics of various neurodegenerative conditions including AD, PD, and Huntington's Disease (HD) (64). Additionally, creatine, utilized to store and supply energy to muscle cells, may influence inflammation and altered neurotransmission after mild traumatic brain injury (65). A clinical trial demonstrated that creatinine had the potential to slow down the progression of PD in patients, leading to considerations for further investigation through Phase III trials (66). In another study, dietary creatine supplementation was associated with increased survival rates and delayed accumulation of huntingtin-positive aggregates in a mouse model of HD (67). Although previous studies suggest a connection between creatine and neurodegenerative diseases, there is no evidence to support that the creatinine degradation II pathway is directly associated with neurodegenerative diseases. However, based on the observed functions related to these pathways, three of the four significant pathways that were upregulated in male FD mice than female FD mice may potentially be linked to neurodegenerative diseases like FD.

A Spearman correlation analysis was performed on the relative abundances of significant genera in both sexes of FD mice and four key metabolic pathways identified from functional analysis. Notably, there was a strong correlation between genus *Romboutsia* and creatinine degradation pathway II. Serum creatinine, which is linked to impaired glucose regulation of both sexes in humans (63), shows similar biochemical interactions in the gut, particularly with *Romboutsia ileaslis CRIB*^{T} (68). This gut bacterium, a crucial component of the small intestine in rats, is involved in glucose degradation (68). Interestingly, in the context of other neurological disorders, *Romboutsia*, known for SCFA production, shows decreased levels in the gut microbiomes of human subjects with traumatic brain injury (69) and in fecal samples associated with AD (70). Overall, a strong correlation exists between genus *Romboutsia* and creatinine degradation II in impacting glucose metabolism, with implications for its potential role in FD and other neurodegenerative diseases.

Limitations While we identified sex as a significant contributor to microbial composition differences in FD mice, the extension of these findings to FD in humans requires further investigation and validation. A notable constraint in this study was the limited number of male samples in the rarified dataset. While statistical interpretations can be obtained between the gut microbiota of FD male and female mice, an unbalanced dataset can lead to biases towards the dependent variable with higher abundance (i.e. FD female mice). Another constraint in this study was the lack of cohoused samples in the dataset. While there was not a distant gut microbiome separation across mice housed in different cages, we cannot rule out the possibility that the cage effect is an additional factor that contributes to the differences in gut microbial composition. Collectively, these limitations may reduce the strength of the conclusions that can be drawn regarding sex differences and their biological relevance in FD mice.

Conclusions This study investigated and identified host variables that significantly contributed to variations in gut microbial diversity and metabolic pathways between FD mice and their healthy counterparts. Sex was determined to be the primary variable associated with gut dysbiosis in FD mice that were housed separately. Interestingly, the richness and evenness of gut microbiota were similar between sexes. However, we observed greater variations in taxonomic composition between sexes than genotypes. Moreover, indicator species analysis revealed eight species exclusive to male FD mice, with microbial relative abundances

indicating four downregulated and six upregulated species in male FD mice compared to their female counterparts. Despite differences in microbial taxonomic compositions, investigation into microbial function suggested no distinct disparities in predicted metabolic pathways between sexes. However, three of the four significant pathways upregulated in male FD mice may be linked to pathways associated with neurodegenerative diseases like FD. Among the four pathways, there was a significant correlation between the abundance of genus *Romboutsia* and creatinine degradation pathway II. While sex influences microbial composition in FD mice, it may not be the primary driver of changes in microbial function. Given the variations in the factors influencing gut microbiome alterations in humans and mice, as well as disparities in experimental settings, we cannot conclude that the mouse model can accurately represent FD in humans. However, future studies comparing FD in both species under controlled conditions could provide clearer insights into the relevance of mouse models for human FD. Since sex influences microbial composition in FD mice, exploring its impact on FD patients could guide the development of sex-specific treatments.

Future Directions Based on our microbial function results, there is no strong evidence for distinctive metabolic pathway predictions between FD mice of different sexes (Fig. 4A). This is likely a consequence of working on a dataset with imbalanced FD male and female mice samples. Future studies can recruit an even distribution of FD male and female mice and repeat methods presented in this study to more accurately infer functional patterns in the gut between FD male and female mice.

Having observed gut microbiome differences between FD male and female mice (Fig. 2 and 3), future studies could explore how genetic and hormonal variations between sexes influence the gut microbiome, with emphasis on identifying any sex-dependent patterns that may have therapeutic implications. Comparative analyses of the gut microbiota and functions between FD mice and humans can further be performed to explore whether FD mouse models are a good representation of FD humans. Such research could inform practices for different treatments in FD males and females, if some levels of similarities are found. As this study highlights the complexity of microbiomes in FD, identifying additional host variables that may contribute to the metabolic functionality of the gut microbiome will be essential for advancing the understanding of FD and improving patient outcomes.

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CONTRIBUTIONS

M: ran PICRUSt2 functional analysis, completed code for downstream R analysis, completed code for correlation analysis, generated corresponding figures in Figure 4A, 4B, and 4C,, wrote methods, results, and discussion, section relating to PICRUSt2 and correlation analysis, and contributed to study limitations and future directions.
O: contributed to QIIME2 pipeline denoising and clustering, wrote code for ISA and DESeq2, generated corresponding figures in Table 1, Figure 3, and Supplementary Figure 3, wrote methods, results, and discussion section relating to ISA and DeSeq2, and contributed to abstract and references.
A: contributed to QIIME2 pipeline taxonomic analysis, alpha-rarefaction, generation of the phyloseq object and rarecurve, completed code for univariate regression analysis, generated corresponding figures in Figure 1A, wrote methods for familial dysautonomia dataset - univariate regression analysis, results for univariate regression analysis and beta and alpha diversity, discussion section relating to univariate regression analysis and beta diversity, and contributed to the conclusion.
Z: contributed to QIIME2 pipeline demultiplexing, contributed towards writing code for taxonomic analysis, beta and alpha diversity, and generating corresponding figures in Figure 1B, 1C, 2, and

Supplementary Figure 1 and 2, wrote methods, results, and discussion section relating to taxonomic composition, and contributed to abstract and the conclusion.
K: contributed to writing code for beta and alpha diversity, and generating corresponding figures in Figure 1B, 1C, and Supplementary Figure 1 and 2, wrote discussion section relating to alpha and beta diversity, introduction, contributed to study limitations, future directions, and completed references.
All members contributed to editing and refining the manuscript.

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