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Increased pathological severity of Familial Dysautonomia enriches murine gut microbial composition

Ashleen Kaur Khatra, Jenny Shee, Aidan Wang, Wei Chuan Kevin Wang

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Familial dysautonomia (FD) is the most prevalent type of Hereditary Sensory and Autonomic Neuropathies, which are rare genetic neurological disorders that affect both the peripheral and central nervous systems. Previous studies have found significant alterations in the gut microbiome and metabolome in FD patients compared to healthy individuals. However, it is unclear how gut microbial composition differs with varying levels of FD severity. Here, we aim to investigate whether there are compositional differences among murine gut models with mild, moderate, and severe FD. First, we showed a significant increase in species richness as severity increases. Moreover, beta diversity analysis indicated increasing compositional differences as severity increases. Second, a differential abundance analysis revealed significant downregulation of core commensal microbes in mice with severe FD compared to healthy controls. Additionally, Indicator Species Analysis unveiled the presence of unique pathogenic species, such as select *Clostridium* spp., underlining the relationship between specific bacterial species and FD pathologies. Overall, our findings suggest that increases in gut microbial richness observed in murine models with severe FD is due to the downregulation of some commensal microbes and the introduction of unique pathogenic species. These results accentuate the gut-brain and gut-metabolism axes as promising therapeutic targets for FD.

INTRODUCTION

Hereditary Sensory and Autonomic Neuropathies (HSANs) are a group of rare genetic disorders that affect the autonomic nervous system (ANS) in addition to sensory nerves. The varying groups are classified by their clinical presentation, but often include sensory loss and autonomic dysfunction. Among these subtypes, HSAN type III, also known as Riley-Day syndrome or Familial Dysautonomia (FD), is by far the most prevalent (1). FD is driven by a defective *ELP1*, encoding a core subunit of the elongator complex (ELP1-6). ELP1 is responsible for the proper translation of genes within the central and peripheral nervous system, which is necessary for supporting neuron development and activity (1, 2). Patients who are homozygous recessive for *ELP1* experience impaired neuron function specific to sensory and autonomic systems, many of which have been linked to the dysregulation of the gut microbiome (3, 4). Consequently, changes in metabolism arising from the crosstalk between the gut and brain can alter the metabolic profile of FD patients, such as lower lipid content (5, 6, 7). Gut dysbiosis is also known to drive changes beyond inflammation and metabolism, including gastrointestinal (GI) symptoms which diminishes the quality of life among FD patients (3, 8, 9). Many FD patients presenting GI symptoms are limited to fundoplication and gastronomy as current treatment options (8, 10).

Many other neurological diseases and disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Autism Spectrum Disorder (ASD), Amyotrophic Lateral Sclerosis

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Address correspondence to: https://jemi.microbiology.ubc.ca/

(ALS), Multiple Sclerosis (MS), and Huntington's disease (HD) are often characterized by gut dysbiosis (11, 12, 13, 14, 15, 16). Recent studies have shown that resident microbiota play an important role in regulating inflammatory processes, and an altered microbiome may contribute to inflammatory signatures typical of many neurodegenerative diseases (17). However, microbial species pertinent to each disorder will differ depending on environmental factors and pathological interactions (18, 19), stressing the significance of distinct species on pathophysiology. Higher propensities for disease progression have been linked to the dysregulation of key bacterial species, including increases in select *Clostridia* species, or reduction in some commensal species such as *Lactobacillus* and *Bifidobacterium* (20, 21, 22). Such imbalances are associated with the downregulation of key metabolites such as short chain fatty acids (SCFA), which hold neuroprotective roles against gut permeability (13, 23). Furthermore, Cheney et al. recently highlighted the role of gut dysbiosis and its effects on the gut-brain axis in FD patients (3). Thus, metabolic regulation may potentially be a critical player in dysbiosis-mediated pathogenesis. Overall, these findings suggest that gut and brain crosstalk is involved in many neurological diseases and disorders. Thus, modulating the gut microbiota may be a promising therapeutic strategy for these disorders.

In our study, we explored whether increasing severities of FD is driven by changes in the microbiome. Here we characterized compositional differences by analyzing alpha and beta diversity metrics. We expect that with increasing pathology, more unique species are identified with an overall decrease in diversity (17). In examining the relationship between pathology scores and microbiome composition, we determined whether greater abundances of pathogenic bacteria compared to commensal bacteria triggers progression of the disease. Unexpectedly, we found that at higher severities, there was an overall increase in bacterial richness. While the majority (50%) of core microbiota appeared to be shared, there were a few taxa that were unique to each severity category. Specifically, we identified that select commensal species were less abundant while species linked to pathogenesis were more abundant in severe FD.

METHODS AND MATERIALS

Data information. The raw Fastq files used in this study were obtained from a previous study by Cheney et al., containing stool samples from mice with FD pathology scores ranging from 0 (no disease) to 12 (severe disease) (3). These files included mice from multiple cohorts of the Cheney et al. study, such as the mice from the cohoused study, the succinate study and general experiments (3). The Specific Pathogen Free (SPF) C57BL/6 mice with the *Tuba1a-Cre*+; *Elp1loxp/loxp* mutation were used to model FD, while the *Tuba1a-Cre−; /Elp1+/loxp* littermates were used as the controls. Both male and female mice were included in this study with ages ranging from 21 to 485 days. Age was not further categorized in our study. DNA was extracted from murine stool samples using a DNeasy Powersoil kit.

Metadata manipulation. The original metadata categorized the pathological severity of FD in mice through a scoring system based on eight factors, including hind limb clasping, evidence of grooming, presence of cataracts, presence of kyphosis, motor function/movement, presence of tremors, observed jumping activity, and body condition. The pathology scores ranged from 0 to 12, where 0 represented the presence of the mutation without observable symptoms, and 12 represented severe disease. However, the highest FD pathology score observed in the metadata was 10, while the lowest score present was 0. Thus, we manually edited the metadata file by binning pathology scores of 0-4, 5-7, and 8-10, into mild, moderate and severe FD groups respectively. Since mice with a pathology score of 0 still had the mutation that causes FD, they were grouped into the mild severity group. The control mice without the mutation were not assigned a pathology score and were binned separately.

Data processing using the QIIME2 pipeline. The data processing steps performed through Quantitative Insights into Microbial Ecology version 2 (QIIME2) (24) are detailed in the supplemental QIIME2 script (QIIME2Script). The raw pair-ended sequence reads were imported and demultiplexed, along with the modified metadata table, into QIIME2 (version

2022.2). A truncation length of 251 base pairs was applied to the reads to ensure a minimum median Phred score of 30. The Divisive Amplicon Denoising Algorithm 2 (DADA2) (25) plugin was used as a quality control and to cluster the resulting high-quality reads to amplicon sequence variants (ASVs). Denoising techniques were used to produce ASVs instead of clustering techniques that result in operational taxonomic units (OTUs), enhancing accuracy beyond 97% identity OTUs and better distinguishing biological variations from sequencing errors (26). The ASVs were taxonomically assigned using the Naive Bayes classifier (27, 28) pre-trained on truncated full-length sequences (251 base pairs) from the SILVA 138 99% database (29, 30, 31, 32) for the V4 region of the 16S rRNA gene (33). The 515F primer (GTGCCAGCMGCCGCGGTAA) and 806R primer (GGACTACHVGGGTWTCTAAT) were used for amplification (34). Based on the alpha-rarefaction curve, a sampling depth of 9,000 was selected (Figure S1), which retained 151 (73.66%) samples overall (74 samples in control, 22 samples in mild, 33 samples in moderate, 22 samples in severe).

Features table filtering. The non-bacterial ASVs, such as those corresponding to chloroplasts and mitochondria, were removed from the feature table during QIIME2 analysis. The feature table was also filtered to remove any ASVs from FD mice that did not have an assigned pathology score. Additional feature table filtering was performed in R (version 2022.12.0) (35) using the phyloseq package (version 1.42.0) (36) as detailed in the supplemental R script (RScript). The low-abundance ASVs that may be a result of sequencing errors or background noise were filtered by setting a minimum relative abundance threshold of 0.05%. Low-quality samples were also filtered by removing any samples with less than 100 reads.

Data processing using R packages. The rooted phylogenetic tree (37, 38), feature table (39), and taxonomic data (40) generated through QIIME2, along with the processed metadata, were imported into R using the read_delim function of the tidyverse package (version 2.0.0) (41). The information from these files were combined into a single phyloseq object using the phyloseq package (36), which was used in all downstream analyses, including the diversity, core microbiome, differential abundance and indicator species analyses. The tidyverse (41), ape (version 5.0) (42), and vegan (version 2.6-4) (43) packages were also used for processing or filtering data in downstream analyses.

Alpha and beta diversity analyses. Visualization of alpha diversity between the microbial gut composition of mice with varying FD severity was done by creating Chao1 and Shannon diversity boxplots using the ggplot2 (version 3.4.1) (44) package (Figure 1). The Kruskal-Wallis test was applied to both metrics to assess whether differences in microbial diversity were statistically significant.

A pairwise permutational analysis of variance (PERMANOVA) was performed for the Jaccard, Bray-Curtis, unweighted UniFrac, and weighted UniFrac metrics in R using the vegan (43) and phyloseq (36) packages to determine the beta diversity between murine microbial gut compositions with varying FD severity. Each beta diversity metric was conducted using 10,000 permutations and a p-value cutoff of <0.05, which defined statistical significance. The betadisper function (version 2.6-4) (43) was used to analyze the multivariate homogeneity of the group dispersions for each of the beta diversity metrics. To visualize the beta diversity analysis between the control, mild FD, moderate FD, and severe FD mice, we generated principal coordinate analysis (PCoA) plots using the ggplot2 (44) package.

Core Microbiome analysis. Core microbiome analysis was performed in R using the microbiome (version 2022-11-01) (45) package, detailed in the supplemental R script (RScript). The detection and prevalence thresholds were set to 0.001 and 0.75 respectively. A Venn diagram was made using ggVennDiagram (version 1.2.2) (46) to visualize the similarities between core members in each group by the number of shared taxa and their respective percentages.

Differential abundance analysis. The samples were subdivided into three groups: control and mild FD, control and moderate FD, and control and severe FD. Differential abundance

analysis was performed on each of these groups to determine the change in abundance of shared taxa using the DESeq2 package (version 01/23/2023) (47), as detailed in the supplemental R script (RScript). The log fold differences for significantly different ASVs were visualized using a volcano plot and a dot plot, which were generated with ggplot2 (44). For the volcano plot, a log2foldchange>1.5 and an adjusted p-value<0.05 were used as the cutoffs. For the dot plot, only the adjusted p-value<0.05 was used as the cutoff. All p-values were adjusted with the Benjamini–Hochberg procedure through the DESeq2 package (47). Differentially abundant genera that were unable to be identified at the genus level were removed from the analysis.

Indicator species analysis. Indicator species analysis was performed in R using the indicspecies package (version 2022-03-05) (48), as detailed in the supplemental R script (RScript). Data was filtered to control, mild, moderate, and severe for indicator analysis at each FD severity. The ASVs were filtered at the species level, and only outputs with a p-value<0.05 are shown in the taxonomy table with their corresponding observed indicator value (Table 2).

Data availability. The microbiome 16S rRNA sequencing data used in this project can be found on the National Center for Biotechnology Information (NCBI) BioSample database (49) with the accession number PRJNA785599. The custom metadata table, QIIME2 command line scripts for data processing and filtering, and the R scripts for alpha diversity, beta diversity, core microbiome, differential abundance and indicator species analyses can be found within the supplementary materials.

RESULTS

Mice with increasing FD severity displayed greater richness in microbial gut composition. Alpha diversity analyses, including the Chao1 and Shannon's diversity metrics, were performed to determine the impact of FD severity on gut microbial diversity in mice. We hypothesized that microbial diversity would decrease with increasing disease severity due to disruption of the normal microbial balance in the body, leading to a state of dysbiosis in the severe FD group. However, the Chao1 metric, which is a quantitative measure of microbial richness, showed an increase in microbial diversity with increasing FD severity levels, with the most severe FD mice displaying the greatest richness (Figure 1A).

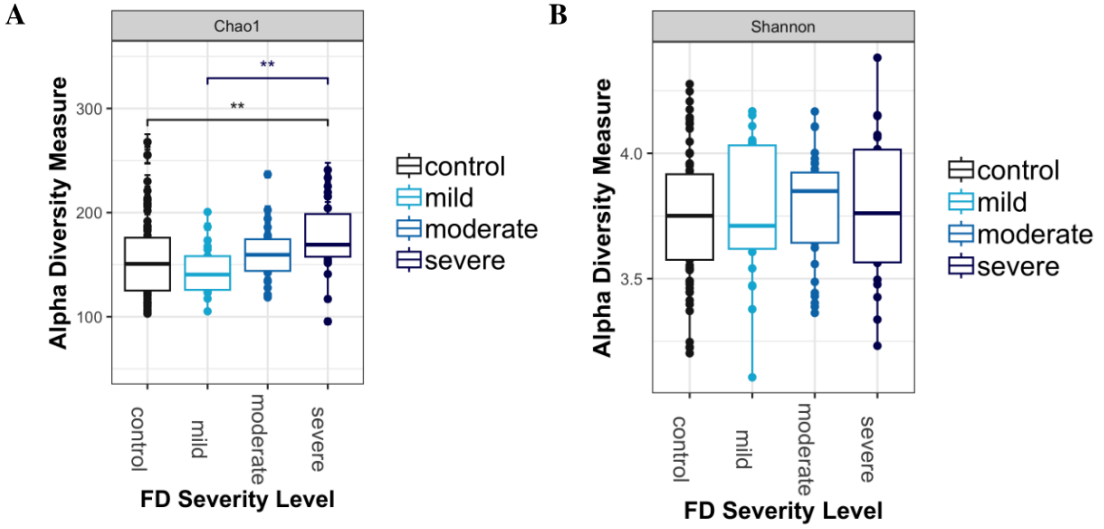

FIG. 1 The richness of microbial composition increases with increasing FD severity as indicated by alpha diversity metrics. (A) Quantification of microbial alpha diversity richness (Chao1) across different FD severity levels and healthy control mice, error bars indicate mean ± SE, p=0.001743 (Kruskall-Wallis Test). (B) Quantification of microbial alpha diversity evenness (Shannon) across different FD severity levels and healthy control mice, error bars indicate mean ± SE, p=0.9484 (Kruskall-Wallis Test). Control n=74, Mild n=22, Moderate n=33, Severe n=22. ** indicate a significant difference between control and FD severity subjects p<0.05.

The Shannon diversity metric, which is a quantitative measure of microbial evenness, did not display distinguishable microbial differences between the varying severity levels (Figure 1B). Statistical analysis using the Kruskal-Wallis test revealed only Chao1 and not Shannon diversity metrics as statistically significant. Although the alpha diversity results were initially not what we expected, other sources of literature have reported similar trends (50).

A comparison between healthy controls and FD severity displayed significantly different microbial gut compositions. The weighted UniFrac beta diversity analysis was performed to assess between-group microbial differences for the healthy control mice and mice with varying levels of FD severity. Through this analysis, we expected a significant difference in the murine gut microbiome for all the FD severity groups compared to the healthy control. As expected, the pairwise PERMANOVA test revealed statistically significant microbial composition differences between the control and all the FD severity levels (Table 1). Other beta diversity metrics, including the Jaccard and unweighted UniFrac metrics, did not meet the assumption of multivariate homogeneity and, therefore, could not be used for diversity analysis. However, the PCoA plots generated for the weighted UniFrac metric did not show clear clustering patterns between healthy control mice, mice with mild FD, mice with moderate FD, and mice with severe FD (Figure S2).

TABLE. 1 Significant changes in beta diversity of the gut microbiome as a result of disease severity. Pair-wise comparison between each FD severity level and the healthy control indicates significant differences in beta diversity. Statistical analysis was performed using the PERMANOVA test. Control n=74, Mild n=22, Moderate n=33, Severe n=22. The threshold of significance is $p<0.05$.

	R^2 value	p-value
Control vs. mild	0.040	0.0042
Control vs. moderate	0.057	9.9e-05
Control vs. severe	0.057	2.0e-04

The core microbiome was consistent regardless of FD severity in mice. A core microbiome analysis was performed to determine the degree of shared taxa between the healthy control and mild, moderate and severe FD groups. Prior to analysis, we expected the FD severity groups would have a low proportion of shared core taxa and a high proportion of unique taxa specific to each group. However, this analysis showed that 50% of taxa were shared amongst all groups, with 4% unique to each of the control and mild groups, and 8% unique to the moderate and severe groups (Figure 2). Contrary to our initial expectations, there was a high proportion of shared taxa between the control and different FD severity groups and a low proportion of unique taxa.

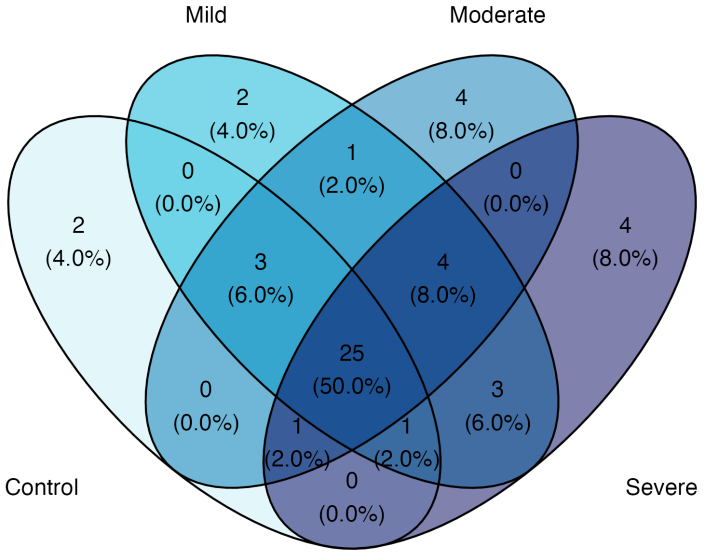

FIG. 2 The core microbiome is consistent across FD severity at the phyla level. Core microbiome analysis was conducted on 51 murine samples in four conditions of no FD (control) and FD severity of mild, moderate, and severe. Analysis was done with a detection threshold of 0.001 and a prevalence threshold of 0.75. Results show that most of the taxa were shared amongst the four conditions and few taxa are unique to each condition, as 50% of the taxa were shared in all groups.

Differential abundance analysis showed a reduced abundance of 4 major phyla in mice with severe FD. Differential abundance analysis was conducted to determine the change in abundance of the shared taxa between the control and each FD severity group. A volcano plot was constructed to visualize the differential abundance between the control and severe FD group based on the negative log10 adjusted p-value, in which 9 ASVs were downregulated and 1 ASV was upregulated (Figure S3). A volcano plot was also created for the control and mild FD groups, but no significant results were found (Figure S4A). While a volcano plot comparison of the control and moderate FD group demonstrated downregulation of 4 ASVs and an upregulation of 1 ASV (Figure S4B). Since the control and severe FD groups had the most differences in the abundance of shared taxa, these differences were selected to be explored further. The shared taxa between the control and severe FD group were found to be less abundant in the severe FD mice, as illustrated by a statistically significant negative log2 fold change in the shared taxa (Figure 3). In comparison to the control group, 11 taxa belonging to the phyla of Actinobacteria, Bacteroidota, Firmicutes, and Proteobacteria were found to be downregulated in severe FD mice (Figure 3). Specific genera for each phylum include *Dubosiella, Lactobacillus, Bifidobacterium, Faecalibaculum, Eubacterium, Parasutterella* and *Paraprevotella* (Figure 3). These findings support our hypothesis that reduced abundance of commensal-associated bacteria are linked to higher disease severities compared to the control group, as reported in other literature.

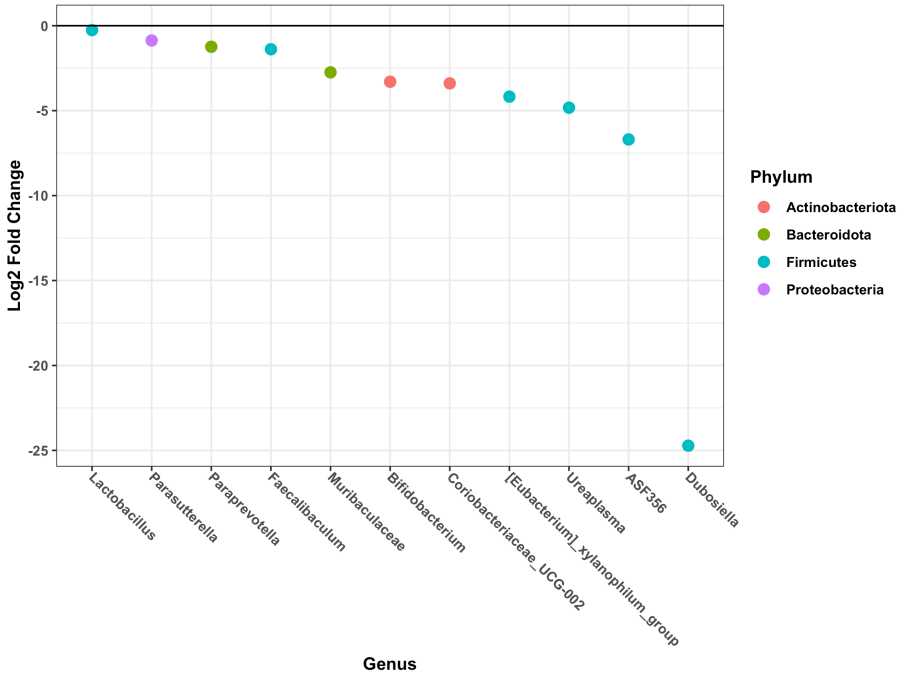

FIG. 3 Differential abundance analysis (DESeq2) unveiled reduced abundance of 4 major phyla in mice with severe FD compared to healthy controls. Taxonomic data was filtered and coloured at the genus and phylum levels, excluding any unidentified taxa. Taxa were then ordered based on decreasing log2 fold change, and only those with a significant difference in abundance (adjusted $p \leq 0.05$) are illustrated. Statistical analysis was performed by the DESeq2's median of ratios method. Each point in the plot represents a unique genus and is colour-coded by phylum. Points below the line indicate negative differential abundance, meaning they are less abundant in severe FD mice compared to control mice. Control n=74, Severe n=22.

Indicator species analysis revealed a unique signature of *Clostridia* spp. in severe mice. The indicator species analysis was conducted to further explore the unique taxa found in the FD severity groups through core microbiome analysis. *Escherichia-Shigella* was found to be unique in mild FD mice, no species were unique to moderate FD mice, and 8 species were unique to severe FD mice (Table 2). Amongst the 8 indicator species in severe FD mice, 4 are found to be *Clostridia* species, which are known to be opportunistic pathogens in the gut microbiome. These results align with our expectations of finding unique pathogenic species in the severe FD group that may be driving the progression of the disease.

DISCUSSION

The present study investigated the role of increasing FD severity in gut dysbiosis to determine whether compositional irregularities in the microbiome contributed to disease state. In our study, we demonstrated that disease progression was positively correlated with bacterial richness in the gut of murine models, owing largely to the upregulation of pathogenic bacteria, and downregulation of normal commensal species (Figure 4). As most core taxa appear to be shared, differences in microbiome between disease and non-disease conditions

TABLE. 2 Unique ASVs resolved to the class, order, family, genus, and species level by indicator species analysis. Indicator species analysis was conducted on murine samples with FD severity of mild, moderate, and severe (n=214). Taxonomic level was filtered at the species level, and only those with a significance in uniqueness are illustrated with decreasing observed indicator value ($p<0.05$). Observed indicator value (IV) represents the probability that a given ASV will be present in all samples of the FD severity group. Results show 1 species unique to the mild FD severity group and 8 species unique to the severe FD severity group.

Taxon Class:Order:Family:Genus:Species	***FD Severity***	***Observed Indicator Value (IV)***	***P-value***
Phylum *Proteobacteria*			
Gammaproteobacteria:Enterobacterales:Enterobacteriaceae:Escherichia-Shigella	mild	0.23	0.04
Phylum *Firmicutes*			
Bacilli:Erysipelotrichales:Erysipelotrichaceae:Turicibacter	severe	0.52	0.005
Clostridia:Clostridiales:Clostridiaceae:Clostridium sensu stricto 1	severe	0.51	0.01
Clostridia:Christensenellales:Christensenellaceae	severe	0.49	0.01
Bacilli:Lactobacillales:Streptococcus	severe	0.34	0.025
Clostridia:Oscillospirales:Ruminococcaceae	severe	0.32	0.005
Clostridia:Lachnospirales:Lachnospiraceae:Roseburia:Eubacterium plexicaudatum	severe	0.29	0.005
Phylum *Actinuboacteriota*			
Coriobacteriia:Coriobacteriales:Eggerthellaceae	severe	0.29	0.03
Phylum *Bacteroidota*			
Bacteroidia:Bacteroidales:Tannerellaceae:Parabacteroides	severe	0.34	0.005

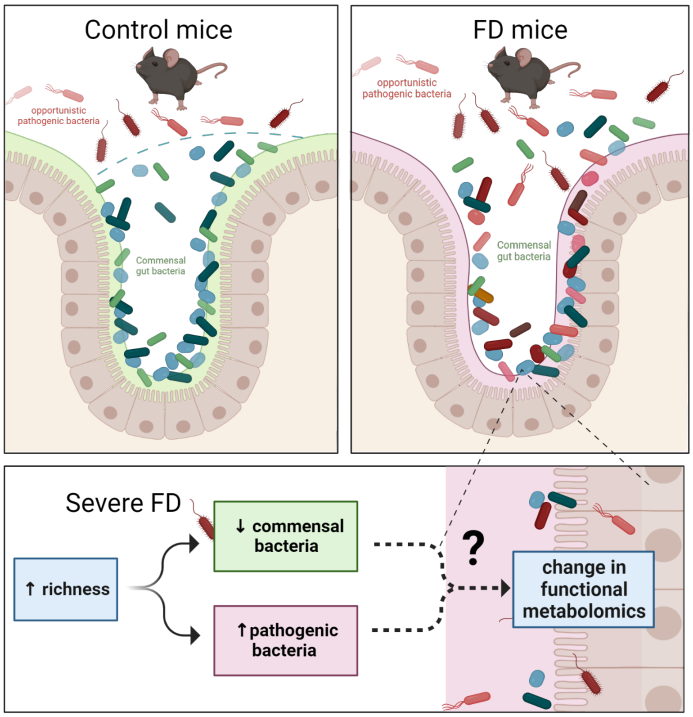

FIG. 4 Proposed model of microbial gut dysbiosis in mice with severe Familial dysautonomia. The bottom panel shows the proposed model that increased richness in mice with severe FD is associated with decreased abundance of commensal bacteria and the presence of pathogenic bacteria. Whether these alterations in the microbiome are associated with changes in functional metabolomics remain undefined. Commensal gut bacteria are shown in green and blue, while pathogenic bacteria are shown in pink and red. FD refers to Familial dysautonomia.

highlight the contributions of specific bacteria and the role they play in advancing disease. Of the commensal bacteria, reduction was observed among 11 genera, including *Lactobacillus, Parasutterella, Paraprevotella, Faecalibaculum, Muribaculaceae, Bifidobacterium, Coriobacteriaceae, Eubacterium, Ureaplasma, ASF356, and Dubosiella*. Comparatively, 8 pathogenic species belonging to *Bacilli, Coriobacteriia, Bacteroidia* and *Clostridia* were observed in severe FD mice, with many belonging to the *Clostridia* class.

Through the weighted UniFrac beta diversity analysis, we found that gut microbial diversity is significantly different between healthy controls and mice with FD (Table 1), supporting our hypothesis that FD severity has an effect on gut microbial composition. These results are also consistent with studies that have explored the effects of other neurological diseases and disorders, such as ASD and PD, on the gut microbial composition (11, 51). Similarly, the association between gut dysbiosis and FD severity suggests a potential role of the gut-microbiome-brain axis in the development of disease. As such, there is a need to further explore the gut-microbiota-brain axis in FD patients, which can provide a better understanding on the development of therapeutics in targeting gut composition in FD patients.

The correlation between poor health prognosis and loss of microbial diversity is frequently observed in cases of gut dysbiosis, as seen in neurodegenerative diseases such as HD. Based on this understanding, we originally hypothesized that an increase in disease severity would result in a decrease in microbial composition (14, 52). Contrary to this perspective, an increasing trend between richness and disease severity was identified in our alpha diversity analysis (Figure 1). However, this trend is not unique to FD. In PD, increased microbial richness has also been associated with increased disease severity (50). A plausible explanation for this trend is that an increase in pathogenic microbes in the higher disease severity groups can be driving the observed increase in microbial richness. This presents the possibility that bacterial populations driving gut dysbiosis may be shared between neurodegenerative diseases such as FD and PD (53).

After observing differences in gut composition between varying FD severity levels, we expected each severity group to possess more unique taxa than shared. However, closer evaluation of our murine model revealed that 50% of taxa were common to all disease conditions, while each individual condition consisted of a few unique taxa (4-8%) (Figure 2). Although we found a high proportion of shared taxa between the healthy group and each FD severity group, changes in the abundance of the shared core taxa could be contributing to the changes in disease severity. However, these interpretations have inherent restrictions, since homogenization of murine microbiomes via coprophagy is a potential confounding variable among cohoused mice which was not addressed in our analysis (3, 54).

In comparison to the healthy control group, we identified 11 downregulated genera within the most severe group that aligned with commensal roles (Figure 3). Of the 11 genera, we examined well-studied commensal bacteria that are actively involved in metabolic homeostasis. Notably, *Dubosiella* is known to be downregulated in other neurological diseases such as AD (55). Lower abundances of this microbe are responsible for an unhealthy gut environment by promoting oxidative stress and its associated decrease in *Lactobacillus* and *Bifidobacterium* (56). Since we also found a decrease in the abundance of *Lactobacillus* and *Bifidobacterium* in mice with FD, the downregulation in *Dubosiella* may be driving this reduction and contributing to the decrease in gut motility and functioning (57). *Lactobacillus* and *Bifidobacterium* are known probiotic constituents that can treat GI symptoms while maintaining gut function and motility (58, 59). Furthermore, *Lactobacillus* spp. are known to enhance gut function by providing an intestinal barrier through increased mucus production which can limit pathogenic bacteria from infiltrating the gut (58). *Bifidobacterium* play a similar role in gut function by supporting intestinal impermeability (11). As seen in a study with Irritable Bowel Syndrome (IBS), supplementation of both commensal microbes was shown to preserve microbial composition (59, 60). This supplementation technique may be useful in mitigating the effects of FD severity.

In addition to GI issues, metabolic changes are also commonly disrupted in FD patients (1, 61). Another beneficial bacterium that contributes to a healthy gut is *Faecalibaculum* (62). Gut dysbiosis is promoted when *Faecalibaculum* is downregulated due to lower SCFA production (62). These metabolites play protective roles in the body, strengthening gut barriers and regulating immunomodulatory functions (63). In our study, low levels of *Faecalibaculum* were detected in mice with severe FD, which imply reduced production of SCFA. Likewise, a common SCFA metabolite, butyrate, may have been diminished in mice with severe FD due to the downregulation of butyrate-producing microbes, such as the *[Eubacterium] xylanophilum group*. Butyrate has anti-inflammatory properties and can restore gut composition and functions, as observed in neurodegenerative diseases such as ALS (15). Therefore, the absence of *Eubacterium* and its associated reduction in butyrate

production may contribute to gut dysbiosis, inflammation, and impaired gut regulation in severe FD conditions. Given that the abundance of SCFAs has been linked to favorable impacts on the gut-brain axis in PD and AD (64, 65), it may also play a role in mitigating the outcomes of FD severity.

Impairment of gut regulation is also impacted by changes in lipid and carbohydrate metabolism. Regulation of lipid metabolism is known to be mediated by two commensal bacteria, *Parasutterella* and *Paraprevotella*, as identified in a study exploring ASD (66, 67). Therefore, the reduction of *Parasutterella* and *Paraprevotella* that was observed in the severe FD mice may be associated with altered lipid regulation in FD patients. Our study also revealed a reduction in *Muribaculaceae*, which is known to regulate carbohydrate metabolism (68). Taken together, downregulation of these commensal bacteria may lead to mediated metabolic landscapes in FD patients which could potentially exacerbate disease severity.

Through indicator species analysis, a selection of 8 bacterial classes were found to be associated with severe FD, suggesting that they may play a role in the progression of the disease. Studies investigating the relationship between the gut microbiome and nervous system communication continue to build evidence towards a neuropathology model instigated by gut dysbiosis (69). Of the taxa that were associated with severe FD, *Clostridia* stood out as the class of interest because 50% of identified taxa were represented under this classification, and many branches of *Clostridia* such as *Lachnoclostridium,* are upregulated in other neurological diseases such as AD (70, 71). Most notably of the 4, both *Lachnospiraceae* and *Ruminococcaceae* are known to modulate serotonin biosynthesis and regulation in intestinal enterochromaffin cells (20). Tryptophan, a precursor to serotonin, is also metabolically regulated by certain divisions of *Clostridia* (72). This suggests that an imbalance of these bacterial families may contribute to disruption of neurochemical homeostasis, which is highly correlated with neurodegenerative disorders (73). In addition, higher prevalence has been noted in patients with IBS, as the two families are thought to be involved in gut motility and sensorimotor detriments (10), a commonly affected process in FD patients. It is also observed that FD patients undergoing pneumonia treatment are more prone to *C. difficile* infection (71).

Among the more pathogenic strains identified, *Clostridium sensu stricto 1* possesses intestinal inflammatory capabilities and is associated with lower concentrations of SCFAs (74). Proportional imbalances of pathogenic and beneficial bacteria can exacerbate systemic inflammatory responses, and reversal of dysbiosis is predicted to suppress secretion of proinflammatory cytokines via supplementation of SCFA-producing bacteria (75). In FD patients, degenerative changes in the nervous system are often accompanied with signs of inflammation (1). Thus, one of the factors that contributes to the pathogenesis of FD could be metabolic in nature, and most likely represents a complex interplay with other bacteria present in the gut microbiome. Many neurodegenerative diseases are characterized by neuron damage via metabolite buildup (70), and it is plausible that the gut microbiome can contribute to the accumulative effect of these proteins. For instance, *C. perfringens* toxins B and D can induce symptoms related to motor dysfunction, which is typical of MS patients (22). Certain species belonging to *Firmicutes* and *Bacteroidota* can also produce amyloids that promote secretion of proinflammatory cytokines IL-7A and IL-22, causing chronic inflammatory responses typically seen in AD (22). The three previously mentioned taxa all mapped to severe FD in our species analysis. While not causal, this affirms current studies underscoring the importance of the gut-metabolism axis on neurological pathologies.

In sum, lower numbers of commensal bacteria, along with greater abundances of pathogenic species observed in FD mice could conceivably be driving gut dysbiosis among FD patients. However, the functions of these commensal and pathogenic bacteria should be further explored in future studies to emphasize the impact of these metabolite producing bacteria (Figure 4). A better understanding of key metabolites may inform their relationship to dysbiosis, and at a broader scale, to the gut-brain axis.

Limitations One caveat of our experiment was the low sample size of mice binned into each severity category relative to control samples. While still statistically feasible, the external validity of these results may be limited to the specific samples that we studied. Thus, it may not be possible to make reliable predictions based on our data alone, and the scope of analysis

remains quite limited. Additionally, the housing style (cohoused vs. non-cohoused) may have influenced the homogeneity of murine gut microbiomes but was not accounted for in our analysis. Cheney et al. highlighted that cohousing appeared to lower pathology scores across FD mice (3). Since the majority of our samples were designated as cohoused, our results may underestimate the full effect of gut dysbiosis on FD severity. Likewise, alpha diversity metrics may have been a lot higher if mice were housed separately. As such, it is probable that cohousing offers an inaccurate depiction of the gut microbiome in FD mice. On a similar note, the overall analysis was performed on murine fecal samples only, which may not fully represent the true diversity of bacterial species.

Assigning pathology scores to mice was based on arbitrary metrics chosen by Cheney et al. (3). It is fair to argue that interpretation of severity is highly subjective and can influence sample sizes between disease states. Until a standardized scoring system is implemented, results may not be easily replicable between studies. For instance, if future studies revealed correlations between certain metabolites and FD progression, we could define concentration ranges that map to specific severity levels. In a related context, human samples were provided in the metadata but did not have an assigned pathology score. Analysis of human gut microbiomes could have been more generalizable and representative, and it was unexpected that Cheney et al. did not define metrics for measuring FD pathology in humans when the symptoms associated with the disease are widely reported and known.

Conclusions Our study investigated the effects of FD severity on murine gut microbiomes. It was observed that the severity of disease and the richness of gut microbiota were positively correlated. While many of the bacterial taxa were shared between disease states, notable pathogenic species, as well as commensals, were increased and decreased respectively. These findings have important implications for the development and progression of FD. The observed differences between pathogenic and commensal bacterial abundance highlights the probable role of dysbiosis in FD pathogenesis. Thus, the identified species in the severe FD category may pose as a promising target for future therapeutic research, raising the suggestion that supplementation or selective depletion of key bacteria could affect FD pathology. The present work emphasizes the need for additional research regarding the multidimensional relationship between the gut microbiome and FD pathology. Development of novel therapeutic strategies to improve neurodegenerative outcomes could further highlight the gut microbiota as a potential target for treatment.

Future Directions In future studies, metabolites associated with the identified commensal and pathogenic bacteria can be explored by conducting a metabolomics analysis. This analysis could reveal regulatory differences in metabolic pathways associated with commensal bacteria, as well as pathways involved in inflammation, cytokine production and gut dysmotility in pathogenic bacteria. Determining the functions of these bacteria can provide evidence for the role they play in the microbiome at each severity. Additionally, exploring the positive correlation between unique species and disease severity by considering the taxonomic bar analysis can reveal whether similar species are identified at less than 1% threshold. This would bolster the credibility of our data and improve our understanding of the trend established between pathology and gut dysbiosis in FD. Furthermore, the effects of pathology can be isolated by separating the cohoused and non-cohoused mice to determine whether the observed trends in microbial diversity are maintained when homogenization of the microbiome is accounted for. The comparison between non-cohoused and cohoused mice is expected to present similar trends albeit with greater variability and lower number of shared taxa. Alternatively, additional murine samples from different sites such as the colon should be contrasted with the fecal samples used in the study. Exclusively using fecal samples may eliminate other bacterial species only present in specific niches in the gut. By evaluating samples from different sites, the true diversity of gut species may be more represented (76). Lastly, to assess the generalizability to the human metabolome, a comparison can be made between the metabolites found in FD mice and human samples. The expected outcome is that metabolites downregulated during gut dysbiosis would be shared in both animal and human models, which has implications for the development of potential therapeutic targets for patients with FD.

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CONTRIBUTIONS

Aidan Wang (AW) and Wei Chuan Kevin Wang (WCKW) performed the QIIME2 analysis while Ashleen Kaur Khatra (AKK) and Jenny Shee (JS) conducted most of the analysis for R. AKK and JS focused on explaining our results and figures, as well as outlining our methods for our experiment. AW and WCKW collaborated on the discussion and introduction. All authors contributed to the editing of the manuscript.

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