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Mentally distressed and non-distressed Parkinson's Disease patients have similar gut microbial compositions

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SUMMARY Parkinson's disease (PD) is a neurodegenerative disease with symptoms of motor function loss and psychological changes. In the early stages of PD, patients display symptoms of depression and fatigue, which may be indicative of the disease. Studies have shown that both depression and fatigue are independently related to the gut microbiome of PD patients, however their combined effect on the gut microbiome remains largely unknown. In this study, we aimed to determine if there is a relationship between the gut microbiome of PD patients and their reported level of fatigue and depression. Here we define distressed patients as those with high fatigue and high depression scores, and non-distressed patients with low fatigue and low depression scores.

While linear correlation and alpha and beta diversity analysis showed no relationship between patients' fatigue and depression scores, an indicator species analysis revealed a higher abundance of *Romboutsia* and *Erysipelatoclostridium* genera in distressed PD patients and *Parasutterella* in non-distressed PD patients. These findings improve our understanding of how the severity of depression and fatigue in PD patients changes the gut microbiome. Further studies should investigate other mental health disorders faced by patients to provide a better understanding of the relationship between the gut microbiome, mental health, and PD.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative disorders in the world and is characterized by the loss of motor function (1). PD patients also display a variety of non-motor symptoms including mental health disorders, gastrointestinal (GI) symptoms, and comorbidities which may display before the onset of motor symptoms.

In recent years, studies have discovered a connection between the gut microbiota and the brain, established differences in the gut microbiota of PD patients and healthy controls, and found that gut microbiota imbalance affects the development and occurrence of Parkinson's disease via association with increased intestinal permeability (2). According to the Parkinson's Foundation, PD patients exhibit mental health symptoms more frequently than healthy individuals. Common disorders reported by patients include anxiety, depression, and apathy, and are often overlooked and undertreated in PD patients (3). Previous studies have associated dysbiosis, an imbalance of the microbial community, with mental health disorders such as depression (4, 5). Others have found that PD patients with depression have altered gut microbiota compositions compared to patients without depression (4, 6).

Fatigue is another non-motor symptom of PD common in early stages of the disease which can occur on its own or with other symptoms including depression. Previous studies reported alterations and reduced diversity in the gut microbiome in cancer patients with fatigue symptoms compared to healthy, non-fatigued control groups (7, 8). Some researchers have also found that the accumulation of D-lactic acid from bacterial fermentation in the gut can lead to an excess in the blood and brain which may lead to fatigue (9). These findings are important to the study of PD as fatigue is one of the most common symptoms, occurring in over half of patients (10, 11).

Moreover, previous studies identified specific phyla and genera of gut microbes linked to PD. These studies found a reduced abundance of Proteobacteria (12) and reduced counts of Faecalibacterium among PD patients with depression compared to healthy individuals (13). Although research is lacking around gut microbiome alterations from fatigue in PD patients

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specifically, past studies have proven that chronic fatigue syndrome can be treated with fecal microbiota transplants (14). There is a gap in research regarding the combined impact of depression and fatigue on the gut microbiome of PD patients. Therefore, the purpose of this study was to investigate how changes to the gut microbiota of PD patients reporting fatigue and depression relates to patients who do not report these symptoms. We hypothesized that patients displaying these symptoms will have altered abundances of certain microbial genera compared to non-symptomatic patients. To test this hypothesis, we analyzed the microbiota composition of patients who reported high fatigue and high depression scores to see whether this affected their microbiota composition. A difference in gut microbiota at the genus level was observed. This research may be helpful in using gut microbes for the prevention, detection, and treatment of PD.

METHODS AND MATERIALS

Dataset and metadata. The dataset used in this study is from Cirstea *et al*. which analyzed 197 PD patients alongside 103 non-PD controls (37). Serum was provided by the subjects for metabolomics. Fecal samples were collected from patients for microbial sequencing and serum for untargeted metabolomics (n=125). The 16S rRNA v4 region was amplified and collected using Illumina MiSeq platform sequencing to produce the raw sequences used for analyses in this paper.

The purpose of the original study was to find associations between microbiota composition, stool consistency, constipation, and systemic microbial metabolites in Parkinson's disease and determine how intestinal microbes contribute to gastrointestinal disturbances typically seen in patients. To aid in their study, the researchers collected data regarding a variety of Parkinson's symptoms, including diet, demographics, and other variables.

Metadata filtering and grouping. In the original dataset, depression scores were reported on the Beck's Depression Inventory (BDI) scale ranging from 0-25, where patients are self-scored based on a questionnaire, with a higher score indicating higher signs of depression (15). According to the scale those with a score of 17 and over are deemed to have "borderline clinical depression". Fatigue scores were reported using the Fatigue Severity Scale (FSS) score metrics, which ranges from 1-7, with higher scores indicating higher fatigue and scores over 4 indicative of significant fatigue (16).

First, the metadata file was downloaded in excel format and all control subjects (patients without PD) were removed. Using the remaining PD patients, FSS fatigue scores (y-axis) were plotted against BDI depression scores (x-axis) to see whether or not these scores are correlated (Fig. 1). A plot was made using the ggplot (17) function on R studio and the R squared value was reported. In order to control for the variation in the sample and to focus on patients most severely affected by both fatigue and depression, we further grouped samples according to both scores. Two categories of patients were created (Table 1). The first included those with high depression and fatigue scores categorized as "Distressed". This category consisted of patients who had a depression score of 15 or over and a fatigue score of 5 and

TABLE. 1 Depression and fatigue score ranges for distressed and non-distressed patients. Depression and fatigue scores were assessed by patient self-reports and recorded by Cirstea *et al*.

	Distressed	Non-Distressed
BDI Depression Score	15+	0-4
FSS Fatigue Score	5+	1-2

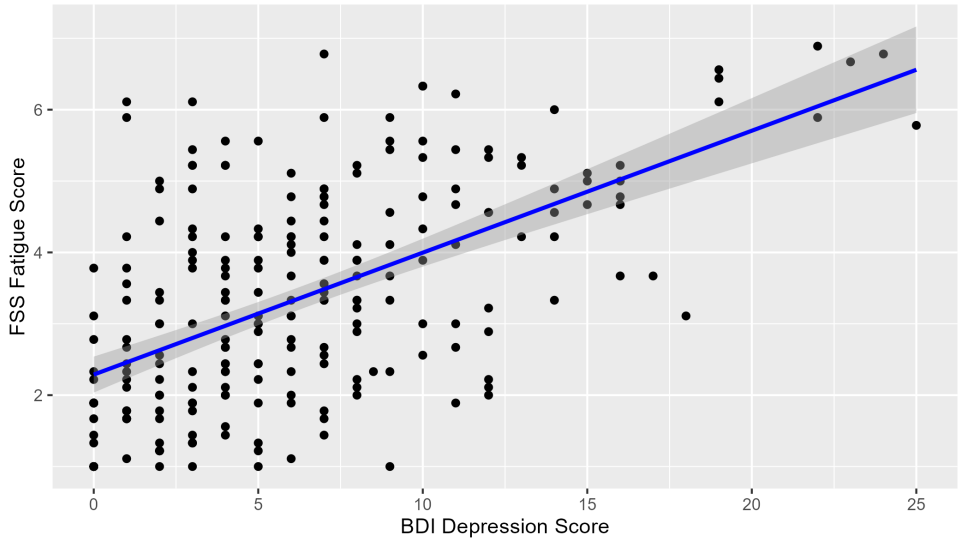

FIG. 1 BDI depression score and FSS fatigue score are weakly correlated in PD patients. Considerable variation is observed between patient depression and fatigue scores, with some patients expressing high fatigue and depression. R^2=0.34. R^2 signifies the strength of the correlation by determining how much of the variance in the dependent variable (FSS fatigue score) is explained by the independent variable (BDI depression score).

above. A second category, which we labelled 'Non-Distressed' consisted of patients who had low scores for both fatigue and depression. This included patients with BDI scores of 0-4 and fatigue scores of 1-2. A new column was added to the metadata in which "Distressed" patients were labelled as "high" and "Non-Distressed" were labelled as "low". Patients who had scores for either depression or fatigue which fell between distressed and non-distressed score ranges were excluded from further analyses and assigned "NA". Using this method, 12 distressed samples and 14 non-distressed samples were obtained. The metadata was then saved as a text file and exported to QIIME2 for data processing.

QIIME2 data processing pipeline. Microbiome bioinformatics were performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) 2017.4 (18). The raw sequence data were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with DADA2 (19) (via q2-dada2). The demultiplexed samples were exported and viewed using the QIIME2 view website. Quality control was performed on the sample using the demultiplexed sequence counts summary. The truncation length was determined to be 251 (demux.qzv). Amplicon sequencing variants (ASVs) were identified and aligned in the different samples (table.qzv). Taxonomy was assigned to ASVs using the q2-feature classifier (silva-138-99-515-806-nb-classifier.qza) (20) and taxa bar plots were generated from the taxonomy files. The taxonomy tables were then filtered to remove mitochondria and chloroplast sequences to create a filtered taxonomy table. The resulting filtered table (table-no-mitochondria-no-chloroplast.qza) was viewed using QIIME2 view and the alpha rarefaction curve was generated using a sequencing depth of 7563. This depth was selected because it allowed us to retain 56% of the samples and have enough sequences and samples to work with, with only one sample eliminated from the distressed group. A total of 14 'low' (non-distressed) and 11 'high'(distressed) samples were retained.

Alpha-diversity metrics (Observed Features and Shannon's Phylogenetic Diversity (21), Faith's Phylogenetic Diversity (22)), beta diversity metrics (Weighted UniFrac (23), Unweighted UniFrac (24), Jaccard distance (25), and Bray-Curtis dissimilarity (26)), and Principle Coordinate Analysis (PCoA) (27) were generated. Data outputs from QIIME2 were exported into R (v4.2.2) and R studio (v2022.12.0.353) (28).

Alpha and beta diversity analysis in R. Using previously imported files from QIIME2 including the metadata, feature table, taxonomy table, and phylogenetic tree outputs, a phyloseq object was created with the phyloseq (29), ape (30) and tidyverse (31) packages in R. The resulting phyloseq object was then filtered using the phyloseq package (29) to filter out non-PD samples and remove any NAs. To determine differences in gut microbiome diversity between distressed and non-distressed PD patients, we ran alpha and beta diversity analyses. For alpha diversity, "Distressed" and "Non-distressed" PD patients were assessed using the Shannon diversity metric. Results were visualized with a boxplot using the phyloseq (29) and ggplot2 (17) R packages. For beta diversity, "Distressed" and "Non-distressed" PD

patients were assessed using the Weighted UniFrac, Unweighted UniFrac, Jaccard, and Bray-Curtis beta diversity metrics. The data for each metric was plotted on principal component analysis (PCA) plots using the phyloseq (29) package in R. Only the Weighted UniFrac PCA plot showed possible clustering and was therefore chosen for subsequent analyses. Statistical analysis was performed on the chosen Weighted UniFrac diversity metric using PERMANOVA testing with 999 permutations and a significance level of $p < 0.05$, using the phyloseq (29) and vegan (32) packages in R.

Taxa bar plot in R. The phyloseq (29) package in R was used to create a taxonomic bar plot at the phylum level to begin assessing taxonomic differences between distressed and non-distressed PD patients and to get an idea of differences in the microbial composition between the two groups. First, the ASV table was converted to relative abundance to transform raw counts into percentages. Next, we clustered by phylum and faceted by PD patient status ("Distressed" vs "Non-distressed").

Indicator Species Analysis in R. To determine if there were species that were more prevalent and/or abundant in distressed and non-distressed PD patients, we ran an indicator species analysis on distressed and non-distressed patients using our existing phyloseq object and the phyloseq (29) and indicspecies (33) packages in R. First, we converted the phyloseq table to relative abundance. Then, we used multipatt to cluster samples into distressed or non-distressed groups. Results were viewed using the summary command to generate a list of all indicator species for each group. The output revealed indicator bacteria at the genus level that were differentially abundant between the two treatment groups as signified by the calculated indicator values and significant P values ($p<0.05$). Only statistically significant genera ($p < 0.05$) were considered indicators. We then converted these results into a table.

RESULTS

The alpha diversity of distressed and non-distressed PD patients was similar. Alpha diversity metrics were analyzed to investigate the gut microbiome diversity of distressed vs non-distressed PD patients. The Shannon alpha diversity metric, which accounts for species richness and evenness, displayed similar diversity indices for distressed and non-distressed groups (Fig. 2). These results suggest that the diversity of species in PD patients is high for both distressed and non-distressed groups and that patient status does not affect the within-group microbial richness and abundance. The alpha diversity of non-distressed patients also had a greater range than distressed patients. Since the IQR is resistant to change, outliers were not removed for future analysis and were considered biological variation between samples.

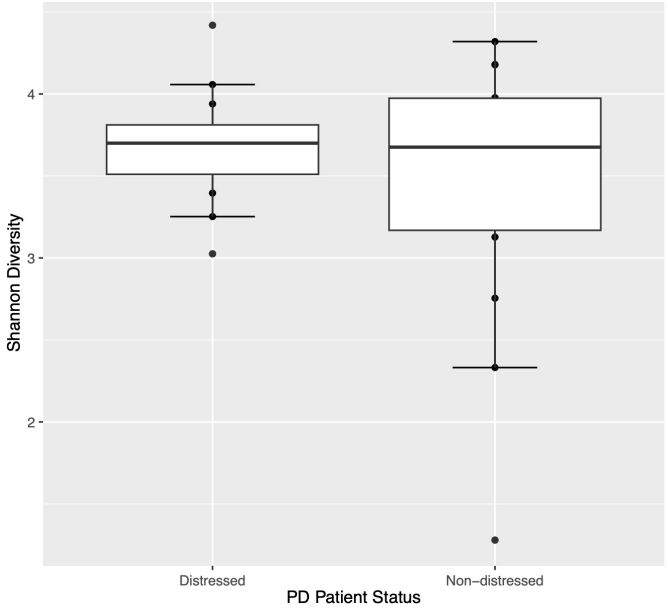

FIG. 2 Patient status does not alter alpha gut microbial diversity. Shannon alpha diversity metrics grouped by PD patient distressed (Shannon's index = 3.70) or non-distressed (Shannon's index = 3.68) status. Whiskers represent the mean +/- IQR*1.5.

Statistical significance was not calculated as the median appeared similar upon visual inspection.

No differences in gut microbiome diversity between PD patients who are distressed and non-distressed according to Weighted UniFrac beta diversity. To determine how patient status affects the gut microbiome diversity of PD patients, Weighted UniFrac beta diversity metrics were analyzed. We observed no distinct clustering between distressed and non-distressed groups on the PCA plots, and many samples were clustered together (Fig. 3). Jaccard, Bray-Curtis, and Unweighted UniFrac beta diversity metrics were also analyzed but showed no clustering (Fig. S1). These results suggest that patient status may not affect microbial diversity.

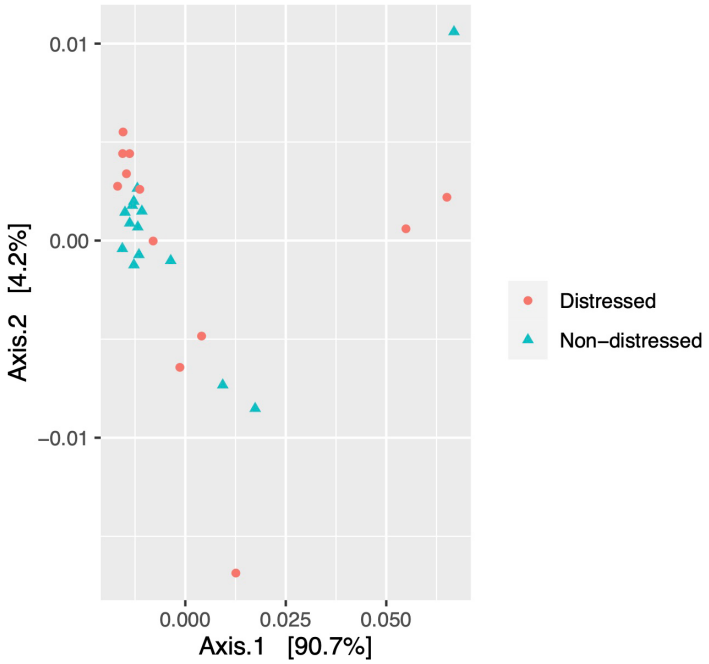

FIG. 3 No distinct microbial communities observed based on distressed and non-distressed status in PD patients. Principal component analysis plot using Weighted UniFrac beta diversity metric. Microbial communities did not separate based on distressed (red circle) and non-distressed (cyan triangle) conditions in PD patients. Corresponding PERMANOVA results show no significant difference between groups (P-value = 0.614).

Relative abundances of bacterial phyla are similar for distressed and non-distressed PD patients. We compared the relative abundance of bacterial phyla for each patient grouped by their status (Fig. 4). There were no noticeable differences in abundant phyla between the two groups. The most abundant phyla for both groups were Firmicutes and Bacteroidota.

Indicator taxa analysis reveals 2 indicator genera for distressed patients and 1 for non-distressed patients. We performed an indicator taxa analysis at the genus level to identify bacterial genera that were more abundant and prevalent for each patient status group (Table 2). We found that *Romboutsia* and *Erysipelatoclostridium* were indicator genera for distressed patients and *Parasutterella* was an indicator genus for non-distressed PD patients. All three genera have high indicator values which are close to the maximum value of 1. This means the ASVs for these groups have both high relative abundance and high relative frequency.

TABLE. 2 Indicator species analysis reveals 3 indicator genera for distressed and non-distressed PD patients. Indicator taxa analysis was performed at the genus level. Observed indicator values and p values are shown alongside indicator genera and phyla. Indicator value scores ASVs based on their abundance and prevalence to assign association within a group. High indicator values represent both high abundance and high prevalence of an ASV within a particular group. All p values were below the threshold set for analysis ($p \leq 0.05$).

Patient Status	Phylum	Genus	Observed Indicator Value (IV)	P-value
Distressed	Firmicutes	*Romboutsia*	0.732	0.035
Distressed	Firmicutes	*Erysipelatoclostridium*	0.689	0.025
Non-Distressed	Proteobacteria	*Parasutterella*	0.863	0.035

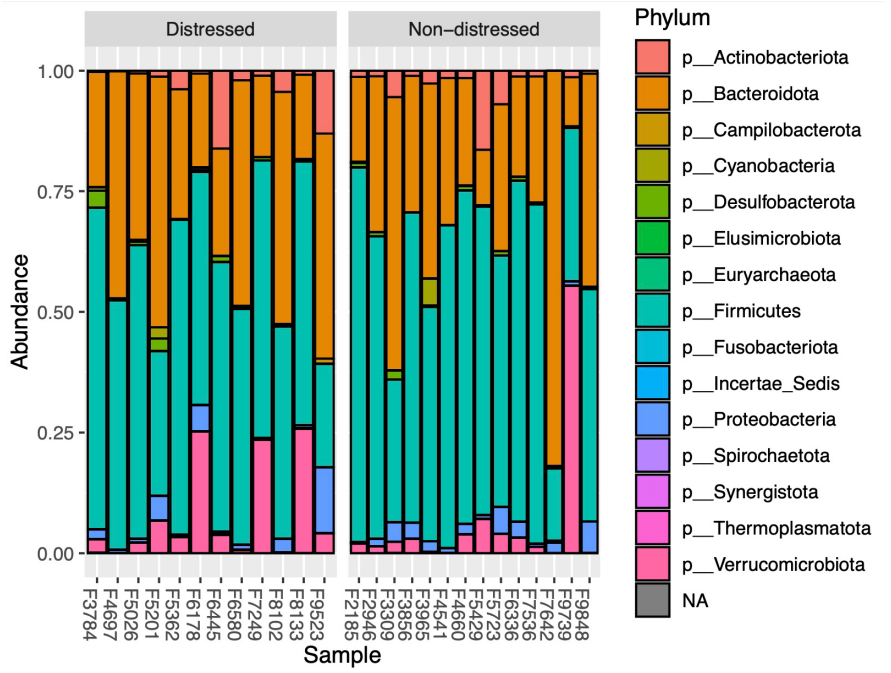

FIG. 4 Relative abundances of bacterial phyla are similar for both patient statuses. The most abundant bacterial phyla were identified for distressed (n=12) and non-distressed (n=14) patients. Bars are grouped by patient status and labelled with individual sample IDs.

DISCUSSION

Depression and fatigue are weakly correlated in PD patients. Before performing diversity analyses and deciding how to bin samples, we aimed to determine the correlation between depression and fatigue scores in PD patients. Previous studies have found that there is a strong correlation between depression and fatigue in PD patients (36). However, it was important to examine this relationship for our dataset specifically. We found that depression and fatigue scores for our dataset were weakly correlated. This may have differed from the strong correlation found by Santos-García *et al.* (36) due to the limited size of the dataset provided by Cirstea *et al.* (2020), as well as the presence of other confounding variables in patient samples such as age (37).

Alpha and beta diversity analyses show no differences in gut microbiome diversity between distressed and non-distressed PD patients. We observed no differences in gut microbiome diversity between distressed and non-distressed PD patients according to alpha and beta diversity analyses. This indicates that the microbiome composition between distressed and non-distressed PD patients is similar. Comparably, previous studies have shown that there is a strong association between microbiome composition and depression via the bidirectional gut-brain axis (6, 14, 38). These studies noted that bacteria in the gut microbiome produced metabolites like glutamate, butyrate, serotonin, and gamma amino butyric acid (GABA), which are important neurotransmitters for depression, suggesting the influence of these microbial communities on an individual's mood and behaviours (39). Previous studies also reported alterations and reduced diversity in the gut microbiome in individuals with fatigue symptoms compared to healthy, non-fatigued control groups (7, 8). However, we did not find a notable reduction in diversity between PD patient groups, which may suggest that depression has more of an effect on gut microbiome diversity than fatigue in distressed and non-distressed PD patients.

However, these studies looked at an isolated association between depression and the gut microbiome or fatigue and the gut microbiome, whereas our study examined the effect of distressed status, a combination of depression and fatigue, on PD patients' gut microbiota. Hence, our results are not directly contradicted by previous findings, but may indicate that the microbiome of a distressed patient more closely resembles that of a non-distressed patient, where both present with high microbial diversity. This may be because a combination of both depression and fatigue may negate the associations of one variable alone. Alternatively,

Parkinson's disease was previously reported to have an association with changes in gut microbiome composition (40). Therefore, depression and fatigue may not be as strongly associated with microbiome diversity compared to Parkinson's disease. However, we would need to conduct a follow-up study comparing control patients without PD with our current PD model to confirm these results.

Taxonomic Differences between distressed and non-distressed PD patients. We found *Romboutsia* and *Erysipelatoclostridium* were indicator genera for distressed patients and *Parasutterella* was an indicator genus for non-distressed patients. Previous studies have found *Romboutsia* to be more abundant in children with autism (41), demonstrating an impact on the brain similar to the neurological impacts of depression and fatigue. *Erysipelatoclostridium* produces the gut metabolite ptilosteroid A, which was enriched in patients with radio-induced intestinal injury (42). This suggests a link between the genus and gut dysbiosis, which is associated with PD. This genus has also been associated with injury to the gut epithelium. Comparably, *Parasutterella* is found in higher levels in healthy patients compared to those with social anxiety disorders (43). This implies *Parasutterella* may have protective effects against mental health conditions like PD.

Looking at the phyla level, both indicator species belong to the Firmicutes phyla. This is one of the highly abundant phyla observed in Fig. 4 which indicates the prevalence of these types of bacteria in the gut microbiota. Previous studies have found that two dominant phyla, Firmicutes and Bacteroidetes, represent over 90% of the gut microbiota (44). A study conducted by Hou *et al.* in 2018 on mice found that depletion of the Firmicutes phyla in gut microbiota was associated with elevated levels of the Osteocalcin protein (45). When injected into the PD mice, the Osteocalcin protein has shown to effectively ameliorate motor defects and neuronal loss (45). The elevation of Firmicutes phyla found in distressed patients in our study may indicate decreased levels of Osteocalcin protein equivalents in gut microbiota. This has neurodegenerative implications, which may explain the mental health symptoms of distressed subjects. Additionally, the Proteobacteria phylum, which makes up a minor proportion of the PD gut microbiota, has been linked with inflammatory bowel disease due to accumulation of common proinflammatory interleukins (46). Mouse studies have further found positive correlations of Proteobacteria abundance to colitis, and Crohn's disease (47). Despite this, the abundance of Proteobacteria has not been evidenced to affect the brain or nervous system. This is in line with non-distressed patients who do not report mental health issues such as depression and fatigue which were studied in this experiment. Further analysis of non-distressed patients may indicate that they do in fact report other symptoms related to inflammation, however that is beyond the scope of this study.

Limitations Several limitations restricted the scope and results of our study. One factor is the origin of the metadata used in our analysis. The original metadata was collected for the purpose of supplementing the Cirstea *et al.* study (37) and was not used as a main focus for their investigation. The metadata only allowed for correlation studies, as this paper has done, by looking at the correlations between patients' mental status, and does not allow investigation into causation. Another limitation was the fact that the metadata had more male patients than female, which was not accounted for in our study. This is also true for other variables in the dataset which were not accounted for in our analyses, some of which may have acted as confounding variables. This limits the ability to establish a causal link between microbial composition and patient status. Additionally, this study was limited by a small sample size which limits the generalizability of findings. Finally, BDI (15) and FSS (16) scores come from patient self-reports, so they may not accurately represent patients' conditions due to a self-reporting bias.

Conclusions The aim of this study was to investigate the combined effect of depression and fatigue on the gut microbial composition of Parkinson's Disease patients. A combined effect of depression and fatigue was defined as distressed and non-distressed patient status. Our study found that depression and fatigue scores were weakly correlated. Alpha and beta diversity analytics revealed no difference in gut microbiome diversity between PD patients who are distressed and non-distressed. Similarly, the abundance of bacterial phyla was generally similar between distressed and non-distressed PD groups. However, using the

indicator species analysis output, we found three indicator genera, *Romboutsia*, *Erysipelatoclostridium* and *Parasutterella* that were differentially abundant between distressed and non-distressed PD patients. These findings confirm our hypothesis that there are differences in the gut microbiota of patients who report fatigue and depression at high levels.

Future Directions We suggest repeating the analyses to include control patients who are distressed and non-distressed. These subsets of sample patients do not have PD but are distressed or non-distressed. This analysis will allow us to compare distressed and non-distressed status between PD and control patients, which may reveal new relationships. Alternatively, we suggest a repeat analysis of the association between depression and fatigue in PD patients, along with a third variable like a mental health disorder (e.g., anxiety) or specific patient factors (e.g., sex, age of disease onset). Previous studies report that the risk of developing PD is twice as high in men than women, however women have faster progression and higher mortality rates (48). The female sex is also associated with more severe anxiety and profound depression (48), hence the relationship between distressed conditions and patient sex could be a new direction for study.

Another future direction would be to carry out a differential abundance analysis on significant bacterial genera like *Romboutsia, Erysipelatoclostridium,* and *Parasutterella* to determine if patient status affects the relative abundance of genera in the gut microbiome of PD patients. Additionally, we suggest performing data collection on a new group of PD patients with additional categories (e.g., bipolar disorder, patient history). A new data set can allow for a wider and more even distribution of depression and fatigue among patients, a repeat analysis with a larger sample size, and new variables to examine novel relationships. If the new data set allows, devising a new binning strategy that more closely aligns with the BDI (15) and FSS scales (16) is another future direction. Using this approach, distressed patients will have severe depression defined by the BDI (15) and severe fatigue defined by the FSS (16), rather than high depression and high fatigue relative to available samples.

Specifying the type of depression (major depressive disorder, persistent depressive disorder, depressive disorder due to another medical condition, etc.) (49), can allow for a more specific hypothesis to test for differences in microbiome diversity in specific distressed and non-distressed PD patients.

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CONTRIBUTIONS

Each team member was an integral part of this study. The contributions of each team member are as follows. All authors contributed to the background research, planning, and design of this study. S.Z. conducted the analyses in QIIME2, and E.D., K.D., and S.R. conducted the analyses in R. The abstract was written by S.Z., introduction by S.R. and S.Z., methods by E.D. and S.Z., results by K.D. and S.R., limitations by S.R. and S.Z., conclusion by K.D. and S.Z., and future directions by K.D. The discussion was co-written by all four authors. All members were involved in the editing of this manuscript. Co-authorship should be considered equal for all team members.

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