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Constipation Severity Affects the Gut Microbiome Composition of Parkinson's Disease Patients

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SUMMARY Parkinson's disease (PD) is a complicated brain disorder caused by a variety of environmental and genetic factors. Besides the neurological deficits associated with PD, there are many physiological changes associated with the disease, such as digestive issues. In fact, PD patients are particularly vulnerable to suffering from constipation, and studies have shown that there is a bi-directional relationship between the gut microbiome of PD patients and constipation. However, the variables that contribute to changes related to PD, and constipation in the gut microbiome are largely unknown. In this study, we aimed to determine if there is a relationship between the gut microbiome of PD patients and the severity of their constipation. First, we used alpha and beta diversity analysis to determine a relationship between constipation severity and the gut microbiome of PD patients. Differential and relative abundance analysis of the amplicon sequence variants (ASVs) present in the highest and lowest constipation severity categories also indicate that particular genera may drive some of the differences identified in high constipation categories compared to lower severity categories in this study. We found that there were less unique amplicon sequence variants (ASVs) identified in the gut microbiome samples of higher constipation severity groups as compared to lower constipation severity groups. Abundance analysis of ASVs showed three distinct genera: *Lachnospiraceae*, *Faecalibacterium*, and *Bifidobacterium* that are differentially abundant in the high constipation severity group compared to the no constipation group. These insights have improved our understanding of the relationship between constipation severity and the gut microbiome of PD patients.

INTRODUCTION

Parkinson's Disease (PD) is the world's second-most-common neurodegenerative illness, with symptoms including bradykinesia, stiffness, involuntary movements, and tremor (1). Clinical and scientific focus has recently switched to additional nonmotor symptoms that have previously gone unnoticed, with constipation being one of the main concerns. Constipation affects up to 66 percent of all PD patients, having a higher prevalence than in the general population (2).

Constipation is defined as infrequent bowel movements or difficult bowel movements that persist for several weeks or longer, as well as having less than three bowel movements per week (2). Though constipation is quite prevalent, occurring in 16% of the people all over the world (3), some people suffer from chronic constipation as a symptom of PD, causing great discomfort to some and greatly debilitating others. The gut microbiome influences the severity of constipation and the relationship between the two is likely bi-directional, which could be explained by multiple possible mechanisms (4). The microbiome contributes to the development of functional constipation by metabolic activities such as bile acids, short-chain fatty acids, 5-hydroxytryptamine, and methane (4). Additionally, the slow speed of transit of bowels due to constipation selects for bacteria that require lower nutrient availability, have slower growth rates and use diverse energy sources (5). For chronic constipation, fecal microbiota transplantation (FMT) has been advocated as a treatment option. Researchers conducted a randomised, controlled trial by which they found that in adults with slow-transit constipation (STC), 6 days of FMT increased complete spontaneous bowel movements per week, soften stool, speed up transit, and improve symptoms of constipation, with a cure rate of 30% higher than conventional treatment (1). This illustrates the big impact the microbiome could have on constipation.

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Previous studies have also identified a relationship between constipation and microbial diversity (5). For example, *Dorea, Oscillospira,* and *Ruminococcus* genera were found to be positively associated with constipation in one study, whereas *Faecalibacterium* and total butyrate producers were reported to be negatively associated with constipation in another (5). Additionally, as per 16S rRNA sequencing and culture studies, the number of butyrate-producing genera *Roseburia, Coprococcus,* and *Faecalibacterium* was low in the gut microbiomes of constipated patients compared to control patients (5).

Although the relationship between constipation and microbial diversity has been identified, the relationship between the gut microbiome to the severity of the patients' constipation has not yet been studied. Our study addresses the research question: What is the relationship between constipation severity and the microbiome in patients with relationship to PD? We hypothesized that there exists a significant difference between the gut microbiome diversity of PD patients with severe constipation compared to those with mild constipation, and that this is accompanied by changes in the microbial composition of the gut microbiome. The dataset our team used to address this was collected by the Finlay Lab at the University of British Columbia to study microbiome composition, metabolism, and gut function in PD (7).

METHODS AND MATERIALS

Dataset and metadata. The dataset our team used for our project was collected by the Finlay Lab at the University of British Columbia to study microbiome composition, metabolism, and gut function in PD (7). These data were obtained through a cross-sectional cohort study that included 300 participants (197 PD patients and 103 healthy controls). Participants provided 300 fecal samples for microbiome sequencing. Motor and nonmotor PD symptoms, medications, diet, and demographics were all examined.

Metadata filtering and grouping. This analysis focused on the relationship between the gut microbiome and constipation severity in PD patients, so samples from healthy controls were filtered out using Quantitative Insights into Microbial Ecology 2 (QIIME2) (2). In the original dataset, constipation severity was reported as a range of values from 0 to 30(3). These scores were based on a standardized symptom-based questionnaire that is commonly used in assessing constipation severity in clinical settings (4). To control for the variation in sample sizes between each constipation category score, we further grouped samples according to their constipation severity scores, and a new column was introduced in the metadata using RStudio: constipation severity category. Values for this column were 0, 1-5, 6-10, 10-15, and 15+ (see Table 1 for sample distribution among categories). The steps for creating the new constipation severity category column and the metadata-based filtering steps on QIIME2 are outlined in Script#1. For the purpose of this study, samples in the 0 constipation severity category are considered to have no constipation. Category 1-5 is considered mild constipation, 6-10 = moderate constipation, 11-15 = moderately severe constipation, and 15+ = severe constipation. Grouping the samples into these categories which are categorical variables, unlike the severity scores which were continuous, also allowed us to perform more comparison-type analyses that better address our research aim.

QIIME2 data processing pipeline. Using the QIIME2 pipeline (2), data were demultiplexed to remove barcodes, followed by denoising and sequence quality control using DADA2 (5) to detect and correct sequencing errors. The truncation length was determined to be 250 (Script #1) using the parametric seven-number summary from the quality plot (demux.qzv) data on QIIME2 View. This length was selected because the median quality score is 37, meaning that the sequence accuracy is over 99.9%. To select ASVs, the rarefaction parameters using the table with ASVs identified in the different samples (table.qzv) and the alpha rarefaction curve were generated to determine the values of maximum depth and sampling depth. A maximum depth of 24076 was chosen because it is the maximum feature count as seen in table.qzv. Next, the sampling depth was fixed to standardize the library size of each sample to reduce sampling depth bias. From the alpha rarefaction curve, a sampling

TABLE 1. Sample Distribution Based on Constipation Severity Category and Disease Status.

Constipation Severity Category	Total	PD	Control
0	65	23	42
1-5	148	96	52
6-10	56	48	8
11-15	26	25	1
15+	5	5	0

depth of 8000 was chosen because that is the point where most samples plateaued, thus it was optimal.

Alpha and beta diversity analysis of disease status based on constipation severity category. Alpha and beta diversity metrics were produced and analysed using QIIME2 and R with the following R package: tidyverse, vegan, ape, phyloseq, DESeq2, ggplot2, dplyr, readxl, and VennDiagram (6–14). For the alpha diversity analysis of Faith's phylogenetic diversity (15) and Pielou's evenness (16), the Kruskal-Wallis test (17) was applied to assess statistical significance. Beta diversity analyses using Jaccard (18), Bray-Curtis (19), Unweighted and Weighted Unifrac measurements (20), were analysed for statistical significance with permutational multivariate analysis of variance (PERMANOVA) all group and pairwise tests (21). The detailed steps in this analysis are outlined in Script #2.

Differential and relative abundance analysis of PD samples based on constipation severity category. Following export of the table with taxonomy, sample metadata and tree from QIIME2 into RStudio, differential abundance analysis and relative abundance analysis were performed using the phyloseq (9) and DESeq2 (10) packages on R and visualized with ggplot2 (11). Preliminary processing steps included rarefaction, removal of low-abundance features, and setting the taxonomic level to the genus level.

RESULTS

Constipation severity has no association with the shift in gut microbial diversity of PD patients. We investigated the effect of constipation severity on gut microbial diversity using the dataset from individuals with PD. Faith's phylogenetic distances revealed that the gut microbial communities within each constipation category had no statistically significant difference (Supplemental Figure 1A) and Pielou's evenness also showed similar diversity regardless of constipation severity (Supplemental Figure 1B). The results of the beta diversity analysis were similar. A PCoA plot of weighted UniFrac distances visualized the similarities of gut microbial abundance at all constipation categories by ellipses and clustering and showed that phylogenetic distances and abundance of gut microbiota were not impacted by the altered metabolic host-gut microbial interaction despite the difference in constipation severity (Figure 1). Statistical comparison of the unweighted Unifrac distances concluded that the phylogenetic distance was not significant using PERMANOVA all group statistical analysis (Supplemental Figure 2). These results suggest that different constipation severity categories are not associated with significant changes in the gut microbial diversity of PD patients.

Less unique ASVs identified in the gut microbiome of PD patients with higher constipation severity. While there was no overall difference in the alpha and beta diversity of the gut microbiome of PD patients with different constipation severities, it is possible that the difference between higher constipation severity categories and lower constipation severity categories are driven by specific unique taxa. In order to explore this, we quantified the unique and shared ASVs between the two lowest constipation severity groups and the two highest constipation severity groups. We found that there were less unique ASVs identified in the higher constipation groups compared to the lower constipation groups (Figure 2A). To further elucidate this relationship, we also quantified the unique and shared ASVs between the no constipation category and the severe constipation category specifically. Similar to the group comparison, the results show that there were fewer unique ASVs identified for the severe constipation category (Figure 2B).

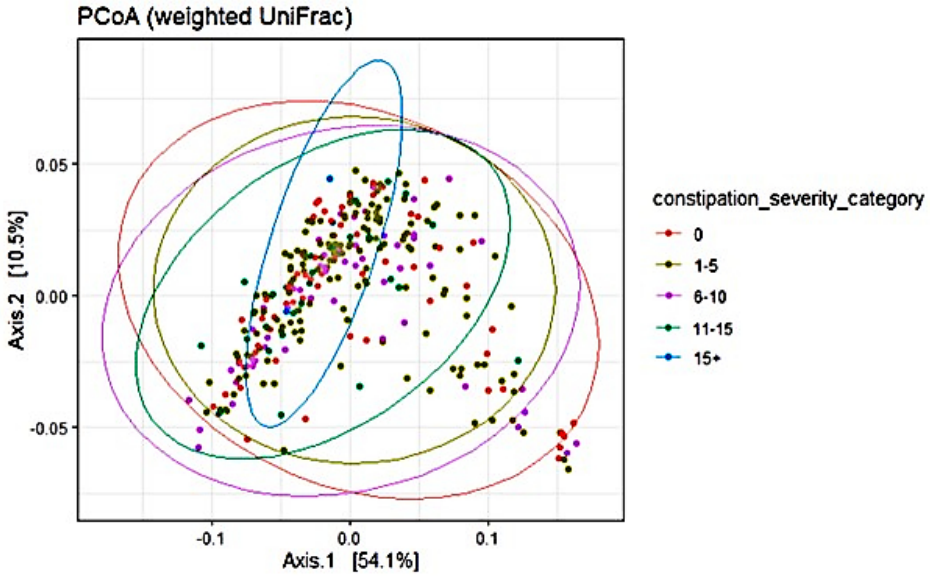

FIG. 1 Beta diversity between different constipation severity groups did not differ in gut microbial abundance. Principal coordinates analysis (PCoA) plot based on weighted UniFract distances showed that there were no differences in the gut microbial communities between the different constipation severity categories. Total sample size of n=175 was divided into five groups (n=19 for 0, n=89 for 1-5, n=42 for 6-10, n=20 for 11-15, and n=5 for 15+).

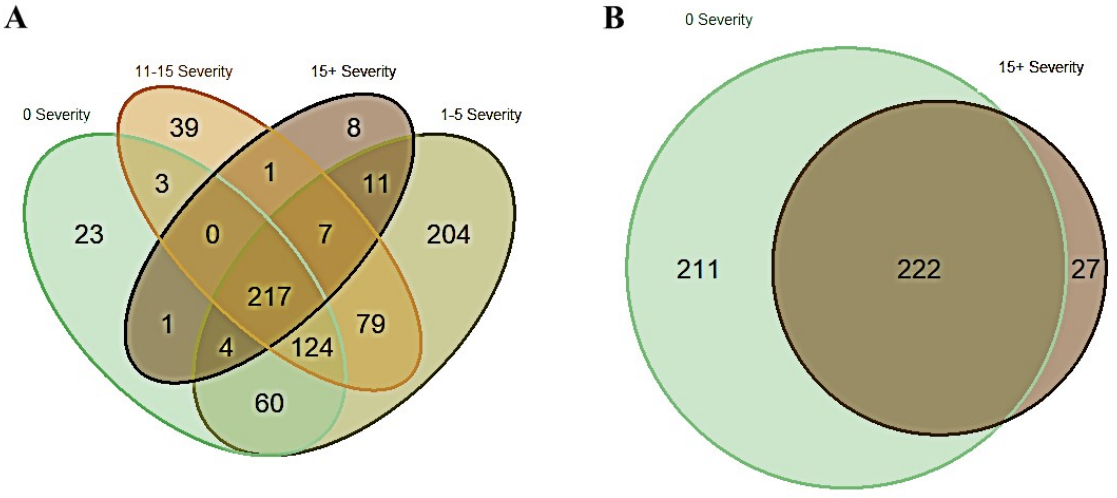

FIG. 2 Unique and Shared ASVs Between Different Constipation Severity Groups. A) Venn diagram comparing the number of unique and shared ASVs identified in the gut microbiome of PD patients from the constipation severity category groups 0 (n = 23 samples), 1-5 (n = 96), 11-15 (n = 25), 15+ (n = 5) and **B)** Venn diagram comparing only 0 vs. 15+ categories.

***Bifidobacterium, Faecalibacterium sp.*, and *Lachnospiraceae sp.* are differentially abundant in PD patients with constipation.** Given that there were fewer unique ASVs identified in the higher severity categories, the next step in our analysis was to determine if there are any differentially abundant genera in the severe constipation category. Three genera, *Faecalibacterium, Lachnospiraceae, and Bifidobacterium*, were differentially abundant between no constipation and severe constipation groups (Figure 3). *Bifidobacterium* had a higher relative abundance in higher severity categories. *Lachnospiraceae* showed the

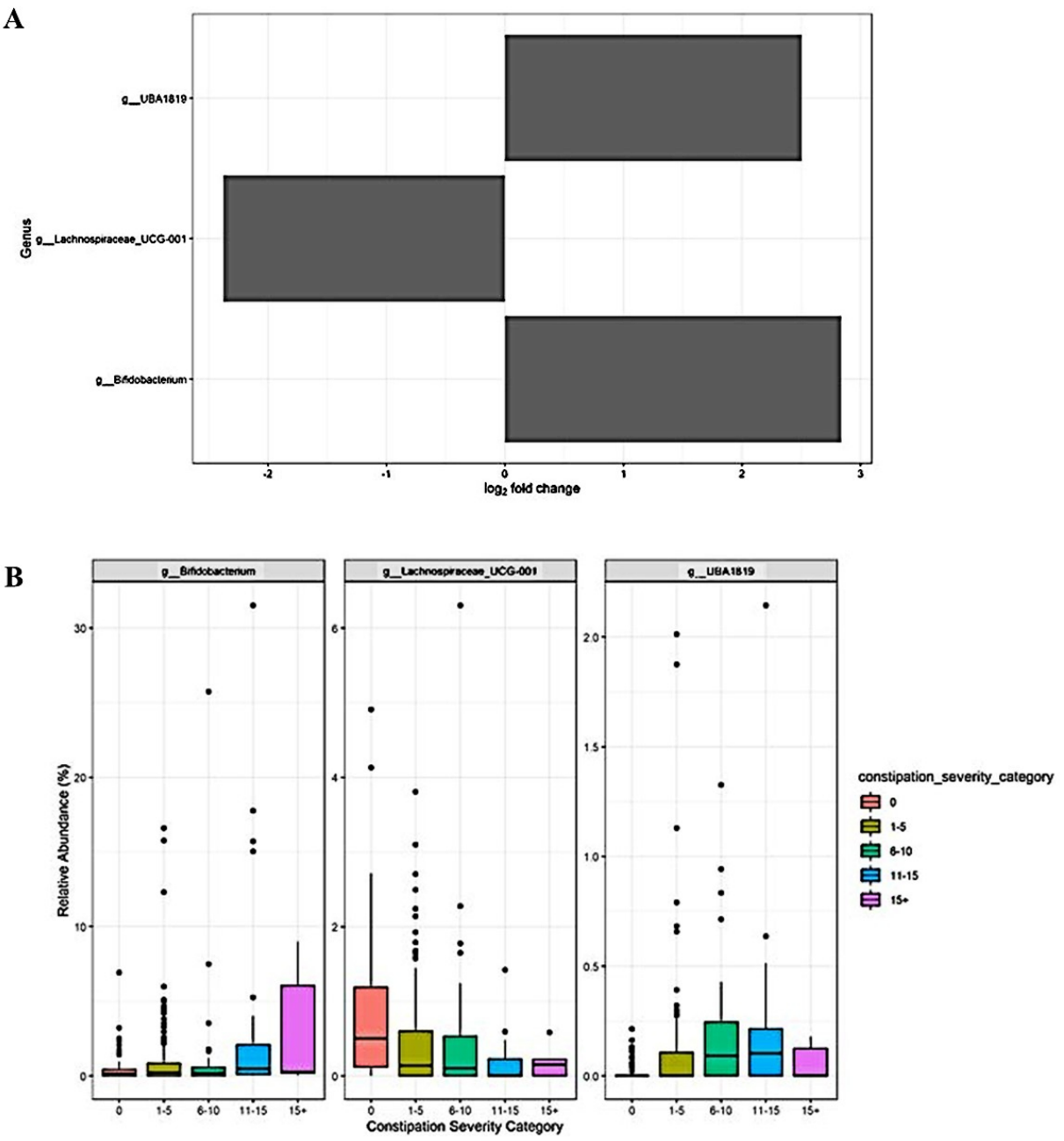

FIG. 3 Significant difference in abundance between genera between low and high constipation severity. A) Differential abundance analysis between constipation severity categories 0 (control) and 15+. Positive Log2 fold change indicates higher abundance in constipation severity category 15+ and negative Log2 fold change indicates higher abundance in severity category 0. The genera shown here are all differentially abundant ($p<0.05$). **B)** Relative abundance of 3 differentially abundant genera, calculated across all 5 constipation severity categories in PD patients for *Bifidobacterium*, *Lachnospiraceae* and *Faecalibacterium*. The genera shown here are all differentially abundant ($p<0.05$).

opposite trend, where relative abundance was highest for the no constipation group and was lower in the higher constipation severity categories. *Faecalibacterium* also had increased abundance, but it had the highest relative abundance in samples from the moderate, moderately severe, and severe categories.

DISCUSSION

Gut Microbial Diversity. Analysis of both alpha and beta diversity of the gut microbiomes of PD patients revealed no difference among different constipation severity groups (Supplemental Figure 1, 2 and Figure 1). A previous report by Mancini *et al.* did show that an osteopathic manipulative medicine (OMM) treatment to improve the symptoms of constipation due to intestinal movement disorder in PD patients improved the constipation severity and the abundance of gut microbiota (22). The difference between the previous research and our study was that the authors used pre-post analysis that tracked the shift in gut microbial communities of the same individuals with the improvement of constipation symptoms by the 4 weekly OMM treatment while our study investigated the tendency of gut microbial abundance for participants with different constipation severity. Altogether, our results revealed no association between the degree of constipation severity and the gut microbial communities; however, this conclusion would help another perspective to the previous study using a different approach.

Unique Genera in PD Patients with Higher Constipation Severity. While we found that constipation severity did not significantly affect the gut microbial diversity of PD patients, we did find that there were less unique ASVs identified from the gut microbiome samples of higher constipation severity groups. This is consistent with findings from other studies on the relationship between the gut microbiome and functional constipation. In literature, functional constipation is often associated with reduced diversity of the gut microbiome (23). As a result, it is possible that this decrease leads to a greater loss of specific genera and species in severely constipated patients. Most studies investigate the increase and decrease in abundance of certain bacterial phyla, such as the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria phyla (24–26) due to their well-studied, functional roles in gut homeostasis and metabolic activity. While many studies look at higher taxonomic levels, some unique taxa have been found in comparative analyses looking specifically on the genus level. For example, one study identified 17 unique genera present in samples from functionally constipated patients and 23 unique genera in their control samples (27). While the differences in unique genera identified were not as large between their constipated samples and their healthy control samples, it similarly identified less unique genera in the samples from constipated patients. It is also important to note that this study grouped all samples from constipated patients together, while in our comparison we compared the highest severity categories to the unconstipated and lowest severity categories. This could explain why we found a larger difference between the number of unique genera identified in our analysis.

In addition to the decrease in unique ASVs identified in the higher constipation severity categories, we found that *Lachnospiraceae, Faecalibacterium, and Bifidobacterium* were differentially abundant in PD patients with higher constipation severity compared to those in low constipation severity and no constipation groups. Many other studies investigating constipation have highlighted the same genera and showed similar relative abundance trends concerning constipation severity. For *Lachnospiraceae*, which belongs to the *Firmicutes* phylum, we found that relative abundance was highest for the no constipation category and decreased in higher constipation severity categories. This is consistent with the findings in the literature (31, 32). For *Faecalibacterium,* we found that relative abundance was low for the no constipation category, but had the highest relative abundance in moderate-high constipation severity categories (Figure 3). Other studies showed mixed results in terms of increases and decreases in constipation. Cirstea *et al*. (2020) and Yarullina *et al.* (2020) found that the abundance of *Faecalibacterim* was low in patients with severe chronic constipation (24). On the other hand, Zhang *et al.* (2021) and Avelar Rodriguez *et al.* (2018) found that *Faecalibacterium* is elevated in functional constipation (33, 34). Our results also showed that Bifidobacterium had a higher relative abundance in high severity categories. This is in agreement with other studies that refer to higher levels of Bifidobacteria with constipation (35, 36).

Limitations Because PD is a heterogeneous disease with many different phenotypes (8), and because the scoring system used to determine the constipation severity scores of the patients

in the study was subjective, these scores are not a definite identification of the severity of the patients' severity. Additionally, the samples sizes were different between the severity categories. Particularly, the severe category had a much smaller sample size than the others, so those samples may not have been as representative of the gut microbiome of all patients with severe constipation. However, the difference between the no constipation category and the severe constipation category was quite large, particularly for the comparison of unique ASVs identified: 17(no constipation) v.s. 211(severe constipation).

Conclusions The results of our study suggest that there is no direct relationship between constipation severity category and the gut microbial diversity of PD patients. However, the quantification of unique ASVs and the differential abundance analysis results indicate that certain genera may affect the severity of constipation in PD patients. These results would need to be further validated due to the limitations of our study, such as uneven sample sizes between constipation severity categories. Nevertheless, these findings have increased our understanding of constipation severity and the gut microbiome in PD patients, who are particularly vulnerable to suffering from constipation. Further investigations into the unique ASVs identified in the highest constipation category could also elucidate if they contribute to worsening the symptoms of functional constipation.

Future Directions Our abundance analysis revealed three unique genera that are differentially abundant in the no constipation category compared to the severe constipation severity category. Previous studies supported our analytical results about the association of constipation with *Lachnospiraceae* UCG-001 (33), *Faecalibacterium* sp. UBA1819 (34, 35) and *Bifidobacterium* (31). For future studies, functional investigation of the three unique genera using PICRUSt2 (36) could clarify the role of identified genera in the host-gut microbiome's metabolic interaction in PD patients and how they affect constipation severity. Future studies could also look at the applicability of these findings beyond PD patients by looking at constipated patients as a group and not only constipated PD patients.

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