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Dopaminergic Therapeutics for Treating Parkinson's Disease Were Associated with a Shift in the Gut Microbiota to Resemble Healthy Individuals

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SUMMARY Parkinson's Disease (PD) is a neurodegenerative disorder associated with dopaminergic neuron loss, leading to dopamine dysregulation. Dopaminergic therapeutics are often administered to restore dopamine levels and have been associated with changes to the gut microbiota. Through a secondary data analysis of a cross-sectional cohort of PD patients, we aimed to investigate changes in the gut microbiome associated with the use of four dopaminergic drugs (entacapone, pramipexole, rasagiline, amantadine). Although the use of dopaminergic therapeutics was not associated with compositional alterations to the microbial diversity of PD patients, we observed changes to specific taxa. Amantadine and pramipexole therapeutics were both associated with a core microbiome that contains *Faecalibacterium* – a genus contained in the core microbiome of healthy individuals but absent in untreated PD patients. Furthermore, entacapone and amantadine use was associated with taxa that are indicative of a healthy gut microbiome, including *Lachnospiraceae* and *Colidextribacter*. We also identified three genera that were differentially abundant with dopaminergic drug use. Dopaminergic therapeutic use was generally associated with increased *Bifidobacterium*, decreased *Prevotella*, and increased *Akkermansia*. While increased *Bifidobacterium* is associated with a healthier gut microbiome and *Akkermansia* is associated with gut dysbiosis, the effects of *Prevotella* remain unclear. Our findings suggest that dopaminergic therapeutics are associated with alterations in the gut microbiome of PD patients that provide an overall benefit to the host. Future studies could incorporate higher resolution analysis at the species level and explore causational effects of dopaminergic drugs in a prospective study.

INTRODUCTION

Parkinson's disease (PD) is the second most common age-associated neurodegenerative disorder in the world, with a prevalence of 1% in individuals 60 years of age and older (1). PD is a complex and multifaceted neurodegenerative condition with symptoms such as tremors, stiffness, and slowed movement, often accompanied by postural instability as the disease progresses (2). While many different neurotransmitters have been suggested to play a role in the development of PD, most current therapeutic approaches are focused on addressing neuronal dopamine dysregulation, a hallmark symptom of PD (3, 4). One major outcome of PD is dopaminergic neuron loss, which eventually leads to dopamine dysregulation (2, 3). Given that dopamine signaling is essential for core motor functions in healthy individuals, this has become a significant concern in PD (4). To address this, treatments such as levodopa and other dopamine agonists are frequently used to restore dopamine levels (2). Other examples of commonly used drugs include the catechol-*O*-methyltransferase (COMT) inhibitor, entacapone, dopamine agonists such as pramipexole, amantadine, and the monoamine oxidase-B (MAO-B) inhibitor, rasagiline (5). These drugs

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ameliorate motor dysfunction caused by PD and can partially relieve symptoms, but the side effects are often drastic (6). These side effects include gut issues such as constipation, which may be caused by interactions between the drugs and the gut microbiome (6). Furthermore, it has been shown that certain bacteria in the gut microbiome such as *Lactobacillus* can severely limit the efficacy of dopaminergic therapeutics including levodopa. This is due to their ability to decarboxylate the drug using tyrosine decarboxylase (TDC), interfering with levodopa availability (7). Similarly, increases in *Lactobacillus* and *Enterococcus* may lead to ineffective levodopa treatment as TDC harboured by these genera will decarboxylate levodopa, potentially rendering it ineffective (7).

Previous research suggests that the gut microbiome of PD patients is significantly different compared to non-PD patients (8). Some taxonomic differences include decreased *Faecalibacterium* and *Lachnospiraceae* in PD patients. Furthermore, common PD drug treatments may also drastically alter the gut microbiome. For instance, the COMT inhibitor, entacapone is associated with changes the gut microbiome composition and reduced fecal short-chain fatty acids (SCFA). This leads to the death of certain beneficial bacterial groups, such as the phylum Bacteroidetes and the family *Prevotellaceae* (9). Finally, dopamine agonists also significantly decrease small intestinal muscle contractility and lead to a shift in the microbiome of the ileum, leading to bacterial overgrowth (10). This makes dopaminergic therapeutics an interesting group of drugs to investigate regarding their effects on the gut microbiome of PD patients versus healthy individuals.

Existing studies focus heavily on either describing the differences in the microbiota between PD patients and healthy individuals, or the effect of levodopa on specific bacterial families. As such, there remains a lack of understanding regarding the specific impacts of each drug on the microbiome of PD patients. To address this gap, compared six different groups of individuals, including PD patients who may be receiving levodopa but no other dopaminergic therapeutics (PD-untreated), healthy individuals, and PD patients receiving entacapone, pramipexole, rasagiline, or amantadine. A more comprehensive understanding of the interactions between dopaminergic treatments and the gut microbiome of PD patients may help inform the prescription of these medications, leading to more favourable clinical outcomes and minimization of side effects. With the strong associations between PD and an altered gut microbiota composition through the gut-brain axis (2), we hypothesized that PD medications capable of restoring patients' neurological health status to that of healthy individuals may also change the microbiome composition to more closely resemble that of healthy individuals.

A study by Cirstea *et al.* (8) previously examined novel associations between PD patients and factors impacting the gut microbiome, including the overall effect of dopaminergic therapeutics in a cross-sectional cohort. However, specific changes associated with each drug were not analyzed in detail in this study. Through further analysis of this dataset, we found that dopaminergic therapeutics did not significantly alter gut microbiota diversity. However, the core microbiome of pramipexole and amantadine treated patients shared taxa found in healthy individuals, suggesting these therapeutics may restore microbial balance. Furthermore, amantadine and entacapone treatment had indicator amplicon sequence variants (ASVs) that were associated with a healthy gut microbiota. Finally, the differential abundance taxa analysis indicated that dopaminergic therapeutic usage was associated with significant changes in the abundance of multiple genera that play a role in PD. Ultimately, treatment with specific dopaminergic drugs was associated with beneficial changes in the gut microbiota of PD patients.

METHODS AND MATERIALS

Dataset and metadata filtering. The Parkinson's Disease (PD) dataset was generated by Cirstea *et. al.* at the University of British Columbia (UBC) (8). Fecal samples were collected from 103 healthy and 197 PD individuals (300 total). PD individuals were within the age range of 40-85 years, with PD onset from age 40-80, and with less than 12 years since PD onset. The bacterial 16S rRNA V4 region was amplified from fecal sample DNA, using barcode 515F (GTGCCAGCMGCCGCGGTAA) / 806R (GGACTACHVHHHTWTCTAAT) primers. The library was pooled and sequenced using an Illumina MiSeq platform. The dataset contained information collected from PD patients and

healthy individuals found in the original research paper (8). Notably, this included information of interest, such as therapeutic use (entacapone, pramipexole, rasagiline, or amantadine) and Parkinson's severity score (using the Unified Parkinson's Disease Rating Scale), which were factors of interest in this study. For our analyses, only patients using a single PD medication were included in our treatment cohorts; 11 patients using multiple medications were excluded from our analyses. No healthy and PD-untreated individuals were eliminated for downstream processing. Metadata was updated to include a new category that classified samples according to their status and medication use: Healthy, PD-untreated, entacapone, pramipexole, rasagiline, and amantadine. Despite treatment in PD-untreated patients with other therapeutics, such as levodopa, we did not control for those variables to preserve sufficient statistical power for downstream analysis. Therefore, our PD-untreated cohort includes all PD patients untreated with the therapeutics of interest. All samples with therapeutic use also possessed a positive PD status.

Data processing via QIIME2. The QIIME2 pipeline 16S rRNA samples from Cirstea *et al*. were imported and demultiplexed (8, 11). Denoising and clustering were conducted in QIIME2 using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (12). Using a minimum mean Phred quality score of 30, no trimming was required; this resulted in 251 nucleotides for all read lengths. The Silva 138-99 database was used to train a classifier for taxonomic analysis, and ASVs were assigned taxonomic information (13). The feature table, rooted tree, taxonomy, and metadata were exported for downstream analysis.

Data processing via R (v4.3.2) (14). Data from QIIME2 was exported (feature table, rooted tree, taxonomy, and metadata). Using phyloseq (v1.46.0) (15) and tidyverse (v2.0.0) (16) packages, data was processed into a phyloseq object. Filtering steps were conducted to remove mitochondrial sequences, chloroplast sequences, and ASVs containing less than or equal to 5 counts.

Alpha and beta diversity analysis. Alpha and beta diversity metrics for our six conditions were analyzed using R (v.4.3.2) (14) with the phyloseq (v1.46.0) (15), ape (v5.7-1) (17), picante (v1.8.2) (18), vegan (v2.6-4) (19), dunn.test (v1.3.6) (20), and FSA (v0.9.5) (21) packages. A maximum sampling depth of 5421 was used to retain at least 5 samples in all of our conditions (Supplemental Table S1). An alpha rarefaction curve was used to confirm that our sampling depth fell within the plateau of unique ASVs. 279/300 samples were retained at this sampling depth. All alpha and beta diversity metrics using the plot_richness() and distance() functions in phyloseq (v1.46.0) (15) were initially evaluated. For alpha diversity analysis, Shannon's diversity index, which considers microbial abundances (22), and Faith's phylogenetic diversity (23), which accounts for phylogenetic distances, were chosen. Alpha diversity metrics were visualized using box plots overlaid with violin plots using the ggplot2 package (16). Statistical significance between groups was assessed using the Kruskal-Wallis test, followed by pairwise testing using Dunn's test. For beta diversity analysis, the weighted UniFrac distance metric, which considers both microbial abundances and phylogenetic distances was used (24). Beta diversity was visualized using principal coordinate analysis (PCoA) plots. Statistical significance was assessed using permutational multivariate analysis of variance (PERMANOVA) with 10000 permutations. All p-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg method to control the false discovery rate (FDR).

Core microbiome analysis. Core microbiome analysis was conducted using the microbiome (v1.23.1) (25) package in R (v.4.3.2) (14). Samples counts were converted to relative abundance and data was transformed as compositional data. Analysis parameters were determined after generating prevalence heatmaps for each group (Supplementary Figure S1). A minimum relative abundance of 2% and a minimum relative prevalence of 50% were used for analysis of core genera.

Indicator species analysis. Indicator species analysis was conducted using the indicispecies (v1.7.14) (26) package in R (v.4.3.2) (14). Phyloseq object was grouped at the genus and

family level and sample counts were converted to relative abundance. Indicator ASVs were filtered for a p-value of less than 0.05. Pair-wise analyses were conducted comparing healthy to PD-untreated conditions and comparing all conditions (Healthy, PD-untreated, entacapone, pramipexole, rasagiline, and amantadine).

Differential abundance analysis. Differential abundance analysis was conducted using the DESeq2 package (v1.42.1) (27) to identify taxa with differential abundance in comparing conditions. Healthy and PD-untreated samples were used as reference groups for comparisons with PD patients using dopaminergic therapeutics (entacapone, pramipexole, rasagiline, and amantadine). Significant differential abundance was determined using an adjusted p-value < 0.01 and |log2FoldChange| > 2.

Predictive regression analysis. Simple linear regression models were created in R (v.4.3.2) (14) using the Unified Parkinson's Rating Scale (UPDRS) score (28) as the response variable and bacterial abundance as a predictor variable. Analysis was conducted on six genera of interest from our core microbiome, indicator taxa, and differential abundance analyses. 116 patients without a UPDRS score were filtered out, leaving a total of 173 samples. Depending on the distribution, bacterial abundance was log(x+1) transformed to retain samples with zero abundance.

Git repository. The code used to complete this study is included here: https://github.com/Xpado-star/MICB-475-Team-2

RESULTS

Dopaminergic therapeutics did not affect the gut microbial diversity of PD patients. We first examined the impact of dopaminergic drug use on the gut microbiome composition through alpha and beta diversity. There were no significant differences in alpha diversity between healthy, PD-untreated, and therapeutic groups as calculated using Shannon's diversity index (Figure 1A) or Faith's phylogenetic diversity index (Figure 1B). Beta diversity

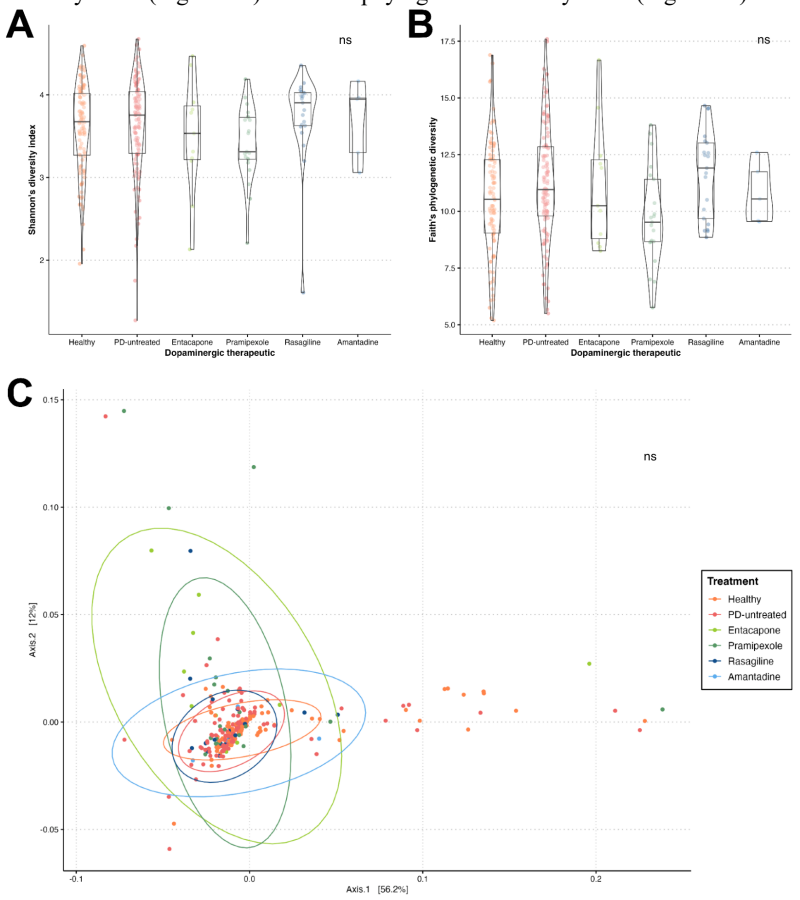

FIG. 1 Dopaminergic therapeutic use did not alter the gut microbiota composition of PD patients. Box plots with violin plot overlay comparing **(A)** Shannon's diversity index and **(B)** Faith's phylogenetic diversity for Healthy, PD-untreated, and all dopaminergic therapeutic groups. No significant differences were found using the Kruskal-Wallis test or pairwise Dunn's test ($q > 0.05$). **(C)** PCoA plot of Weighted UniFrac distance. Ellipses represent 95% confidence interval for clusters. No statistically significant clusters were found for therapeutic groups using PERMANOVA ($q > 0.05$).

analysis revealed a significant pairwise difference only between healthy and PD-untreated groups using Weighted Unifrac distance (P = 0.045), suggesting compositional changes driven by both abundance and phylogenetic relatedness (Figure 1C). Taken together, the lack of significant compositional differences in alpha and beta diversity between dopaminergic therapeutic and control groups suggests that gut microbiome changes related to dopaminergic therapeutic use may be subtle and require further investigation into specific taxa.

Amantadine and pramipexole use were associated with increased overlap of core taxa with healthy controls. We performed a core microbiome analysis to investigate shared or unique core taxa between healthy individuals, PD-untreated individuals, and PD patients treated with dopaminergic therapeutics. Venn diagram comparisons between conditions revealed a common core genus between all groups (Figure 2). This common core genus was found to be *Bacteroides*. Compared to the PD-untreated group, both amantadine and pramipexole groups had an increase in core taxa from one to three. These additional taxa, both found to belong to the *Faecalibacterium* genus, were identical to two other core taxa in the healthy group. Furthermore, the ASVs were absent as a core taxon of the PD-untreated group. This suggests that amantadine and pramipexole usage may be associated with beneficial alterations to the core microbiome in PD patients, as indicated by the presence of additional core taxa resembling those found in healthy individuals.

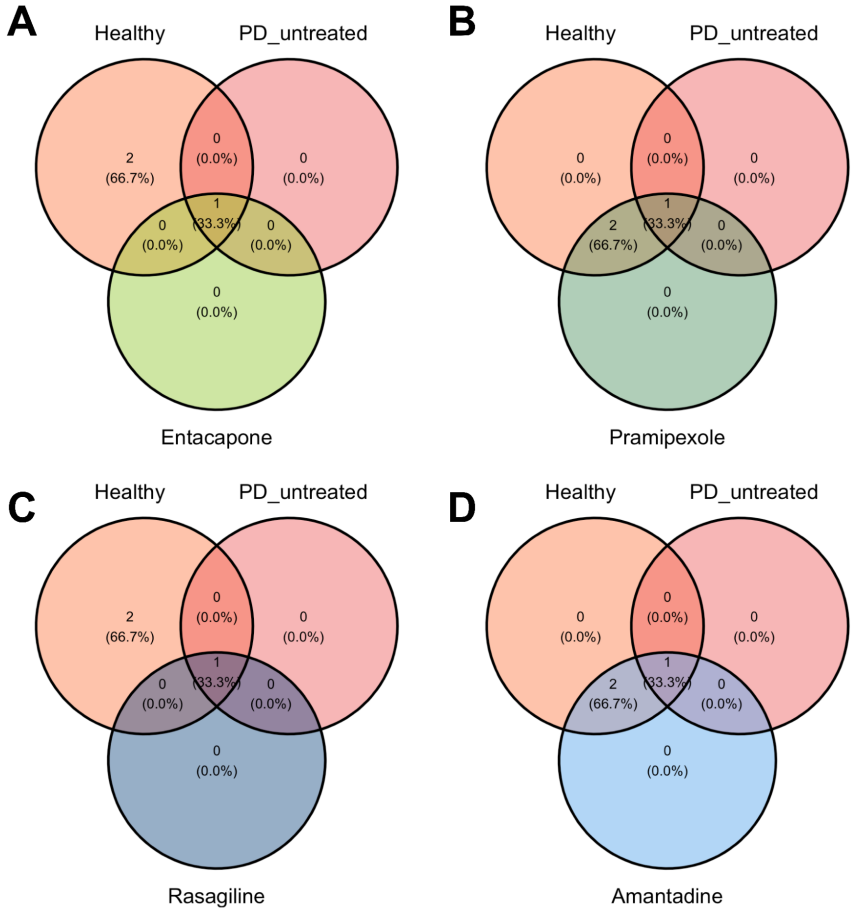

FIG. 2 Select dopaminergic treatments shift the core microbiome of PD patients to resemble healthy individuals. Core microbiome analysis was performed using a relative abundance of threshold 2% and prevalence of 50%. Core taxa were resolved at the genus level. Healthy and PD-untreated groups were compared to PD patients using **(A)** entacapone, **(B)** pramipexole, **(C)** rasagiline, and **(D)** amantadine.

Entacapone and amantadine use were associated with unique beneficial ASVs absent from healthy or PD-untreated patients. Indicator species analysis was performed to further determine taxa associated with dopaminergic therapeutic groups. Each drug-treated group was compared to the healthy and PD-untreated control groups to identify unique ASVs.

Initial indicator species analysis found multiple indicator ASVs for both healthy and PD-untreated patients, serving as unique identifiers strongly associated only with their respective groups (Table 1). Upon inclusion of dopaminergic therapeutic groups, neither healthy nor PD-untreated groups maintained unique taxa (Table 1). Meanwhile, entacapone and amantadine groups had the highest unique ASVs among therapeutic groups at 57 and 71, respectively (Table 1). Further analysis of the specific ASVs in those treatment groups revealed several taxa with suggested benefits such as those from the *Lachnospiraceae* family and *Colidextribacter* genus (Supplemental Table S2). Overall, entacapone and amantadine treatments were associated with a notable increase in the number of unique ASVs, including taxa with characteristics commonly associated with a healthy microbiome.

TABLE. 1 Entacapone and amantadine are associated with indicator taxa not present in healthy or PD-untreated patients. The number of unique ASVs per condition with dopaminergic treatments included or excluded in indicator species analyses (ISA). All analyses included Healthy and PD-untreated groups. Results were filtered to include only indicator ASVs with a p-value < *0.05*.

Dopaminergic Treatment Inclusion	Healthy	PD-untreated	Entacapone	Pramipexole	Rasagiline	Amantadine
Excluding Treatments	18	30	0	0	0	0
Including Treatments	0	0	57	1	3	71

Dopaminergic therapeutics altered abundance profiles of multiple genera of interest in PD patients. To examine how individual taxa were differentially abundant across different dopaminergic therapeutic groups, differential abundance analysis was performed using DESeq2 (27). Each drug-treated group was compared to the healthy and PD-untreated control groups. In every comparison, we observed more ASVs that were significantly depleted than those that were significantly elevated (Supplemental Table S3, Supplemental Figure S2). Additionally, out of the four dopaminergic therapeutics, entacapone, and pramipexole yielded a greater number of differentially abundant ASVs than rasagiline and amantadine (Supplemental Table S3). Notably, we identified three genera that were differentially abundant across multiple comparisons: *Bifidobacterium*, *Prevotella*, and *Akkermansia*. These genera of interest also exhibited the greatest increase or reduction in at least one comparison (Figures 3, 4). *Bifidobacterium* levels increased in the entacapone (Figure 3A), pramipexole (Figure 3B), and rasagiline (Figure 3C) groups, representing the most elevated genus when entacapone was compared to the healthy control group (Figure 4, *'Bifidobacterium'*). *Prevotella* levels decreased in every drug-treated group. *Prevotella* represented the most reduced genus in the pramipexole (Figure 3B) and amantadine (Figure 3D) groups compared to the healthy control, while also representing the most reduced genus in entacapone (Figure 3E), pramipexole (Figure 3F), and amantadine (Figure 3H) groups compared to the PD-untreated control (Figure 4, *'Prevotella'*). *Akkermansia* levels increased when comparing the entacapone (Figure 3A), pramipexole (Figure 3B), and rasagiline (Figure 3C) groups to the healthy control, and represented the most elevated genus in the entacapone versus healthy comparison (Figure 4, *'Akkermansia'*). Taken together, these results indicate that dopaminergic therapeutic usage is associated with significant changes in the abundance of multiple genera.

DISCUSSION

This study aimed to explore the effect of the dopaminergic therapeutics, entacapone, pramipexole, rasagiline, and amantadine, on gut microbial diversity using a dataset collected by Cirstea *et al* (8**)**. Alpha and beta diversity analyses did not yield any significant compositional differences between the therapeutic groups and PD-untreated or healthy controls. Cirstea *et al.* (8) also previously examined beta diversity for dopaminergic therapeutics and found that only entacapone is associated with a significant shift in diversity. However, our study's focus on patients using a single PD medication and comparison between

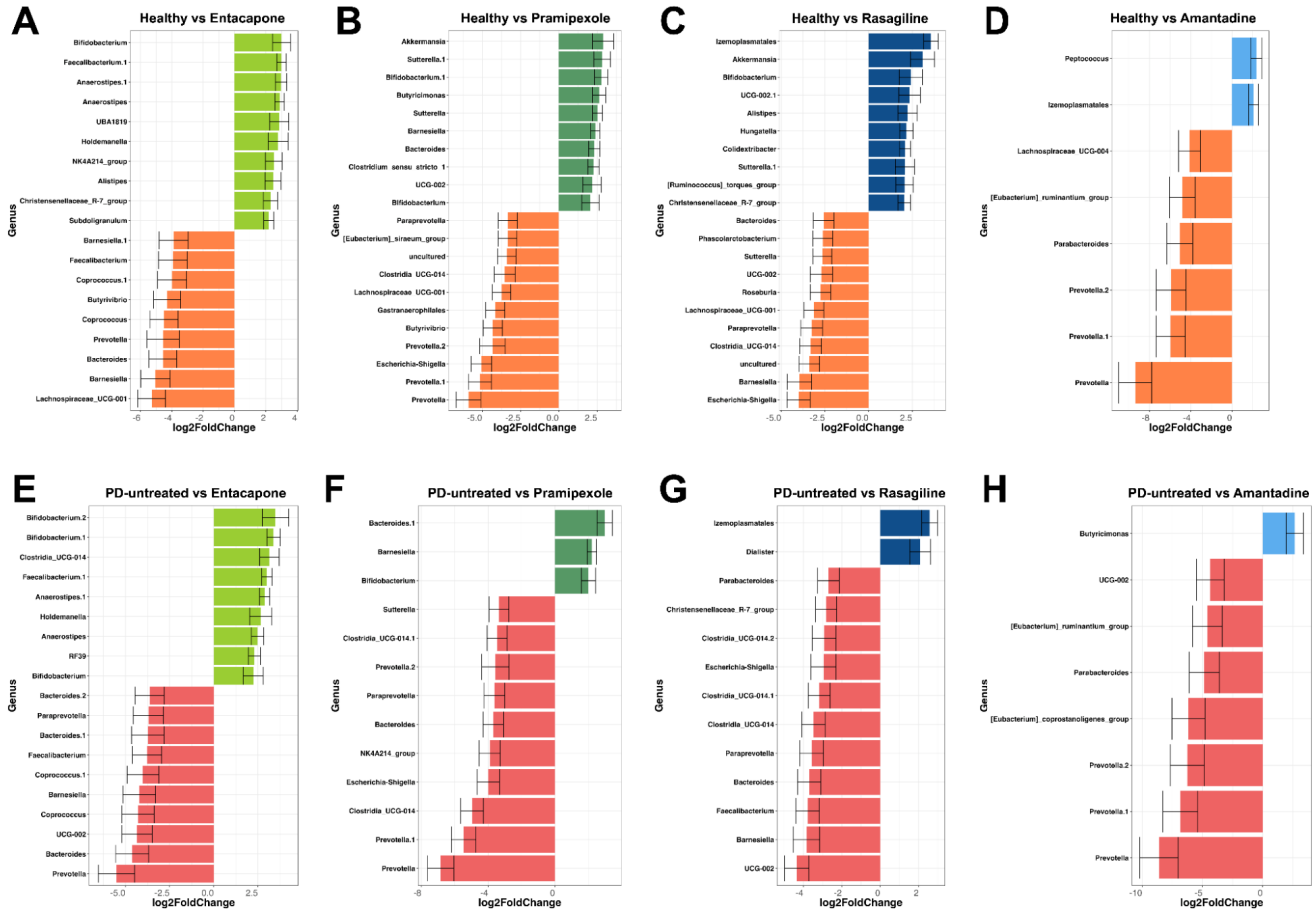

FIG. 3 Dopaminergic therapeutics are associated with differential abundances of multiple ASVs compared to healthy controls and PD-untreated patients. Differential abundance analysis (DESeq) was performed. The healthy group was used as a reference for identifying ASVs that were elevated or reduced in PD patients using **(A)** entacapone, **(B)** pramipexole, **(C)** rasagiline, and **(D)** amantadine. The PD-untreated group was also used as a reference for identifying elevated and reduced ASVs in PD patients using **(E)** entacapone, **(F)** pramipexole, **(G)** rasagiline, and **(H)** amantadine. Results shown include the top ten most elevated genera and most reduced genera that were also identified as significant. Significance was defined with an adjusted p-value < 0.01 and |log2FoldChange| > 2. Error bars represent standard error of the log2 fold change for each genus.

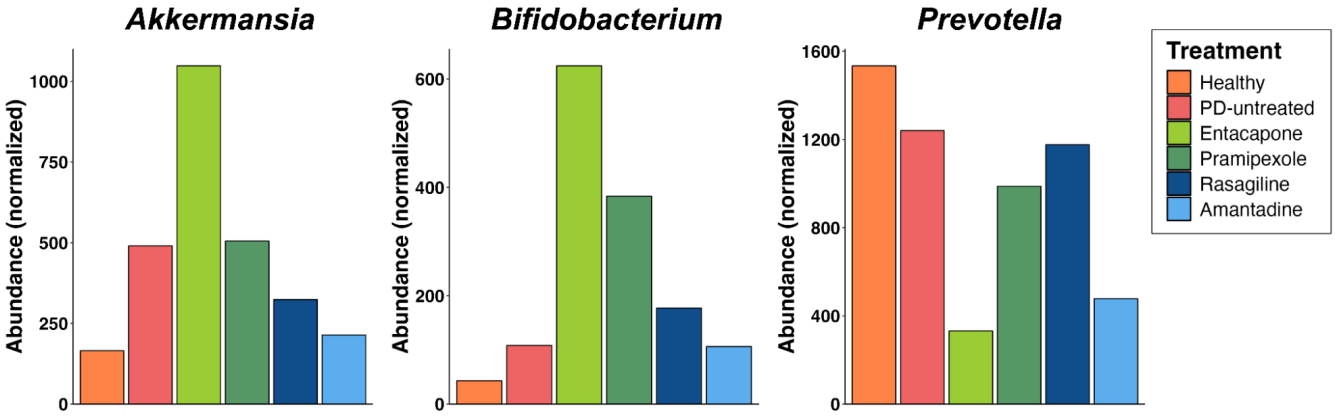

FIG. 4 *Bifidobacterium*, *Prevotella*, and *Akkermansia* increase or decrease in abundance in multiple dopaminergic therapeutic groups. Normalized abundance bar plots for *Bifidobacterium*, *Prevotella*, and *Akkermansia* across all conditions analyzed. Normalized abundances were calculated as the sum of raw ASV values for each genus in each treatment group divided by the number of samples in each treatment group.

both healthy and PD-untreated individuals may have contributed to the absence of significant differences. Additionally, core microbiome, indicator taxa, and differential abundance analyses revealed that dopaminergic therapeutics were associated with changes in specific taxa that made the gut microbiota more similar to that of healthy controls.

Core microbiome analysis suggested that select dopaminergic treatments shift the core microbiomes of PD patients to resemble those of healthy individuals. In the human colon, approximately 25 percent of anaerobic bacteria are species of the *Bacteroides* genus (29). This is consistent with our data that showed that *Bacteroides* was part of the core taxa of every comparison group and was unaffected by dopaminergic therapeutic usage. The two additional core taxa present in PD patients treated with pramipexole and amantadine both belonged to the *Faecalibacterium* genus. Weis *et al.* previously observed a relative decrease of specific bacterial taxa in PD patients, including *Faecalibacterium*, which are associated with beneficial effects such as reducing inflammation, supporting epithelial barrier integrity and promoting overall health (30). Previous research also suggests that the gut microbiome of PD patients is consistently associated with the depletion of health-associated SCFA-producing bacterial genera, one of them being *Faecalibacterium* (31). Furthermore, depletion of this genus has been linked to the development of other neuro-inflammatory and neurodegenerative disorders such as multiple sclerosis in part due to a loss of butyrate (31). Firmicutes are major producers of butyrate, and *Faecalibacterium* is one of the notable genera contributing to this (32). Butyrogenic microbes play a major role in maintaining adequate dopamine concentrations by protecting against dopaminergic neuronal loss, which is a symptom of PD (32). With *Faecalibacterium* being associated with the core taxa of amantadine and pramipexole treated PD patients, our analysis suggests that dopaminergic treatment may lead to the increase of core taxa associated with a healthy gut microbiome. Core taxa, such as *Faecalibacterium,* could synergize with PD treatments to provide PD patients with butyrate, which may prevent dopaminergic neuron loss. Further research is needed to explore this potential interaction.

We also performed indicator species analysis to identify unique taxa at the genus and family level. Indicator species analysis uses the relative abundance and relative frequency of occurrence of species in defined groups to identify a select few species that are the most characteristic of each group. Unique ASVs were present when comparing only healthy and PD-untreated groups (Table 1). However, we found that neither the PD-untreated nor healthy individual treatment groups had any remaining indicator taxa specifically associated with those groups when all conditions were compared (Table 1). The reduction in indicator taxa to zero in the healthy and PD-untreated groups, when including all conditions in the analysis, raises questions regarding their impact on the gut microbiome of PD patients. This suggests that treatment with dopaminergic therapeutics may affect the presence and frequency of the indicator taxa found in both healthy and PD-untreated individuals, therefore, these taxa are no longer indicators for these two groups when all conditions are compared. Notably, treatment of PD patients using entacapone and amantadine was associated with high amounts of uniquely upregulated ASVs, when comparing all conditions (Table 1).

Here, we want to highlight two key families and genera of interest that were identified as indicator taxa in the entacapone and amantadine drug groups. Notably, *Lachnospiraceae* was not a unique indicator for healthy or PD-untreated individuals when compared to the drug treatment groups. However, *Lachnospiraceae* ASVs were unique to entacapone and amantadine groups and were an indicator for the healthy control before therapeutic groups were considered. *Lachnospiraceae* are producers of SCFAs which have a beneficial effect on gastrointestinal function and are typically associated with a healthy microbiome (33). Research has shown a decrease of *Lachnospiraceae* is associated with an increase in PD severity and motor impairment (34). This was also observed through a significant correlation between UPDRS score and *Lachnospiraceae* abundance in our cohort (Supplemental Figure S3). Despite the modest effect size, the observed relationship aligns with existing literature, suggesting that even small changes in Lachnospiraceae abundance could have biological relevance in the context of PD. With a high indicator value, we can infer that *Lachnospiraceae* is both relatively frequent and abundant in amantadine and entacapone treatment compared to other conditions (Table 2, 3). This could suggest a shift towards a beneficial gut microbiome, as *Lachnospiraceae* is also associated with beneficial effects on gastrointestinal

muscle strength and function (31). Considering our findings and the role of *Lachnospiracae* in the gut microbiome, entacapone and amantadine may improve PD outcomes through their increased abundance and frequency in the gut.

TABLE. 2 Amantadine treatment is associated with a high number of unique ASVs compared to healthy and PD-untreated individuals. All ASVs unique to the amantadine treatment group after ISA. Comparisons include all drug treatments, healthy individuals, PD-untreated patients.

ASV	Indicator Value	p.value	Family
15c4a7a11ad1466019c3b85fb85559a3	0.43	0.01	Eggerthellaceae
178de850c1e748fe250e593be9ce617f	0.41	0.03	Gastranaerophilales
d4777d06d938847950232bccbb9673cb	0.38	0.04	Izemoplasmatales
1504a42c204ef50d648add61958e01ce	0.36	0.02	RF39
b63fee1b7681fedb989b36e4ff800786	0.42	0.01	Anaerovoracaceae
d33cce23c91790d0a5d8b630121b8057	0.41	0.03	Anaerovoracaceae
9f4be7f4678f30623c3e2ea0bec18a5a	0.36	0.04	Anaerovoracaceae
b4c2a2a806198d49c649b48770777bc9	0.41	0.03	Anaerovoracaceae
dedec428ce46a13d419a28fd01601fb3	0.37	0.05	Anaerovoracaceae
327e928d80b137060bd55c0515d64b2a	0.41	0.03	Erysipelotrichaceae
c6f1865ef6b1ff4c8585f4507ba9a661	0.38	0.04	Erysipelatoclostridiaceae
ae3fe60bea5c557da726d66d9596d349	0.43	0.02	Peptococcaceae
64d94620fe0f5db1cd211f24f6a6d659	0.47	0.02	Peptococcaceae
a5cc73dbd1b4afe438d0380b13f01857	0.41	0.03	Clostridia_vadinBB60_group
f48da462142571db71709c96e739cf72	0.51	0.03	Clostridia_vadinBB60_group
d214be7deecbf3f6f850bc1a73b4a1a7	0.36	0.04	Clostridia_UCG-014
f9a31f0e20a6345322d37d2a8b73e833	0.41	0.04	Enterobacteriaceae
7f33cd53f9f1ef07f7cec81651b793e1	0.41	0.01	Christensenellaceae
e829420ef3778bbbae247504afe2019d	0.41	0.02	Christensenellaceae
ddf803d692c52753f5e92e669fc042d2	0.39	0.04	Clostridiaceae
80739ec8ad6cbd28d375b0dc6bd09a8e	0.39	0.04	UCG-010
30dfdea9ad684383de9a7e1cca18b1cd	0.41	0.03	Rikenellaceae
144b3e2e574d758db8a9ea10961be6dc	0.41	0.02	Rikenellaceae
5da13fc04eb63d3792b4cc8f4e348034	0.39	0.05	Butyricicoccaceae
e6d2abe005b4a3116ff8432eda81d182	0.41	0.02	Oxalobacteraceae
09d37fca29ea27c0bd0664057c057144	0.41	0.02	Sutterellaceae
3f6165c36fd490c9f5a857f4417ceef6	0.41	0.04	Lachnospiraceae
bfab02a86c5187ed451db10d9b81b8d5	0.53	0.02	Lachnospiraceae
7284f7e75125bff0fe5a7a3817c34e97	0.41	0.02	Barnesiellaceae
ec5201550254279bac4f38913e984f18	0.45	0.04	Oscillospiraceae
738791239e12526b592035d66e55f33c	0.41	0.03	Oscillospiraceae
df02d44679528d8270cbfc09b344ebf3	0.41	0.02	Oscillospiraceae
e1f26b79b6ce326e13f3a61897f6fe23	0.41	0.03	Oscillospiraceae
608212bdf1db91f32b7165156a4ac897	0.41	0.02	Oscillospiraceae
a1d2121c62e6bf9b716c7192a8f97b01	0.37	0.05	Oscillospiraceae
e040af5d92076b918ba8bc5d6ec91d63	0.41	0.02	Ruminococcaceae
550b7d227c9d10ece4ab89914bb750f2	0.40	0.03	Ruminococcaceae
71be7d7d2b9ed4fe37319691c42f668d	0.41	0.03	Ruminococcaceae
e520e89c24875a66bda7489559c4ccc3	0.41	0.03	Ruminococcaceae
13e13995d803a26f01a0f96c7eebc5eb	0.41	0.04	Ruminococcaceae
baa6071bb0d26f15591c0c52228c7cdf	0.48	0.02	Ruminococcaceae
9ecef158fa74ffbafd4f2ddff29e06ab	0.41	0.04	Ruminococcaceae
2ef7fe71bae4918953b041ff085d418a	0.41	0.02	[Eubacterium]_coprostanoligenes_group
fb368bda4b32025d51637c2fc1a0ba9a	0.57	0.02	[Eubacterium]_coprostanoligenes_group
4b76b63ee8e2dcfb090fa20c339ccbd7	0.41	0.03	[Eubacterium]_coprostanoligenes_group
a538de005e4aebaed58f64e377c0edb3	0.41	0.02	Oscillospiraceae
b7f6717f0fa8107b00445288cfd7bedc	0.41	0.03	Barnesiellaceae
302453da7fdf3d0fc7c898a56d1902cc	0.63	0.01	Barnesiellaceae
ea367ce0729a4a95193663cae994b4da	0.45	0.03	Marinifilaceae
906fc76bd280eaa61cbf55df7f0b87e3	0.41	0.02	Tannerellaceae
4062729b5455e007de16e1a0c5ff89da	0.41	0.01	Tannerellaceae
5cb4975a299f98d45c785c1ee117aada	0.40	0.03	Bacteroidaceae
b0b77c9bb41edca8168951f829da58db	0.41	0.02	Bacteroidaceae

TABLE. 3 Entacapone treatment is associated with a high number of unique ASVs compared to healthy and PD-untreated individuals. All unique ASVs to the entacapone treatment group after ISA. Comparisons include all drug treatments, healthy individuals, PD-untreated patients.

ASV	Indicator Value	p.value	Family
281041dc55d7f999ab812c2025391ab6	0.73	0.01	Bifidobacteriaceae
386622a3b30e3e131e44a1db5038508d	0.43	0.01	Streptococcaceae
899a17421c7f1ed9d9f12dd5325d715b	0.42	0.01	Lactobacillaceae
b24aef84189c706af6b00adb76a70029	0.43	0.01	Enterococcaceae
4facd15f124cae4b6cd3e19a4756d2de	0.30	0.04	RF39
c6412b8612a901ee7542cf8698c2dcf0	0.45	0.02	Anaerovoracaceae
9ed8e6c3f69e2795a5171c930ba6ac48	0.30	0.05	Lachnospiraceae
7ef9038eed4579d894598c97df238dea	0.30	0.05	Peptococcaceae
b837ddf72a92f744bf1f979a63915b7d	0.30	0.04	Clostridia_vadinBB60_group
81872e80795f3e9242d26051e87b67e5	0.30	0.04	Clostridia_vadinBB60_group
1231748d56f761bfe9a3b94d63854054	0.46	0.04	Clostridia_UCG-014
510f2bd9396b8fd55432eadd617d1cbf	0.30	0.04	Clostridia_UCG-014
4c8288bfbd76958c0c094d87b97650f8	0.72	0.02	Enterobacteriaceae
ee0861406fb645b1a12b9fe687dc04d7	0.46	0.03	Desulfovibrionaceae
5deb0b73cfb92b0e68196ea947737f0f	0.30	0.05	Christensenellaceae
7aea13ac258d1cee20f352f86e061059	0.30	0.05	Christensenellaceae
cce0edb04a54ff09e005fbb67806129e	0.30	0.05	UCG-010
c6e99ec4d58c8811e6dbcb421f8b2d40	0.30	0.04	Monoglobaceae
f7d697a9254ea01210734d773714255d	0.30	0.05	Monoglobaceae
796e1a6f917bff78d10d1fe989858d0e	0.30	0.04	Rikenellaceae
25364150e51732580dd49be259653f01	0.30	0.04	Rikenellaceae
21a65e313b6782e8a9f41dbdc3d8525c	0.30	0.05	Rikenellaceae
fdf55d99ba80fcc19b28c1e0c50b74f4	0.30	0.05	Rikenellaceae
19af8760014dd2160d4cbe087d2ed976	0.56	0.01	Rikenellaceae
4c7c04878b5585a68cf5561aea24b743	0.39	0.02	Sutterellaceae
ed8381d1057ee57ffac38835c0e8626a	0.30	0.05	Lachnospiraceae
0360e1c32e3e3084c11ac4507b47ab73	0.30	0.04	UCG-010
78d87e17c83a3dec1aa0ae113a117362	0.30	0.05	Oscillospiraceae
9db6a6db0729637457520f7e24c38ba1	0.30	0.05	uncultured
76e1aee1145042620c020a2d5ab4b976	0.43	0.01	Ruminococcaceae
1656243ea96802940efa7db08f7a4410	0.42	0.01	Ruminococcaceae
0bee493e1e1aa441ce8d31e684943748	0.41	0.01	Ruminococcaceae
183fd78cda98f039e55d87a2aa384c08	0.43	0.01	Ruminococcaceae
4d6bf17e07206c175cb8a41d3d907bcc	0.30	0.04	Ruminococcaceae
5a8d812f6cb57762dda495a37f1ec620	0.30	0.04	Muribaculaceae
4ede98cba52e7e0f5c4cecf1cd165f23	0.30	0.05	Lachnospiraceae
9a4d329af518c3c86816cdee835222d7	0.30	0.04	Tannerellaceae
1f018a634006e50829465cbe12baebfe	0.30	0.04	Bacteroidaceae
cb645d9e303b0e2d761969abb610d210	0.40	0.03	Prevotellaceae
038605015f118d4b83f1dd1b9035b769	0.30	0.05	Lachnospiraceae
39460aec9268f9c5077fa5e3a6d5c1f4	0.30	0.04	Lachnospiraceae
bff6893546293740c858e5efd4499fa6	0.39	0.04	Lachnospiraceae
9f3c0ad19c03d8211539f3ad3fd31bd7	0.30	0.04	Lachnospiraceae

Interestingly, all dopaminergic therapeutic groups and healthy individuals had *Colidextribacter* as an indicator taxon. This genus was not represented in PD untreated patients, and low abundance and frequency, or absence of *Colidextribacter* as is often associated with PD (35). Both entacapone and amantadine, and other drug therapeutic use led to a similar relative abundance and frequency of *Colidextribacter* in healthy individuals (Supplemental Table S2). This reinforces the general trend observed with *Lachnospiraceae* that entacapone and amantadine are associated with the restoration or increased abundance of

beneficial genera and families. This shift in the composition of the gut microbiome may suggest a move towards a healthier state.

From the differential abundance analysis, we identified three genera of interest: *Prevotella, Bifidobacterium*, and *Akkermansia*. Elevation of *Bifidobacterium* and reduction of *Prevotella* was consistently observed in multiple treatment groups.

Previous research exploring the effects of the gut microbiome on PD development found that *Bifidobacterium* levels are higher in PD patients compared to healthy controls (36-38). These observations were recapitulated in our study (Figure 4). Although this association between PD patients and elevated abundance of *Bifidobacterium* may suggest that the genus is harmful or contributes to the development of PD, this has not been supported by any available scientific literature. Rather, investigations have reported that *Bifidobacterium* may have disease-alleviating or neuroprotective effects (39-41). Species of the *Bifidobacterium* genus are known to produce various beneficial vitamins as well as lactic acid, which play a role in regulating intestinal microbial homeostasis, inhibiting the growth of pathogenic bacteria, and modulating immune responses (42). One study found that *Bifidobacterium* counts are negatively correlated to worsening thought disorder, while another study using a PD mouse model listed multiple benefits conferred by *Bifidobacterium* including protection of dopaminergic neurons, suppression of neuroinflammation, and alleviation of oxidative stress (39, 42). A third study found that *Bifidobacterium breve Bif11* supplementation is effective in attenuating the cognitive and motor changes in a rat PD model through its role in reducing oxidative stress and intestinal epithelial permeability (40). While *Bifidobacterium* is generally considered beneficial, its higher abundance in PD patients compared to healthy controls might reflect a compensatory mechanism or reflect the altered gut environment associated with the disease and its treatments. This could explain why, despite its potential benefits, *Bifidobacterium* levels are not as elevated in healthy individuals who do not require such protective effects. Taking into account the past literature and the results of this study, the elevation of *Bifidobacterium* that is associated with certain dopaminergic therapeutics may contribute to the alleviation of PD as a potential adaptive response.

However, our study also found that each dopaminergic therapeutic investigated was associated with a reduction in the abundance of *Prevotella* (Figures 3, 4). Since this genus is largely regarded as beneficial, these observations indicate that while the dopaminergic therapeutics are effective in elevating the abundance of certain beneficial genera or reducing certain harmful ones, they may also induce negative downstream effects resulting in the reduction of beneficial genera (36, 38, 43). *Prevotella*, a genus within the *Prevotellaceae* family, is widely associated with PD; however, findings concerning this genus have varied across studies (36). A potential reason for these discrepancies may be that changes in abundance depend on the species type, and more in-depth investigations must be done with higher taxonomic resolution to resolve these effects at the species level and suggest more specific associations. Nonetheless, the majority of studies examining *Prevotella* indicate that it is present at lower levels in PD patients compared to healthy controls (36, 38, 43). Additionally, studies report that *Prevotella* provides benefits to its host by aiding in the digestion of high-fiber diets and secreting hydrogen sulfide which has been shown to exert a protective effect on dopaminergic neurons in multiple mouse and rat PD models (36, 44). Reduction of *Prevotella* is associated with decreased arginine and glutamate metabolism as well as decreased mucin synthesis, which contributes to the development of PD (36, 44). Overall, while dopaminergic therapeutics may effectively alter the gut microbiome by reducing harmful bacteria, their potential to inadvertently diminish beneficial genera like *Prevotella* highlights the need for careful consideration of their broader impact on gut health in PD patients.

The last genus of interest that was found in our study to be significantly elevated across multiple differential abundance comparisons while also exhibiting the greatest increase in at least one comparison was *Akkermansia* (Figure 3). In our study, while *Akkermansia* abundance was significantly elevated by entacapone, rasagiline, and amantadine relative to the healthy control, they were also reduced by rasagiline and amantadine relative to the PD-untreated group. *Akkermansia* is a genus of the *Akkermansiaceae* family that has consistently been found to be enriched in PD patients compared to healthy controls and has been classified as a harmful genus that contributes to the development and progression of PD (45-47). Under

deprivation of dietary fiber, *Akkermansia* contributes to the degradation of the gut's mucus layer, which normally serves as the first protective layer of the epithelium; degradation of this layer promotes intestinal permeability, inflammation, and oxidative stress (46-48). Therefore, rasagiline and amantadine appear to reduce the harmful, elevated levels of *Akkermansia* in PD patients, bringing them closer to the intermediate levels seen in healthy controls..

Taking these results together, while the use of dopaminergic therapeutics was associated with significant elevations and reductions in the abundance of multiple ASVs, the net effect of each drug is unclear. Although entacapone is associated with the greatest increase of beneficial bacteria like *Bifidobacterium*, it is also simultaneously associated with the greatest increase of harmful bacteria like *Akkermansia*. Conversely, while amantadine yields the smallest change in *Bifidobacterium* abundance, it is the most effective in modifying *Akkermansia* levels to resemble that of healthy individuals. Therefore, these findings partially support our hypothesis, as some dopaminergic therapeutics are associated with a shift in the abundance of select genera from levels found in PD-untreated patients to levels more closely resembling those of healthy individuals. However, not all results matched the expected findings, and further investigations should be undertaken to address these inconsistencies.

Limitations A prominent limitation of this study was the sample size of each condition. Certain PD patients were on multiple dopaminergic treatments, and those samples were removed to avoid confounding factors such as drug interactions. Furthermore, the proportion of samples in each condition analyzed was different by magnitudes of up to 20-fold (Supplemental Table 1). Rarefaction further reduced the number of samples per condition. After data processing, amantadine contained only five samples for analysis (Supplemental Table 1). The relatively low and varying sample numbers may not be representative of actual changes in the gut microbiomes. Significant conclusions may also have been missed in our analyses due to low and varying sample numbers.

Several confounding variables were not accounted for in our study. One important confounding variable was the use and dosage of levodopa which varied across PD-untreated and PD patients treated with the dopaminergic therapeutics we analyzed. To maintain a higher number of samples for statistical significance, we did not control for levodopa use. Studies have demonstrated the association of levodopa with changes in the gut microbiome (49). Therefore, observed gut microbiota changes in our study may have been attributed to levodopa use and dosage. Moreover, the dopaminergic therapeutics analyzed in our study did not state dosage and duration of use in the original metadata; it was not stated whether therapeutics were administered close to the date of sample collection or whether each patient had a higher or lower dose. As a result, due to the lack of clarity of these critical factors, conclusions drawn may be due to relationships between drug use, dosage, duration of use, and sample collection, not all of which are controlled for.

Furthermore, the patient cohort presents more confounding factors. The disproportionate and greater number of male patients compared to female patients was not controlled to maintain significant sample sizes. As a result, conclusions drawn from our results may be more representative of male PD compared to female PD. Distinct representation is important, as women tend to experience more rapid progression, lower survival rates, and more clinical manifestations in PD, despite having half the risk of developing PD compared to males (50). Since PD is associated with an altered gut microbiome, altered PD manifestations due to sex may also affect gut microbiota composition. Healthy patients were often spouses of the PD patients. This introduces a confounding factor of familial microbiota similarities in healthy spouses, as they are likely to share a more similar microbiota to PD patients than non-spouses. This may reduce the reliability of our healthy controls and lead to less significant differences in our results. Since a majority of PD-patients were male, many spouses were female and a large proportion of the healthy control group consisted of females. Our healthy controls may be more representative of healthy females, therefore, our results may be more representative of PD microbiomes compared to healthy females than healthy males. Additionally, the samples drawn from the original dataset only contained patients from British Columbia (8). Our results, therefore, may be more indicative of PD in British Columbia than PD globally.

Very few of the ASVs that were identified in this study could be classified at the species level. For example, our analyses revealed a significant and consistent reduction in the

abundance of the *Prevotella* genus across multiple drug-treated groups compared to both the PD-untreated and healthy control groups. However, previous research has demonstrated that different species of *Prevotella* may exhibit distinct protective or negative effects on PD development and may decrease or increase in abundance differently as PD progresses (37). Therefore, our study findings are primarily confined to observations at the genus level. Due to this, we were unable to make nuanced or specific comparisons with previous literature, limiting the depth of our conclusions.

Lastly, these findings represent a retrospective analysis based on a cross-sectional cohort. Due to the observational nature of the original study (8), the conclusions drawn from our results are all correlational, not causal.

Conclusions Our study aimed to investigate the impact of four dopaminergic therapeutics (entacapone, pramipexole, rasagiline, and amantadine) on the gut microbiota of PD patients. With the strong association between PD and changes in the gut microbiota, we hypothesized that dopaminergic therapeutics to treat the neurological health of PD patients may also alter the gut microbiota to resemble that of healthy individuals. Diversity analyses revealed that dopaminergic therapeutics were not associated with a significant alteration in the gut microbiota diversity of PD patients. The core microbiome of PD patients treated with pramipexole and amantadine contained taxa shared with healthy patients, suggesting a "rescue" effect of these two drugs. Additionally, entacapone and amantadine usage was associated with predictive indicator taxa that were related to positive health benefits. PD patients treated with dopaminergic therapeutics also presented a differential abundance of taxa. Notably, compared to untreated PD patients, the use of certain dopaminergic therapeutics displayed increased beneficial bacteria like Bifidobacterium, decreased harmful bacteria like *Akkermansia*, and decreased *Prevotella* which are generally considered beneficial but may exert varying species-specific effects that require higher taxonomic resolution for analysis. Therefore, our results support our hypothesis to an extent, as dopaminergic therapeutic usage was often associated with beneficial changes to the PD gut microbiota. However, not all results matched the expected findings, and further research is necessary to resolve inconsistencies.

Future Directions This study demonstrated that PD drugs potentially affect not only the neurological aspects of PD but also the patient's gut health. To further enhance the findings presented in this study and address the limitations, it would be ideal to perform a study where PD patients and healthy individuals were more balanced for sex, gender, therapeutic usage, and geography to offer more representative results. A prospective study would enable longitudinal analyses, with gut microbiome samples being collected from each patient at multiple time points. This would allow researchers to study the temporal dynamics of the gut microbiome in PD, including how the composition changes as PD progresses and disease severity increases. Furthermore, future studies could integrate more patient data to draw statistically significant conclusions from a larger and more balanced cohort throughout various conditions analyzed. Future studies may also control for levodopa effects and other confounders, and include information on the duration and dosage of other dopaminergic therapeutics.

Multi-omic analyses could be performed on gut microbiome samples of PD patients. This expanded methodology could involve analyzing the transcriptome, proteome, and metabolome of bacterial samples, thus allowing for a more comprehensive understanding of the biological mechanisms underlying PD as well as how the microbiome functions and interacts with the host at a molecular level. Additional analyses that could be performed in such a study could include functional pathway analyses to identify enriched or dysregulated pathways, and metabolomic profiling to gain insights into the metabolites that are altered or can potentially serve as PD biomarkers for disease diagnosis.

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CONTRIBUTIONS

JG carried out QIIME2 pipeline, formatted all figures, created Supplemental Table 1 - patient samples per condition, wrote the abstract, all methods, conclusion, limitations, future directions, and acknowledgments. **DT** wrote the introduction, performed indicator species analysis in R (Table 1 - all unique ASVs; Supplemental Table 2 - notable ASVs), wrote corresponding results and discussion, and formatted all references. **CY** performed differential abundance analysis in R (Figure 3 - DESeq bar plot; Figure 4 - genus abundance plot; Supplemental Figure 2 - volcano plot, Supplemental Table 3 - DESeq2 summary), wrote corresponding results and discussion sections, wrote discussion summary. **AZa** created the phyloseq object and performed data processing in R, performed diversity analysis in R (Figure 1 - alpha box plot and beta PCoA plot), created heat maps for core microbiome analysis (Supplemental Figure 1), performed linear regression analysis (Supplemental Figure 3), wrote corresponding methods, results, discussion sections. **AZh** performed core microbiome analysis in R (Figure 2 - Venn diagram) and wrote the corresponding results and discussion, wrote the introduction.
All authors contributed to refining and editing the final draft of the manuscript.

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