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The effects of coffee consumption and antibiotic use on gut microbial community structure of Parkinson's disease patients

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SUMMARY Parkinson's disease (PD) is a complex brain disorder involving a multitude of contributing factors ranging from environmental to genetic. Interestingly, it has been found that the gut microbial community is altered in PD patients when compared to that of non-PD individuals. However, the factors contributing to altered gut microbiota remain largely unexplored. As such, the first objective of our study was to determine whether a difference exists between the gut microbial community structure of PD patients and controls in the current data set. Subsequently, we aimed to analyze the relationship between the consumption of coffee and antibiotics and the observed alteration in the gut microbial communities in PD patients. We confirmed, in the current data set, that PD patients possess significantly different microbial community structures when compared to controls. While some previous studies have suggested antibiotics to be a potential trigger for alterations in gut microbiota characteristic of PD, we found that antibiotic consumption does not induce significant alterations to the gut microbial community structure. However, although overall microbial community structure is not significantly affected, it was found that *Bifidobacterium* abundance is altered by antibiotic consumption. Additionally, coffee consumption, regardless of frequency, did not result in significant differences in gut microbial composition. Therefore, research examining other factors should be pursued as an understanding of the cause of altered gut microbial community structure in PD patients could allow for the development of diagnostic treatment methods which could be critical in combating PD.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder involving the loss of dopaminergic neurons in the substantia nigra and is marked by the deposition of alpha-synuclein protein aggregates named Lewy bodies in the brain (1, 2). Importantly, the deposited Lewy bodies can lead to neuroinflammation in the brain resulting in the death of dopaminergic neurons (1). The death of these neurons decreases dopamine to the striatum portion of the brain (3). This results in the loss of neural connections, leading to disruption in voluntary movements (3). In addition to causing motor dysfunctions, PD can also lead to a range of non-motor symptoms, some of which have been identified to be gut-associated such as constipation, slow colonic transit time, and small intestinal bacterial overgrowth (4). In general, it is known that the microbiota composition of PD patients differs significantly from that of control individuals; specifically, less anti-inflammatory butyrate-producing bacteria such as the genera *Faecalibacterium, Coprococcus, Roseburia, Blautia,* and *Prevotellaceae*, are present in the microbial communities of PD patients when compared to controls (1, 4, 5). Additionally, research has shown that PD patients have an increase in *Akkermansia*, *Bifidobacterium* and *Enterobacteriaceae* (1, 4, 5).

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Among the factors that influence gut microbial community structure, the effects of antibiotics have been extensively studied (6, 7, 8, 9, 10). However, there remains much debate regarding the role antibiotics may play. On the one hand, previous studies have identified antibiotics as a potential trigger of dysbiosis in Parkinson's disease (6) and caution the public about their potential role in elevating the risk of PD (7). On the other hand, studies have identified the potential of antibiotics as an anti-inflammatory treatment for PD patients (8, 9). Specifically, a study shows that when PD patients are treated with rifaximin, an antibiotic commonly used to treat diarrhea, a decline in bacterial overgrowth is observed which is followed by improvements in gut symptoms and motor function (10).

In addition to antibiotics, the consumption of coffee has also been identified to possess an impact on gut-associated microbial organisms (11). Besides being known to help improve alertness and productivity with the help of its main component, caffeine, coffee also comprises other active ingredients such as minerals, phenolic polymers, polysaccharides, and chlorogenic acid (12). Some of these components have been shown to possess special properties such as antibacterial or anti-diabetic activity that may benefit health, while other components may pose certain health risks such as high blood pressure and diabetes if consumed in high amounts (12). Specifically, chlorogenic acid has been linked to increase the diversity of the gut microbiome in humans (11). Regarding gut health, coffee has also been demonstrated by various studies to help improve bowel movement. One study shows that the postoperative intake of coffee significantly reduces the time to first bowel movement, first flatus, and solid diet tolerance (13). Additionally, coffee has been associated with preventing neurodegenerative diseases such as PD and Alzheimer's disease (12). One study specifically shows that different combinations of caffeine and Eicosanoyl-5-hydroxytryptamide — a naturally occurring fatty acid in coffee — improves motor symptoms in a mouse model of PD by reducing alpha-synuclein clumping in the brain as well as less brain inflammation (14).

Taking into consideration the results of previous studies, we aimed to further explore the variables associated with the altered gut microbiota in PD patients by 1) confirming the differences in gut microbiota composition between PD patients and controls and examining how microbial communities differ between the two groups, 2) testing the effects of antibiotic consumption on gut microbial community structure and 3) exploring the effects of coffee consumption on the gut microbiome. To do so, we used a dataset that was compiled by Cirstea *et al*. (2020) that included fecal samples collected from PD patients and controls, along with their associated coffee intake and antibiotic usage. As gut symptoms are early signs of PD, an understanding of the gut composition of PD patients and the factors that contribute to observed differences in gut microbial community structure could potentially provide new approaches that allow for the detection and treatment of the disease.

METHODS AND MATERIALS

Metadata and quality control in QIIME2 pipeline. The dataset used in our study was obtained from Cirstea *et al*. (2020) which consisted of 300 human participants (197 PD patients and 103 controls) aged 40 to 85. One fecal sample was supplied in a sample collection tube by each participant after an initial meeting with the research team. While many data categories including dietary and medical information were collected in the original metadata, we specifically focused on data regarding antibiotics and coffee consumption. According to Cirstea *et al*. (2020), the DNA sequences were first extracted. Then, the V4 region of the 16S rRNA gene was amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVHHHTWTCTAAT-3') (4). Next, Illumina sequencing was performed on these amplified sequences to get the raw sequencing data (4). Further information regarding sample collection and gene sequencing can be found in the paper by Cirstea *et al*. (2020) which is published on public domains with the accession number: PRJNA594156 (4).

The demultiplexed V4 16S rRNA sequences were provided by the authors of Cirstea *et al*. (2020) and imported into QIIME2 (v2020.8) (15). Subsequently, a truncation length of 249 was applied. No other parameters were specified. The sequences were then denoised using Divisive Amplicon Denoising Algorithm 2 (DADA2) for the detection of amplicon

sequence variants (ASVs) for sequence quality control (16). A features table was subsequently generated. The steps involved with DADA2 can be found in "script1.sh".

Metadata filtering and grouping. Our paper focused on the difference between the gut microbiota of PD and non-PD patients as well as the effects of antibiotic usage and coffee consumption. No metadata category (removal of samples) was done when exploring the difference between gut microbiota of PD patients and controls. However, when examining the effects of antibiotic usage and coffee consumption, data was first grouped into two categories: PD patients (n = 186) and controls (n = 99). Those two categories were kept separate for subsequent analysis to control for the potential of the disease condition to act as a confounding variable. Data on antibiotic usage within the last five years was provided as the quantified number of doses ranging from 0 to 20, yet there was a lack of information regarding the frequency and specific timeframe of usage. As such, antibiotic usage within the last five years was grouped into either "Yes", "No", or "null" with 0 categorized as "No" and number of doses>0 categorized as "Yes". The "null" data type was shown if no data was collected on the patient. We decided to remove samples that contained "null" data types from downstream analysis. Out of 143 PD patients, 55 were "No" samples and 88 were "Yes" samples that went through the analysis. Out of the 79 controls, 25 were "No" samples and 52 were "Yes" samples. Similarly, PD patients were first separated from controls and further stratified based on coffee consumption frequency. The frequency of coffee consumption was grouped into five categories in the original metadata, namely "infrequent", "daily", "multiple cups daily", "no consumption", and "null," and these groupings were kept for our study. The "null" group consisted of individuals who did not answer the coffee consumption frequency survey question. The "null" in both PD and controls were removed prior to analysis. As such, our analysis only looked at PD patients and controls categorized as being "infrequent", "daily", "multiple cups daily", and "no" consumers of coffee. Out of the PD patients, 53 were considered "daily" consumers, 33 were considered "daily multiple cups" consumers, 34 were considered "infrequent" consumers, and 50 were considered "No" consumers. Out of the controls, 24 were considered "daily" consumers, 28 were considered "daily multiple cups" consumers, 10 were considered "infrequent" consumers, and 24 were considered "No" consumers. The separation of PD patients and controls, removal of "null" data categories, and stratification of samples based on antibiotic usage and coffee consumption frequency were performed in QIIME2 (14) and R (v4.0.5) (17). The stratification steps in QIIME2 are outlined in "script1.sh" while the steps done in R can be found in "script2.r", "script3.r", and "script4.r".

Data processing using QIIME2 pipeline. An alpha rarefaction curve (Fig. S1**)** was generated to identify an optimal sampling depth; a sampling depth of 6000 was chosen. At this sampling depth, 285 samples were retained while 15 were removed. . Importantly, the sampling depth chosen for this paper is different from that of Cirstea *et al*. (2020) who chose a sampling depth 3797. Subsequently, the Greengenes 13_8 99% (18, 19) identity reference tree backbone was used to create phylogenetic trees to run alpha and beta diversity analyses. This allowed for the amplicon sequence variants (ASVs) in the data to be compared against the reference to investigate sequence variability/relatedness. Steps in the QIIME2 workflow can be found in "script1.sh".

Alpha diversity analysis. QIIME2 was used to evaluate alpha diversity metrics including Faith's phylogenetic distance and Pielou's evenness. Statistical significance between groups analyzed with alpha diversity analyses was evaluated using Kruskal-Wallis pairwise testing and was determined using a significance level of $p < 0.05$. Details regarding these steps are included in "script1.sh" in the supplemental text files document.

Beta diversity analysis. QIIME2 was used to evaluate beta diversity metrics including Jaccard index, Bray-Curtis dissimilarity, weighted UniFrac distance, and unweighted UniFrac distance. Statistical significance was evaluated using permutational analysis of variance (PERMANOVA) and was determined using a significance level of $p < 0.05$. Details regarding these steps are included in "script1.sh".

Taxonomic classification using QIIME2. To classify ASVs taxonomically, the q2-feature-classifier plugin was used. The pre-trained naive Bayes machine-learning classifier was used to differentiate taxa based on a 250bp portion of the V4 hypervariable region within the Greengenes 13_8 99% reference sequence (18, 19) which was generated from the same primers used by Cirstea *et al*. (2020), 515F and 806R. Then information from the taxonomy classifications was used for subsequent analyses in R (17). All steps in this process are outlined in "script1.r".

Differential abundance analysis in R. The software R (17) was used to create differential abundance plots. Specifically, all analyses required *tidyverse* (20)*, vegan* (21)*, ape* (22)*, phyloseq* (23)*,* and *DESeq2* (24) packages. Filtering the data to a sequencing depth of 6000 was performed in R (16). There was no filtering of the raw metadata when comparing gut microbial communities of PD to controls; however, when examining the effects of coffee and antibiotic consumption, PD and control participant data were separated to reduce the effects of confounding variables. After filtering the data, removal of ASVs that had < 0.1% relative abundance was done to observe highly abundant taxa only. At the genus level, only the organisms that had significant differential abundance (FDR-corrected $P < 0.05$) were presented on the graph. All steps in the analysis are outlined in the R scripts ("script2.r", "script3.r", and "script4.r").

Relative abundance analysis in R. R (17) was also used to create relative abundance plots. First, relative abundance of genera were calculated. Removal of low abundant samples was done by only including genera that had > 0.1% relative abundance. Then, taking all the significantly differentially abundant bacteria identified by *DESeq2* (24) a relative abundance graph was generated for each taxon using the ggplot2() function in the *tidyverse* (20) package. All other steps are outlined in the R scripts ("script2.r", "script3.r", and "script4.r").

RESULTS

PD patients had a significantly different gut microbial community structure compared to controls. Alpha diversity metrics (Pielou's evenness and Faith's phylogenetic diversity) were first evaluated and analyzed. It was found that the abundance and evenness of gut microbial communities of PD patients and controls were not significantly different for both Pielou's evenness (Kruskal-Wallis p = 0.8479) and Faith's phylogenetic diversity (Kruskal-Wallis p = 0.3579) (Fig. S2). However, while alpha diversity results indicated that samples of the two conditions possess similar within sample structure, the evaluation and analysis of beta diversity metrics (Jaccard, Bray-Curtis, and weighted/unweighted UniFrac distance) showed that the two groups are statistically different from each other (Fig. 1). Specifically, there was a significant difference in terms of phylogenetic distance, as indicated by unweighted UniFrac distance (p = 0.001), and abundance, as indicated by Bray-Curtis dissimilarity (p = 0.001) (Fig. 1). Following the identification of significant differences in microbial community structure, we investigated which genera were contributing to these differences. Through differential abundance analysis, it was found that at the genus level, PD patients possess a significantly higher abundance of *Bifidobacterium, Akkermansia, Collinsella,* and *Bilophila* while possessing a significantly lower abundance of *Faecalibacterium* and *Roseburia* when compared to the controls (Fig. 2). To further visualize the distribution of the differential abundance data, relative abundance analysis was carried out; the spread of the relative abundance of each bacterium can be observed in the relative abundance graphs (Fig. S3) and these results further support the conclusion drawn from the differential abundance analysis.

Antibiotic consumption did not significantly alter gut microbial community structure, but *Bifidobacterium* abundance varied with antibiotic consumption. The effect of antibiotics was analyzed first by alpha diversity analysis to determine whether antibiotics disrupted the normal balance of the microbial community. Faith's phylogenetic distance and Pielou's evenness were evaluated; it was found that antibiotic consumption did not have a significant impact on the microbial communities of samples from either PD patients or controls, suggesting that antibiotic consumption does not alter the within-group evenness and

abundance (Fig. S4, S5). Subsequently, beta diversity metrics (Jaccard, Bray-Curtis, and weighted/unweighted UniFrac distance) were evaluated to determine the potential impacts of antibiotics on the overall microbial community structure. There was no statistical significance observed (PERMANOVA $p > 0.05$) when evaluating the difference between the samples of Yes-Antibiotics VS No-Antibiotics under either PD or control conditions using the previously mentioned beta diversity metrics (Fig. 3, S6). These results provided no evidence for the association between antibiotic consumption and changes in gut microbial community structure in the current dataset. In addition to the above-mentioned diversity metrics, differential abundance analysis was evaluated to further compare the microbiota under the Yes VS No conditions. When comparing control participants that consumed or did not consume antibiotics in the last five years, there were no differentially abundant organisms; however, PD patients were identified to possess one differentially abundant bacterium, namely *Bifidobacterium* ($p = 0.0175$, log2 fold change = -1.757763). The negative Log2 Fold-Change is indicative of lower abundance in the antibiotics consumption group. It was found that *Bifidobacterium* is significantly more abundant in the PD patients that had not taken antibiotics in the last five years when compared to those that had.

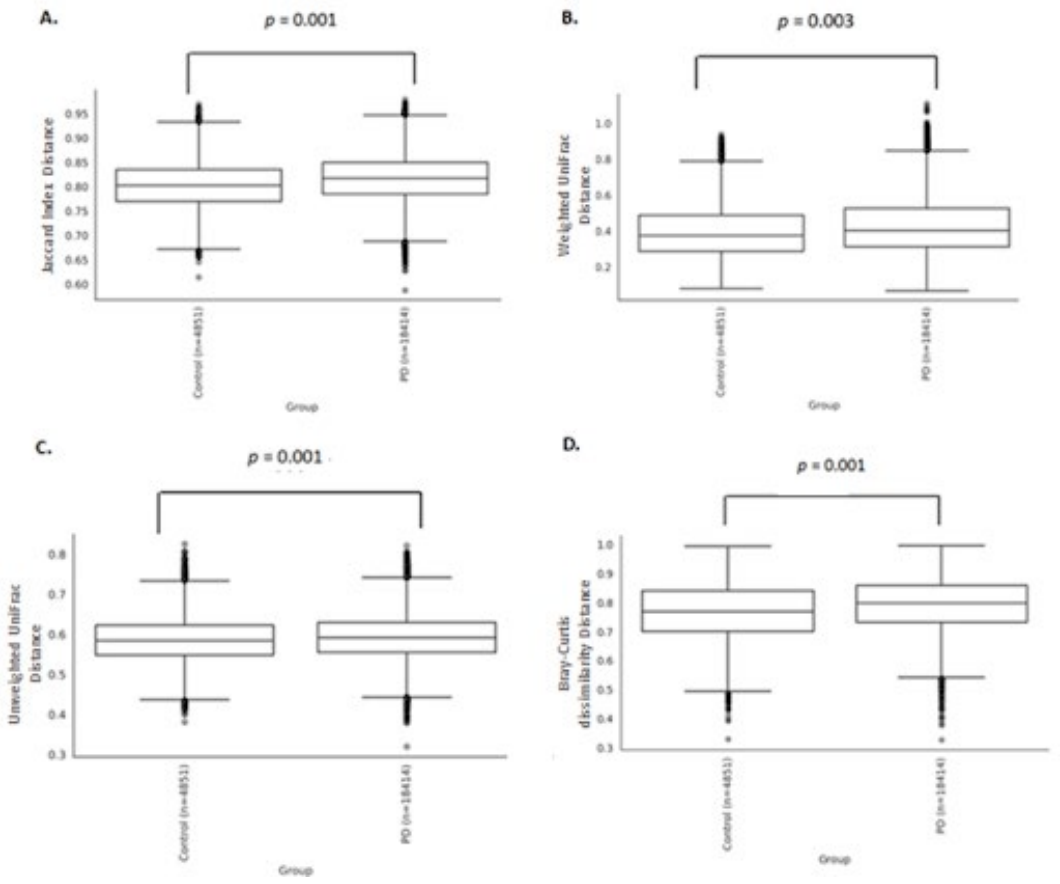
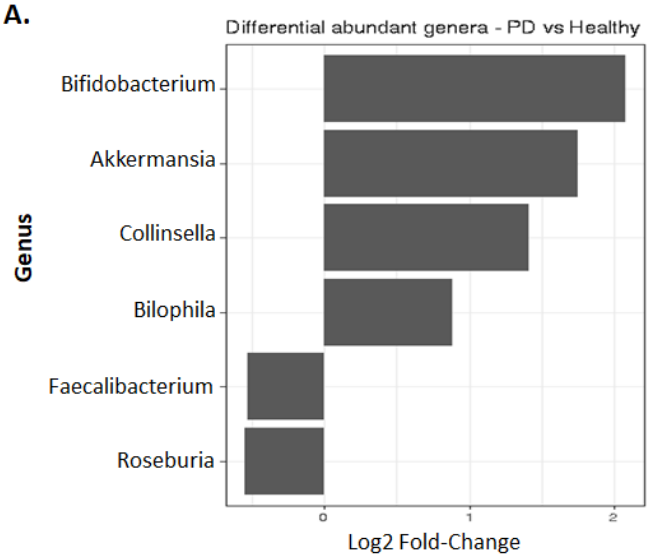


FIG. 1 Boxplots showing significant differences between gut microbial community structure in controls and PD patients using beta diversity analyses. Samples are grouped based on disease condition. Samples from the two groups display statistically significant differences, determined using PERMANOVA ($p < 0.05$), when evaluated using **(A)** Jaccard Index, **(B)** weighted UniFrac distance, **(C)** unweighted UniFrac distance and **(D)** Bray-Curtis dissimilarity. *n* is the number of permutations calculated by PERMANOVA.

Coffee consumption of varying frequency did not have significant impacts on gut microbial community structure. The effect of coffee consumption was then analyzed using alpha diversity analysis. Coffee consumption was categorized into four categories based on frequency: “infrequent”, “daily”, “multiple cups daily” and “no consumption”. It was found that coffee consumption, regardless of frequency, possesses no statistical significance on within-sample evenness and relatedness of PD patients (Fig. S7, Table S1) or controls (Fig. S8, Table S2) (PERMANOVA; $p > 0.05$) in Pielou’s evenness and Faith’s Phylogenetic Distance, respectively. Beta diversity metrics including Jaccard, Bray-Curtis, and weighted/unweighted UniFrac Distance were subsequently evaluated. Statistical analyses of the evaluated beta diversity metrics indicated no significant difference between the microbial community structure of individuals (PD and controls) who consumed coffee at varying

frequencies in terms of between-group abundance or phylogenetic distance (Fig. 4, S9). The results suggested that there is no evidence that coffee consumption has an impact on gut microbial community structure in this dataset.

A.



B.

Genus	Padj value
Bifidobacterium	1.197817e-06
Akkermansia	2.397737e-05
Collinsella	8.842204e-06
Bilophila	3.423256e-02
Faecalibacterium	2.578514e-03
Roseburia	2.574151e-03

FIG. 2 Significant difference in abundance between gut microbial community structure of controls and PD patients. (A) Differential analysis examining the difference in abundance of bacterial genera between PD patients and controls. Positive Log2 Fold-change indicates higher abundance in PD patients and negative Log2 fold-change indicates higher abundance in controls. **(B)** The Wald test was used to determine statistical significance; FDR adjusted p-values display the padj value column ($p < 0.05$).

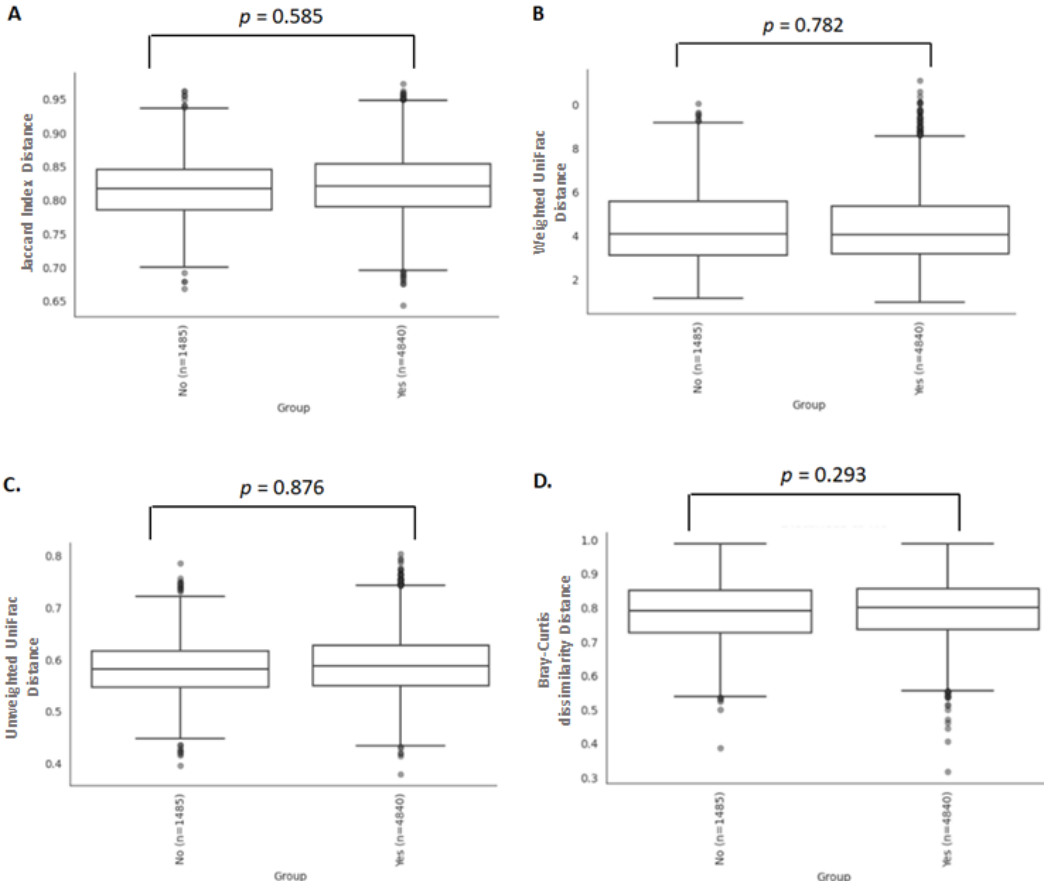


FIG. 3 Boxplots showing that antibiotic consumption does not have a significant impact on gut microbial community structure of PD patients using beta diversity analyses. Samples are grouped based on antibiotic usage within the last 5 years. Samples from the two groups (Yes vs. No) display no statistically significant differences, determined using PERMANOVA ($p > 0.05$), when evaluated using **(A)** Jaccard Index, **(B)** weighted UniFrac distance, **(C)** unweighted UniFrac distance and **(D)** Bray-Curtis dissimilarity. *n* is the number of permutations calculated by PERMANOVA.

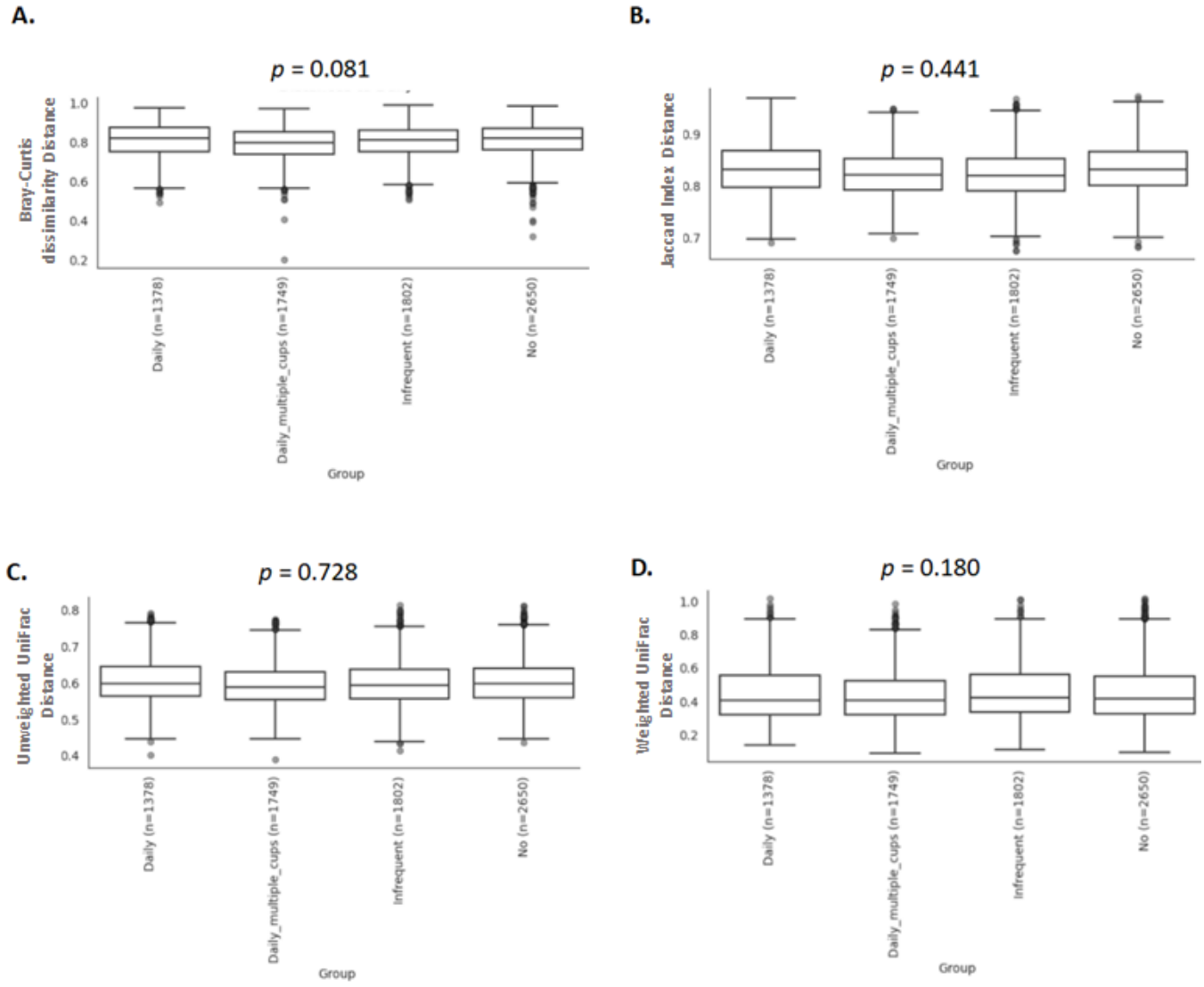


FIG. 4 Boxplots showing that coffee consumption does not have a significant impact on gut microbial community structure of PD patients using beta diversity analyses. Samples are grouped based on coffee consumption frequency (Daily, Daily multiple cups, Infrequent, No). Samples from the four groups display no statistically significant differences, determined using PERMANOVA ($p > 0.05$), when evaluated using **(A)** Bray-Curtis dissimilarity, **(B)** Jaccard Index, **(C)** unweighted UniFrac distance and **(D)** weighted UniFrac distance. *n* is the number of permutations calculated by PERMANOVA.

DISCUSSION

Comparing the gut microbiome compositions of PD patients and controls . While Cirstea *et al*. (2020) has determined that significant differences do exist between the samples of PD patients and controls in the current dataset, we aimed to confirm the observed differences by applying a different set of parameters and stratification techniques than Cirstea *et al*. (2020). To decide the truncation length, we first analyzed the quality distribution scores. While the quality score distributions were evenly distributed throughout the sequence and were all above the quality score of 20, it was observed that some sequences only possessed 249 nucleotides while most others possessed 251 nucleotides. It was determined that retaining all samples outweighed the loss of information from the removal of 2 nucleotides and, as such, a truncation length of 249 was applied as opposed to the truncation length of 250 as determined by Cirstea *et al*. (2020). Additionally, a sampling depth of 6000 was applied as opposed to a depth of 3797 applied by Cirstea *et al*. (2020). We observed that at a sampling depth of 6000, the rarefaction curve (Fig. S1) was plateaued, indicating that such sampling

depth would provide a good representation of the microbial community which includes both abundant and rare species. While Cirstea *et al*. (2020) retained 30.88% of ASVs, we were able to retain 46.51% of ASVs using our sampling depth. Although 15 samples were excluded as a result of applying a sampling depth of 6000 as compared to 1 sample being lost when applying the sampling depth determined by Cirstea *et al*. (2020), we believe that the higher number of retained ASVs from a sampling depth of 6000 would provide a more accurate analysis while still maintaining sufficient numbers of samples (n=285) to provide statistical power. Using the public metadata provided by Cirstea *et al*. (2020), we found that, in accordance with Cirstea *et al*. (2020), there was no significant difference in alpha diversity metrics between PD patients and controls; however, significant differences were observed in all the evaluated beta diversity metrics (Jaccard, Bray-Curtis, unweighted UniFrac, and weighted UniFrac) when comparing PD patients to controls. These results supported our hypothesis and indicated that PD patients possess significantly different microbiota as compared to controls. Additionally, in agreement with Cirstea *et al*. (2020), we identified that PD patients had an increase in the abundance of the genera *Bifidobacterium, Collinsella, Bilophila,* and *Akkermansia* and a decrease in the abundance of *Roseburia* and *Faecalibacterium*. However, in addition to the previously mentioned genera, Cirstea *et al*. (2020) found the bacterial family *Christensenellaceae* to be significantly differentially abundant; while we did not identify this family as being significantly differentially abundant. The difference between the results of our paper and that of Cirstea *et al*. (2020) is likely due to differences in the filtration methods and parameters; specifically, these differences are likely due to the differing sample sizes as a result of the application of differences in sampling depth.

In terms of the broader literature, there have been many studies that have examined the difference in the gut microbiome of PD patients and controls (1, 4, 5, 25, 26, 27, 28, 29, 30). In some of these studies, alpha diversity was not significantly different between PD and controls (25, 26), which agrees with our results. However, one study by Romano *et al*. shows differing results and found that alpha diversity is significantly different between PD patients and controls (1). In this paper, a meta-analysis was performed which found that PD patients had higher overall species richness compared to controls; however, they did mention that this significance seemed to be from only three out of the ten datasets (1). In terms of beta diversity, there is an overwhelming amount of evidence that suggests the gut microbiomes of PD patients and controls are different (1, 4, 25, 26). These findings are further supported by our results. To further determine how the microbiota differ between PD and controls, we carried out differential abundance analysis which identified bacterial genera that were differentially abundant in PD compared to control groups.

We identified *Bifidobacterium, Collinsella, Bilophila*, and *Akkermansia* as being more abundant in PD patients compared to controls. In addition, we observed that *Roseburia* and *Faecalibacterium* were less abundant in PD patients. In past research, there is an overwhelming amount of data that supports that *Akkermansia* bacteria are more abundant in PD patients (1, 25, 26) and that *Faecalibacterium* and *Roseburia* bacteria are less abundant in PD patients (1, 5, 26, 27, 28). *Akkermansia* has been shown to degrade mucin proteins at the intestinal epithelial barrier (1). This degradation can lead to loss of intestinal barrier integrity which can terminally lead to inflammation and potentially lead to PD (1). *Faecalibacterium* and *Roseburia* are considered anti-inflammatory bacteria as they are butyrate-producers. Butyrate has been suggested to help improve intestinal barrier integrity, potentially influence microglial development in the brain, and have systemic anti-inflammatory properties (1). Since we and others have observed significant losses in these two bacteria, it suggests a potential role for altered immune homeostasis which could potentially lead to systemic inflammation which has been correlated to inducing/exacerbating PD symptoms (1).

Bifidobacterium is considered a health-promoting probiotic (29) but has also been implicated in several studies as being elevated in PD patients. Wallen *et al*. (2020) interestingly found that *Bifidobacterium* levels in the gut are correlated to doses of a PD-specific medication called levodopa (L-dopa) (5) and levels of this specific bacteria may just be elevated because of medications prescribed to PD patients. In the Cirstea *et al*. (2020) dataset, 91.4% of PD patients were on L-dopa. In contrast to the Wallen *et al*. (2020) paper

(5), Cirstea *et al*. (2020) mentioned that the only medication that induced significant alterations in the gut microbiome composition was entacapone in their dataset. As a result, in the dataset we used, it appears that the *Bifidobacterium* elevation is likely due to a factor other than L-dopa consumption. Since other studies have observed a link between L-dopa and *Bifidobacterium*, we believe more research into this area is needed. The genera *Collinsella* and *Bilophila* were bacteria that have not been commonly associated with PD patients' microbiomes. Both these bacteria are known to be pro-inflammatory which agrees with previous literature that PD patients tend to have more systemic inflammation and pro-inflammatory gut bacteria (31).

Antibiotics did not affect the diversity of the gut microbiome but did impact the relative abundance of *Bifidobacterium* in PD patients. We did not observe a significant difference in alpha or beta diversity metrics between the antibiotic consumption group and the no consumption group in either PD patients or controls. Our results reject our hypothesis regarding the effects of antibiotics on the gut microbiota. Furthermore, our results are different from most of the previous research done on antibiotics which have found that antibiotic consumption causes significantly diminished levels of microbial diversity (31, 32). However, it is important to recognize potential variables related to antibiotic consumption that may be at play.

Elvers *et al*. (2019) observed that some people take approximately 2 - 6 months to recover their gut microbiome to its initial state after antibiotic treatment, while most recover mere weeks after their antibiotic treatment. Unfortunately, we had limited information regarding antibiotic consumption. Besides the number of doses taken within the last five years, we had no information on the length of treatment, length of time off treatment, or the type of antibiotic used for each participant. It is possible that, in our dataset, most of the participants who consumed antibiotics had enough time to recover, thus resulting in no observed significant difference in alpha or beta diversity between antibiotic consumption groups or non-consumption groups.

Elvers *et al*. (2019) also observed that the antibiotics doxycycline and clarithromycin reduced *Bifidobacterium* levels while the antibiotics phenoxymethylpenicillin, nitrofurantoin, and amoxicillin had very little effect on the gut microbiome composition (31). It is worth noting that we also found that our antibiotic consumption group had reduced *Bifidobacterium* levels which agrees with the Elvers *et al*. (2019) study. This may indicate that some PD patients did take doxycycline and clarithromycin but cannot be confirmed due to lack of information gathered on antibiotic consumption in Cirstea *et al*.'s (2020) study. In future studies, we believe gathering more specific information on antibiotics will provide a clearer picture for the mechanism of *Bifidobacterium* changes in terms of antibiotic consumption.

Although we did not see any alpha or beta diversity differences, we found that *Bifidobacterium* had a lower relative abundance in the PD antibiotic consumption group when compared to the no consumption PD group, which suggested that antibiotic consumption may have had an impact on this genus. As previously mentioned, *Bifidobacterium* is associated with L-Dopa intake and is generally considered a good health-promoting bacterium. Interestingly, Tamtaji *et al*. (2019) found that PD patients had reduced PD symptoms (decrease in MDS-Unified Parkinson's Disease Rating Scale) when they consumed probiotics containing *Lactobacillus* and *Bifidobacterium* (33). Further, they found that the probiotic consumption group had lower levels of C reactive protein which is a common systemic inflammatory marker (33). This suggests that *Bifidobacterium* may have a positive role in maintaining good health in PD patients. Taken together with our results, antibiotic consumption likely leads to a decrease in *Bifidobacterium* which could potentially lead to more inflammation and exacerbation of PD symptoms within PD patients. More research is needed to confirm this.

Coffee consumption did not impact the gut microbial structure in PD patients. We sought to determine the relationship between the consumption of coffee and its effects on gut microbial community structure. Consumption of coffee has been demonstrated by multiple studies to improve bowel movement and intestinal muscle contractility (13, 34). However, the question of whether drinking coffee impacts the gut microbiota remains an area of active

study that has received various findings which support both sides of the argument. A study by Gurwara *et al*. (2019) found that in high caffeine consumers, the gut microbial abundance was highest (Shannon index of $p < 0.0001$). Using beta diversity analysis, they also found a significant difference in the gut microbial composition between low and high caffeine consumers ($p = 0.0001$) (34). These results largely contrast with what we found in our study, in which no significant difference in microbial abundance was found within each of the coffee consumption frequency groups (all $q > 0.05$). Additionally, for both PD patients and controls, we did not find any significant difference in microbial structure between the frequency groups in all beta diversity analyses (all $p > 0.05$). These results rejected our hypothesis and suggested that coffee consumption may not play a significant role in altering gut microbial community structure. However, interestingly, a study by Hedge *et al*. (2019) found that higher coffee content inhibits the growth of rats' gut microbiome both *in vitro* and *in vivo* in a caffeine-independent manner. A 3-day coffee treatment reduced the number of viable colonic bacteria up to more than 1.6 times *in vivo*, whereas a 3% coffee content agar reduced the number of colonic bacteria up to almost 20,000 times *in vitro* compared to a regular 0% coffee agar (35). Overall, the effect of coffee on the microbiome remains inconclusive.

Jaquet *et al*. (2009) also investigated the effects of coffee consumption on the gut microbiota in human volunteers, who took 3 cups of coffee daily for a period of 3 weeks. However, unlike previously mentioned studies, they found that faecal profiles of the dominant microbiota were not significantly impacted after the consumption of coffee (36). These findings align with the results of our study, suggesting that coffee consumption has no significant effect on gut microbiome community structure.

It is quite interesting to observe the diversity of results and findings across different studies on essentially the same area of investigation. This makes the topic of coffee consumption and its relationship with the gut microbiome rather controversial and hence, follow-up studies are needed to fully clarify and confirm the actual effect of coffee consumption on the diversity of the gut microbiome. Although it can be very challenging to accomplish, it is also important that follow-up studies are able to standardize the methodology as different confounding variables, such as alcohol consumption, can greatly impact how the results are interpreted.

Limitations A major limitation of this study is the lack of details included in the antibiotics data. Aside from the number of doses consumed within the last five years, there is a lack of information on important parameters such as the type of antibiotics used, the frequency of consumption, and whether antibiotics were consumed close to the date of sample collection. It is expected that different antibiotics and the timing of consumption in relation to sample collection date have different impacts on the gut microbiome. As such, due to a lack of critical information, the conclusions drawn using this metadata set should be confirmed by future experiments which take into consideration the above-mentioned factors.

Furthermore, the metadata contained many categories (99 categories). While we have attempted to control for confounding factors by analyzing the effects of antibiotic usage and coffee consumption frequency of PD patients and controls separately, many other variables may be contributing simultaneously to the differences observed in the gut microbial community structure observed between the PD patients and controls. Additionally, while all participants lived in the Greater Vancouver region during the time of the study, the difference in climate and condition of the participants' residence may contribute to changes in gut microbiome composition. Such discrepancies are difficult to control for and pose a major limitation to this study.

Conclusions The first goal of this study was to confirm the results of previous studies and compare the PD patient microbiome with control volunteers by applying a different set of parameters than Cirstea *et al*. (2020). Furthermore, we aimed to investigate whether antibiotic or coffee consumption would significantly alter the microbiomes of PD patients compared to controls. In our comparison of the gut microbial community structures of PD patients and control volunteers, we found no significant difference in the evaluated alpha diversity metrics, indicating PD does not significantly alter the within-group community structure. However, when directly comparing the PD and control participants using beta diversity, we found that

the microbial community structure between the two groups was significantly different. Subsequent differential analysis furthered this conclusion by identifying six bacterial genera that are significantly differentially abundant. Specifically, PD patients possessed a higher abundance of *Akkermansia, Bifidobacterium, Bilophila,* and *Collinsella* and a lower abundance of *Faecalibacterium* and *Roseburia*. The differences in the abundance of the identified genera of microbes may be linked to the progression of PD symptoms. Furthermore, while antibiotic consumption was not found to significantly impact overall gut microbial community structure in either PD patients or controls, it is related to the alteration of the abundance of *Bifidobacterium* in PD patients. PD patients that consumed antibiotics within the last five years possessed a significantly lower abundance of *Bifidobacterium* — an important probiotic involved in digestion — indicating that antibiotic consumption may negatively impact beneficial gut microbes. Coffee consumption did not significantly alter gut microbial community structure in either PD or controls and does not appear to significantly impact the abundance of specific bacteria. These results are contrary to some of the results found by previous papers and indicate that coffee consumption may not play a significant role in gut microbiota structure.

Future Directions As mentioned in the limitations subsection, there is a lack of information regarding antibiotic usage. As the frequency and duration of antibiotic usage and the type of antibiotics consumed were not known, future studies could consider repeating the investigation on the effects of antibiotics. Particularly, information on the type of antibiotics consumed would allow for a better understanding of the bacterial organisms that are targeted as well as other key qualities inherent to the antibiotic such as anti-inflammatory properties. Importantly, it is possible that a difference in the type of antibiotics consumed may lead to different outcomes, whether it be improvements or exacerbations in PD symptoms. As we have identified that there is a significant difference between *Bifidobacterium* abundance between antibiotic groups, an antibiotic of interest may be doxycycline which has been previously identified to reduce *Bifidobacterium* diversity (31). It would be beneficial to observe the differences in gut microbiota composition in mouse models that have either been given or not given doxycycline in a future study. More detailed antibiotic information will allow for the better categorization and filtration of the antibiotic data, thereby allowing for better control of potentially confounding variables.

While we did not identify the factors that are contributing to the difference in gut microbial community structure of PD patients and controls, many other components were included in the original metadata file and remain to be explored. The contribution of microbial community structure to the differences in sleep problems and Fatigue Severity Score (FSS) may be interesting to explore in addition to the effect of coffee consumption which was examined in this study.

Wallen *et al*. (2020) and Weis *et al*. (2019) have shown that some of the identified differentially abundant bacteria in PD patients are correlated to the number of administered doses of L-dopa and entacapone (Parkinson's disease-specific medications). The two studies together observed that L-dopa and entacapone may enhance levels of *Bifidobacterium* but lower *Faecalibacterium* levels. Interestingly, our results identified these two bacteria to be differentially abundant between PD patients and controls; *Bifidobacterium* was found to be more abundant and *Faecalibacterium* was found to be less abundant in PD patients. Since we identified these two bacteria as being differentially abundant between PD patients and controls, it may be interesting to identify if L-dopa and entacapone may be drivers of the differential abundance that we observed in our results. While we did not carry out a differential abundance analysis based on coffee consumption frequency, we believe that such analyses could yield interesting results in a future study. In addition to grouping coffee consumption based on frequency (i.e. "infrequent", "daily", "multiple cups daily", and "no"), we feel it may be beneficial to also group coffee consumers into either "Yes" or "No" groups in a future study; such grouping could provide a more general understanding of the overall effects of coffee consumption and improve statistical power.

For the diversity analyses regarding antibiotic consumption, we feel that it would also be interesting to combine both PD and control participants and do a diversity analysis on the "Yes" vs "No" antibiotic consuming individuals to determine if there is an overall antibiotic

effect on the diversity of the gut microbiome in humans. Our p-value within this study is quite close to being significant and we believe that, by combining the PD and control groups in a future study, statistical power could be improved and yield results regarding the general effects of antibiotics on gut microbiomes.

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CONTRIBUTIONS

This paper is a result of the collective effort of all the co-authors. We received help from each other through the process, but the general contributions are as follows. M.H. was responsible for the initial data filtration and quality control, evaluated the diversity metrics and taxonomic bar charts comparing PD patients to controls, performed differential/relative abundance analysis in R, and contributed to the writing of the introduction, discussion, study limitations, conclusions, future directions, and references. T.T. filtered the data and evaluated diversity metrics to explore the effect of antibiotics and coffee consumption, was responsible for compiling the figures and writing the abstract, introduction, methods, and results, and contributed to the writing of study limitations, future directions, and conclusion. N.L. generated taxonomic bar charts for both the coffee and antibiotic questions and contributed to the writing of the introduction, discussion, study limitations, conclusions, and future directions. All co-authors contributed to the editing of this manuscript.

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