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Effects of dietary fiber intake on gut microbial diversity and the abundance of short-chain fatty acid producing and proteolytic bacteria in Parkinson's disease patients

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SUMMARY Parkinson's disease (PD) is a neurodegenerative disorder that affects more than ten million people worldwide. Research shows that gut microbiome imbalances are a common feature of PD; specifically, a decrease in short-chain fatty acid (SCFA) producing microorganisms and an increase in proteolytic microorganisms. There are many studies that highlight the link between diet and the gut microbial population in healthy individuals. However, this link is unclear in Parkinson's disease patients. Since diet is a significant factor affecting the gut microbiome, the aim of this study was to assess how fiber, a macronutrient, affects the gut microbial community in Parkinson's disease patients. We performed an *in silico* analysis using gut microbiome sequences and associated dietary information to assess whether fiber affects the gut microbial diversity and the relative-abundance of SCFA-producing and proteolytic bacteria. We observed higher microbial diversity in Parkinson's disease patients with higher fiber intake. In high and low fiber intake groups, we observed no difference in the relative abundance of SCFA-producing and proteolytic bacteria. These results suggest that fiber may not play a significant role in changing the SCFA-producing and proteolytic bacteria population in PD patients and may not be of great importance to affect the gut microbiome imbalances experienced by these patients.

INTRODUCTION

The human intestinal microbiota harbours trillions of microbes that are often in a symbiotic relationship with their host (1). The host provides the microbes with food and a suitable environment; and in return, the microbes play important roles in vitamin production, dietary fiber breakdown, and immune function (1). As of recently, microbiome imbalances have been recognized as a consistent feature in Parkinson's disease (PD) (2). While little progress has been made in efforts to prevent PD progression, diet has gained importance as being a potential factor that helps reduce the motor and non-motor symptoms associated with PD (3,4,5). Understanding how specific dietary macro and micro-nutrients affect the composition of the microbiome and associated symptoms in PD patients may serve as a possible therapeutic strategy and emphasize the need for future controlled, experimental studies.

Diet is recognized as an important influential factor on the composition of gut microbiota (6). The intestinal microbiota metabolizes indigestible fat, fibers, proteins, and uses them as energy sources (7). Primarily, gut microbiota obtain their energy by fermenting indigestible fibers into short chain fatty acids (SCFA) (2). SCFA, such as butyrate, play a key role in maintaining a healthy gut (2). Butyrate provides energy to colonocytes, regulates gut inflammation by moderating a few inflammatory transcription factor levels, and regulates gene expression in colonocytes through its histone deacetylase activity (8). Fiber intake has direct effects on the amount of microbial diversity in the gut, as a reduction in fiber intake decreases bacterial diversity (9). This is because the presence of fiber increases fiber-metabolizing, and SCFA-producing, bacteria to grow in population, dominating in terms of abundance, which modulates the bacterial community (9). When looking at diet and microbiota composition, literature has stated the main goal is to enhance SCFA production (10). In addition to the previously mentioned benefits of SCFA, they also help in maintaining the function of the intestinal barrier (11). Thus, this demonstrates the importance of SCFA for the gut, and given that fiber is directly associated with the concentration and amounts of

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SCFA present in the gut, it indicates a possible avenue to investigate its influence on PD patients (9).

Fiber consumption also has an influence on the abundance of proteolytic, or protein-degrading, bacteria. When there is a reduction in fiber, a shift occurs as gut bacteria start metabolizing protein instead, which is a much less favorable substrate (9). It's considered less favorable due to the production of detrimental metabolites such as p-cresol and ammonia (9). That being said, a diet consisting of high fiber will likely be able to combat the influence of protein as SCFA-producing bacteria will still be present in high abundance (9). Specifically, this can be seen in the two diets; Mediterranean and Western. In a Mediterranean diet, protein and fat intake tends to be low and dietary fiber intake tends to be high compared to a Western-based diet (12). Adherence to a Mediterranean diet, as opposed to a western diet, is associated with lower risks of developing PD (12). Thus, fiber's key role in regulating SCFA-production and combatting proteolytic metabolites demonstrates its importance for gut health (9).

Cirstea et al. set out to look at the community composition of the gut microbiota and its association with the gut function in PD patients (2). Cirstea et al. identified two distinct microbial communities, A and B, that were highly associated with PD (2). Community A was dominated by SCFA-producing bacteria and was less abundant in PD patients (2). In contrast to this, community B bacteria had a higher abundance of proteolytic bacteria and were more abundant in PD patients (2). Community B was also positively associated with firmer stools and constipation severity, while the SCFA-producing community was inversely associated with those variables (2). These associations indicate that the shift in bacterial composition is directly related to GI symptoms in PD patients.

The influential role of diet on the composition of the microbiome has gained an increasing amount of importance in the last decade (13,14). Several studies have explored the effects of general diets and food groups on the risks of PD progression, however; studies exploring the direct effects of specific nutrients on the microbial composition and GI abnormalities in PD are limited (15,6). Cirstea et al. investigated dietary intake (2), yet their study did not extensively investigate the effects of diet on the gut microbial composition. Given that Cirstea et al. highlighted how GI abnormalities in PD are linked to microbiota alterations, we decided to explore how diet could influence these alterations. Understanding how diet affects the microbiota may open doors to future intervention strategies that could modify the disease course of PD. For that reason, we used the dietary information collected by Cirstea et al. to investigate if the variability in dietary fiber consumption would alter the abundance of SCFA-producing and proteolytic bacteria. We anticipated that this would likely change the stool firmness and constipation severity in PD patients.

In our study, we aim to determine the effects of fiber on the composition and diversity of the gut microbiota in PD patients. Cirstea et al. had determined two clusters which primarily had SCFA-producing and proteolytic bacteria, respectively. Therefore, we studied how dietary intake affects both SCFA and proteolytic bacteria production in PD patients. We chose to specifically focus on fiber because of its crucial role in maintaining gut microbiota health and diversity (9). We hypothesize that an increase in dietary fiber intake would increase the abundance of SCFA-producing bacteria. We predict that the variability in fiber intake would alter the intestinal microbial diversity and changes to the microbial diversity would affect the severity of GI abnormalities common in PD. We also predict that a decrease in proteolytic bacteria abundance will occur in response to an increase in SCFA-producing abundance. Understanding a possible mechanistic link between diet and the intestinal microbiome may open doors for future therapeutic strategies, as diet could indirectly affect the GI abnormalities by altering the microbiota. These findings may also help establish diet's role in optimizing gut microbiota in PD patients.

METHODS AND MATERIALS

Data acquisition and metadata filtering. All data used in this study were acquired from the Cirstea *et al*. study and specifics of their methods can be found in their paper; however, a general summary will be included here (2). Cirstea et al. acquired data is from 197 PD patients and 103 controls, from which fecal samples were collected. The 16S rRNA V4 region was amplified and sequenced on an Illumina platform, with 515F/806R primers, giving us the FASTQ files required for our own analysis. Cirstea et al. also collected extensive amount of

metadata. Dietary information was collected using the EPIC-Norfolk Food Frequency Questionnaire (FFQ), then using the FETA software (17), specific values were generated for 46 nutrients and 14 food groups. Constipation severity was measured using the Rome III constipation criteria (18). Consistency of stool samples was assessed using the Bristol Stool Scale (19). Metadata were filtered using R (20) (v4.0.2) with the *tidyverse* package (21), from which we filtered the nutrient groups of interest: Proteins and Non-Starch Polysaccharides. New columns for each of these nutrients were created that distinguished them as "high" or "low" based on their values as above or below the median value per nutrient collected.

QIIME2 Processing using filtered metadata. The raw 16S rRNA sequences obtained from Cristea et al. were processed using DADA2 (22) within QIIME2 (v2020.8) (23) to give the frequencies of each unique amplicon sequencing variant (ASV) and to map each ASV to the sample that they represent. Using the Greengenes 99% OTU database, a Naive Bayes classifier was trained with the 16S rRNA sequences, and taxonomy was assigned to each sequence. In QIIME2, low frequency ASVs that had less than 0.005% of the total reads (less than ~230 counts), and mitochondrial sequences were filtered out. Finally, the processed sequences and the taxonomy data were merged and exported to R for further analysis.

Alpha and Beta diversity analysis in R. The processed files from QIIME2 were imported to R using the *qiime export tools* code, and the *phyloseq* package (24) was used to subset PD and Control samples. Samples were rarefied at the 5558 sampling depth to ensure maximum diversity within each sample. Alpha and beta diversity analyses were performed on both Control and PD samples. For alpha diversity, the Shannon's and observed feature metrics were chosen to assess sample diversity, and box plots were generated for high/low fiber consumption for both PD and Control samples. For beta diversity, the weighted and unweighted UniFrac distances were chosen to assess differences in microbial composition between high and low fiber consumption samples in both PD and control samples, and the data was plotted on principal coordinates analysis (PCoA) plots.

Differential abundance and correlation analyses. The *phyloseq* (24) and *DEseq2* (25) R packages were used to create relative abundance plots. Bacterial species used for relative abundance plots were selected based on the bacterial communities, A and B, presented in Cristea *et al*. study. Additional known metabolizers of SCFAs and proteolytic bacteria such as members of the *Bacteroides* genus were included in this analysis. These SCFA metabolizers and proteolytic bacteria plotted against high and low fiber consumption groups to create differential abundance plots, and analysis of variance (ANOVA) tests were used to determine significance. Values were plotted using the ggplot2 package (26). Correlation analysis was completed using the *ggpubr* (27) package in R to plot constipation and Bristol-stool scores relative to fiber consumption.

RESULTS

The alpha diversity of high fiber PD groups were higher relative to low fiber PD groups. We set out to determine the effects of fiber consumption on the alpha diversity of the gut microbiota in PD based on Shannon's and observed feature metrics. Statistical significance for our data was determined using ANOVA tests. Shannon's and observed feature metrics were higher for both high fiber Control and PD groups (Fig. 1). It is important to note that results were statistically more significant for PD samples (Fig. 1B, observed p-value = 0.0098, Shannon's p-value = 0.074). Nonetheless, these results suggest that fiber intake affects both healthy and PD patients similarly, as increased fiber intake was associated with higher gut microbial diversity in both PD and Control.

Gut microbial composition is similar between low and high fiber consumption groups in both Control and PD patients. We investigated how fiber consumption affects gut microbial composition of PD patients. We plotted unweighted and weighted UniFrac distances (Fig. 2) for Control and PD patients. We observe no distinct microbial communities between high and low fiber consumption samples in any of the PCoA plots and all samples

are clustered together. These observations suggest that the microbial composition is similar between high and low fiber consumption individuals for both Control and PD samples.

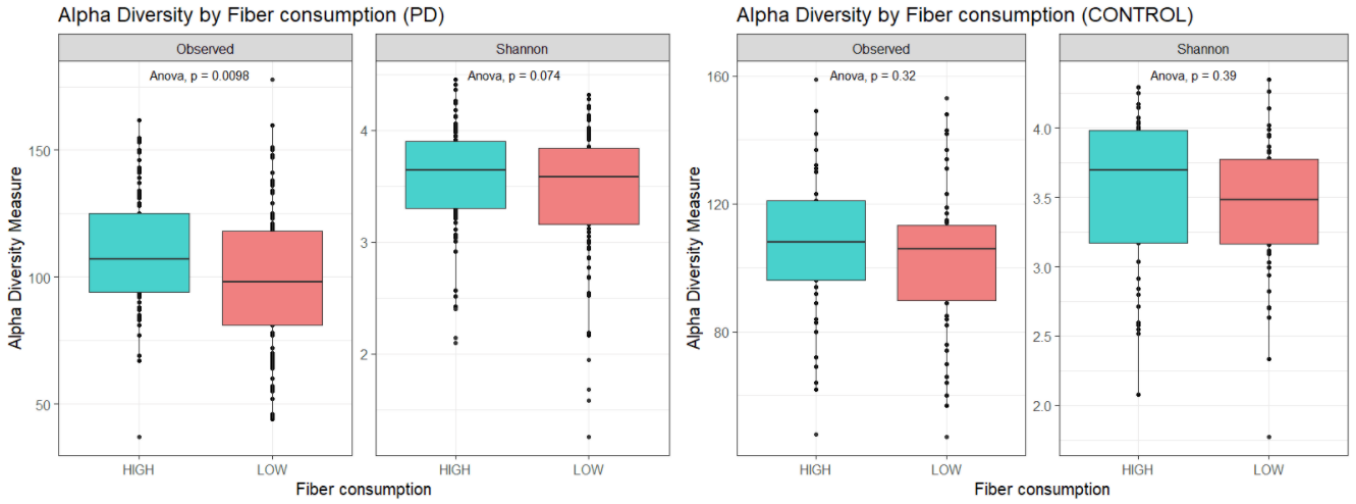

FIG. 1 Higher sample diversity in high fiber consumption groups of PD patients. Shannon's and observed features alpha diversity metrics grouped by fiber consumption (high/low)for **(A)** control and **(B)** PD patients. Sample diversity is higher in high fiber consumption groups in both control and PD samples. However, it is statistically more significant in PD patients (p = 0.0098 for observed features, p = 0.074 for Shannon's).

Relative abundance of SCFA bacteria did not change significantly between low and high fiber PD groups. We asked how variable fiber consumption affected the relative abundance of SCFA-producing bacteria in PD patients. Differential abundance analysis was performed in R and statistical significance was assessed using ANOVA. Fig. 3 shows the relative abundance of SCFA-producers at the genus level; *Roseburia*, *Faecalibacterium*, and the family Lachnobacterium. *Roseburia* had higher relative abundance in low fiber PD patients. While the difference relative to high fiber PD patients was not statistically significant (p = 0.054), it did show a large negative trend. Similar trends were seen in Lachnobacterium, as their relative abundance were generally higher in low fiber groups although the differences between low and high fiber groups were not statistically significant (p > 0.05). Finally, *Faecalibacterium* showed no significant change in abundance for both groups.
Control samples were also examined to provide strength to our results. Only *Roseburia* relative abundance was significantly different (P = 0.048), finding higher abundance in low fiber diets. The remaining bacteria showed very little variance between diets, and were not statistically significant. Additional differential abundance plots can be found in the supplemental figures (Fig. S1) regarding the genera *Blautia*, *Corprococcus* and *Lachnospira*. However, none of these bacteria displayed any significant difference between high and low fiber groups in either PD or Control.

Significant change in differential abundance of *Bacteroides* genera, and no change in other proteolytic bacteria. We asked how variable fiber consumption affected the relative abundance of proteolytic bacteria in PD patients. To investigate this, we followed the same procedure as described above. Figure 4 shows the relative abundance of proteolytic bacteria at various taxa levels. In PD patients two bacterial families *Ruminococcaceae* and *Akkermansia* both displayed no significant changes in abundance (p = 0.057 and p = 0.640 respectively), although a slight increase in *Ruminococcaceae* is observed (Fig. 4B). Alternatively, Bacteroides relative abundance decreased significantly in Control samples (P = 0.014) from low to high fiber conditions. The median change is from roughly 17 percent in the high fiber condition to close to 27 percent in the low fiber condition. The relative abundance of Bacteroides in the PD group was not statistically significant (P = 0.170), although the median abundance was once again lower in the high fiber group. Additional genera were screened as present in supplemental figures (Fig. S2), however none displayed significant differences in relative abundance when compared between fiber groups.

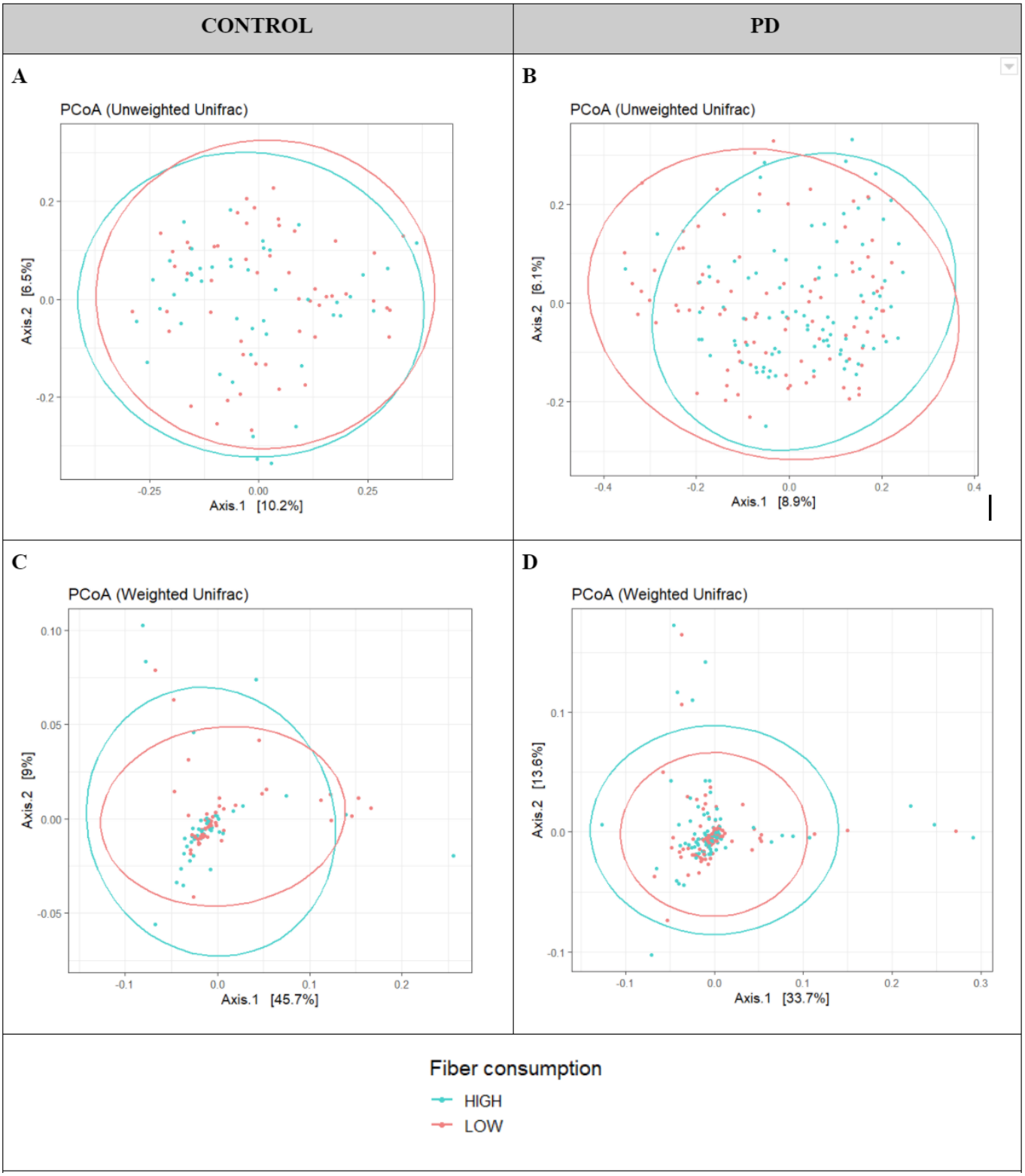

FIG. 2 No distinct microbial communities observed between high and low fiber consumption groups. Principal coordinates analysis plots using weighted UniFrac. **(A)** Control and **(B)** PD samples are coloured by fiber consumption (blue = high, red = low). There are no distinct microbial communities observed between high/low fiber consumption groups for both Control and PD samples

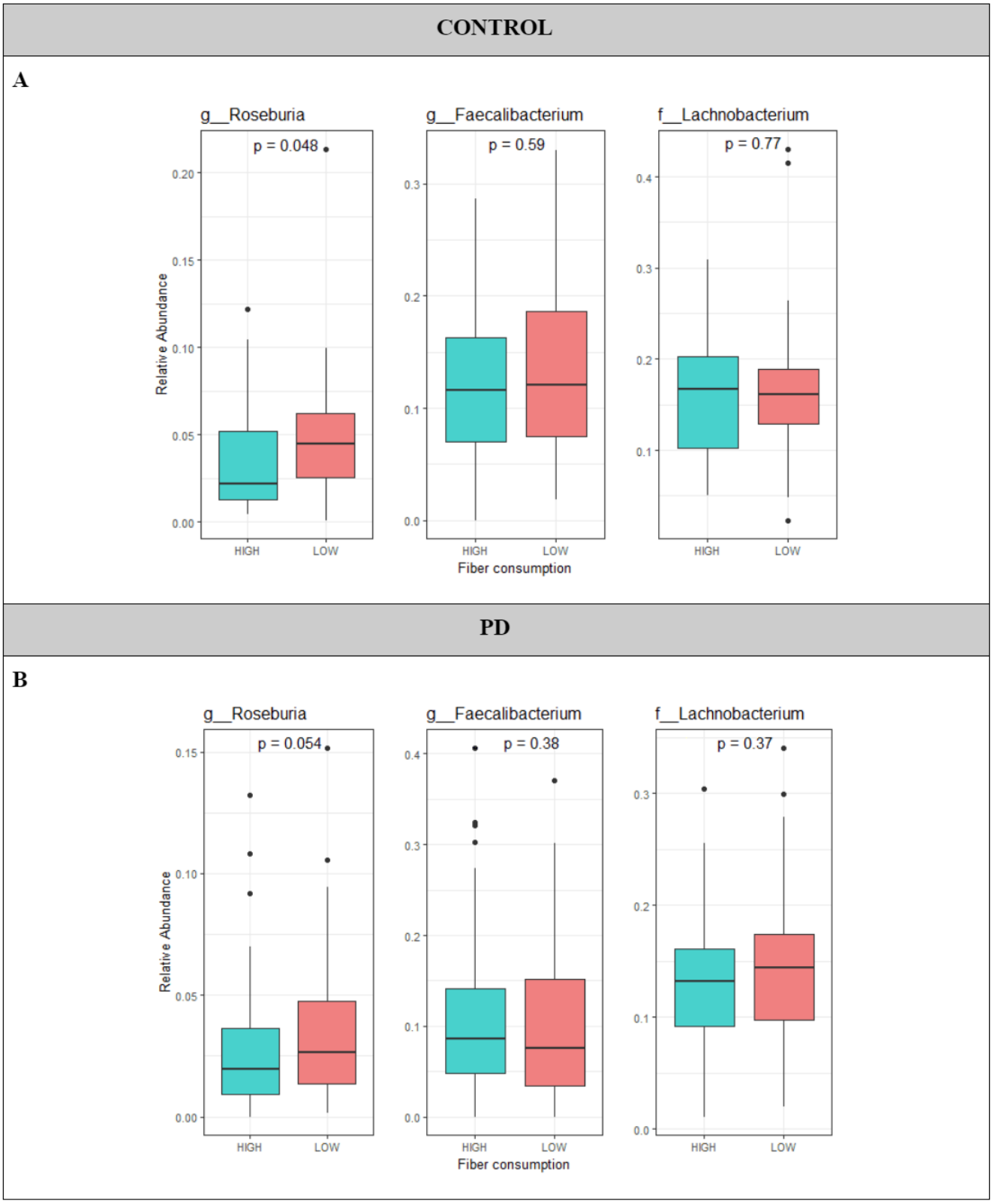

FIG. 3 Increase in *Rosburia sp*. in low fiber diets, no significant change in other SCFA producers. Relative abundance of SCFA producing bacteria in (**A**) Control and (**B**) PD patients in high (blue) and low (red) fiber conditions. The only statistically significant change is in Control group *Roseburia* abundances, which was less abundant in high fiber samples.

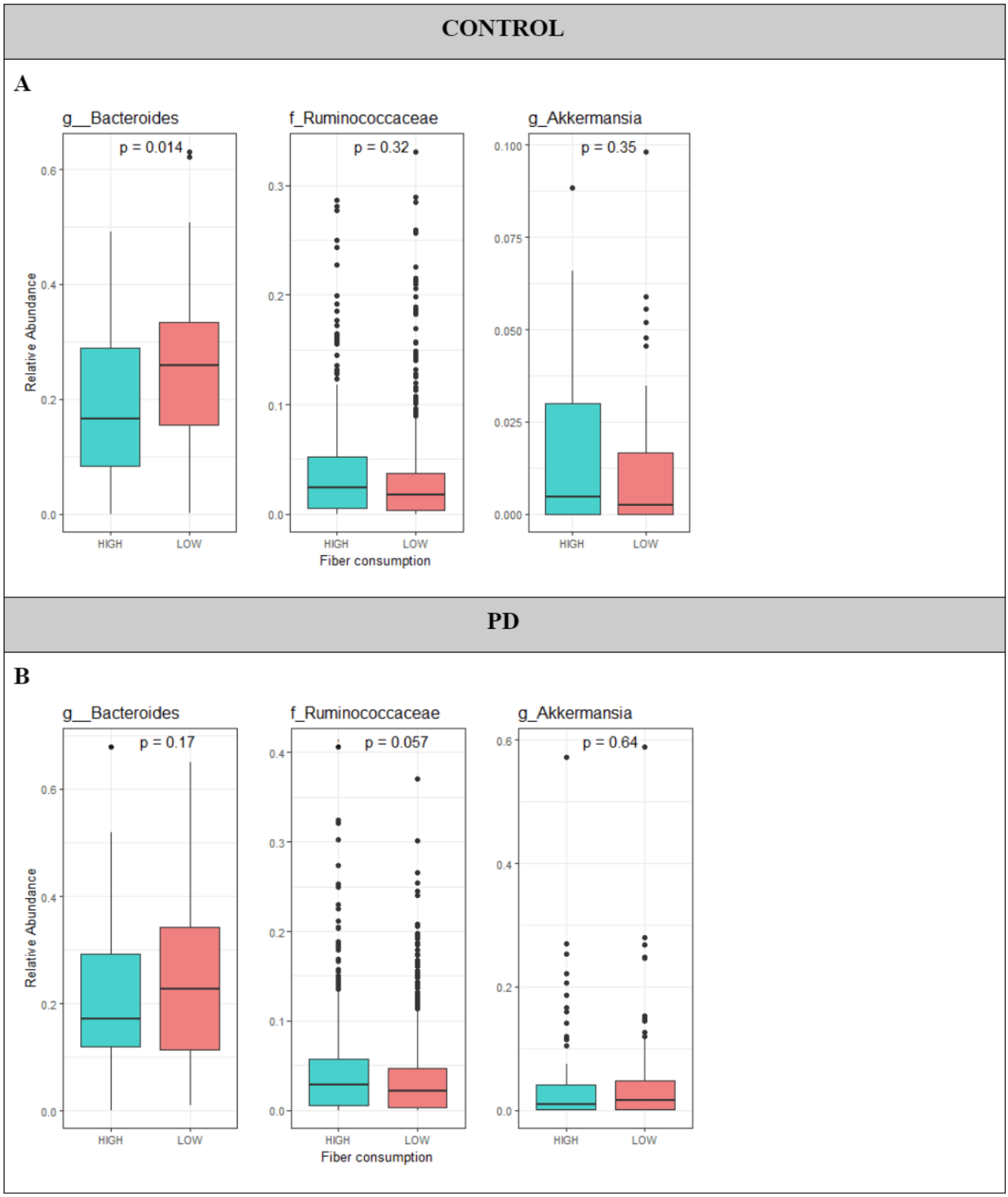

FIG. 4 Increase in *Bacteroides sp.* in low fiber diets, no significant change in other proteolytic bacteria. Relative abundance of Proteolytic bacteria in (**A**) control and (**B**) PD patients in high (blue) and low (red) fiber conditions.

No correlation between fiber consumption and constipation severity score. We decided to do a correlation analysis between our explanatory variable of fiber (in grams) and constipation severity (on a scale of 0-28), our response variable. Our data showed a very weak negative correlation between increased fiber consumption and constipation severity score in

the Control group (r = -0.035) (Fig. 5A). Similarly, based on the r-value of -0.053, our data showed a very weakly-associated negative correlation between increased fiber consumption and constipation severity score in PD patients (Fig. 5B). The low r-value and p-value of 0.480 indicate our results are not statistically significant . Thus, an increased intake of fiber consumption showed to have little effect on the constipation severity score in PD patients. These results are consistent with those observed for Bristol-stool scores. Bristol-stool scores were also plotted against fiber consumption (Fig. S3), however much like these results no correlation was observed.

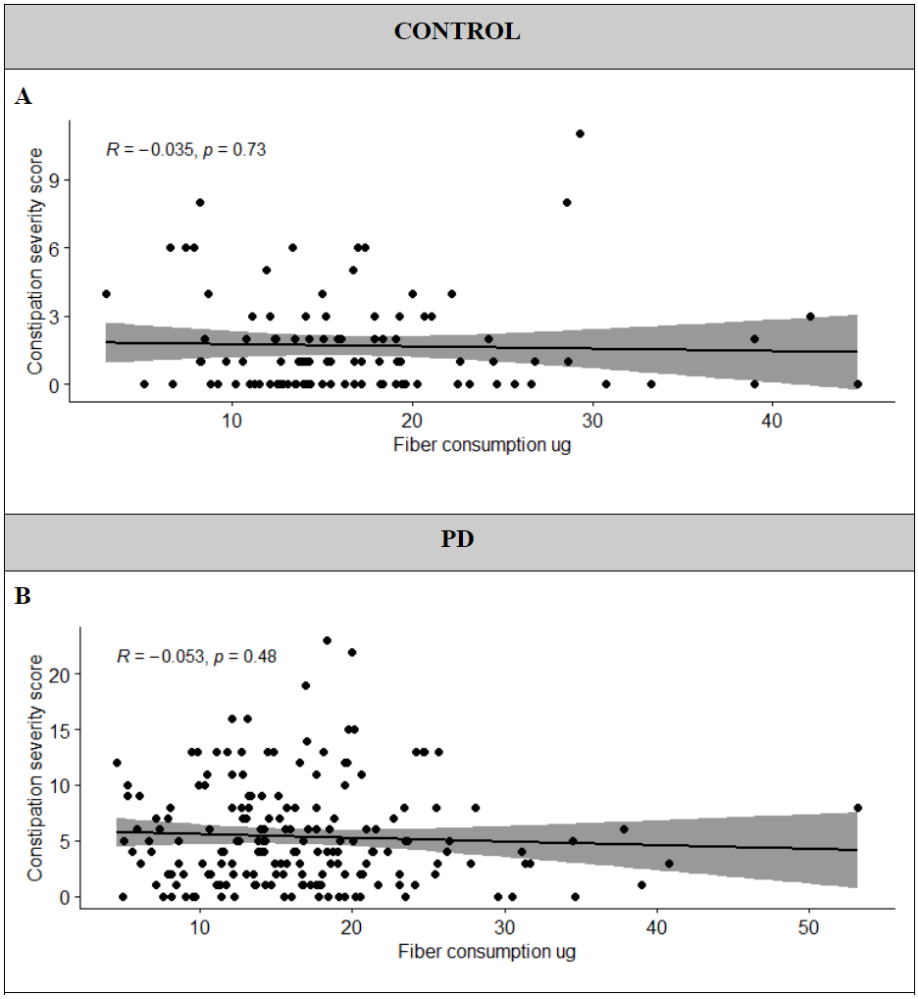

FIG. 5 No significant correlation between constipation and fiber consumption. Correlational analysis of Constipation severity to fiber consumption of (**A**) Control and (**B**) PD groups. Both control and PD groups were slightly negatively correlated (-0.035 and -0.053 respectively) but neither was statistically significant (P >0.05) signifying no correlation existing between these factors

DISCUSSION

In our study, we investigated the effects of fiber consumption on the gut microbial diversity and relative abundance of proteolytic and SCFA-producing bacteria. We grouped PD patients and Control groups into high and low fiber intake groups using the dietary information collected by Cirstea *et al*. Upon analysis of our alpha diversity plots, they supported our hypothesis that high fiber consumption would cause an increase in alpha diversity. The gut microbial alpha diversity was significantly higher in the high fiber PD patients compared to the low fiber group. In our Control, although statistically insignificant, these same trends were observed as high fiber intake also led to higher alpha diversity. Meanwhile, when looking at the effects of fiber on the relative abundance of proteolytic and SCFA-producing bacteria, the results did not support our hypothesis that high fiber would cause an increase in SCFA-producing bacteria while causing a decrease in proteolytic bacteria. In low fiber PD patients, there was a higher relative abundance of some SCFA-producing bacteria, while high fiber PD groups had a higher relative abundance of some proteolytic bacteria.

Several studies have associated the gut microbiota of PD individuals with significantly less microbial diversity (28, 29). Reduced microbial diversity has been associated with other

diseases, such as inflammatory bowel disease or type 1 diabetes; this inevitably highlights how important an increased microbial diversity is to one's overall health (30). A species-rich gut ecosystem benefits our immune system by exposing it to a variety of pathogens, which contributes to a more stable and resilient immune system (30). A decline in the functionality of the immune system has been seen as PD progresses, which further illustrates a key link between microbial diversity and immune function (31). As diet is a major influencer of microbial composition, we analyzed the microbial diversity between low and high fiber intake PD individuals. Our data showed that high fiber intake significantly increased the richness of the microbiota (Fig. 1B). Increased microbial diversity in high fiber PD groups is consistent with previous studies that looked at the effects of fiber consumption in healthy individuals **(**17). When looking at the Control group, the difference in microbial diversity between low and high fiber groups was not statistically significant. However, the median of the high fiber Control group was higher than low fiber Control groups, which could be suggesting similar effects of fiber as seen with PD patients. Thus, our results highlight the importance of dietary fiber intervention as it may regulate gut diversity, which in turn would improve both gut and immune function.

The beta diversity results showed no distinct microbial communities between high and low fiber diets in both PD and Control individuals. This finding alludes to there being similar bacterial communities between individuals partaking in these two diet types. However, these similarities indicated that we would expect to find key differences in the relative abundance of only a few taxa between low and high fiber intake individuals. Our data did not support our hypothesis as the relative abundance of SCFA-producing bacteria did not significantly increase in high fiber PD individuals. However, the relative abundance of the SCFA-producer, *Roseburia*, is significantly higher in low fiber control compared to the high fiber control group (Fig. 3A)**.** Despite the PD results not being significant, the median for *Roseburia* relative abundance was clearly higher in the low fiber PD group. Interestingly, previous studies have associated the abundance of *Roseburia* species with improved colonic motility (32). In fact, species belonging to *Roseburia* were highly abundant in healthy gut samples, which on the contrary, were depleted in constipated gut samples (32). Other studies have highlighted that *Roseburia* species play a role in gut inflammatory processes and through butyrate, contribute to immune system maturation and improved gut barrier function (33). Overall, the importance of *Roseburia,* highlighted by previous studies and our results, introduces the idea of dietary low fiber intervention as a way of potentially regulating the relative abundance of *Roseburia.* Increasing the abundance of *Roseburia* species could improve constipation severity present in PD patients, which can encourage future well-controlled studies to investigate the link between low fiber and increased *Roseburia* abundance.

As for the relative abundance of proteolytic bacteria, our data did not support our hypothesis since we did not observe a global change in the relative abundance of proteolytic bacteria with different levels of fiber consumption except in the case of the genus *Bacteroides*. We observed a statistically significant lower relative abundance of *Bacteroides* in high fiber consumption Control samples, but not PD samples. Since it is known that *Bacteroides* constitute most of the proteolytic bacteria in the gut, higher fiber consumption could shift the gut microbial community to favour the SCFA producers over the proteolytic organisms which could explain the lower relative abundance of *Bacteroides* in Control samples (34). However, unlike Control samples, high fiber consumption in PD samples was not associated with lower relative abundance of *Bacteroides*. This could be related to the microbial dysbiosis that is associated with PD status and the shift towards proteolytic organisms in the gut microbiome of PD patients (6). In other words, the shift towards proteolytic bacteria in PD patients could mask the ability of fiber to decrease the proteolytic population which could explain the lack of difference in *Bacteroides* between high and low fiber consumption PD samples.

Limitations Cirstea et al. aimed to look at the link between gut microbiota, metabolism, and Parkinson's Disease (6). Their main goal was to look at metabolism and how it affects the gastrointestinal (GI) tract's function (6). We, however, utilized their unexplored diet data to look at how diet directly affects and is related to gut microbiota and composition. Given the difference in our research aim, this may have played a role of limitation with the data we

used. Cirstea et al. retrieved food and nutritional data through the EPIC-Norfolk Food Frequency Questionnaire (FFQ). As this was a questionnaire, the intake values for each nutritional item were average estimates of one's daily intake. This may not be entirely reflective or even accurate of their actual intake amount because people may over or under exaggerate their intakes. If the data had been collected differently, for example by collecting data from patients with controlled diets, we feel that our study would have fared much more meaningful results. Specifically, if Cirstea et al. had given a set and controlled amount of each micronutrient and macronutrient to each individual, our take on investigating diet would likely have returned different results.

Another limitation of our study is our lack of control for confounding factors. We did not run any linear regression models to assess the differential contributions of confounders such as other dietary nutrients, age, disease progression, weight and medications. Other potential confounding variables include how the Control patients were selected (6). Specifically, 43 of the 103 controls were spouses of PD patients which may not be suitable for a study of diet, because couples tend to have more similar gut microbial communities compared to unrelated individuals (35). This may explain, in part, the high similarity observed between PD and Control groups throughout our analysis. Overall, these confounding variables weaken the findings of our study, and must be taken into consideration when considering the results discussed in this paper.

Conclusions We set out to determine the effects of fiber on the gut microbial composition and diversity of PD patients. Additionally, we aimed to determine the effect of fiber on the relative abundance of both SCFA-producing and proteolytic bacteria. Diversity analyses revealed higher microbial diversity in Parkinson's Disease patients with high fiber diets. Relative abundance analysis found no change in SCFA producing or proteolytic bacteria between high and low fiber diets in PD patients, although some change was found in *Roseburia* and *Bacteroides* genera in Control groups. These results demonstrate a departure from other published works, in questioning the importance of dietary fiber in the alteration of the gut microbiome, specifically it finds that dietary fiber quantity may not play a role in determining the abundance of these bacteria. Current knowledge gaps are prevalent in the comprehension of macronutrients and their role in the gut function of PD patients. This paper helps illuminate the role of fiber on the microbiome, helping reinforce potential downstream treatment options for PD.

Future Directions The low strength nature of our results increases the need for further study of diet in PD patients. The limitations of our study can easily be corrected by changing the experimental design. Specifically, diet regiments would need to be implemented in PD patients, both a low fiber diet and a high fiber diet, to better understand its role on the gut microbiome. Future research would also require controlling for confounding variables which we were not able to do for this study. Together these two changes would look at the same components of the gut that we did but would generate much more reliable results. Other areas worth studying include examining the effects of different kinds of fiber. Studies have found the chemical properties of different types of non-starch polysaccharides (fiber) affect both microbial composition and SCFA production (36). These studies would allow for further analysis of some notable findings we produced in this paper, specifically checking the abundance differences of *Roseburia* and *Bacteroides* genera in response to fiber, to help give strength to our findings.

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David Oliver contributed through teaching the majority of the course, aiding in understanding of scientific principles. Stephan Koenig provided the majority of the R-script used to filter the metadata, create beta diversity PCoA plots, and undergo differential abundance analysis.

CONTRIBUTIONS

Each individual on the team was integral to the completion of this draft manuscript. The following are some of the key contributions of each team member. All 4 authors contributed to the planning and design of the study and all were involved in doing the background research for this project. P.A. and A.A. conducted the analyses in QIIME2, and B.K. and N.D. conducted the analyses in R. Abstract was written by P.A. and A.A., introduction by A.A. and B.K., methods by P.A. and N.D., results by P.A. and N.D. The discussion and conclusion sections were written by all 4 authors. Co-authorship should be considered equal for all authors.

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