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Parkinson's Disease Dampens the Impact of Mental Health on Microbial Predicted Metabolic Functions

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SUMMARY Parkinson's Disease (PD) is a progressive neurodegenerative disorder characterized by motor and non-motor symptoms. Out of all the neurodegenerative diseases investigated to date, PD has the strongest association with gut microbiota alterations. The gut microbiota of PD patients consistently shows a reduction in carbohydrate fermentation and increased production of deleterious amino acid metabolites compared to healthy controls. Interestingly, mental health problems such as depression, anxiety, and poor sleep often co-occur with PD, on top of being prodromal symptoms that can appear a decade prior to disease onset. Although depression, anxiety, and poor sleep each have gut microbiota alterations in the absence of PD, the impact of these conditions on the predicted gut function of individuals with PD is not well known. This present study investigates the compositional and functional metabolic alterations underlying depression, anxiety, and poor sleep co-occurring with PD. Alpha and beta diversity analysis showed no significant difference for all mental health factors. Minor alterations were observed at the taxonomic level, with *Verrucomicrobiota* being uniquely identified in PD patients experiencing co-occurring poor sleep. Functional analysis using PICRUSt2 showed a reduction in metabolic pathway alterations for PD patients with co-occurring depression. Anxiety was associated with subtle functional changes that were more similar than different between PD individuals and healthy controls. Lastly, significant alterations were identified for individuals experiencing poor sleep. Overall, this study suggests that PD dampens the functional impact of mental health conditions on the gut microbiota, highlighting the volatility of the PD microbiota and need for further function-based investigations.

INTRODUCTION

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder, with a wide spectrum of motor and non-motor symptoms (1). PD motor dysfunctions are propagated by the accumulation of protein alpha-synuclein in Lewy's bodies in the substantia nigra pars compacta of the midbrain, which leads to a loss of dopamine-containing neurons (1). Classical motor symptoms of PD include resting tremors, rigidity, bradykinesia, reduced colonic transit time, and constipation, as well as non-motor symptoms such as fatigue and REM sleep behavior disorder (1). Compared to other chronic neurological disorders, PD has undergone the fastest growth in disability and prevalence, with an estimated 60,000+ Canadians affected (2, 3). The exact etiology of PD remains unknown, although genetic and environmental factors have been postulated to influence PD development (1, 4). The gut microbiota has been increasingly recognized as a feature of neurological disorders, and PD is no exception. Gastrointestinal comorbidities, constipation, combined with a skew towards decreased short-chain fatty acid (SCFA) concentrations are hallmarks of the PD gut microbiota (4–6). Moreover, PD is associated with an overall decrease in carbohydrate fermentation and butyrate synthesis, in addition to elevated proteolytic fermentation and deleterious amino acid metabolite production (6). These changes are observed in both microbiota composition and predicted metabolite functions in PD versus control cohorts. Meta-analysis of the PD gut microbiome showed that the most consistent gut microbiota alterations are enriched *Lactobacillus*, *Akkermansia*, *Bifidobacterium*, accompanied by depleted *Lachnospiraceae* and *Faecalibacterium* (7). Both *Lachnospiraceae* and *Faecalibacterium* are essential in gut SCFA production (7).

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One factor that complicates teasing apart the PD microbiota is mental health. Depression, anxiety, and poor sleep each have distinct gut microbiota alterations in addition to being prodromal, non-motor symptoms of PD (8–12). In healthy individuals, correlational studies on the gut microbiota alterations associated with depression or anxiety identified a general increase in pro-inflammatory bacteria, particularly *Proteobacteria* (5). Furthermore, depression and anxiety are correlated with the kynurenine pathway, which is closely regulated by the gut microbiota and participates as a messenger in gut-brain communication (13). Poor sleep further contributes to the correlation between gut microbiota and depression or anxiety. Sleep issues not only hold predictive value for determining PD onset, but also further exacerbate depression and anxiety symptoms (10–12). In healthy individuals, diurnal fluctuations of *Clostridiales*, *Lactobacillales*, and *Bacteroidales*, which make up 60% of the gut microbiota, cause time-of-day specific taxonomic configurations that are altered in individuals with poor sleep (14). When looking at the role of both mental health and PD on the gut microbiota, studies have found similar microbiota compositions between depressed and non-depressed patients (15). There is limited work on the functional alterations associated with depression, and no investigations have been carried out on the microbiota perturbations associated with anxiety and poor sleep in individuals with PD (8, 9).

Therefore, using data collected from Cirstea et al.'s cross-sectional cohort study (197 PD patients and 103 controls), our research aims to characterize the compositional and functional alterations associated with PD when co-occurring with depression, anxiety, or poor sleep (6). By characterizing functional metabolic alterations of PD patients with co-occurring mental health conditions, we aim to identify contributors to PD pathophysiology. A lack of compositional difference does not correlate with a lack of functional alterations. Given mental health problems can present up to 10 years prior to disease onset, understanding the impact of co-occurring mental health conditions is key to furthering our knowledge of PD development.

We found minimal compositional differences across all three mental health variables based on diversity and taxonomic analyses, although there were differences at the functional level. The results of our diversity analyses are consistent with literature, as previous investigations on depression and poor sleep also found no significant compositional difference (15). From a functional perspective, the metabolic pathway changes in PD patients with co-occurring depression or poor sleep were drastically reduced compared to healthy controls. To our surprise, the metabolic changes associated with anxiety were similar regardless of disease status. Taken together, our study suggests there is interplay between PD and mental health at the functional level, even in the absence of compositional differences. Given the high incidence of concurrent mental health conditions in PD patients, this study highlights the need for further investigations on the associated metabolic alterations, as this is linked to PD pathophysiology despite the lack of significant compositional differences.

METHODS AND MATERIALS

Parkinson's Disease dataset. The dataset was generated by Cirstea et al., who collected fecal samples from 300 individuals (103 healthy, 197 with PD) aged 40-85 using take-home collection kits (6). The DNA from these samples was collected, and barcoded 515F (GTGCCAGCMGCCGCGGTAA) / 806R (GGACTACHVHHHTWTCTAAT) primers were used for amplification of the V4 region of bacterial 16S rDNA before raw sequences were obtained using an Illumina MiSeq platform. The researchers who created the dataset collected data on the participants' mental health status as well as many other pieces of demographic information that can be found in the original paper (6). The factors of interest for this analysis consist of depression, anxiety, (BDI_depression_score, STAI_anxiety_score) and poor sleep (Sleep_problems). Severity scores for depression were obtained using Beck's Depression Inventory (BDI) and scores for anxiety were obtained using the State-Trait Anxiety Inventory (STAI). For our analysis, depression and anxiety scores were binned to a binary "yes" or "no" category determined using a score cutoff of "11" for depression and "80" for anxiety, where values above the cutoff went in "yes" and scores below went in "no".

Preliminary data processing via QIIME2 pipeline. Using the QIIME2 (v2023.7) pipeline, we imported and demultiplexed the 16S rRNA samples for the data provided by Cirstea et al.

(6, 16). Denoising and clustering was carried out using Divisive Amplicon Denoising Algorithm 2 (DADA2) (17). We trimmed the reads to retain base pairs 13-250 as consistent with the trimming done by Cirstea et al (6). The mitochondrial and chloroplast sequences were removed, and a feature table was generated using the output table from DADA2. Taxonomic classification was done using DADA2 to assign each ASV at the species level. Finally, rarefaction was carried out using a sampling depth of 3797 based on the alpha-rarefaction curve of the observed features as well as the code from Cirstea et al. in the supplementary files (Supplementary Figure 1) (6). 299 out of 300 samples were retained for each category. ASVs less than 5 were filtered out, and the resulting feature table was used in all downstream analysis in RStudio.

Alpha and beta diversity analysis. Phyloseq objects were generated using the custom metadata, features table, taxonomy classification, and phylogenetic tree for downstream analysis. Alpha-diversity metrics (Shannon) and beta-diversity metrics (Jaccard, Bray Curtis, Unweighted and Weighted UniFrac) were computed on R 4.3.2 using the tidyverse (v2.0.0), vegan (v2.6-4), and phyloseq (v1.44.0) packages (18–20). PD and control groups were analyzed separately. 1-way ANOVA was used to compute statistical significance for alpha-diversity metrics. PERMANOVA was used to compute statistical significance for beta-diversity metrics.

Taxonomic analysis. Taxonomic bar plots were generated on R 4.3.2 using the tidyverse (v2.0.0), vegan (v2.6-4), and phyloseq (v.1.44.0) packages (18–20). The metadata, OTU data, and taxonomic data were used to generate relative abundance, and the average relative abundance was presented in bar plots. Phyla that represent a relative abundance of less than 1% were filtered out. Only the poor sleep variable was examined to generate the bar plot for the *Verrucomicrobiota* phylum. The relative abundance for each genus in different groups was calculated, then filtered to keep only bacteria in the *Verrucomicrobiota* phylum. Genera with a relative abundance of less than 1% were filtered out before generating the final bar plot.

DESeq2 analysis. DESeq2 was conducted for differential expression analysis of ASVs between study participants who were classified as “Yes” or “No” for the mental health variables depression, anxiety, and poor sleep. The DESeq2 package (v1.40.2) was used to calculate the relative library depth of each sample, the dispersion of counts for each gene, and the significance of coefficients in a negative binomial generalized linear models using size and dispersion outputs. An adjusted p-value of 0.01 and an absolute $\log_2$ fold change greater than 2 were used to define significant hits. The ggplot2 package (3.4.4) was used to visualize the differential abundance for each variable of interest as a volcano plot (21). Bar plots that show different taxonomic groups were also generated via ggplot2 to visualize the differential abundance of participants who had (“Yes”) depression, anxiety, or poor sleep compared to those without (“No”) mental health problems.

PICRUSt2 analysis. Functional abundances were predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software (v 2.5.2) (22). Using QIIME2 (v2023.7), all features with 5 counts or lower were removed from the feature table prior to running PICRUSt2 (16). The PICRUSt2 pipeline created by Douglas et al. was followed to align ASVs to reference sequences from the MetaCyc database, generating an abundance table with functional pathway annotations (22, 23). DESeq2 package (v 1.40.2) was used as the differential abundance analysis method (24). For each variable of interest, PD or control samples were filtered out to generate respective PCoA and $\log_2$ fold plots. All plots were visualized using the ggpicrust2 package (v1.7.3), with a significance cut off of $p < 0.05$ (21).

RESULTS

Mental health problems have no significant impact on taxonomic composition in PD patients. Beta diversity analyses were performed to investigate the impact of mental health factors on microbial community composition. No significant difference was observed in

microbial diversity for people with or without anxiety and poor sleep in both PD and healthy control cohorts (Table 1). In addition, anxiety had no significant impact on microbial diversity in the PD cohort, while more variation was seen in the control cohort (Table 1). We also showed that PD disease status is a major driver for beta diversity differences across all distance matrices (Table 1).

TABLE. 1 Mental health problems have no significant impact on microbial diversity in PD patients. PD disease status had the most significant impact on microbial diversity, while depression, anxiety and sleep problems show no significant impact. Beta diversity analysis was performed across all four variables (PD disease status, depression, anxiety, and sleep problems) using four matrices. The PERMANOVA test was used to determine the significance of each variable (**$p \leq 0.001$, *$p \leq 0.05$).

	Jaccard	Bray Curtis	Unweighted Unifrac	Weighted Unifrac
PD vs. Healthy Control	0.001**	0.001**	0.001**	0.038*
PD				
Depression	0.82	0.94	0.922	0.56
Anxiety	0.989	0.992	0.997	0.974
Sleep Problems	0.075	0.178	0.164	0.646
Control				
Depression	0.92	0.413	0.504	0.564
Anxiety	0.059	0.028*	0.191	0.115
Sleep Problems	0.082	0.205	0.824	0.337

Taxa bar plots were generated to investigate microbial diversity composition at the phylum level for different mental health problems in both PD and control cohorts. The phyla of *Bacteroides* and *Firmicutes* were enriched across all mental health variables (Figure 1). The *Verrucomicrobiota* phylum was present in PD patients without depression but not in PD individuals with depression (Figure 1A). In contrast, similar taxonomic composition was observed in PD patients with or without anxiety (Figure 1B). The *Verrucomicrobiota* phylum was found in both PD and control cohorts with co-occurring poor sleep (Figure 1C). This suggests that poor sleep might be associated with taxonomic composition changes despite the presence of PD, therefore we generated a genus-level taxa bar plot for the *Verrucomicrobiota* phylum to further investigate poor sleep (Figure 1D). *Akkermansia* was the only genus observed in this phylum, making up around 2% relative abundance in the healthy control cohort with poor sleep, as well as both groups for poor sleep in the PD cohort (Figure 1D). Notably, this phylum was not observed in healthy individuals without sleep problems. Taken together, this suggests that mental health conditions are not the main factor underlying changes in *Verrucomicrobiota* abundance, rather it is PD onset that is associated with the presence of this phylum.

Depression is associated with fewer metabolic pathway alterations in PD patients. As our diversity and taxonomic analyses indicated minimal contributions from depression, we investigated whether depression alters the PD gut microbiota at a functional level. Principal Coordinates Analysis (PCoA) plots were made based on metabolic pathways in depressed versus non-depressed individuals for both PD and healthy control cohorts. In the healthy controls, distinct clusters are observed for depressed and non-depressed individuals (Figure 2A). In the PD cohort however, depressed and non-depressed patients exhibited similar clustering, suggesting the impact of depression on the functional gut microbiota is reduced by co-occurring PD (Figure 2B).

To further investigate the metabolic changes associated with depression, we generated $\log_2$ fold change plots for both PD and control cohorts. Samples from non-depressed individuals were set as the control to identify significantly upregulated or downregulated

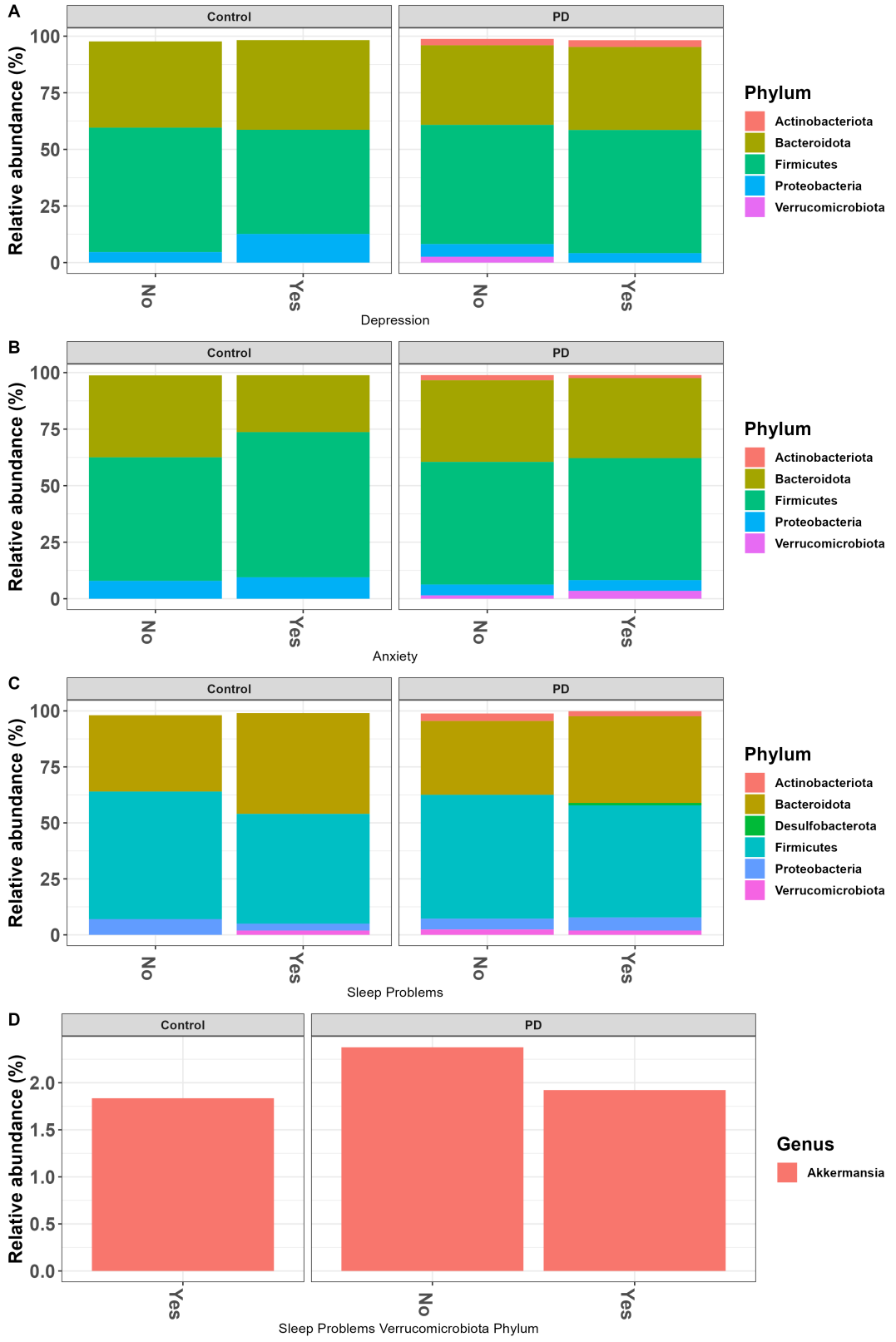

FIG. 1 Depression, anxiety, sleep problems do not affect the taxonomic composition in PD patients. (A-C) Taxa bar plots showing microbial composition between PD and control cohorts with and without depression (A), anxiety (B), and poor sleep (C) at the phylum level. Colour indicates phylum and the height of the bar indicates relative abundance. Differences were not significant between each of the cohorts. (D) Taxa bar plot at the genus level for the phylum *Verrucomicrobiota* for individuals in both cohorts with and without sleep problems.

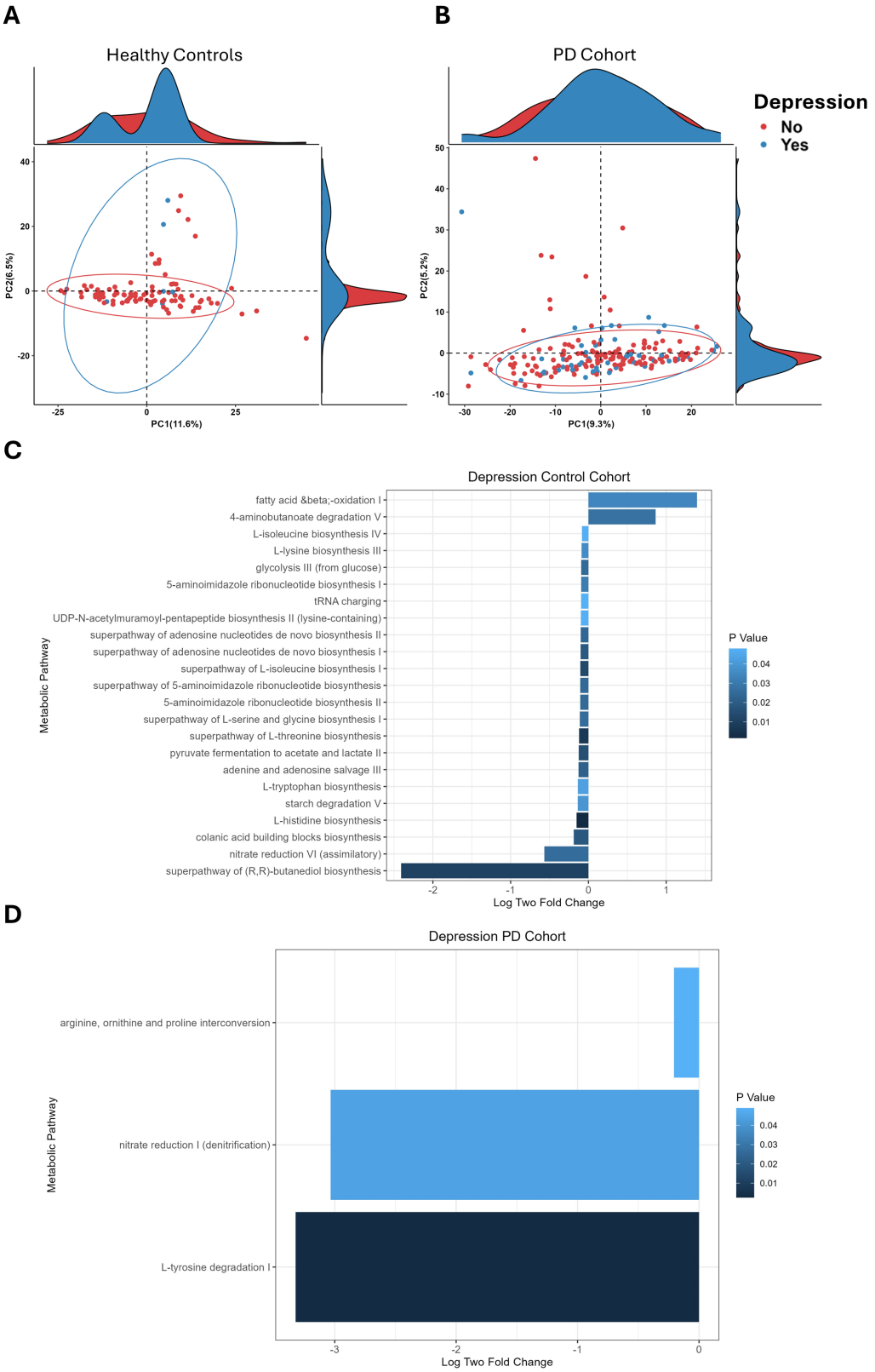

FIG. 2 Depression is associated with fewer metabolic pathway alterations in PD patients. (A-B) Principal Coordinates Analysis (PCoA) plots for control cohort (A) and PD cohort (B) with and without depression showing clustering of predicted metabolic pathways. Colour of dots indicates presence (blue) or absence (red) of depression. (C-D) Log_2 fold change plots for the control cohort (C) and PD cohort (D) showing pathways that are up and downregulated in people with depression. Only pathways with a P value < 0.05 are included. Bar colour shows P-value with darker colours indicating higher significance.

pathways in depressed participants. For the control cohort, depression is correlated with downregulation of 21 different pathways and upregulation of 2 metabolic pathways (Figure 2C). The PD cohort had three significantly upregulated pathway alterations (Figure 2D). There was no overlap in metabolic pathways between the two cohorts (Figure 2C-D). Taken together, this shows that the functional metabolic changes associated with depression are greatly reduced when co-occurring with PD.

Anxiety-associated metabolic changes are similar between PD patients and healthy individuals. To examine the functional effects of anxiety on the gut microbiota, we generated PCoA plots and graphed out significantly altered pathways on $\log_2$ fold plots for PD patients and healthy controls. Distinct clustering can be identified between healthy controls with or without anxiety (Figure 3A). Within the PD cohort, both anxious and non-anxious groups displayed similar metabolic composition (Figure 3B). Despite the distinct clustering pattern

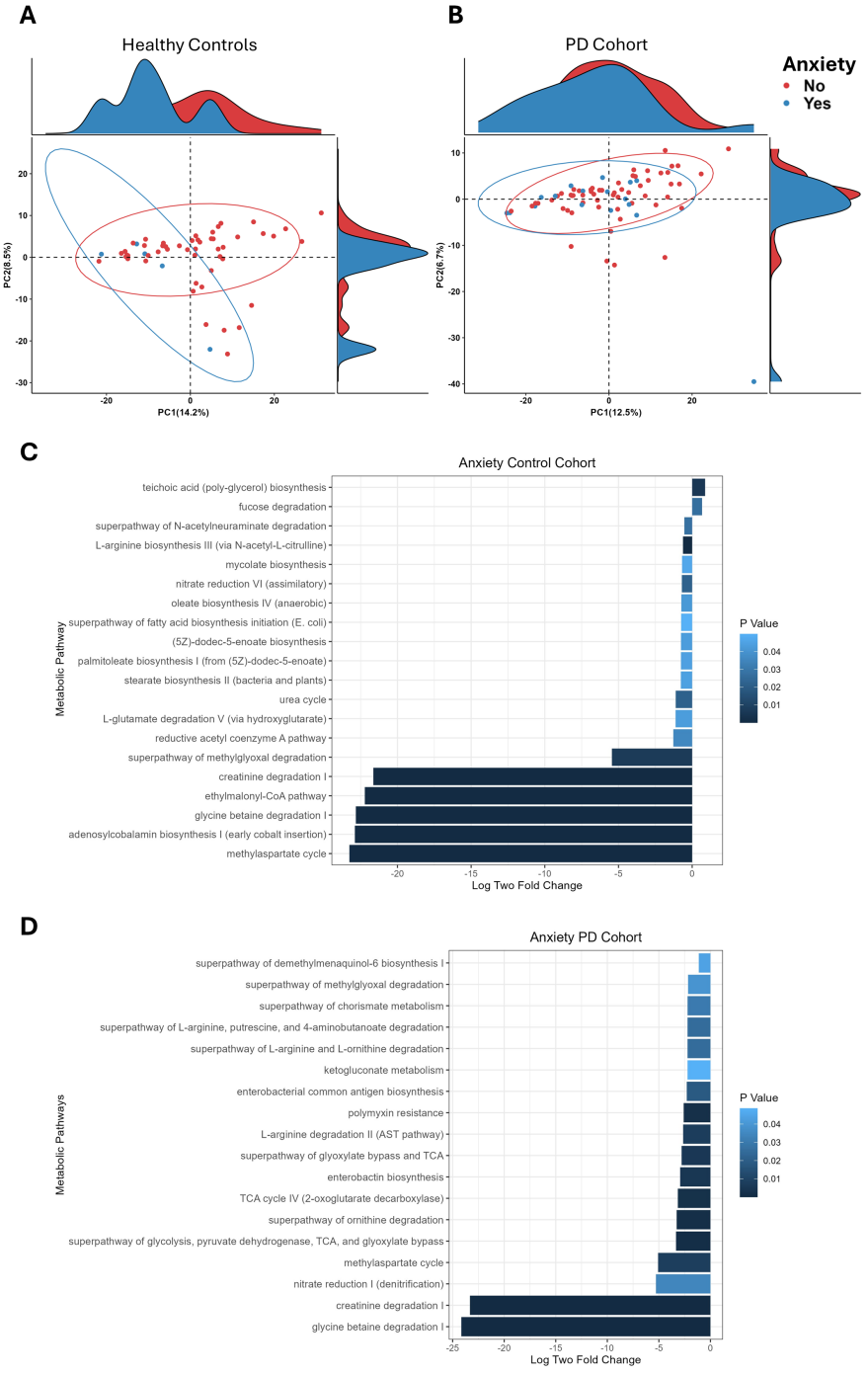

FIG. 3 Anxiety-associated metabolic changes are similar between PD patients and healthy individuals. (A-B) Principal Coordinates Analysis (PCoA) plots for control cohort (A) and PD cohort (B) with and without anxiety showing clustering of predicted metabolic pathways. Colour of the dots indicates presence (blue) or absence (red) of anxiety. (C-D) Log_2 fold change plots for the control cohort (C) and PD cohort (D) showing pathways that are up and downregulated in people with anxiety. Only pathways with a P-value < 0.05 are included. The bar colour shows P-value with darker colours indicating higher significance.

between cohorts on PCoA plots, the overall trend in both $\log_2$ fold change graphs is similar in terms of significant number of pathways detected (Figure 3C-3D). The control cohort had significant alterations in 20 metabolic pathways, compared to 18 in PD patients. No pathways were upregulated in PD patients, with two pathways being slightly upregulated in the controls. The most significantly altered pathways, as well as the magnitude of change for these pathways, was similar between PD and healthy control cohorts. Five pathways were downregulated by more than 10 log units with a p-value < 0.001 in the control group. The PD cohort had two pathways that met this criterion, both of which were found in the five most downregulated pathways for healthy controls. Despite slight differences in the PCoA plots and $\log_2$ fold change graphs, the metabolic alterations appeared more similar than different when examining the impact of anxiety on the functional gut microbiota.

Poor Sleep is associated with fewer changes to gut metabolic pathways in PD patients than healthy individuals. While the taxonomic analysis and diversity metrics also showed that poor sleep had a minimal effect on both healthy and PD cohorts, we generated PCoA plots and $\log_2$ fold change plots to see if there was any effect of sleep on the functional makeup of the gut microbiota. PCoA plots for both healthy and control cohorts showed that those with poor sleep and those without poor sleep have functional metabolic pathways clustered together very tightly. This indicates that the gut microbial metabolic functions between PD and control cohorts are similar in people with poor sleep (Figure 4A-B). While the clustering of the metabolic pathways does not differ significantly between individuals with and without poor sleep for either cohort, there are significant changes to the number of pathways that we see differentially regulated between the control and PD groups. In the control cohort, we see that 59 pathways total are altered, including a wide array of biosynthesis and degradation pathways, with 36 of these being downregulated and 23 being upregulated in control individuals who exhibit poor sleep (Figure 4C). In the PD cohort, we see fewer pathways being altered with 16 total alterations consisting of 10 downregulated pathways and 6 upregulated pathways (Figure 4D). Most of the pathway alterations in the PD cohort are biosynthesis pathways such as queuosine biosynthesis, L-tryptophan biosynthesis, pantothenate biosynthesis and coenzyme A biosynthesis. Both the magnitude and significance of such changes are largest in the downregulated pathways within the control cohort with a $\log_2$ fold change of around 3 for most of these pathways, and a consistently lower p-value than the PD pathway alterations.

DISCUSSION

PD, depression, anxiety, and poor sleep are associated with particular gut microbiota alterations (8–12). In this study, we aimed to identify if there was an association between mental health and PD at both the compositional as well as functional levels. To our knowledge, this is the first study that emphasizes functional investigations on the link between mental health and PD. While few compositional differences were found, functional analysis identified different trends in the metabolic changes associated with PD and each mental health variable of interest.

Based on the beta diversity analyses and taxonomic bar plots, mental health problems such as depression, anxiety, and poor sleep do not affect PD patients' microbiota composition. Previous research showed that the gut microbiota is highly correlated with the pathophysiological mechanisms of mental health through microbiota-gut-brain bidirectional interactions, without considering PD (25, 26). The gut microbiota is postulated to regulate brain functions by either affecting the synthesis and metabolism of neurotransmitters or generating neurotransmitters by themselves, thereby acting as a potential contributor to mental health problems (25). Previous research also explored the effect of depression on gut microbiota, and significantly higher microbial diversity was observed in the fecal samples of patients with major depressive disorder compared to healthy controls (27). Furthermore, previous research shows that the gut microbial composition of Generalized Anxiety Disorder (GAD) patients was also altered compared to healthy control (28, 29). Similarly, participants with sleep deprivation exhibited significantly reduced microbial diversity as assessed by beta diversity analysis (30). The aforementioned findings were not carried out in comparison to PD patients. Our research in this present study showed that, in the context of PD, there is no correlation between depression, anxiety, or poor sleep with gut microbiota compositional

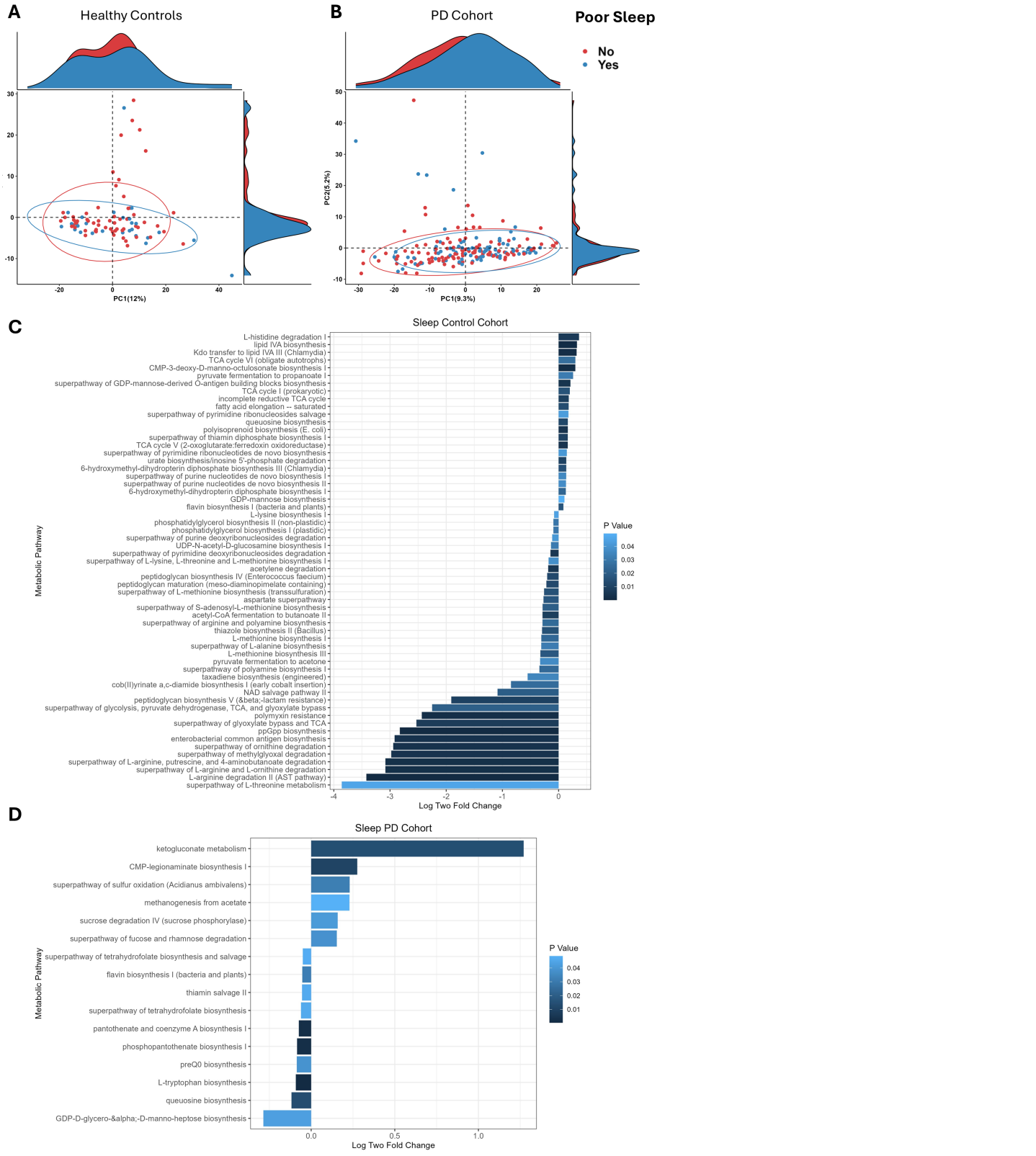

FIG. 4 Sleep problems are associated with fewer changes to gut metabolic pathways in PD patients than in healthy individuals. (A-B) Principal Coordinates Analysis (PCoA) plots for control cohort (A) and PD cohort (B) with and without sleep problems showing clustering of predicted metabolic pathways. Colour of dots indicates presence (blue) or absence (red) of sleep problems. (C-D) Log_2 fold change plots for the control cohort (C) and PD cohort (D) showing pathways that are up and downregulated in people with sleep problems. Only pathways with a P value < 0.05 are included. Bar colour shows P value with darker colours indicating higher significance.

alterations (Figure 1A, 1B, 1C). This suggests that PD may dampen the effect of mental health problems on the gut microbiota.

Another difference between our study and previous investigations is the presence of *Verrucomicrobiota* in PD and healthy control cohorts. *Verrucomicrobiota* was previously found to increase significantly in patients with severe PD compared to healthy controls and patients in earlier stages of the disease. In our study, we found comparable levels of *Verrucomicrobiota* abundance in healthy controls with poor sleep, and both groups in the PD cohort (31, Figure 1D). The presence of *Verrucomicrobiota* in the control cohort could be attributed to the fact that 43 control participants are the spouses of PD patients. These individuals may have similar gut microbiota relative to non-spouse controls due to comparable environmental exposure and leading similar lifestyles (6, 32). *Akkermansia,* the only genus representing the *Verrucomicrobiota* phylum, may contribute to the boost of "gastrointestinal barrier function and anti-inflammatory immune stimulation" previously found in literature (33).

To summarize the impact of mental health on the composition of the PD gut microbiota, we found that PD disease status, as opposed to mental health conditions, is the main driving factor for differences in microbial composition between PD and healthy control cohorts (Table 1, Supplementary Figure 2 & 3). This finding is not surprising given that previous research showed that PD is associated with a distinct shift in the gut microbiota, whereas few investigations have linked mental health to the PD gut microbiota.

While our study found no significant differences for depressed controls compared to depressed PD patients, our PICRUSt2 analyses indicated that PD reduces the functional changes associated with depression. Distinct clusters are observed for depressed individuals compared to those without PD in the healthy control cohort. When depression co-occurs with PD, the two clusters for depressed versus non-depressed individuals appear functionally similar, suggesting the volatile microbiota of PD hides any functional alterations observed in healthy individuals with depression.

Beyond the number of altered pathways, the types of functional alterations appear distinct between healthy controls and PD patients. Neither cohort presented with the characteristic increase in proteolytic pathways accompanied by decreased short-chain fatty acid and reduced carbohydrate fermentation as mentioned in literature (4, 6, 7). The control depression cohort had several metabolic alterations that have been previously established as contributors to depression. Fatty acid and beta oxidation is enriched in the control cohort. While there is no consensus on whether fatty acid metabolism is upregulated or downregulated, lipid metabolism is correlated with depression scores (34–36). Moreover, the second most enriched pathway in the control cohort, 4-aminobutanoate degradation V, is a key player in depression pathophysiology. 4-aminobutanoate is more commonly known as gamma-aminobutyric acid (GABA). This neurotransmitter is deficient in individuals with depression, thus it is not surprising to see an enrichment of GABA degradation when comparing the functional profile of non-depressed healthy individuals to those with depression (37, 38). PD has shown dysregulation of the GABAergic system of neurotransmission in addition to lipid metabolism alterations, however neither of these two pathways were significantly different enough in the PD cohort to be detected with a p-value of 0.05 (39).

Within the PD cohort, the most significant metabolic pathway alteration is decreased L-tyrosine degradation. This is characteristic of PD as the expression of tyrosine hydroxylase, an enzyme that breaks down L-tyrosine to a dopamine precursor, is preferentially lost in the dopaminergic neurons of the substantia nigra (40, 41). Depression does not have clear associations with L-tyrosine. Depleting L-tyrosine alters dopaminergic activity; however, it does not improve perceptions of mood amongst depressed individuals (42). L-tyrosine degradation was not among the significantly altered metabolic pathways for healthy controls. Interestingly, no associations for nitrate reduction or superpathway of (R,R)-butanediol biosynthesis were found in literature for either PD or depression, despite these pathways showing $\log_2$ fold changes that were greater compared to the other pathways in each respective cohort. Altogether, the PCoA plots and $\log_2$ fold plots suggest that PD masks the functional alterations that are typically observed in depression. The metabolic pathways that

have comparatively large $\log_2$ fold changes appear related to either depression or PD, with minimal associations between the two.

In terms of anxiety, functional PICRUSt2 analysis did not show strong evidence for a relationship between PD and anxiety. Based on PCoA plots alone, healthy controls with anxiety appeared to be functionally different from non-anxious participants, whereas PD patients showed minimal differences. This is not conclusive evidence for PD masking anxiety-associated functional alterations, as the $\log_2$ fold change plots show a similar trend overall in pathway changes for both cohorts. The absence of statistical testing, combined with the small sample size of anxious healthy controls (n=5) used to generate the PCoA plots, indicates the distinct clustering in the PCoA plots may not be a true underlying difference that is lost when anxiety co-occurs with PD. This observation is further supported by the most significantly altered pathways having a change of more than 20 log units for both cohorts in addition to p-values < 0.01. For comparison, in our PICRUSt2 analysis on depression and sleep the most significant pathway changes did not exceed a magnitude of 5 log units. The consistency in the overall trend of pathway alterations, in addition to the magnitude of change suggests that regardless of whether anxiety co-occurs with PD, the associated functional alterations remain similar.

The pathways that are altered in anxiety do not show the characteristic enrichment of protein related pathways with downregulated carbohydrate metabolism that is reported in PD literature (6). The five pathways most significantly associated with anxiety in control participants include the methylaspartate cycle, adenosylcobalamin biosynthesis I, glycine betaine degradation I, ethylmalonyl-CoA pathway, and creatinine degradation I. In the PD cohort, glycine betaine degradation I and creatinine degradation I remain the two most significant pathway alterations. With the exception of glycine betaine degradation, none of the above pathways appear consistently implicated in anxiety nor PD. Glycine betaine (GB), otherwise known as trimethylglycine or betaine, has broad roles in neurodegenerative, cardiovascular, and renal diseases (43). Downregulation of GB metabolism is linked to accumulation of tau proteins and amyloid-β, both of which are classical characteristics of Alzheimer's pathology, although increasing evidence suggests these proteins are connected to PD (44–46). GB is involved in GABA production and recycling. In stress-related disorders such as depression, anxiety, and post-traumatic stress disorder, GABAergic neurotransmission is dysregulated (47). The large $\log_2$ fold change observed for both cohorts in GB degradation suggests this pathway may be a key functional change associated with anxiety, however given this pathway is a feature of PD as well, no conclusions can be drawn as to whether glycine betaine degradation is solely associated with PD or anxiety.

From PICRUSt2 analysis on both the control and PD cohorts that aimed to investigate the impact that poor sleep has on metabolic function and diversity, we see that the metabolic pathways observed are altered in both groups, although more pathways are differentially regulated in the control cohort than in the PD one. The reduced number of pathway alterations in the PD cohort compared to the healthy control cohort for individuals with poor sleep suggests that there is some component of PD pathology or some confounding variable within the PD cohort that dampens the impact of poor sleep on the metabolic function of the PD gut microbiota. An example of such a confounding variable may be the use of Parkinson's medication such as Levodopa, which has been linked to gastrointestinal dysfunction and altered gut microbiota composition in rat models (48).

Looking at the specific pathways that were altered by the presence of poor sleep in the PD cohort, the most upregulated pathway is ketogluconate metabolism. This precedes the pentose phosphate pathway, and one study has found a link between pentose phosphate pathway dysfunction and neurodegeneration similar to PD, although the specifics of this relationship are unknown and the pentose phosphate pathway dysfunction in the study was glucose-6-phosphate dehydrogenase-mediated (49). This may suggest that poor sleep in individuals with Parkinson's can accelerate the progression of the disease by increasing the rate of neurodegeneration, although no causal relationship can be determined from this analysis and future work would need to be done in order to determine the accuracy of this prediction. We also see the downregulation of L-tryptophan biosynthesis in the PD cohort with poor sleep, which is a precursor to serotonin, a neurotransmitter. Multiple studies have investigated the relationship between serotonin dysregulation and Parkinson's, and have

concluded that it is linked to the development of both motor and non-motor symptoms (50, 51). Again, this may suggest that poor sleep in individuals with Parkinson's could contribute to the worsening of disease symptoms, this time due to changes in serotonin levels rather than alterations to levels of ketogluconate metabolism and subsequent neurodegeneration.

Limitations The sex ratios in the PD and healthy control cohorts of this study are unbalanced. Parkinson's disease predominantly affects male individuals in a 2:1 ratio, and the PD cohort in Cirstea et al.'s analysis is no exception (6). 62% of the PD cohort was male, but over half of the healthy controls were female. The recruitment method for healthy controls further exacerbated this imbalance. Nearly half of the 103 healthy controls were the spouses of participating PD patients, and as PD mostly affects males, the composition of the control group was skewed towards females. Cirstea et al. found that sex had no major impact on the composition of the PD gut microbiota, but our present investigation was on functional alterations, and we did not account for this disproportionate sex ratio (6). Furthermore, our three mental health variables of interest predominantly affect females, with a male to female ratio of 1:2 (8-10). Given the functional changes we observed in our analysis were done by comparing healthy controls to PD patients, our findings may be overturned if both PD and healthy control cohorts had equal sex ratios.

The recruitment of spouses as healthy controls is another limitation beyond unbalanced sex ratios. Spouses likely share similar diets, lifestyles, and living environments as PD patients. While the PD microbiota has highly distinct changes, the gut microbiota alterations associated with depression, anxiety, and poor sleep are much more variable (8-11). These environmental factors may not mask the PD microbiota, but they may hide gut microbiota changes associated with mental health. The data in this analysis is sourced entirely from the UBC Pacific Parkinson's Research Centre. It is highly likely that healthy controls live in similar environments, making it difficult to detect mental health related gut microbiota alterations when comparing individuals with to those without depression, anxiety, or poor sleep. Healthy was defined as the absence of PD, but the controls used in this study could have other co-morbidities that mask the gut microbiota associated with our three variables of interest, especially considering that mental health produces more subtle microbiota changes.

Sample size is another major limitation of our study. The sample size of healthy controls with anxiety or depression is comparatively low after filtering out observations with NAs and binning the metadata. Only 5 healthy control individuals had anxiety, and only 6 had depression. Having such a small sample size greatly limits power in statistical analysis. Furthermore, small sample size contributes to increased uncertainty in interpreting the PICRUSt2 PCoA plots, of which had no statistical tests done. We had to deviate from the precise scales defined in the original paper for depression and anxiety in order to retain some individuals with anxiety and depression for our healthy control cohort. Moreover, by binning our categories of interest into a binary output of "Yes" or "No", we lose information that is associated with having numerical scores for depression and anxiety.

Our study is limited by confounding variables. One of the major known confounding variables is antidepressant drug usage, which is linked to changes in gut microbiota composition that are unique to PD patients compared to controls with depression only (53). Furthermore, PD medication usage such as Levodopa is another major confounding variable that causes gastrointestinal dysfunction. We did not control for these variables in our study due to sample size limitations. Again, as this dataset was sourced from the UBC Pacific Parkinson's Research Centre, most study participants are from British Columbia, thus our results in this paper cannot be generalized for all PD patients.

There are some limitations inherent to PICRUSt2. 16S rRNA Gene Amplicon Sequencing approach detects alterations in community diversity on a large time scale. However, compared to the metagenomic data, the 16S rRNA gene has limited resolution among phylogenetically proximate species, lower sensitivity, and PCR amplification biases (52). Any sequences or pathways that are not yet documented cannot be identified through the tools we utilized in this study. That said, this is the nature of our analysis, and these limitations apply in any context where inferential tools are used.

Conclusions The main motivation of this study was to investigate the impact of mental health problems such as depression, anxiety, and poor sleep on PD patients. Based on the diversity analysis, as well as the statistical analysis for each research parameter, our mental health factors of interest do not contribute to compositional microbial alterations. Functional metabolic pathway analysis inferred that the impact of depression on the functional gut microbiota is reduced by concurrent PD. The functional impacts of anxiety are still observed to some extent regardless of disease status, although there is a slight reduction in metabolic diversity in PD patients. For sleep, metabolic functional analysis suggests that having poor sleep might accelerate PD progression. However, this study cannot draw a direct causational conclusion on whether poor sleep would drive the acceleration of PD progression. From the results generated in this study, we conclude that the impact of mental health problems such as depression, anxiety, and poor sleep on compositional and functional metabolic alterations is dampened by PD.

Future Directions As mentioned in the study limitations, the data collected for the original study was not gathered specifically for studying the microbiome alterations and functional changes of mental health problems in PD patients. As a result, our study had unbalanced sample sizes for mental health parameters in the healthy control group, as well as unbalanced gender ratios in both PD and control cohorts. To address this, future research could be designed to target PD patients and their mental health problems for validation of our current study results. Future downstream analysis should employ well-defined depression and anxiety scales that are not binary, with a large enough sample size to divide participants into cohorts while allowing adequate detection of potential alterations in PD patients when compared to healthy controls. Moreover, future studies should aim to include a more diverse group of healthy controls. Healthy spouses are an excellent control to characterize what differences exist in the PD gut microbiota as they likely share similar lifestyles to PD patients, but a more diverse group is needed for thorough investigation on the impact of mental health.

Due to limited sample size, we were unable to control confounding variables, despite our dataset having many known and potential confounders. Beyond controlling confounding variables, future studies could carry out functional analysis on related mental health variables such as antidepressant or levodopa usage. Functional data may provide more insight into PD development that compositional data cannot capture.

Further research can also be carried out on the significantly altered pathways we identified in our present study. Glycine betaine degradation is significantly downregulated in both PD and healthy control cohort with concurrent anxiety. GB degradation has been linked to PD and anxiety separately, but no studies have examined the relationship between this pathway in PD patients with co-occurring anxiety. This pathway had a very large change in log units for both cohorts, thus further studies can conduct comparative analysis across anxiety, PD, and healthy control cohorts to better understand the role of GB degradation in PD when co-occurring with anxiety. To further explore the implications of the ketogluconate metabolism pathway that is associated with poor sleep, future research can examine the interactions between ketogluconate metabolism and the pentose phosphate pathway that could potentially be contribute to neurodegenerative mechanisms in PD patients with poor sleep. Such an investigation could provide therapeutic implications for improving sleep quality of PD patients, as poor sleep has the highest correlation with PD development out of all our mental health variables of interest. Ultimately, a deeper understanding of the impact of concurrent mental health conditions will provide more effective care for individuals with PD.

Data availability. All code used to generate data is available here: https://github.com/zhang17s/MICB475-Parkinsons-Mental-Health.git

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