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# Dietary vitamin B1, B2, and B6 intake influence the microbial composition and functional potential of the gut microbiome in Parkinson's disease

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**SUMMARY** Parkinson's disease (PD) is the second-most common neurodegenerative disorder of the central nervous system, and its prevalence is on the rise. Gut microbiome dysbiosis has been linked to PD symptoms and pathogenesis, while certain nutrients (polyunsaturated fatty acids (PUFAs), saturated fatty acids (SFAs) and vitamins A, B1, B2, B3, B6, B12, C, D, and E) have shown protective effects against the disease. However, whether nutrient intake improves PD outcomes by alleviating gut microbiome dysbiosis remains unclear. In this study, we investigated whether dietary intake of the nutrients described above was associated with the microbial composition and functional potential of the PD gut microbiome. We analyzed the fecal microbiome of 285 participants with (n=184) or without (n=101) PD using 16S rRNA sequencing and compared microbiome composition to reported dietary nutrient intake. We found that high vitamin B2 intake is associated with increased Faith's phylogenetic diversity of the PD gut microbiome. Vitamin B1 intake is associated with changes in both PD and non-PD microbiome composition, while vitamin B2 and B6 intake are uniquely associated with changes in PD microbiome composition. Further investigation found that low vitamin B1, B2, and B6 intake are associated with changes in the functional potential of the PD gut microbiome, with the potential to aggravate PD pathology. Our findings suggest that vitamin B1, B2, and B6 could play a role in remediating the PD gut microbiome, with the potential to also treat PD symptoms and progression via the gut-brain axis.

## INTRODUCTION

Parkinson's disease (PD) is the second-most common neurodegenerative disorder of the central nervous system, and its incidence is increasing worldwide (1). PD is characterized by aggregation of alpha-synuclein proteins (Lewy bodies), leading to the loss of dopaminergic neurons in the brain (2, 3). Motor symptoms of PD are attributed to the loss of dopaminergic neurons in the basal ganglia (4), and include rest tremor, bradykinesia, rigidity, and gait disorder (5). Non-motor symptoms of PD–which include cognitive impairment, sensory disturbances, sleep disorders, and gastrointestinal dysfunction (6)–can similarly impair a patient's quality of life (1).

Gut microbiome dysbiosis has been associated with PD symptoms and pathogenesis, particularly through microbiota linked to gastrointestinal inflammation and neuroinflammation (7). It has been shown that the gut microbiota of PD patients can differ significantly from that of neurologically healthy controls (3). More specifically, PD gut microbiota composition is characterized by depletion of short-chain fatty acid (SCFA) producers, such as butyrate producers from the genera *Blautia*, *Roseburia*, *Prevotellaceae*, *Faecalibacterium*, and *Coprococcus* (in the family *Lachnospiraceae*) (7–10). This depletion

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of SCFA producers can lead to inflammation in PD, as SCFAs possess anti-inflammatory properties and butyrate specifically contributes to intestinal wall integrity (11).

At the phylum level, the PD gut microbiome is associated with increased abundances of Firmicutes and decreased abundances of Bacteroidetes (3). Enrichment of the family *Verrucomicrobiaceae* and the genera *Lactobacillus*, *Akkermansia*, *Methanobrevibacter*, and *Bifidobacterium* are also found in the PD gut microbiome (2, 5). Several of these microbes are associated with gastrointestinal disturbances observed in PD: *Akkermansia* is associated with an impaired intestinal barrier, while *Firmicutes*, *Akkermansia* and *Methanobrevibacter* are associated with constipation (3, 7, 12). Some strains of *Lactobacillus* possess enzymes that can break down the PD drug levodopa into dopamine, suggesting their enrichment in patients with PD might be the result of using this medication (7).

Previous studies have shown that supplementation of various vitamins can reduce the risk, symptoms, and/or progression of PD. High doses of vitamin B1 and B2 have been found to promote the recovery of motor functions in PD patients (13, 14). Certain PD symptoms, such as fatigue and sleep dysfunction, can be relieved by increasing B3 dosage (15). Studies report that high dietary intake of vitamin B6 can significantly decrease the risk of PD (16, 17). Although vitamin B12 does not appear to be significantly associated with PD progression, it is associated with lower risk of developing sensory symptoms (18).

Since oxidative stress is a contributing factor to dopaminergic neuronal degeneration in PD, antioxidants such as vitamin E have shown potential for neuroprotective therapy in PD (19). Additionally, high dietary intake of vitamin E is associated with reduction in PD risk (19). Vitamin C has similarly been proposed as a treatment to PD neurodegeneration, with the potential to relieve oxidative stress (20). However, its effect in PD remains controversial: a study by Hughes et al. (21) showed vitamin C intake can significantly decrease risk of PD, while other studies (19, 22) did not support a significant preventive effect.

Dietary vitamin D intake is associated with lower PD risk (23), and metabolites of vitamin D2 (synthesized from diet) are also inversely associated with PD risk (24). The impacts of vitamin A on PD have also been proposed, since vitamin A and its equivalents are found to be involved in signal transduction of the dopaminergic system, which constitutes a pathway for PD development (25). However, a meta-analysis suggests current published data are not sufficient to draw a definite conclusion about the association between dietary vitamin A and PD risk (26).

Polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs) are suggested to play a role in PD development. A diet high in PUFAs is associated with a lower risk for PD (27). Additionally, a diet high in PUFAs decreases the correlation between PD and exposure to neurotoxins such as pesticides, while a diet high in SFAs increases this correlation (28). These results show that PUFAs and SFAs seem to be playing a role in modifying the effects of neurotoxins on PD development (27, 28). PUFAs have also been shown to exhibit anti-inflammatory properties, and may reduce alpha-synuclein accumulation (28). Although these studies suggest neuroprotective properties of PUFAs in PD patients, the potential ability of PUFAs and SFAs to modulate the PD microbiome has yet to be explored.

In this study, we analyzed a dataset originally collected by Cirstea et al. to assess the association between microbiota composition, systemic microbial metabolites, and gastrointestinal disturbances in PD (8). This cross-sectional cohort study collected fecal samples and serum from 300 participants (197 PD patients and 103 non-PD controls). In addition to biological samples, the researchers collected information on PD symptoms, depression/anxiety symptoms, medications, diet, and demographics (8). Recent studies have used this dataset to explore associations between the PD gut microbiome and caffeine consumption, antibiotic use (29), dietary fibre intake (30), alcohol consumption, and body mass index (BMI) (31). These investigations concluded that fiber intake is positively correlated with PD microbiome diversity (30), and the family *Veillonellaceae* is more abundant in overweight PD patients (31). Notably, these studies suggest an unexamined facet of the dataset: how dietary nutrient intake impacts the PD gut microbiome.

Since the gut microbiome can contribute to PD symptoms and pathogenesis (2), it raised our interest to examine the association between dietary nutrient intake and gut microbiome composition in PD patients. While the mechanism by which diet reduces PD symptoms and risk is unclear, dietary modulation of the gut microbiome has the potential to treat gut

symptoms and microbiome dysfunction associated with PD. The possibility of remediating the PD gut microbiome through diet can also potentially treat PD symptoms and progression through the gut-brain axis (28).

In this study, we decided to assess how nutrient intake–specifically PUFA, SFA, and vitamins A, B1, B2, B3, B6, B12, C, D, and E intake–is associated with the microbial composition and functional potential of the PD gut microbiome. We chose to investigate these nutrients given the results of previously published studies describing their impact in PD risk, symptoms, and/or progression (13-28).

Understanding the dietary factors that potentially shift the PD gut microbiome towards a neurologically healthy phenotype can aid in developing new therapies for the disease. In this study, we found that high vitamin B2 intake is associated with increased Faith's phylogenetic diversity in the PD gut microbiome. Vitamin B1 intake is associated with changes in PD and non-PD gut microbiome composition, while vitamin B2 and B6 intake are uniquely associated with alterations to PD microbiome composition. We also found that low vitamin B1, B2, and B6 intake are associated with changes in the functional potential of the PD gut microbiome, with the potential to aggravate symptoms and progression of PD. Our findings suggest that vitamin B1, B2, and B6 could play a role in remediating the PD gut microbiome, with the potential to also treat PD symptoms and progression via the gut-brain axis.

## METHODS AND MATERIALS

**Sample and experimental data collection**. The dataset analyzed in this study was acquired from Cirstea et al. (8). The researchers collected fecal samples and serum from 300 participants (197 PD patients and 103 non-PD controls). For fecal microbiome analysis, the bacterial 16S rRNA V4 region was PCR-amplified using barcoded 515F/806R primers, and sequenced on an Illumina MiSeq platform (8). Diet was reported using the EPIC-Norfolk Food Frequency Questionnaire (FFQ) (32), then converted into daily nutritional intake for 46 nutrients and 14 food groups by FETA (32, 33). Further information about sample collection and experimental methods can be found in the original study (8).

**Nutrient stratification and sample filtering**. The package tidyverse was used for data manipulation (34); readxl (35) and xlsx (36) for data import and export (respectively); and ggplot2 for data visualization (37) in RStudio (v2021.09.0) (38, 39). Nutrient intake (PUFAs, SFAs, and vitamins A, B1, B2, B3, B6, B12, C, D, and E) was stratified into "low," "moderate," or "high" based on the nutrient intake distributions of the non-PD group (38, 39). The first (lowest) quartile of non-PD nutrient intake was defined as "low" intake; the interquartile range was defined as "moderate" intake; and the fourth (upper) quartile was defined as "high" intake. Any samples with "N/A" values for nutrient intake were filtered out of the metadata and manifest, with 285 samples retained after filtering (184 PD patients and 101 non-PD controls).

**Alpha and beta diversity analysis**. The demultiplexed sequences of 285 fecal samples were denoised using DADA2 (40) in QIIME2 (v2021.4) (41). Reads had high mean quality scores (>Q30) throughout the sequence, and were thus not trimmed (retained at 251 nucleotides) prior to the denoising step. Mitochondrial and chloroplast sequences were filtered out of the resulting feature table. In order to perform diversity analysis and taxonomic classification, multiple sequence alignment of the filtered feature table was performed in MAFFT (42) and converted into a rooted tree using FastTree (43) in QIIME2 (41). Alpha rarefaction curves (observed features plotted against sequencing depth) were generated using the filtered feature table. Since the rarefaction curves based on disease status and nutrient intake plateaued at ~6500 sequences/sample, samples were alpha-rarified to 7000 sequences/samples for alpha and beta diversity analysis.

Alpha diversity (Faith's phylogenetic diversity, Shannon's diversity, and Pielou's evenness) and beta diversity (weighted UniFrac and unweighted UniFrac) were analyzed in QIIME2 for PD and non-PD, and for each stratified nutrient intake level (low/moderate/high) in PD and non-PD (41). Faith's metric was chosen as an alpha diversity measure that considers phylogenetic differences, Shannon's metric as an alpha diversity measure that considers microbial abundances, and Pielou's metric as a measure of community evenness.

Pairwise Kruskal-Wallis testing in QIIME2 was used to compare differences in alpha diversity by nutrient intake and disease status (41). Variables with significant results in Kruskal-Wallis testing ($q < 0.05$) were funneled into post-hoc Dunn's pairwise testing, which used the FSA package (44) in RStudio (38, 39) to determine which group (PD/non-PD, low/moderate/high nutrient intake) was associated with increased alpha diversity. For both Kruskal-Wallis and Dunn's testing, p-values were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method.

For beta diversity, weighted UniFrac was chosen as a measure that considers both microbial abundances and phylogenetic diversity, and unweighted UniFrac as a measure that considers only phylogenetic diversity (more sensitive to low-abundance features). The multivariate adonis test was used to compare differences in beta diversity by nutrient intake and disease status, as well as the interaction between the two variables via the vegan package (45) in RStudio (38, 39). Groups with significant results in the adonis test ($p < 0.05$) were funneled into a pairwise PERMANOVA in QIIME2 to confirm whether independent analysis of each variable (PD vs. non-PD, low vs. high nutrient intake) was associated with changes to beta diversity of the gut microbiome (41). P-values for the pairwise PERMANOVA were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method.

**Differential microbial abundance analysis**. Using QIIME2 (41), ASVs (filtered to remove mitochondrial and chloroplast sequences) were taxonomically classified by a naïve Bayes machine-learning classifier that was pre-trained to sort taxa in the SILVA 138 99% identity reference set (45-47). The package tidyverse was used for data manipulation (34); ape for phylogenetic tree manipulation (48); phyloseq for data processing and calculating relative abundance (49); DESeq2 for differential abundance analysis (50); and ggplot2 for data visualization (37) in RStudio (38, 39). Data was filtered to a sampling depth of 7000 sequences/sample and relative abundance was then calculated for each taxa. Relative microbial abundances in PD vs. non-PD were expressed as a proportion of all genera in the dataset, while relative abundances in low vs. moderate vs. high nutrient intake were expressed as a proportion of all genera in either the PD or non-PD microbiome. Taxa below a relative abundance of 0.1% were removed. For differential abundance analysis in DESeq2 (50), microbial abundances (at the phylum and genus level) were compared between the PD vs. non-PD microbiome, and between low vs. high intake of vitamin B1, B2, and B6 in the PD or non-PD microbiome. Differential microbial abundances were compared and significance was assessed by the Wald test, with p-values corrected for multiple testing ($p_{adj}$) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Statistical significance of the identified genera was defined by the adjusted p-value ($p_{adj} < 0.05$), with no $\log_2$(fold change) cut-off.

**Taxonomic classification**. Taxonomic classification of the microbiome was performed to identify taxa unique to or missing from the PD microbiome, as they are filtered out prior to differential abundance analysis. Using QIIME2 (41), ASVs (filtered to remove mitochondrial and chloroplast sequences) were classified by a naïve Bayes machine-learning classifier that was pre-trained to sort taxa in the SILVA 138 99% identity reference set (45-47). Samples with fewer features than the rarefaction threshold (7000 sequences/sample) were filtered out, and taxonomic abundances were tabulated in QIIME2 (41). Tables of taxonomic abundances at the phylum and genus level were imported into RStudio (38, 39) for analysis, where the package tidyverse was used for data manipulation (34). Taxonomic tables were filtered for phyla and genera missing in either the PD or non-PD group (ie. taxa missing from or unique to the PD microbiome).

**Functional potential analysis**. Microbiome functional potential was inferred from 16S amplicon data in PICRUSt2 (v2.4.1) (51). ASVs (filtered to remove mitochondrial and chloroplast sequences and normalized by predicted 16S copy numbers) were aligned to reference sequences using HMMER (52), then placed on a reference tree using EPA-ng (53) and gappa (54). Sequences belonging to each gene family were counted using castor (55), followed by inference of gene products for each ASV. Finally, gene products defined by their

enzyme classification (EC) numbers (56) were mapped to MetaCyc (57) functional pathways using MinPath (58). STAMP was used to compare functional potential of pathways by disease status (PD/non-PD) or nutrient intake (59). Differences in functional pathways were assessed by Welch's two-sided t-test with 95% confidence intervals.

**Scripts**. Refer to supplemental scripts for detailed commands. Scripts for QIIME2 (script1.sh) and R (script2.R) analysis can be found at https://github.com/hesoru/Sokolovska_et_al_2022.

## RESULTS

**The PD gut microbiome is associated with significant changes in microbial composition and functional potential.** Prior to nutritional analysis, we assessed baseline differences between the PD and non-PD gut microbiome. We performed pairwise Kruskal-Wallis testing to investigate the association of disease status (PD/non-PD) and nutrient intake (low/moderate/high) with alpha diversity of the gut microbiome. Evaluation of disease status showed no significant difference in Faith's phylogenetic diversity, Shannon's diversity, and Pielou's evenness between the PD and non-PD gut microbiome ($q > 0.05$, **Table 1**). We then performed a multivariate adonis analysis to examine whether PD status and/or nutrient intake (as well as their interaction) were associated with

**TABLE. 1 PD is not associated with significant changes in alpha diversity of the gut microbiome.** Pairwise Kruskal-Wallis testing based on Faith's phylogenetic diversity, Shannon's diversity, and Pielou's evenness was performed to determine whether PD status (PD vs. non-PD) is associated with the alpha diversity of the gut microbiome. P-values were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Abbreviations: PD = Parkinson's disease, df = degrees of freedom.

| Pairwise Kruskal–Wallis test: alpha diversity for PD vs. non-PD | | |
|---|---|---|
| **Alpha diversity metric** | **H (df=1)** | **q** |
| **Faith's phylogenetic diversity** | 1.81 | 0.18 |
| **Shannon's diversity** | 0.72 | 0.39 |
| **Pielou's evenness** | 0.21 | 0.64 |

compositional changes to the gut microbiome (**Table 2**), which revealed a significant difference between PD and non-PD gut microbiome composition (weighted/unweighted UniFrac, $p < 0.05$). Independent analysis of beta diversity by disease status alone was performed via pairwise PERMANOVA, further confirming the dissimilarity between PD and non-PD gut microbiome composition (weighted/unweighted UniFrac, $q < 0.05$, **Table 3**).

Next we compared abundances of microbial genera in the PD vs. non-PD microbiome, by differential abundance analysis in DESeq2. Analysis showed that patients with PD possessed increased abundances of the genera *Collinsella, Akkermansia, Bifidobacterium,* and *Oscillibacter* relative to non-PD controls, and decreased abundances of *Faecalibacterium* and *Roseburia* (Wald test, $p_{adj} < 0.05$, **Table 4**). Relative abundance was calculated: defined as the proportion of these microbial genera relative to all genera in the dataset. This allowed us to see that the genera *Faecalibacterium* and *Roseburia* (decreased in PD) occupied a larger portion of the gut microbiome (>0.5% in both PD and non-PD) than other differentially abundant genera (**Figure S1**).

Differential abundance analysis removed taxa with a relative abundance below 0.1%–thus if taxa were missing from either the PD or non-PD microbiome, their abundances would not be compared. We identified taxa missing from and unique to the PD microbiome that escaped differential abundance analysis. Taxonomic classification of the PD and non-PD gut microbiome was performed via a naïve Bayes machine-learning classifier that was pre-trained to sort taxa in the SILVA 138 99% identity reference set. Taxa only found in the PD or non-PD gut microbiome were identified: 2 phyla and 14 genera were missing from the PD gut microbiome (**Table S1**), while 39 genera were unique to the PD gut microbiome (**Table S2**).

Comparisons between the functional potential of the PD and non-PD gut microbiome were inferred based on 16S amplicon data. PICRUSt2 analysis identified 87 significantly differentially abundant pathways in PD (Welch's two-sided t-test, $p < 0.05$, **Figure 1**). The

PD gut microbiome was associated with increased functional potential for multiple pathways involved in fermentation, the citric acid (TCA) cycle, amino acid biosynthesis (especially L-

**TABLE. 2 The adonis test shows PD and all investigated nutrients except for SFAs are associated with significant changes in gut microbiome composition.** The multivariate adonis test based on weighted and unweighted UniFrac was performed to determine whether PD status and/or nutrient intake (as well as their interaction) are associated with compositional changes to the gut microbiome. Groups with significant results ($p < 0.05$) in the adonis test were subsequently funneled into a pairwise PERMANOVA, comparing weighted and unweighted UniFrac distance for PD vs. non-PD (**Table 3**) and low vs. high nutrient intake (in PD and non-PD) (**Table 7**). Bold values emphasize groups with $Pr(>F) < 0.05$ (ie. $p < 0.05$). Analyses with non-significant results for nutrient intake are shaded grey. Abbreviations: PD = Parkinson's disease, df = degrees of freedom, PUFAs = polyunsaturated fatty acids, SFAs = saturated fatty acids.

| Adonis analysis of beta diversity | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Nutrient** | **Analysis variable (Disease status df=1, nutrient intake df=2)** | **Beta diversity metric** | | | | | |
| | | **Weighted UniFrac** | | | **Unweighted UniFrac** | | |
| | | **F** | **$R^2$** | **Pr(>F)** | **F** | **$R^2$** | **Pr(>F)** |
| **PUFAs** | Disease status | **4.82** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | **3.03** | **0.023** | **0.003** | **1.32** | **0.010** | **0.045** |
| | Nutrient Intake*Disease status | 1.47 | 0.011 | 0.15 | 1.08 | 0.008 | 0.26 |
| **SFAs** | Disease status | **4.70** | **0.018** | **0.003** | **2.10** | **0.008** | **0.009** |
| | Nutrient Intake | 0.68 | 0.005 | 0.77 | 1.27 | 0.010 | 0.07 |
| | Nutrient Intake*Disease status | 0.44 | 0.003 | 0.96 | 0.89 | 0.007 | 0.71 |
| **Vitamin A** | Disease status | **4.74** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | 0.71 | 0.005 | 0.72 | **1.41** | **0.011** | **0.032** |
| | Nutrient Intake*Disease status | 1.69 | 0.013 | 0.09 | 0.94 | 0.007 | 0.60 |
| **Vitamin B1** | Disease status | **4.73** | **0.018** | **0.003** | **2.12** | **0.008** | **0.009** |
| | Nutrient Intake | 1.47 | 0.011 | 0.14 | **1.79** | **0.014** | **0.002** |
| | Nutrient Intake*Disease status | 0.71 | 0.005 | 0.73 | 1.22 | 0.009 | 0.108 |
| **Vitamin B2** | Disease status | **4.85** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | **2.24** | **0.017** | **0.02** | **1.74** | **0.013** | **0.004** |
| | Nutrient Intake*Disease status | **3.21** | **0.024** | **0.003** | 1.01 | 0.008 | 0.412 |
| **Vitamin B3** | Disease status | **4.72** | **0.018** | **0.003** | **2.12** | **0.008** | **0.009** |
| | Nutrient Intake | 0.96 | 0.007 | 0.42 | **1.32** | **0.010** | **0.03** |
| | Nutrient Intake*Disease status | 0.78 | 0.006 | 0.63 | 0.89 | 0.007 | 0.72 |
| **Vitamin B6** | Disease status | **4.74** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | 1.41 | 0.011 | 0.16 | **1.77** | **0.013** | **0.004** |
| | Nutrient Intake*Disease status | 0.83 | 0.006 | 0.58 | 1.02 | 0.008 | 0.39 |
| **Vitamin B12** | Disease status | **4.85** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | **2.24** | **0.017** | **0.02** | 1.29 | 0.010 | 0.06 |
| | Nutrient Intake*Disease status | **3.21** | **0.024** | **0.003** | 1.04 | 0.008 | 0.33 |
| **Vitamin C** | Disease status | **4.71** | **0.018** | **0.003** | **2.10** | **0.008** | **0.009** |
| | Nutrient Intake | 0.94 | 0.007 | 0.45 | **1.29** | **0.010** | **0.045** |
| | Nutrient Intake*Disease status | 0.65 | 0.005 | 0.79 | 0.73 | 0.006 | 0.99 |
| **Vitamin D** | Disease status | **4.79** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | **1.88** | **0.014** | **0.04** | 1.26 | 0.010 | 0.07 |
| | Nutrient Intake*Disease status | 1.70 | 0.013 | 0.08 | 1.08 | 0.008 | 0.25 |
| **Vitamin E** | Disease status | **4.75** | **0.018** | **0.003** | **2.11** | **0.008** | **0.009** |
| | Nutrient Intake | 1.70 | 0.013 | 0.07 | **1.39** | **0.011** | **0.03** |
| | Nutrient Intake*Disease status | 0.85 | 0.006 | 0.52 | 0.87 | 0.007 | 0.78 |

**TABLE. 3 Pairwise PERMANOVA by disease status confirms PD is associated with significant changes in gut microbiome composition.** Pairwise PERMANOVA based on weighted and unweighted UniFrac was performed to compare gut microbiome composition in PD vs. non-PD. P-values were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Only variables with significant results ($p < 0.05$) in the adonis analysis (**Table 2**) were analyzed. Bold values emphasize groups with $q < 0.05$. Abbreviations: PD = Parkinson's disease, df = degrees of freedom.

| Pairwise PERMANOVA: beta diversity for PD vs. non-PD | | |
|---|---|---|
| **Beta diversity metric** | **F (df=1)** | **q** |
| **Weighted UniFrac** | **4.73** | **0.002** |
| **Unweighted UniFrac** | **2.10** | **0.003** |

**TABLE. 4 Differentially abundant genera associated with the PD gut microbiome.** Differential microbial abundances were calculated at the genus level relative to the non-PD microbiome using DESeq2. Significance was assessed by the Wald test, with p-values corrected for multiple testing ($p_{adj}$) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Only genera with statistically significant changes are shown ($p_{adj} < 0.05$), with no $\log_2$(fold change) cut-off. Abbreviations: PD = Parkinson's disease.

| Change in Abundance | Genus | $\log_2$(Fold Change) |
|---|---|---|
| **Increased in PD** | *Collinsella* | 0.86 |
| | *Akkermansia* | 1.70 |
| | *Bifidobacterium* | 2.04 |
| | *Oscillibacter* | 0.86 |
| **Decreased in PD** | *Faecalibacterium* | -0.65 |
| | *Roseburia* | -0.94 |

methionine), purine nucleotide/nucleobase degradation, menaquinol (vitamin K2) biosynthesis, and thiamine (vitamin B1) biosynthesis/salvage. The PD microbiome was also associated with decreased functional potential for multiple pathways involved in fatty acid biosynthesis (fatty acid biosynthesis initiation, as well as palmitoleate, stearate, oleate, gondoate, and mycolate biosynthesis); sugar (especially sucrose) degradation; and adenosylcobalamin (vitamin B12) biosynthesis/salvage. Having established the differentially abundant pathways associated with PD (ie. the PD phenotype), we were then able to investigate whether nutrient intake can influence these pathways.

**High vitamin B2 intake is associated with increased Faith's phylogenetic diversity in the PD gut microbiome.** We performed pairwise Kruskal-Wallis testing to investigate whether nutrient intake (low vs. high) was associated with alpha diversity of the PD and non-PD gut microbiome. Vitamin B2 intake was associated with significant changes in Faith's phylogenetic diversity in the PD gut microbiome ($q < 0.05$, **Table 5**). To determine which group (low/moderate/high nutrient intake) was associated with increased alpha diversity, variables with significant results in Kruskal-Wallis testing were funneled into post-hoc Dunn's pairwise testing. Analysis determined that high vitamin B2 intake was associated with increased Faith's phylogenetic diversity in PD (Dunn's test, $q < 0.05$, **Table 6**).

**Vitamin B1 intake is associated with changes in PD and non-PD gut microbiome composition, while vitamin B2 and B6 intake are uniquely associated with changes in PD gut microbiome composition.** Next, we assessed whether nutrient intake was associated with alterations in the beta diversity of the PD and non-PD gut microbiome. The adonis test was used to compare differences in beta diversity by nutrient intake and disease status, as well as the interaction between the two variables. All nutrients except SFAs were associated with changes in gut microbiome composition ($p < 0.05$, **Table 2**). Vitamins B2, B12, D, and PUFAs were associated with changes in weighted UniFrac distance, while vitamins A, B1, B2, B3, B6, C, E, and PUFAs were associated with changes in unweighted UniFrac distance ($p < 0.05$). Groups identified in the adonis test ($p < 0.05$) were funneled into a pairwise PERMANOVA to confirm whether independent analysis of each variable (PD vs. non-PD, low vs. high nutrient intake) was associated with changes in gut microbiome composition. Pairwise PERMANOVA only confirmed that vitamin B1 intake was associated with changes in PD and non-PD gut microbiome composition, and vitamin B2

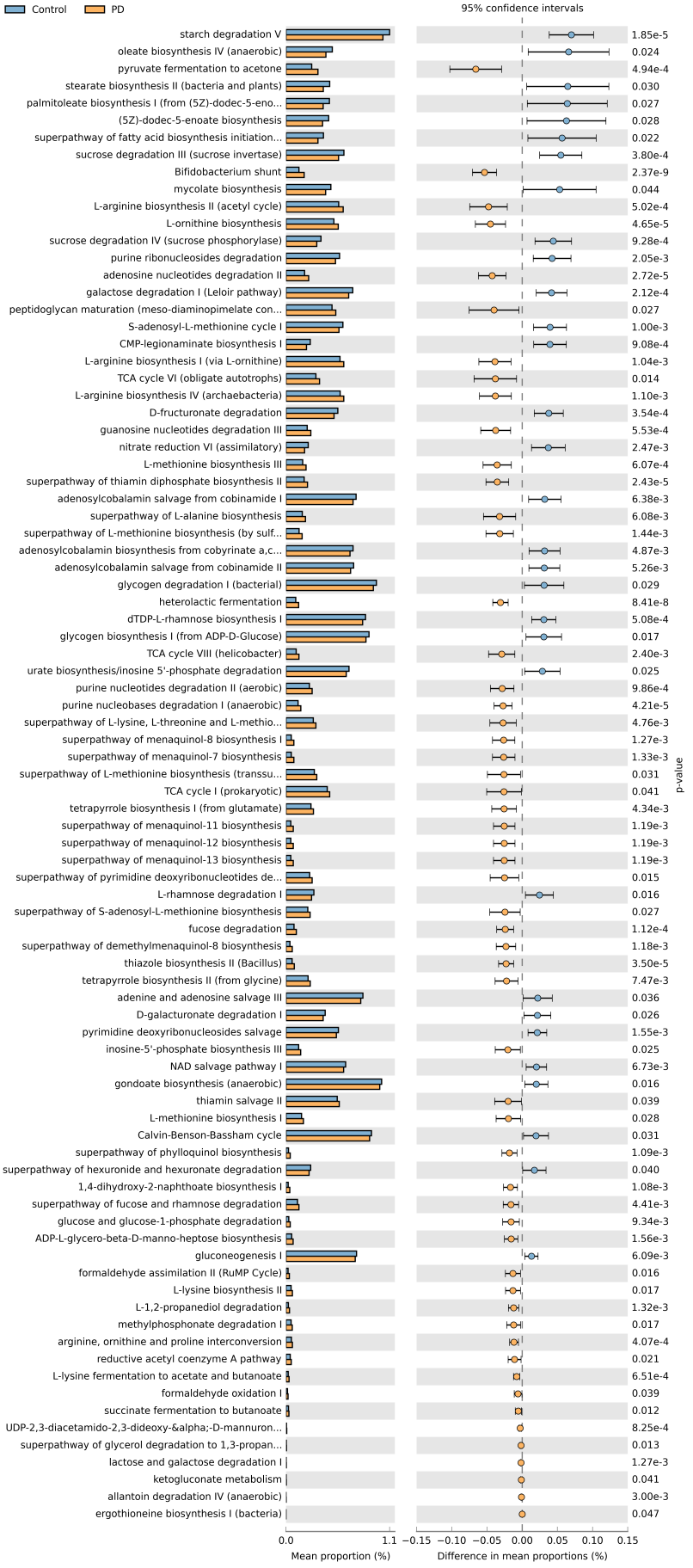


**FIG. 1 Functional potential analysis identified 87 differentially abundant pathways in PD.** Microbiome functional potential was inferred from 16S amplicon data in PICRUSt2, comparing MetaCyc pathway abundances in PD vs. non-PD gut microbiomes. All pathway differences between PD and non-PD (control) groups are significant ($p < 0.05$) according to Welch's two-sided t-test. Pathway abundances are illustrated on the left bar graph, with effect size (ordered from greatest to least) and 95% confidence intervals on the right plot.

**TABLE. 5 Vitamin B2 intake is associated with significant changes in Faith's phylogenetic diversity of the PD gut microbiome.** Pairwise Kruskal-Wallis testing based on Faith's phylogenetic diversity, Shannon's diversity, and Pielou's evenness was performed to determine whether (low vs. high) nutrient intake is associated with alpha diversity of the PD and non-PD gut microbiome. P-values were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Variables with significant results ($q < 0.05$) in Kruskal-Wallis testing were funneled into post-hoc Dunn's pairwise testing, to determine which group (low/moderate/high nutrient intake) was associated with increased alpha diversity (**Table 6**). Bold values emphasize groups with $q < 0.05$, and non-significant results are shaded grey. Abbreviations: PD = Parkinson's disease, df = degrees of freedom, PUFAs = polyunsaturated fatty acids, SFAs = saturated fatty acids.

| Pairwise Kruskal–Wallis test: alpha diversity for low vs. high nutrient intake | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Nutrient: low vs. high intake** | **Alpha diversity metric** | | | | | | | | | | | |
| | **Faith's phylogenetic diversity** | | | | **Shannon's diversity** | | | | **Pielou's evenness** | | | |
| | **PD** | | **non-PD** | | **PD** | | **non-PD** | | **PD** | | **non-PD** | |
| | **H (df=5)** | **q** | **H (df=5)** | **q** | **H (df=5)** | **q** | **H (df=5)** | **q** | **H (df=5)** | **q** | **H (df=5)** | **q** |
| **PUFAs** | 0.55 | 0.86 | 0.36 | 0.86 | 0.93 | 0.67 | 0.05 | 0.90 | 1.56 | 0.80 | 0.14 | 0.82 |
| **SFAs** | 1.79 | 0.39 | 2.76 | 0.36 | 0.24 | 0.75 | 0.96 | 0.74 | 0.01 | 0.97 | 0.14 | 0.97 |
| **Vitamin A** | 2.12 | 0.36 | 2.24 | 0.36 | 0.02 | 0.90 | 2.30 | 0.39 | 0.22 | 0.82 | 2.24 | 0.48 |
| **Vitamin B1** | 7.26 | 0.07 | 3.31 | 0.15 | 1.10 | 0.73 | 0.44 | 0.73 | 0.15 | 0.92 | 0.29 | 0.92 |
| **Vitamin B2** | **9.54** | **0.02** | 3.60 | 0.14 | 5.03 | 0.19 | 1.34 | 0.47 | 2.78 | 0.67 | 0.59 | 0.76 |
| **Vitamin B3** | 2.28 | 0.33 | 3.75 | 0.26 | 0.02 | 0.98 | 0.72 | 0.98 | 0.03 | 0.99 | 0.08 | 0.99 |
| **Vitamin B6** | 7.80 | 0.057 | 3.44 | 0.16 | 1.15 | 0.68 | 1.14 | 0.68 | 0.30 | 0.89 | 0.96 | 0.89 |
| **Vitamin B12** | 0.04 | 0.94 | 3.76 | 0.16 | 0.31 | 0.67 | 1.43 | 0.35 | 0.61 | 0.60 | 0.41 | 0.63 |
| **Vitamin C** | 4.75 | 0.22 | 2.62 | 0.31 | 0.25 | 0.76 | 3.10 | 0.53 | 0.0009 | 0.98 | 3.69 | 0.39 |
| **Vitamin D** | 1.05 | 0.76 | 2.23 | 0.51 | 0.01 | 0.92 | 0.96 | 0.54 | 0.16 | 0.69 | 0.22 | 0.69 |
| **Vitamin E** | 3.89 | 0.48 | 0.12 | 0.92 | 0.21 | 0.88 | 1.85 | 0.87 | 0.01 | 0.95 | 3.17 | 0.85 |

**TABLE. 6 High B2 intake is associated with significantly increased Faith's phylogenetic diversity in the PD gut microbiome, relative to low intake.** Variables with significant results ($q < 0.05$) in Kruskal-Wallis testing (**Table 1** and **Table 5**) were funneled into post-hoc Dunn's pairwise testing, to determine which group (PD/non-PD, low/moderate/high nutrient intake) was associated with increased alpha diversity. P-values were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Bold values emphasize groups with $q < 0.05$. Abbreviations: PD = Parkinson's disease, df = degrees of freedom.

| Post-hoc Dunn's test for multiple comparisons: Faith's phylogenetic diversity by vitamin B2 intake in PD and non-PD | | |
|---|---|---|
| **Comparison** | **Z (df=5)** | **q** |
| high B2 intake / low B2 intake in PD | **3.27** | **0.02** |
| high B2 intake / low B2 intake in non-PD | 1.80 | 1.00 |
| high B2 intake / moderate B2 intake in non-PD | 0.77 | 0.51 |
| low B2 intake / moderate B2 intake in non-PD | -1.28 | 0.34 |
| high B2 intake / moderate B2 intake in PD | 1.59 | 0.21 |
| low B2 intake / moderate B2 intake in PD | -2.02 | 0.13 |

and B6 intake were associated with changes in only PD gut microbiome composition (unweighted UniFrac, $q < 0.05$, **Table 7**).

**High vitamin B1 intake is associated with decreased abundances of genera *Gastranaerophilales*, *Lachnoclostridium*, and *Bacteroides* in the non-PD microbiome.** To further understand the changes in gut microbiome composition associated with vitamins B1, B2, and B6, we performed differential abundance analysis in DESeq2: comparing low vs. high vitamin intake in PD and non-PD microbiomes. No differentially abundant genera were identified in PD based on vitamin B1 and B6 intake, though high vitamin B2 intake was associated with an increased abundance of the genus *Anaeroplasma* in the PD gut microbiome (Wald test, $p_{adj} < 0.05$, **Table 8**). For the non-PD microbiome, high vitamin B1 intake was associated with increased abundances of genera *Muribaculaceae* and *Prevotellaceae NK3B31 group* and decreased abundances of genera *Gastranaerophilales*,

**TABLE. 7 Vitamin B1 intake is associated with significant changes in PD and non-PD gut microbiome composition, while low vitamin B2 and B6 intake are only associated with significant changes in PD gut microbiome composition.** Pairwise PERMANOVA based on weighted and unweighted UniFrac was performed to compare gut microbiome composition for low vs. high nutrient intake in PD and non-PD. P-values were corrected for multiple testing (q) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Only variables with significant results ($p < 0.05$) in the adonis analysis (**Table 2**) were analyzed. Bold values emphasize groups with $q < 0.05$. Abbreviations: PD = Parkinson's disease, df = degrees of freedom, PUFAs = polyunsaturated fatty acids, SFAs = saturated fatty acids.

| Pairwise PERMANOVA: beta diversity for low vs. high nutrient intake | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Nutrient: low vs. high intake** | **Weighted UniFrac** | | | | | | **Unweighted UniFrac** | | | | | |
| | **PD** | | | **non-PD** | | | **PD** | | | **non-PD** | | |
| | **n** | **F (df=2)** | **q** | **n** | **F (df=2)** | **q** | **n** | **F (df=2)** | **q** | **n** | **F (df=2)** | **q** |
| **PUFAs** | 100 | 1.85 | 0.12 | 50 | 0.87 | 0.41 | 100 | 1.15 | 0.24 | 50 | 1.32 | 0.15 |
| **SFAs** | | | | | | | | | | | | |
| **Vitamin A** | | | | | | | 115 | 1.52 | 0.12 | 48 | 1.14 | 0.27 |
| **Vitamin B1** | | | | | | | **95** | **1.75** | **0.02** | **49** | **2.05** | **0.02** |
| **Vitamin B2** | 95 | 2.17 | 0.12 | 50 | 1.23 | 0.30 | **95** | **2.09** | **0.04** | 50 | 1.40 | 0.10 |
| **Vitamin B3** | | | | | | | 79 | 1.02 | 0.38 | 47 | 1.37 | 0.25 |
| **Vitamin B6** | | | | | | | **92** | **1.91** | **0.04** | 47 | 1.42 | 0.08 |
| **Vitamin B12** | 67 | 1.41 | 0.20 | 47 | 0.97 | 0.38 | | | | | | |
| **Vitamin C** | | | | | | | 77 | 1.67 | 0.13 | 49 | 1.12 | 0.36 |
| **Vitamin D** | 87 | 2.83 | 0.06 | 47 | 0.55 | 0.65 | | | | | | |
| **Vitamin E** | | | | | | | 87 | 1.74 | 0.11 | 49 | 0.83 | 0.85 |

**TABLE. 8 Differentially abundant genera associated with high vitamin B1, B2, and B6 intake in the PD and non-PD gut microbiome.** Differential microbial abundances were calculated at the genus level relative to low nutrient intake using DESeq2. Significance was assessed by the Wald test, with p-values corrected for multiple testing ($p_{adj}$) using the Benjamini-Hochberg false discovery rate (BH FDR) method. Only genera with statistically significant changes are shown ($p_{adj} < 0.05$). Abbreviations: PD = Parkinson's disease.

| | **Genus** | | | | |
|---|---|---|---|---|---|
| | **Increased Abundance** | | | **Decreased Abundance** | |
| **Nutrient** | **PD** | **non-PD** | **$Log_2$(Fold Change)** | **non-PD** | **$Log_2$(Fold Change)** |
| **High vitamin B1 intake** (relative to low intake) | | *Muribaculaceae* | 25.79 | *Bacteroides* | -0.91 |
| | | *Prevotellaceae NK3B31 group* | 23.22 | *Lachnoclostridium* | -1.91 |
| | | | | *Gastranaerophilales* | -3.75 |
| **High vitamin B2 intake** (relative to low intake) | *Anaeroplasma* | | 9.16 | *Butyrivibrio* | -23.30 |
| | | | | *Prevotellaceae NK3B31 group* | -23.56 |
| **High vitamin B6 intake** (relative to low intake) | | *Prevotellaceae NK3B31 group* | 23.24 | | |

*Lachnoclostridium*, and *Bacteroides* (Wald test, $p_{adj} < 0.05$). High vitamin B2 intake was associated with decreased abundances of genera *Butyrivibrio* and *Prevotellaceae NK3B31 group*, while high vitamin B6 intake was associated with an increased abundance of *Prevotellaceae NK3B31 group* in the non-PD microbiome (Wald test, $p_{adj} < 0.05$).

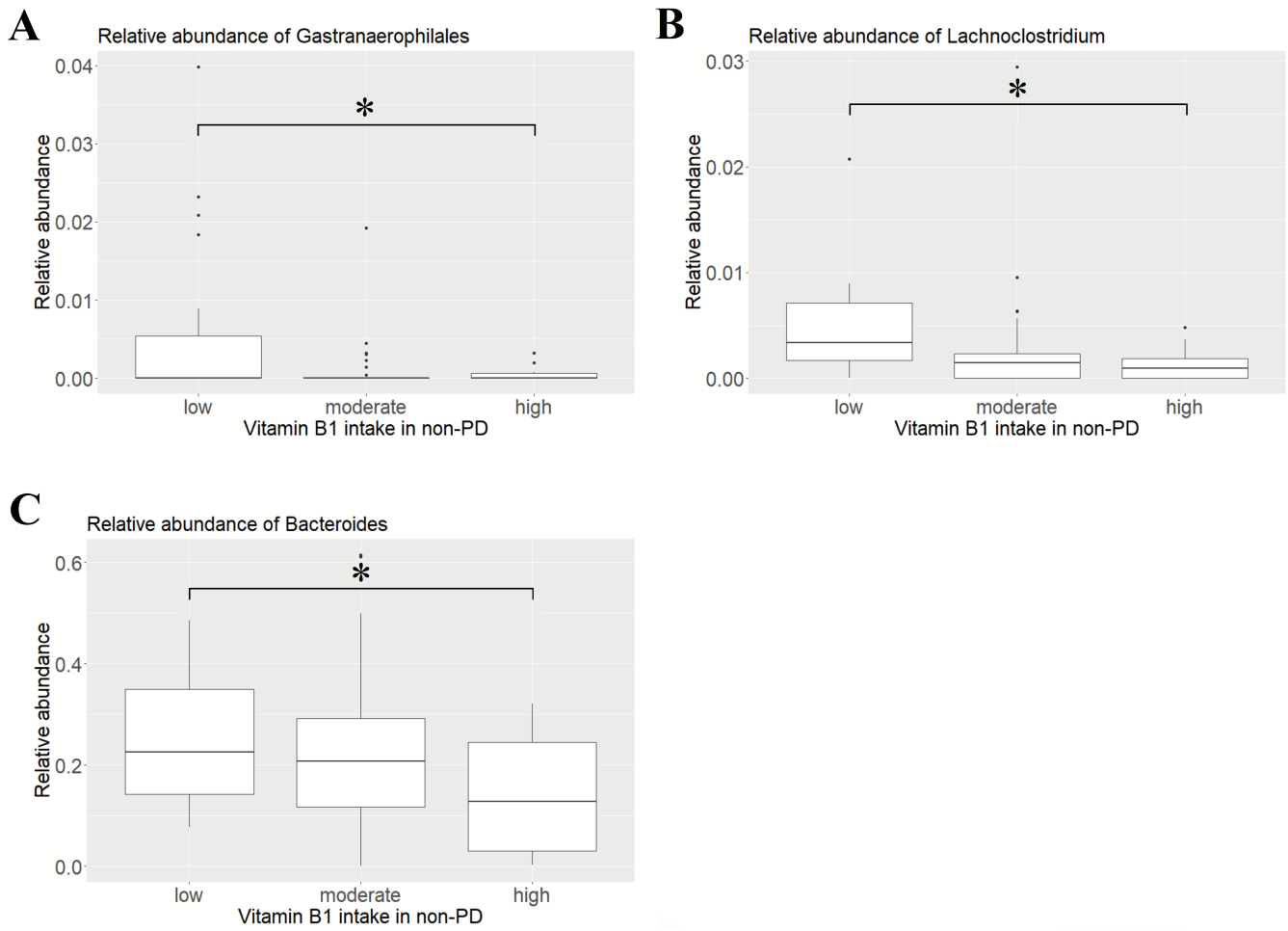


**FIG. 2 High vitamin B1 intake is associated with decreased abundances of *Gastranaerophilales*, *Lachnoclostridium*, and *Bacteroides* in the non-PD microbiome.** Abundance of genus relative to all genera in the gut microbiome: (A) *Gastranaerophilales*, (B) *Lachnoclostridium*, and (C) *Bacteroides*. Asterisk denotes $p_{adj} < 0.05$.

We visualized the proportion of differentially abundant genera relative to all genera in the gut microbiome (relative abundance) ($p_{adj} < 0.05$, **Figure 2**). Note that only the relative abundance plots for *Gastranaerophilales*, *Lachnoclostridium*, and *Bacteroides* are shown in **Figure 2**. Very few (<10%) samples contained *Muribaculaceae, Prevotellaceae NK3B31 group,* and *Butyrivibrio* in the non-PD microbiome and *Anaeroplasma* in the PD microbiome, thus their median relative abundances are ~0 (**Figure S2A-F**). Extremely low relative abundances of these genera explain why they had surprisingly large $\log_2$(fold change) values in differential abundance analysis, and the results in **Table 8** for *Muribaculaceae, Prevotellaceae NK3B31 group, Butyrivibrio* and *Anaeroplasma* are likely artefacts of analysis. This concludes that of all the genera identified by differential abundance analysis, only *Gastranaerophilales, Lachnoclostridium,* and *Bacteroides* (decreased by high vitamin B1 intake in the non-PD microbiome) are present in appreciable quantities.

**Low vitamin B1, B2, and B6 intake are each associated with changes in the functional potential of the PD gut microbiome.** To further explore the association of vitamins B1, B2, and B6 with the composition of the PD microbiome, we performed functional potential analysis in PICRUSt2. Inferred functional potential of low vitamin B1, B2, and B6 intake was compared to high intake in the PD gut microbiome. We identified 30 differentially abundant pathways for low vs. high vitamin B1 intake in the PD microbiome, with the majority downregulated in low vitamin B1 intake (Welch's two-sided t-test, $p < 0.05$, **Figure 3**). Low vitamin B1 intake in PD was associated with increased potential for multiple pathways involving assimilatory sulfate reduction; pyridoxal 5'-phosphate (vitamin B6) biosynthesis; D-galactarate

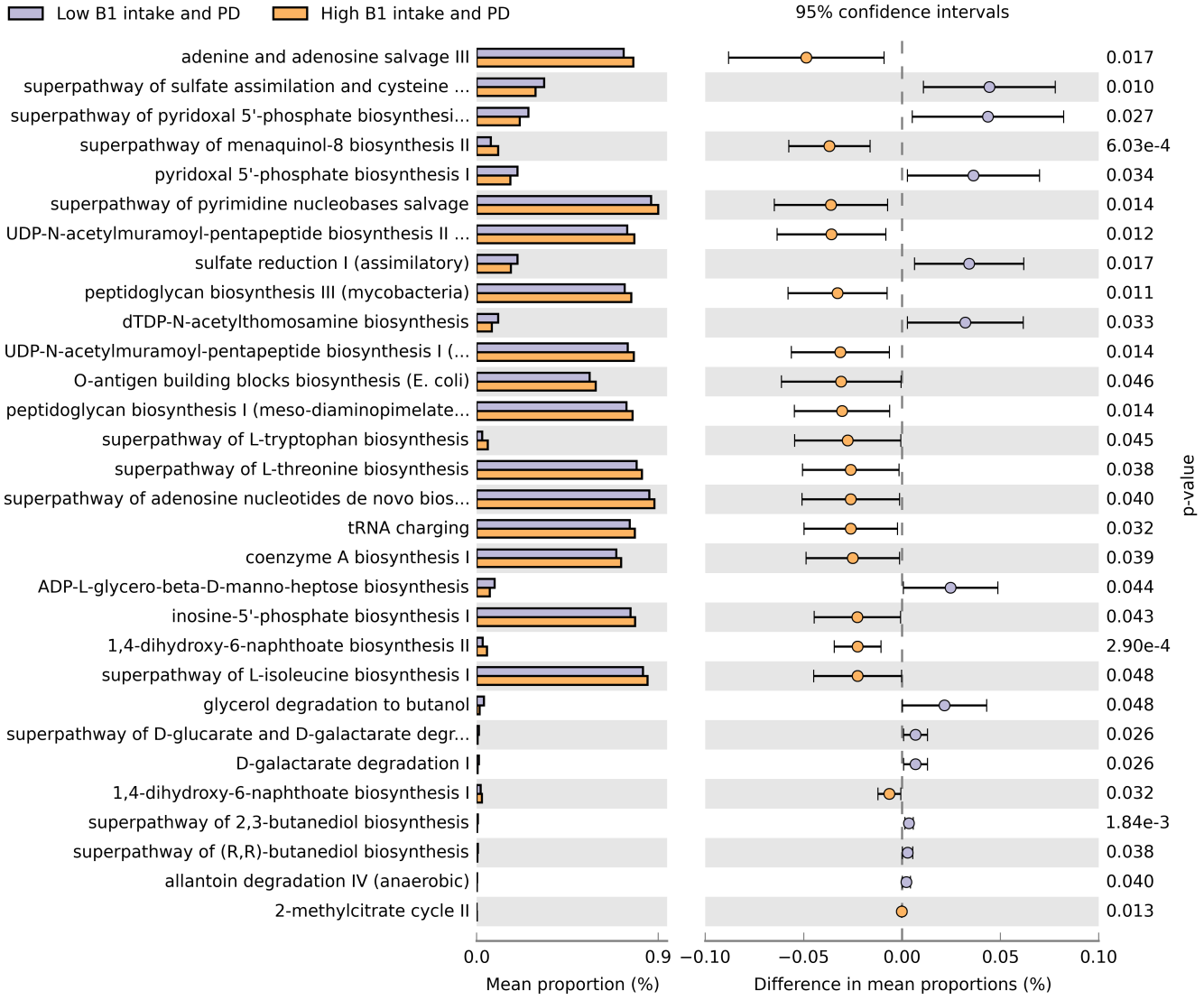


**FIG. 3 Functional potential analysis identified 30 differentially abundant pathways for low vs. high vitamin B1 intake in PD.** Microbiome functional potential was inferred from 16S amplicon data in PICRUSt2, comparing MetaCyc pathway abundances for low vs. high vitamin B1 intake in the PD gut microbiome. All pathway differences between low and high vitamin B1 intake in PD are significant ($p < 0.05$) according to Welch's two-sided t-test. Pathway abundances illustrated on the left bar graph, with effect size (ordered from greatest to least) and 95% confidence intervals on the right plot.

degradation; and butanediol/butanol production (butanediol biosynthesis and glycerol degradation to butanol). Low intake was associated with decreased potential for multiple pathways involving nucleobase/nucleotide salvage (adenine/adenosine salvage and pyrimidine nucleobases salvage); menaquinol (vitamin K2) biosynthesis (menaquinol and 1,4-dihydroxy-6-naphthoate biosynthesis); purine nucleotide biosynthesis (adenosine and inosine-5'-phosphate biosynthesis); peptidoglycan (PG) biosynthesis (peptidoglycan biosynthesis III and UDP-N-acetylmuramoyl-pentapeptide biosynthesis); and amino acid biosynthesis (L-tryptophan, L-threonine, and L-isoleucine biosynthesis).

We identified 38 differentially abundant pathways for low vs. high vitamin B2 intake in the PD microbiome (Welch's two-sided t-test, $p < 0.05$, **Figure 4**). Low vitamin B2 intake in PD was associated with increased potential for multiple pathways involving LPS component biosynthesis (Lipid IVA and CMP-3-deoxy-D-manno-octulosonate biosynthesis); tetrahydrofolate (a form of vitamin B9) and tetrahydrofolate precursor (6-hydroxymethyl-dihydropterin) biosynthesis; inositol degradation; and butanoate/butanediol/butanol

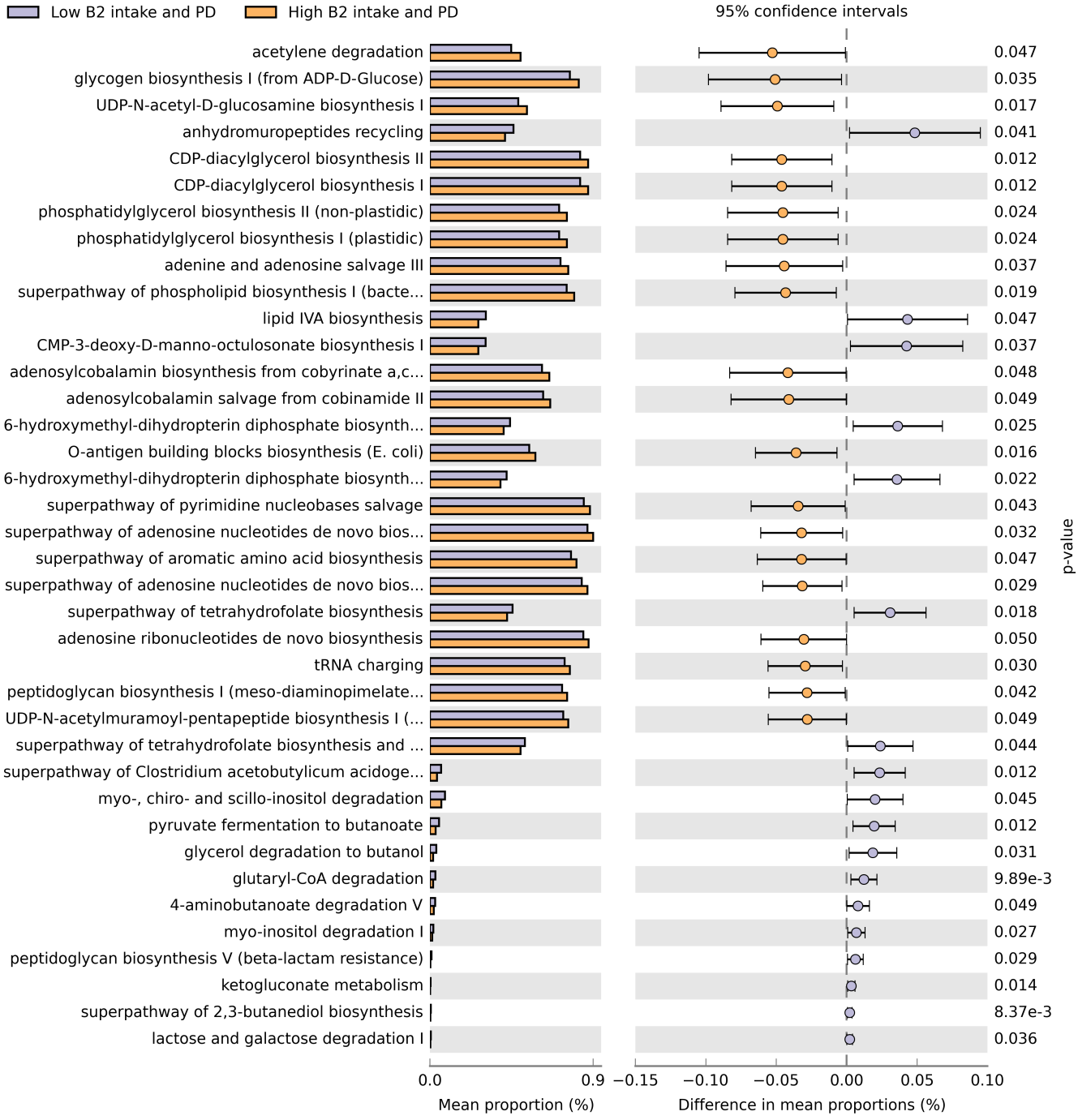


**FIG. 4 Functional potential analysis identified 38 differentially abundant pathways for low vs. high vitamin B2 intake in PD.** Microbiome functional potential was inferred from 16S amplicon data in PICRUSt2, comparing MetaCyc pathway abundances for low vs. high B2 intake in the PD gut microbiome. All pathway differences between low and high vitamin B2 intake in PD are significant ($p < 0.05$) according to Welch's two-sided t-test. Pathway abundances illustrated on the left bar graph, with effect size (ordered from greatest to least) and 95% confidence intervals on the right plot.

production (pyruvate fermentation to butanoate, butanediol biosynthesis, and glycerol degradation to butanol). Low intake was associated with decreased potential for multiple pathways involving PG biosynthesis (UDP-N-acetyl-D-glucosamine/UDP-N-acetylmuramoyl-pentapeptide biosynthesis and peptidoglycan biosynthesis I); membrane lipid biosynthesis (CDP-diacylglycerol, phosphatidylglycerol, and phospholipid biosynthesis); nucleobase/nucleotide salvage (adenine/adenosine salvage and pyrimidine

nucleobases salvage); adenosylcobalamin (vitamin B12) salvage/biosynthesis; and adenosine nucleotide/ribonucleotide biosynthesis.

We identified 58 differentially abundant pathways for low vs. high vitamin B6 intake in the PD microbiome, with the majority downregulated in low vitamin B6 intake (Welch's two-sided t-test, $p < 0.05$, **Figure 5**). Low vitamin B6 intake in PD was associated with increased potential for multiple pathways involving L-methionine and S-adenosyl-L-methionine (SAM) biosynthesis: earlier identified as a phenotype of the PD microbiome (**Figure 1**). Low intake was also associated with increased potential for multiple pathways involving LPS component biosynthesis (UDP-glucose-derived O-antigen building block and dTDP-N-acetylthomosamine biosynthesis); D-galactarate degradation; and butanoate/butanediol production (succinate fermentation to butanoate and butanediol biosynthesis). Low vitamin B6 intake in PD was associated with decreased potential for multiple pathways involving membrane lipid biosynthesis (CDP-diacylglycerol, phosphatidylglycerol, and phospholipid biosynthesis); PG biosynthesis (peptidoglycan biosynthesis I/III/IV and UDP-N-acetylmuramoyl-pentapeptide biosynthesis); nucleobase/nucleotide salvage (adenine/adenosine salvage and pyrimidine nucleobases salvage); purine biosynthesis (adenosine nucleotide/ribonucleotide de novo biosynthesis, 5-aminoimidazole ribonucleotide biosynthesis, and inosine-5'-phosphate biosynthesis); amino acid biosynthesis (L-isoleucine, L-histidine, L-serine, L-glycine, L-threonine, and aromatic amino acid biosynthesis); and long-chain cis-monounsaturated fatty acid (LCMUFA) biosynthesis (gondoate and cis-vaccenate biosynthesis).

## DISCUSSION

Adonis and pairwise PERMANOVA analysis show a significant difference between PD and non-PD gut microbiome composition in terms of community richness, microbial abundances, and/or phylogenetic diversity (weighted/unweighted UniFrac). This is consistent with a meta-analysis which confirmed significant alterations in PD gut microbiome composition across ten studies. On the genus level, the meta-analysis found that the PD microbiome was most often associated with enrichment of *Akkermansia* and *Bifidobacterium* (whose bacterial families drove most of the divergence between PD and non-PD), and depletion of *Faecalibacterium*–a phenotype also observed in our differential abundance analysis (7). Several differentially abundant genera we identified in the PD gut microbiome are associated with PD symptoms and pathogenesis. *Akkermansia* (60) and *Oscillibacter* (61)–both increased in PD–have shown potential in contributing to PD pathogenesis, as the genera are associated with increased gut permeability. Increased permeability can lead to gut inflammation: a common symptom in PD. *Akkermansia* is also associated with exposingintestinal neural plexus to oxidative stress, which can lead to alpha-synuclein aggregation in the intestines (60). Lower abundances of *Faecalibacterium* and *Roseburia* have also been associated with PD pathogenesis (60, 62, 63), as butyrate-producing bacteria that exert anti-inflammatory effects in the gut microbiome (64, 65). Decreased levels of butyrate in the gut can damage the intestinal mucus lining and cause the gut to become more susceptible to inflammation in PD (65). Additionally, we identified 2 phyla and 14 genera missing from the PD gut microbiome, and 39 genera unique to the PD gut microbiome. It should be noted that some of these taxa were rare in the dataset (found in <5% of samples), thus their presence in only the PD or non-PD microbiome may be attributed to chance rather than true microbiome differences.

The PD gut microbiome was associated with increased functional potential for multiple pathways involved in fermentation, the citric acid (TCA) cycle, amino acid biosynthesis (especially L-methionine), purine nucleotide/nucleobase degradation, menaquinol (vitamin K2) biosynthesis, and thiamine (vitamin B1) biosynthesis/salvage. Increased fermentation and menaquinol biosynthesis potential is corroborated by an existing meta-analysis of PD microbiome datasets (7). Surprisingly, menaquinol rescues mitochondrial deficits observed in PD animal models, and thus menaquinol upregulation may be a compensatory mechanism in PD (66). Increased vitamin B1 and decreased vitamin B12 biosynthesis/salvage potential in PD may result from alterations to B vitamin availability in the gut, as our functional

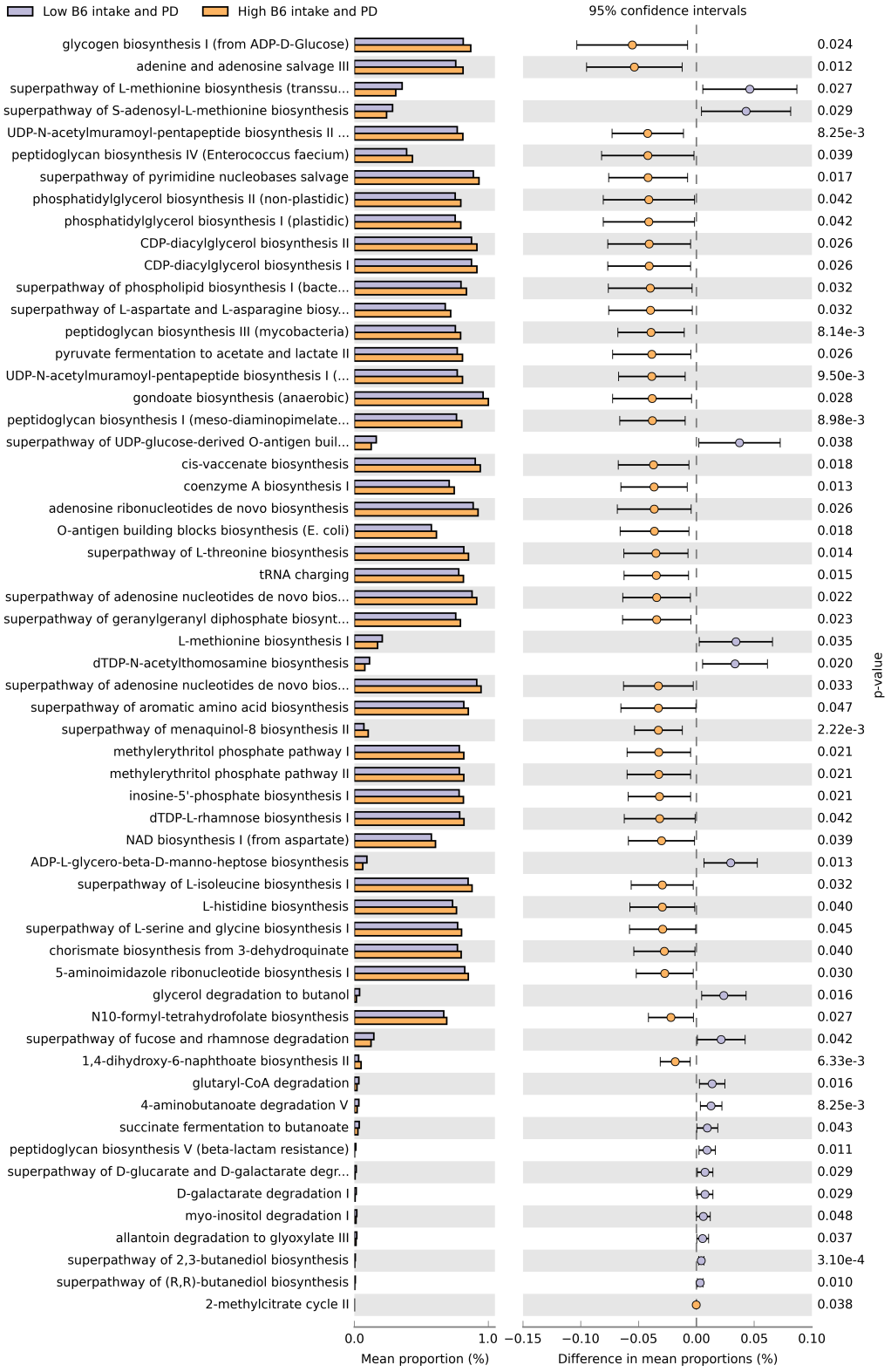


**FIG. 5 Functional potential analysis identified 58 differentially abundant pathways for low vs. high vitamin B2 intake in PD.** Microbiome functional potential was inferred from 16S amplicon data in PICRUSt2, comparing MetaCyc pathway abundances for low vs. high B6 intake in the PD gut microbiome. All pathway differences between low and high vitamin B6 intake in PD are significant ($p < 0.05$) according to Welch's two-sided t-test. Pathway abundances illustrated on the left bar graph, with effect size (ordered from greatest to least) and 95% confidence intervals on the right plot.

potential analysis showed that intake of certain B vitamins (vitamins B1 and B2) in PD is associated with changes in the potential of B vitamin biosynthesis pathways. Purines are anti-inflammatory neuromodulators and low purine levels have been implicated as a biomarker of PD progression, thus the increased purine degradation found in the PD gut microbiome may correlate with worse disease outcomes (67). Previous metabolomics studies identified decreased branched-chain and aromatic amino acids in the PD gut microbiome (68, 69), and so increased amino acid biosynthesis was unexpected in the PD gut microbiome. The PD microbiome was associated with enrichment of several biosynthetic superpathways involving L-methionine, including the biosynthesis of S-adenosyl-L-methionine (SAM). SAM has previously been associated with Parkinson's pathology: injection of SAM into the brain of rats induces PD-like motor impairments. It is hypothesized that SAM-dependent methylation could cause several biochemical changes observed in PD, including decreased dopamine, tyrosine hydroxylase, norepinephrine, serotonin, and melanin pigments, with increased acetylcholinergic activity (70). Decreased potential of fatty acid biosynthesis, sugar (especially sucrose) degradation, and adenosylcobalamin (vitamin B12) biosynthesis/salvage pathways in PD is corroborated by previous functional investigations of the PD microbiome (7, 71). Decreased fatty acid biosynthesis initiation potential aligns with the decrease in SCFA producers observed in the PD microbiome, with the potential to promote gut inflammation (64). Palmitate-induced toxicity in humans–resulting in endoplasmic reticulum stress and apoptosis (72)–is prevented by conversion to palmitoleate, stearate, and oleate: strikingly, biosynthesis of all these fatty acids is decreased in the PD gut microbiome (73). This suggests that the PD microbiome may leave patients more susceptible to SFA-induced toxicity, which has been posited to contribute to neuropathological changes in PD (74).

Pairwise Kruskal-Wallis and subsequent pairwise Dunn's testing associated high vitamin B2 intake with increased Faith's phylogenetic diversity in PD. Vitamin B2 intake did not impact Shannon's diversity/Pielou's evenness (which consider microbial abundances/evenness), therefore the increase in diversity associated with high vitamin B2 intake likely corresponds to increased phylogenetic differences in the PD gut microbiome. These findings are corroborated by previous studies, which found intake of some B vitamins can impact alpha diversity of the non-PD gut microbiome (75, 76). Supplementation of vitamin B2 has been shown to increase community richness and the number of butyrate producers in the gut microbiome (76).

Adonis analysis followed by pairwise PERMANOVA based on unweighted UniFrac distance shows that vitamin B1 intake is associated with significant changes in PD and non-PD gut microbiome composition, while vitamin B2 and B6 intake are uniquely associated with changes in PD gut microbiome composition. Intake of these nutrients was associated with significant differences in unweighted (but not weighted) UniFrac distance, suggesting that changes in microbiome composition associated with vitamins B1, B2, and B6 likely correspond to low-abundance features of the microbiome. The reason that vitamin B2 and B6 intake was uniquely associated with alterations to the PD microbiome remains unclear. Multivariate adonis analysis found no interaction between vitamin B2/B6 intake and PD status on the unweighted UniFrac distance of the gut microbiome. For this reason, PD does not appear to influence vitamin B2/B6 intake, and other factors unique to PD (eg. microbiome composition, physiological differences, or metabolic differences) are likely responsible for the impact of these nutrients on only PD microbiome composition.

Despite associating vitamin B1, B2, and B6 with changes to beta diversity, significant changes in specific microbial genera were only observed for vitamin B1 intake in the non-PD microbiome: high vitamin B1 intake was associated with decreased abundances of *Gastranaerophilales*, *Lachnoclostridium*, and *Bacteroides*. *Lachnoclostridium* is found to be associated with colitis: gastrointestinal inflammation which is often associated with PD development (77). *Bacteroides* also showed the potential to cause inflammation by secreting pro-inflammatory neurotoxins (78) such as LPS, which also induces alpha-synuclein aggregation (79). Meanwhile, *Gastranaerophilales* is potentially beneficial in aiding host digestion and as a source of vitamins B and K (80). These findings suggest that high vitamin B1 intake has mixed effects on the non-PD microbiome. Changes in beta diversity of the PD gut microbiome were likely not reflected in differential abundance analysis because this analysis removes rare or low-abundance taxa, and cannot compare taxa which are not present

in both groups (50). Vitamins B1, B2, and B6 may evade differential abundance analysis in PD if there is large microbiome variation across PD patients, many taxa are introduced/removed between low vs. high intake groups, or there are changes in rare/low-abundance taxa between low vs. high intake groups.

Low vitamin B1, B2, and B6 intake are each associated with changes in the functional potential of the PD gut microbiome. Low intake of vitamin B1, B2, and B6 are all associated with decreased potential for PG biosynthesis (relative to high intake). This may be attributed to replacement of Gram-positive with Gram-negative bacteria–in fact, low vitamin B2 and B6 intake are associated with increased potential for LPS component biosynthesis (a component of Gram-negative bacteria) (81). Increased LPS biosynthesis potential may aggravate PD symptoms and progression, as the LPS endotoxin contributes to alpha-synuclein amyloidogenesis (79). Low intake of vitamin B1, B2, and B6 is also associated with decreased potential for nucleobase/nucleotide salvage and purine nucleotide biosynthesis–processes with parallel the PD phenotype (**Figure 1**) of increased purine nucleotide degradation. As these anti-inflammatory neuromodulators are inversely associated with PD progression, decreased biosynthesis/salvage associated with low vitamin B1, B2, and B6 intake corresponds to worse disease outcomes (67). Low vitamin B2 and B6 intake were associated with decreased membrane lipid biosynthesis (CDP-diacylglycerol, phosphatidylglycerol, and phospholipid biosynthesis) in the PD microbiome. The impact of this phenotype remains unclear: CDP-diacylglycerol is the precursor to phospholipids phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL) (74). CL prevents (82) and PG promotes alpha-synuclein aggregation, while the role of PI remains poorly defined (74). Previous metabolomics studies in PD patients identified decreased branched-chain and aromatic amino acids (68, 69). This parallels our findings of decreased potential for L-tryptophan/L-isoleucine biosynthesis and L-isoleucine/L-histidine/aromatic amino acid biosynthesis associated with low vitamin B1 and vitamin B6 intake in PD, respectively. Given that these changes in functional potential resemble the PD phenotype, low vitamin B1, B2, and B6 may contribute to PD symptoms, progression, and pathogenesis.

Low vitamin B1 intake in PD is associated with increased potential for multiple pathways involving assimilatory sulfate reduction (relative to high intake), which produce cysteine/methionine. We earlier identified increased L-methionine biosynthesis potential as a phenotype of the PD microbiome (**Figure 1**). This pathway could further contribute to production of SAM. Our results suggest that intake of certain B vitamins can impact the biosynthesis of other B and K vitamins in the PD gut microbiome. Decreased menaquinol (vitamin K2) biosynthesis is associated with low vitamin B1 intake, and is detrimental to mitochondrial function in PD (66). Low vitamin B1 intake in PD is also associated with increased potential for pyridoxal 5'-phosphate (vitamin B6) biosynthesis, while low vitamin B2 intake is associated with increased potential for tetrahydrofolate (a form of vitamin B9) biosynthesis and decreased potential for adenosylcobalamin (vitamin B12) salvage/biosynthesis–demonstrating the interaction between different B vitamins in the gut microbiome.

Low vitamin B6 intake in PD was associated with enrichment of several biosynthetic pathways involving L-methionine, including the biosynthesis of SAM. We earlier identified increased L-methionine and SAM biosynthesis potential as a phenotype of the PD microbiome (**Figure 1**), with SAM being capable of inducing PD-like motor impairments (70). Low vitamin B6 intake is additionally associated with decreased LCMUFA biosynthesis potential in PD. Interestingly, the low vitamin B6 phenotype in PD of decreased L-isoleucine, L-threonine, L-histidine, adenosine nucleotide, and LCMUFA biosynthesis potential is shared in obesity, where patients exhibited improved mood and cognition following supplementation with inulin (a fermentable dietary fibre) (83). This suggests that low vitamin B6 intake is associated with metabolic changes in the PD microbiome that could worsen mood and cognition, which vitamin B6 and inulin supplementation can potentially treat.

**Limitations** These findings are based on secondary data analysis of a cross-sectional cohort study. As such, we were unable to control for nutrient intake. All results in this study are correlational, due to the observational nature of this dataset. Causality and mechanistic links would require further experimental testing. Gut microbiome variation across subjects (due to

confounding variables such as genetic background and environmental factors) is likely to mask microbiome changes associated with some nutrients.

In this dataset, PD patients took medications to manage symptoms, which potentially interact with the gut microbiome. Reporting of dietary nutrient intake may be relatively inaccurate, and nutrients that can be produced in the human body (eg. vitamin D) or by gut microbes (eg. vitamin B) may also impact gut microbiome composition. Additionally, diet information collected in a single questionnaire may not reflect the consistent dietary patterns of participants.

If nutrients were not associated with changes in beta diversity of the PD gut microbiome, we did not proceed with analyzing changes in functional potential. We made the assumption that no significant change in beta diversity translates to no significant change in functional potential. A major limitation of PICRUSt2 is that functional potential is inferred from 16S amplicon data. This requires extrapolating the genome of microbes based on their 16S rRNA sequence, and does not consider gene expression (51). Thus, PICRUSt2 results especially require confirmation by further experimental testing, such as metatranscriptomics, shotgun metagenomics, metabolomics, or activity assays.

**Conclusions** The aim of our study was to examine the effect of nutrient intake (PUFAs, SFAs, and vitamins A, B1, B2, B3, B6, B12, C, D, and E) on PD gut microbiome composition and functional potential. We found that the PD gut microbiome has increased abundances of the genera *Collinsella, Akkermansia, Bifidobacterium,* and *Oscillibacter*, and decreased abundances of *Faecalibacterium* and *Roseburia* relative to non-PD controls. We found that high vitamin B2 intake is associated with increased Faith's phylogenetic diversity of the PD gut microbiome. Vitamin B1 intake is associated with significant alterations to PD and non-PD gut microbiome composition, and high vitamin B1 intake is associated with decreased abundances of genera *Gastranaerophilales, Lachnoclostridium,* and *Bacteroides* in the non-PD gut microbiome. We also found that vitamin B2 and B6 intake are uniquely associated with altered PD gut microbiome composition. Low vitamin B1, B2, and B6 intake are also associated with changes in the functional potential of the PD gut microbiome that may aggravate PD. Our findings suggest that vitamin B1, B2, and B6 could play a role in remediating the PD gut microbiome, with the potential to also treat PD symptoms and progression via the gut-brain axis.

**Future Directions** Because gut microbiome variation across subjects may mask some dietary effects, future longitudinal studies should examine microbiome changes in individuals over time in response to modulating vitamin B1, B2, and B6 intake. Metatranscriptomics, shotgun metagenomics, metabolomics, or activity assays can confirm the functional changes to the PD gut microbiome that were inferred from 16S amplicon data. Future studies may also map microbial changes to changes in functional potential, and ultimately elucidate whether vitamin B1, B2, and B6 supplementation are clinically beneficial in remediating the PD gut microbiome.

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## CONTRIBUTIONS

Data analysis in QIIME2 was performed by YZ. Data analysis in R was performed by AF and YL. Nutrient stratification and data analysis in PICRUSt2 was performed by YZ and HS. All authors contributed to the writing of all sections of the manuscript.

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