Title

The Association of Gut Microbiome Composition and Parkinson's Disease in Patient Cohort of Central California

Author

Chou, Hsiang-Chin

Publication Date

2019

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Association of Gut Microbiome Composition and Parkinson's Disease in Patient Cohort of Central California

A thesis submitted in partial satisfaction

of the requirements for the Master of Science

in Epidemiology

by

Hsiang-Chin Chou

2019

ABSTRACT OF THE THESIS

The Association of Gut Microbiome Composition
and Parkinson's Disease in Patient Cohort
of Central California

By

Hsiang-Chin Chou

Master of Science in Epidemiology
University of California, Los Angeles, 2019
Professor Beate Ritz, Chair

The gut microbiome recently has been associated with many diseases, with studies showing that the microbiome can affect aspects of neurological function, brain activity, and behavior. While several digestive symptoms are well-known non-motor features of Parkinson's disease (PD), the role of the gut microbiome in the neurodegenerative process of PD remains underexplored. Here we recruited a cohort of 81 PD patients and 56 healthy controls from central California. We collected ethanol fixed fecal samples and conducted microbial composition analyses. Beta diversity analysis demonstrated compositional differences in the microbiome of PD patients and controls. We also identified specific bacterial genera that were associated with PD status using negative binomial models to determine the differential abundance of taxa (DESeq2 package in R;

B-H adjusted p-value < 0.05). We also identified specific bacterial genera significantly different in abundance between higher levodopa equivalent dose (LED) use PD patients and lower LED use PD patients. Furthermore, several specific bacterial families were found to be highly correlated with a constipation score in PD patients and controls. In conclusion, we found differences in the gut microbiome composition comparing PD cases and controls and identified specific bacterial genera that seemed to be associated with PD status. In the future, investigations are needed to identify underlying pathophysiologic pathways influenced by the gut microbiome in PD.

The thesis of Hsiang-Chin Chou is approved.

Jonathan P. Jacobs

Ondine Solv von Ehrenstein

Beate R. Ritz, Committee Chair

University of California, Los Angeles

2019

Table of Contents

List of Tables

List of Figures

1. Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder with progressive motor impairment and non-motor symptoms, including cognitive decline, mood disorders[1], and gastro-intestinal (GI) features such as constipation and gastro-paresis. The pathologic hallmarks of neurodegeneration in PD, namely Lewy bodies and α-synuclein aggregates, have also been found in the gut of PD patients[2-4]. While constipation, gastroparesis, and other digestive symptoms are well-known non-motor features of PD, the role the gut microbiome may play in the neurodegenerative process remains underexplored, even though several studies have pointed out the importance of the gut microbiome in PD[5-15]. In those previous studies, few of the patients lived in the United States[13,14] and microbiota may have differed due to many regional/cultural influences. Furthermore, studies differed in their protocols and analysis approaches and study results were not entirely consistent[8-15]. Although the gut microbiome is relatively stable in general, it has been shown to be influenced by various factors such as genetic make-up of the host, diet, medication use and the environment in general[16-18].

We conducted a study to investigate the gut microbiome in PD in a largely rural California population with the goal to determine differences in microbiome composition between PD patients and age-matched household and population controls who participated in a case-control study in central California as part of the population-based Parkinson's Environment and Gene study[19,20].

2. Method

2.1 Participant recruitment and data collection

All procedures described were approved by the University of California at Los Angeles (UCLA) Institutional Review Boards (IRBs). Informed consent was obtained from all participants.

PEG is a community-based study located in central California counties (Kern, Fresno, and Tulare)[19,20].

Idiopathic Parkinson's disease patients were first recruited early in disease (within the first 5 years after diagnosis) into the Parkinson's Environment and Genes study between 2001 and 2015 and then followed for disease progression (on average for 5-7 years after initial enrollment); those seen in 2017-2018 were also asked to provide a fecal sample. From 2001-2007, newly diagnosed PD cases were recruited through local neurology medical group, medical centers and the Veterans Affairs hospital. Between 2011-2017, we recruited newly diagnosed PD patients with the help of the California PD registry (CAPDR). Patient eligibility criteria were: (1) PD diagnosis in the past 3-5 years; (2) resident of one of three central California counties (Kern, Fresno, and Tulare); (3) examined and diagnosed by a UCLA PEG study movement disorder specialists; (4) no serious psychiatric conditions (including dementia before motor onset); (5) not terminally ill or institutionalized. We enrolled participants for this gut microbiome study in 2017-2018 and collected fecal samples if they met the following additional criteria: (1) no serious inflammatory bowel disease history (e.g. Crohn's Disease or ulcerative colitis); (2) no antibiotics use history or infections in the past three months before stool sample collection; (3) not currently under immunosuppressant medication[21].

We also recruited unaffected household controls (N = 39) for as many PD cases as possible. These controls were relatively well matched by age but gender disparate (spouses N = 38) and one

daughter. Inclusion criteria for household controls were: (1) no neurodegenerative disease; (2) currently living in the same household as the PD patient; and (3) met the same criteria for fecal sample collection described above for cases. Population-based controls (N = 17) were originally recruited between 2009-2011 from the same three counties, mostly selected randomly from the county's residential tax records and re-contacted for this study in 2017-18. These controls were required to meet the same eligibility criteria as household controls except for living in a patients' household.

In total, we enrolled 137 participants, including 81 Parkinson's disease patients and 56 unaffected controls (39 household and 17 population-based controls).

At the time of sample collection, we also collected additional data including the Geriatric Depression Scale-15 (GDS-15), the Wexner constipation questionnaire (Cleveland Clinic constipation scoring system)[22], and recorded antibiotic usage history and behavioral (e.g. smoking, caffeine, physical activity), medical (comorbidities), and environmental factors from all participants.

2.2 Dietary assessment

We administered to all subjects a validated paper-based Diet History Questionnaire II (DHQ II, https://epi.grants.cancer.gov/dhq2/); a food frequency questionnaire (FFQ) developed by the Risk Factor Assessment Branch (RFAB) in the National Cancer Institute (NCI). The DHQ II consists of 134 food items and 8 dietary supplement questions that asks to report the intake of food items (and portion sizes) in the past month. These data can be analyzed with a Diet*Calc Software (version 1.5.0) developed by NCI that generates nutrient and food group intake estimates for the DHQ II.

2.3 Fecal sample collection

All samples analyzed here were collected between November 2017 and November 2018. Participants were provided with a fecal collection kit to be used at their home at their convenience. Specifically, they are asked to urinate first, then defecate into a Fisher-brand Commode Specimen Collection System, or the Fe-Col® Faeces Collection Paper and transfer the feces to a Para-Pak stool collection cup prefilled with 20 ml of 95% ethanol solution to stabilize the sample, allowing for storage at room temperature for up to two weeks[23]. The stabilized samples were mailed and delivered to UCLA at room temperature in padded envelopes and stored in a -80°C freezer right after having been received.

2.4 Bacterial 16S rRNA sequencing and processing

From the ethanol-preserved feces samples, 0.25g underwent DNA extraction by bead beating with the MO BIO Powersoil kit. The V4 hypervariable region of the 16S rRNA gene was amplified using the 515F and 806R primers (5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTCTAAT-3', respectively) according to a published protocol[24]. PCR products were purified by a commercial kit and sequenced on the Illumina HiSeq 2500 platform. In most microbial species, the V4 region is approximately 253 bp, and only deviates from this length by a few base pairs. Because the HiSeq 2500 rapid run mode enables paired 150-bp reads, the reads overlap to generate high-quality, full-length sequences of the entire region. The 806R primer includes a unique sequence tag to barcode the samples, enabling 200-400 specimen to be run as one batch with a targeted depth of 250,000 sequences/sample.

Sequence data with quality information (i.e. FASTQ) are imported into QIIME 2.0 for sequence quality filtering in Casava 1.8 demultiplexed (paired-end) format. In the pipeline of DADA2, in an effort to remove noise in paired-end sequences and for quality control, no bases

from the beginning of the sequences were trimmed; sequences were truncated from 245 bases forward and 55 bases backward due to the expected decrease in quality.

The taxonomic assignments of sequences are performed using open reference operational taxonomic unit (OTU) picking in QIIME 2.0 against the SILVA 132 99% database (https://www.arb-silva.de/). The range of our demultiplexed sequence counts from the Illumina HiSeq 2500 platform was between 102,017 and 433,935 (median = 185,928), and a total of 5421 OTUs were picked.

2.5 Statistical analysis methods for microbial community composition and abundance

Relative abundance of operational taxonomic units (OTUs, which correspond roughly to species), genera, and also at higher taxonomic levels were computed for each study participant using QIIME 2.0 software. A rooted phylogenetic tree was generated to analyze phylogenetic diversity. To test the alpha diversity (the diversity within each individual sample, expressed by the richness of species) within the PD case group and within a control group, different metrics were used to evaluate each sample's species richness including the measures known as the Observed OTUs, Chao1 index, Shannon index, and Faith's phylogenetic diversity; we also generated visualizations via boxplots. Microbial composition (i.e. between group diversity; beta diversity) will be compared across all samples using unweighted and weighted UniFrac (a phylogenetic distance metric), Bray-Curtis and Canberra distances and visualizations via non-metric multidimensional scaling (NMDS) and principal coordinate (PCoA) plots.

Microbiota composition difference (beta diversity) in relation to PD status (PD vs Control Groups) and covariates was assessed using multivariate permutational analysis of variance (PERMANOVA)[25], which is a non-parametric method based on dissimilarities, and also allows for complex analytical designs adjusting for covariates and for partitioning of variability. The

phyloseq package in R[26], integrated within the DESeq2 package were employed to test the differential abundance of taxa comparing PD with control group samples. Differences were determined at the genus level using a negative binomial distribution (Benjamini-Hochberg adjusted p-value < 0.05) and we provided log2 fold change plots to display significant microbiota differences. To adjust for potential confounders, the microbiota identified from DESeq2 were evaluated after adjustment for covariates (sex and race) using the likelihood-ratio test.

Microbiota composition difference (beta diversity) in relation to disease duration or levodopa equivalent dose among PD patients were also assessed using PERMANOVA. Differences in microbiota at the genus level between those with a longer (10+ years) or shorter disease duration (< 10 years) were also determined with the DESeq2 package in R and displayed with log2 fold change plots.

We also assessed Spearman's correlations between microbiota relative abundance and clinical characteristics.

The 36 cases and their household controls were considered matched pairs and we also assessed differences in microbiota composition using both pair-wise PERMANOVA and DESeq2 to identify bacteria possibly related to Parkinson's disease.

3. Results

3.1. Demographic characteristics

Demographic characteristics of the study cohort are presented in Table 1. The participants are mainly of European ancestry (77.4%), which comprises the majority of the elderly population of the central California counties (i.e. Kern, Fresno, and Tulare). There were more males among PD patients (64.2%) than controls (37.5%), which reflects the higher prevalence of PD in men and the fact that household controls were opposite gender spouses. The PD patients (mean age 72.1

years) were on average 4 years older than the controls (mean age 68.7 years), but had similar education levels (14.4 years vs. 14.9 years in all controls). The PD patients had on average a higher constipation score (mean Wexner constipation score = 6.69 vs. 4.38 in all controls) and a lower BMI (27.36 vs. 29.6 in all controls). Among PD patients 61.7% and among controls 66.0% had never smoked. In terms of the dietary history, PD patients on average reported a higher intake of energy (kcal) and carbohydrates (g) than all controls, while the population controls on average reported a higher intake of total fat (g), protein (g) and dietary fiber (g). Concerning their clinical characteristics, PD patients, on average were first diagnosed at age 62.7 (± 10.9) years, and at the time of the fecal sampling their mean disease duration was 9.4 (± 4.1) years, and their average Levodopa equivalent dose (LED) was 985.35 mg (median = 690 mg). Most of the PD patients (90%) were treated with Levodopa/Carbidopa/Entacapone only or along with dopamine agonist, while 5% used Dopamine Agonists only and 5% used none.

Table 1. Demographic characteristics in PD patients and healthy controls (N = 137)

	Total (N=137)		PD case (N=81)		Household control (N=39)		Population control (N=17)	
Characteristic of Cases	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
PD Diagnosis Age	-	-	81	62.7 (10.9)	-	-	-	-
Disease duration	-	-	81	9.4 (4.1)	-	-	-	-
L-dopa equivalent dose (mg)	-	-	81	985.4 (866.4)	-	-	-	-
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
PD Type								
Probable PD	-	-	27	33.3	-	-	-	-
Definite PD	-	-	54	66.7	-	-	-	-
Motor Subtype								
Tremor dominant	-	-	33	41.3	-	-	-	-
PIGD*	-	-	40	50	-	-	-	-
Indeterminate	-	-	7	8.8	-	-	-	-
Disease duration								
Long (10 years+)	-	-	29	35.8	-	-	-	-
Short (less than 10 years)	-	-	52	64.2	-	-	-	-
L-DOPA and DA								
Only DA	-	-	4	4.9	-	-	-	-
Only L-DOPA or both	-	-	73	90.1	-	-	-	-
None	-	-	4	4.9	-	-	-	-
Characteristic of all participants	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
Age	137	70.8 (9.1)	81	72.14 (10.9)	39	69.1 (8.9)	17	68.2 (6.2)
Education (Years)	134	14.5 (4.4)	81	14.35 (4.7)	36	14.8 (4.2)	17	14.9 (3.8)
BMI	129	28.3 (5.9)	77	27.36 (5.2)	35	29.7 (7.5)	17	29.4 (4.4)
GDS score at fecal collection	129	6.2 (2.1)	78	6.55 (2.4)	34	5.5 (1.1)	17	5.7 (1.5)
Constipation score	123	5.7 (4.2)	68	6.69 (4.1)	38	4.4 (4.1)	17	4.4 (3.7)
Energy (kcal)	108	1892.3 (879.9)	61	1975.8 (919.8)	32	1728.9 (826.3)	15	1901.3 (826.3)
Total fat (g)	108	78.6 (42.4)	61	79.1 (43.9)	32	75.4 (43.3)	15	83.1 (35.9)
Carbohydrate (g)	108	222.3 (103.3)	61	245.8 (110.5)	32	188.0 (82.2)	15	200.3 (93.0)
Protein (g)	108	71.5 (37.9)	61	71.2 (37.3)	32	68.0 (38.3)	15	79.9 (40.4)
Dietary fiber (g)	108	17.5 (9.4)	61	17.6 (8.8)	32	16.9 (10.9)	15	17.9 (8.5)
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Sex								
Male	73	53.3	52	64.2	11	28.2	10	58.8

	Female	64	46.7	29	35.8	28	71.8	7	41.2
Race									
	European/White	103	77.4	65	80.3	27	77.1	11	64.7
	Hispanic	23	17.3	13	16.1	6	17.1	4	23.5
	Asian	5	3.8	2	2.5	2	5.7	1	5.9
	Native American	2	1.5	1	1.2	0	0	1	5.9
Smoking Status									
	Ever (Quit or current)	49	36.6	31	38.3	11	30.6	7	41.2
	Never	85	63.4	50	61.7	25	69.4	10	58.8
Relatives With PD									
	None	100	76.9	53	68.0	33	94.3	14	82.4
	Primary relative	23	17.7	19	24.4	1	2.9	3	17.7
	Non-primary relative	7	5.4	6	7.7	1	2.9	0	0

*PIGD: Postural Instability and Gait Dysfunction
*DA: Dopamine Agonist

3.2. Gut microbiome composition between PD patients and controls

Overall, there were no statistically significant differences in four different indices of alpha diversity (the richness of species) according to the Kruskal-Wallis test for observed OTUs, Chao1 index, Shannon index, or Faith's phylogenetic diversity between PD patients and all controls. The results are visualized with boxplots in Figure 1.

To assess the overall microbial species composition differences between PD patients and controls across all samples, PERMANOVA tests (repeated with 100,000 permutations) were conducted using four different metrics (Bray-Curtis and Canberra distances, unweighted and weighted UniFrac distance metrics). Statistically significant differences were found with three different metrics (Bray-Curtis: P = 0.03, Canberra: P = 0.001, and Unweighted UniFrac: P = 0.02) before adjusting for covariates (Table 2 (A)). Figure 2 visualizes the microbial composition differences via non-metric multidimensional scaling (NMDS) plots and principal coordinate (PCoA) plots.

To adjust for potential confounders, additional PERMANOVA tests were performed adjusting for sex, race and age, and again statistically significant microbial species composition differences were found between PD patients and controls in two metrics (Bray-Curtis: P = 0.02 and Canberra: P = 0.001) and marginally significant in the unweighted UniFrac (P = 0.06) (Table 2 (B)-(C)). Further adjusting for carbohydrate intake and constipation score, the results remained statistically significant Bray-Curtis (P = 0.04) and Canberra (P = 0.002), but not in unweighted UniFrac (P = 0.099).

We also investigated the effect of disease duration on the microbial composition difference among PD patients (N = 81) only after adjusting for diagnosis age and race with PERMANOVA.

However, no differences were found for disease duration in all four metrics (Bray-Curtis: P = 0.98, Canberra: P = 0.98, Unweighted UniFrac: P = 0.93, Weighted UniFrac: P = 0.93; Table 2 (D)).

In addition, we analyzed the microbiome composition among those patients who were currently taking PD medications using PERMANOVA. We quantified each patient's dosage of PD medications by calculating the levodopa equivalent doses (LED)[27]. We found statistically significant associations between microbial species composition and LED with three metrics (Bray-Curtis: P = 0.02, Canberra: P = 0.003, Unweighted UniFrac: P = 0.001; Table 2 (E)).

3.3. Identification of differentially abundant taxa at family or genus level between PD versus controls

Analyses of the differential abundance of taxa at the genus level comparing PD with all control samples and using the negative binomial distribution (B-H adjusted p-value < 0.05) revealed that several bacteria differed statistically significantly between the 2 groups, after adjusting for sex and race. The bacterial taxa with a statistically significant abundance increase among PD cases included 10 genera (*Akkermansia, Alistipes, Parabacteroides, Enterococcus, Family XIII AD3011 group, Hungatella, UBA1819, 3 unclassified at genus level*), which belonged to 9 families (*Akkermansiaceae, Eggerthellaceae, Enterococcaceae, Family XIII, Lachnospiraceae, Muribaculaceae, Tannerellaceae, Rikenellaceae, Ruminococcaceae*) and 4 phyla (*Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia*). Those with a statistically significant decrease in abundance in PD cases included 4 genera (*Butyricicoccus, Erysipelotrichaceae UCG-003, Fusicatenibacter, Lachnospiraceae NK4A136 group*), which belonged to 3 families (*Erysipelotrichaceae, Lachnospiraceae, Ruminococcaceae*) and 1 phylum (*Firmicutes*). Shifts in the identified microbial abundances in PD patients versus controls are displayed with log2 fold change plots in Figure 3 (A).

We further performed analysis to test the differential abundance of taxa at the genus level in PD patients with PD duration time 10 years or longer compared with control group samples. The bacteria with significant abundance increase in longer disease duration cases included 4 genera (*Enterococcus, Lactobacillus, Parabacteroides, 1 unclassified at genus level*), which belonged to 4 families (*Enterococcaceae, Lachnospiraceae, Tannerellaceae, Ruminococcaceae*) and 2 phyla (*Bacteroidetes, Firmicutes*). The bacteria with significant abundance decrease in longer disease duration cases included 3 genera (*Lachnospiraceae NK4A136 group, Roseburia, [Eubacterium] ruminantium group*), which all belonged to the family *Lachnospiraceae* and the phylum *Firmicutes*. Comparing PD patients with disease duration less than 10 years to control group samples, the bacterial taxa with a statistically significant abundance increase included 10 genera (*Enterococcus, Lactobacillus, Family XIII AD3011 group, Hungatella, [Eubacterium] coprostanoligenes group, Erysipelatoclostridium, Turicibacter, Cloacibacillus, 2 unclassified at genus level*), which belonged to 9 families (*Enterococcaceae, Erysipelotrichaceae, Family XIII, Lachnospiraceae, Lactobacillaceae, Muribaculaceae, Ruminococcaceae, Synergistaceae, 1 unclassified at family level*) and 3 phyla (Bacteroidetes, *Firmicutes, Synergistetes*). The bacterial taxa with statistically significant abundance decrease in shorter disease duration cases included 5 genera (*Butyricicoccus, CAG-352, Coprococcus 2, Lachnospiraceae NK4A136 group, Prevotella 9*), which belonged to 3 families (*Prevotellaceae, Lachnospiraceae, Ruminococcaceae*) and 2 phyla (*Bacteroidetes, Firmicutes*). There were 3 genera (*Enterococcus, Lachnospiraceae NK4A136 group, Lactobacillus*) with statistically significant increase or decrease in both the longer and shorter disease duration groups. The results are displayed in Figure 3 (B) and (C) and the taxa differences are presented in Table 3.

We also investigated the differential abundance of taxa at the genus level in PD patients currently under PD medication with LED higher than the median (690 mg) compared with LED lower than or equal to the median (690 mg). We identified 28 genera with significantly different abundance between high LED group and low LED group, in which 26 genera exhibited higher abundance in high LED group, and 2 genera (*[O: Rhodospirillales] unclassified, Pseudomonas*) had higher abundance in the low LED group. The detail information of bacteria genera is presented in Table 4 and Figure 6.

Table 2. PERMANOVA test for association of PD cases and controls with the microbiome, adjusted for potential confounders

PERMANOVA (Permutation = 100,000)	Case	Control	Distance / Matrix							
	N	N	Bray Curtis		Canberra		Unweighted UniFrac		Weighted UniFrac	
			R-square	P-value	R-square	P-value	R-square	P-value	R-square	P-value
(A) PD (Yes vs No)	81	56	0.01024	0.03147 *	0.00968	0.00104 **	0.00959	0.01962 *	0.00481	0.8734
(B) PD and covariates	81	52								
PD (Yes vs No)			0.01091	0.01951 *	0.01	0.00057 ***	0.00923	0.06328 .	0.0096	0.17691
Sex (male vs female)			0.00838	0.23858	0.00848	0.04754 *	0.00921	0.06647 .	0.00889	0.24921
Race (European vs non-European)			0.00923	0.11122	0.00962	0.00223 **	0.01203	0.00127 **	0.01003	0.14954
Age (continuous)			0.00998	0.05260 .	0.00802	0.15708	0.00871	0.12547	0.02072	0.00064 ***
(C) PD and covariates	61	47								
PD (Yes vs No)			0.01269	0.04204 *	0.01196	0.00165 **	0.01085	0.09917 .	0.00259	0.99705
Sex (male vs female)			0.00869	0.60741	0.00964	0.26257	0.01137	0.05323 .	0.00738	0.65383
Age (continuous)			0.0106	0.21411	0.01001	0.13092	0.01124	0.06283 .	0.00984	0.35926
Race (European vs non-European)			0.01792	0.58045	0.02012	0.06143 .	0.02253	0.02559 *	0.01393	0.71397
Carbohydrate (continuous)			0.008	0.77181	0.00809	0.99245	0.00944	0.39299	0.02019	0.02425 *
(D) Patient only covariates	81	-								
Disease duration (continuous)			0.00854	0.98404	0.01127	0.97753	0.01055	0.93011	0.00713	0.93059
Age diagnosed (continuous)			0.0157	0.08493 .	0.01266	0.35811	0.01211	0.57193	0.02136	0.03764 *
Race (European vs non-European)			0.01532	0.10454	0.0145	0.01607 *	0.01535	0.03829 *	0.01425	0.27955
(E) Patient only covariates	79	-								
Levodopa equivalent dosage (continuous)			0.01854	0.01665 *	0.01586	0.0026 **	0.01874	0.00109 **	0.01555	0.218

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 3. Significantly identified taxa at genus level by negative binomial distribution regression model

					PD Case vs Control (Adjusted for sex and race)			
Phylum	**Class**	**Order**	**Family**	**Genus**	**log2Fold -Change**	**Adjusted p-value**		
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Eggerthellaceae*	*unclassified*	2.838	0.02707		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Tannerellaceae*	*Parabacteroides*	0.961	0.02707		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*unclassified*	6.539	0.02707		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*	1.047	0.03581		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Fusicatenibacter*	-1.670	0.00002		
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus**	4.666	0.00307		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*UBA1819*	1.815	0.00321		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Butyricicoccus*	-0.992	0.02707		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospiraceae NK4A136 group*	-1.235	0.02707		
Firmicutes	*Clostridia*	*Clostridiales*	*unclassified*	*unclassified*	2.407	0.02707		
Firmicutes	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Erysipelotrichaceae UCG-003*	-2.018	0.03110		
Firmicutes	*Clostridia*	*Clostridiales*	*Family XIII*	*Family XIII AD3011 group*	1.351	0.03982		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Hungatella*	2.449	0.04323		
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Akkermansiaceae*	*Akkermansia**	1.762	0.00489		
					PD duration 10+ years Case vs Control		**PD duration < 10 years Case vs Control**	
Phylum	**Class**	**Order**	**Family**	**Genus**	**log2Fold -Change**	**Adjusted p-value**	**log2Fold -Change**	**Adjusted p-value**
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Tannerellaceae*	*Parabacteroides*	1.259	0.0140	-	-
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*unclassified*	-	-	2.643	0.00105
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella 9**	-	-	-2.967	0.00047
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	3.601	0.0140	3.130	0.00478
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus**	0.473	0.0156	0.269	0.00239
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospiraceae NK4A136 group*	-1.759	0.0065	-0.395	0.00044
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia**	-1.409	0.0329	-	-

Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*[Eubacterium] ruminantium group*	-1.395	1.17E-12	-	-
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*unclassified*	3.049	0.0140	-	-
Firmicutes	*Clostridia*	*Clostridiales*	*Family XIII*	*Family XIII AD3011 group*	-	-	1.328	0.03493
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus 2**	-	-	-1.777	0.02143
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Hungatella*	-	-	3.562	0.04809
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Butyricicoccus*	-	-	-1.008	0.03046
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*CAG-352*	-	-	-2.848	0.03381
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*[Eubacterium] coprostanoligenes group*	-	-	1.304	0.03046
Firmicutes	*Clostridia*	*Clostridiales*	*unclassified*	*unclassified*	-	-	1.970	0.04495
Firmicutes	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Erysipelatoclostridium*	-	-	0.420	0.04973
Firmicutes	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Turicibacter*	-	-	1.843	0.03815
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*Cloacibacillus*	-	-	2.369	6.90E-05

** same direction of association from previous studies.*

Table 4. Significantly identified taxa at genus level by negative binomial distribution regression model

					PD Case (LED > median, 690 mg) vs. PD Case (LED ≤ median, 690 mg)	
Phylum	**Class**	**Order**	**Family**	**Genus**	**log2Fold-Change**	**Adjusted p-value**
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Actinomyces*	0.841	0.049
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Atopobiaceae*	*Atopobium*	3.623	0.023
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Eggerthellaceae*	*Eggerthella*	2.632	0.004
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	0.856	0.039
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	2.513	0.049
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactococcus*	4.549	0.017
Firmicutes	*Clostridia*	*Clostridiales*	*Family XIII*	*Family XIII AD3011 group*	1.404	0.043
Firmicutes	*Clostridia*	*Clostridiales*	*Family XIII*	*[Eubacterium] brachy group*	3.245	0.009
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Blautia*	1.318	0.002
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Eisenbergiella*	2.025	0.012
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnoclostridium*	1.915	2E-5
Firmicutes	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Romboutsia*	1.723	0.006
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Flavonifractor*	1.611	0.023
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Fournierella*	4.949	4.5E-4
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminiclostridium 5*	1.134	0.029
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*UBA1819*	2.514	1E-5
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*unclassified*	2.136	1.171E-3
Firmicutes	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Turicibacter*	2.104	0.038
Firmicutes	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*[Clostridium] innocuum group*	2.179	0.049
Firmicutes	*Negativicutes*	*Selenomonadales*	*Acidaminococcaceae*	*Succiniclasticum*	0.723	0.000
Firmicutes	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Dialister*	2.768	0.016
Firmicutes	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Megasphaera*	2.970	0.039
Firmicutes	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Veillonella*	2.640	0.001
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*unclassified*	*unclassified*	-2.133	6.49E-14
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Bilophila*	1.610	0.023

Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*unclassified*	0.589	0.016
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	-6.540	0.002
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Akkermansiaceae*	*Akkermansia*	1.785	0.006

3.4. Correlation between taxa and clinical status

We furthermore used Spearman correlations to assess whether disease duration in PD patients and the Wexner constipation score were associated with the gut microbiome abundance difference. Although PD duration was found to be positively associated with *Bacteroidetes* (r = 0.046, P = 0.686) and negatively associated with *Firmicutes* (r = -0.066, P = 0.559), the correlations were weak and far from statistically significant. For the association of taxa and Wexner constipation score, we used the level of family for correlation analysis in order to compare our results with a previously published study[8]. Our results showed that 4 families (*Coriobacteriaceae, Eggerthellaceae, Tannerellaceae, Pasteurellaceae*) were statistically significantly associated with the Wexner score in PD patients (P < 0.05); while in controls, 11 families (*Bifidobacteriaceae, Marinifilaceae, Rikenellaceae, Tannerellaceae, env.OPS 17, [O: Gastranaerophilales] unclassified, Clostridiales vadinBB60 group, Defluviitaleaceae, [O: DTU014] unclassified, Acidaminococcaceae, Puniceicoccaceae*) were significantly associated with the Wexner score. Only *Tannerellaceae* appeared to be significantly associated with constipation in either group. The correlation results are presented in Table 5.

Table 5. Correlation between taxa (family level) and Wexner constipation score

Case (N = 68)	**r**	**P-value**
Family		
Coriobacteriaceae	0.2473	0.0421
Eggerthellaceae	0.4008	0.0007
Tannerellaceae	0.2476	0.0418
Pasteurellaceae	-0.2788	0.0213
Control (N = 55)	**r**	**P-value**
Family		
Bifidobacteriaceae	-0.325	0.0155
Marinifilaceae	0.3474	0.0093
Rikenellaceae	0.4097	0.0019
Tannerellaceae	0.4153	0.0016
env.OPS 17	-0.2656	0.05
[O: Gastranaerophilales] unclassified	0.3792	0.0043
Clostridiales vadinBB60 group	0.2741	0.0429
Defluviitaleaceae	0.3872	0.0035
[O: DTU014] unclassified	0.3077	0.0223
Acidaminococcaceae	0.3449	0.0099
Puniceicoccaceae	0.3086	0.0219

3.5. Paired case and household control comparisons

When comparing PD patients with their paired household controls, the gut microbiome composition showed a greater alpha diversity (OTU counts, Chao 1, ACE, Shannon/Simpson Index) in the paired controls (Figure 4). Overall microbial composition (i.e. beta diversity) was compared between cases and household controls in unweighted and weighted UniFrac, and with Bray-Curtis distances using PERMANOVA, but there were no statistically significant differences found after adjusting for sex and race. However, the pair-wise (N = 36) PERMANOVA showed significant differences (p-value = 0.001).

Using negative binomial models to determine the differential abundance of taxa (B-H adjusted p-value < 0.05), we found 2 genera with statistically significant abundance increase in PD cases (*[O: Rhodospirillales], Lactobacilllus*) and 3 genera with statistically significant abundance decrease in PD cases (*Preveotella 9, CAG-352, [Eubacterium] ruminantium group*). More specifically, 6 genera (*Lachnospiraceae NK4B4 group, Catenibacterium, Catenisphaera, [Eubacterium] nodatum group, [F: Puniceicoccaceae] unclassified, [Eubacterium] ruminantium group*) had statistically significant abundance decreases in longer disease durations PD patients (≥ 10 years). In shorter disease durations PD patients (< 10 years), 2 genera with statistically significant abundance increase (*[O: Rhodospirillales], Lactobacilllus*) and 2 genera with statistically significant abundance decrease (*CAG-352, [Eubacterium] ruminantium group*) were identified. The shifts in the identified statistically significant microbial abundances in PD patients compared with paired household controls are displayed with log2 fold change plots in Figure 5.

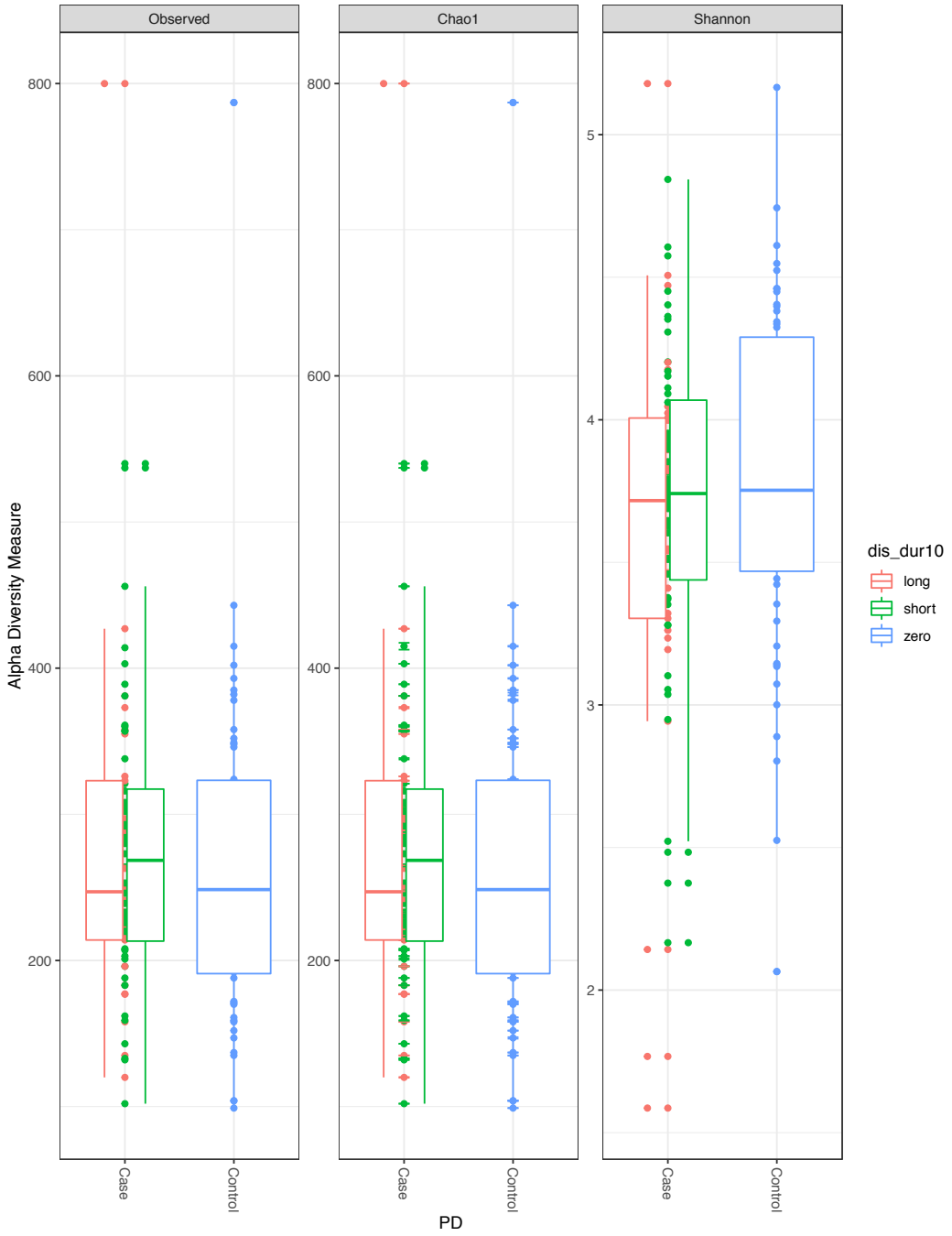


Figure 1. Analysis of alpha diversity using Observed OTU counts (left), Chao1 (middle) and Shannon index (right). Total of 81 PD patients and 56 controls. Color indicates the disease duration of PD patients (Red: disease duration 10+ years, green: disease duration <10 years, blue: disease duration zero known as controls).

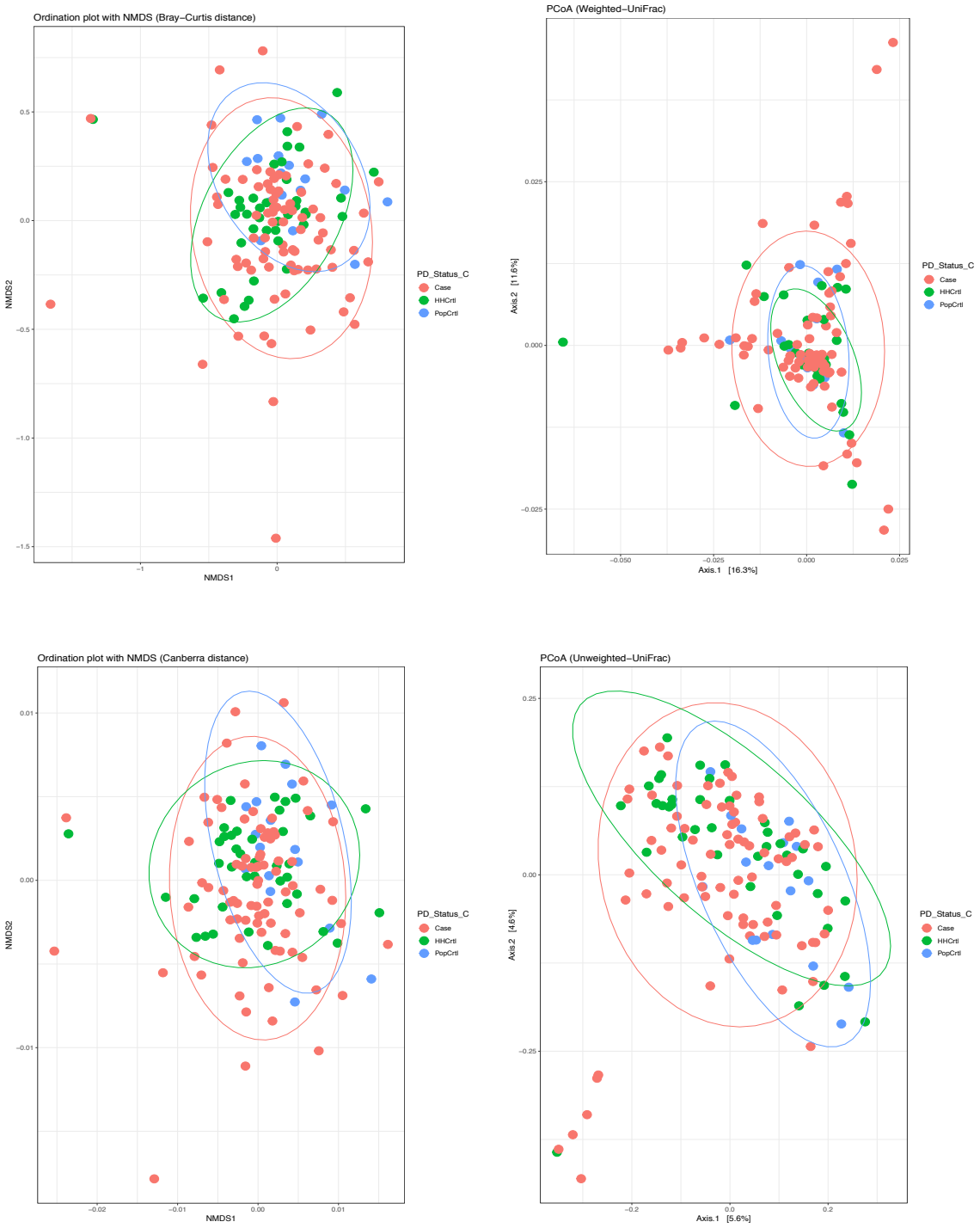
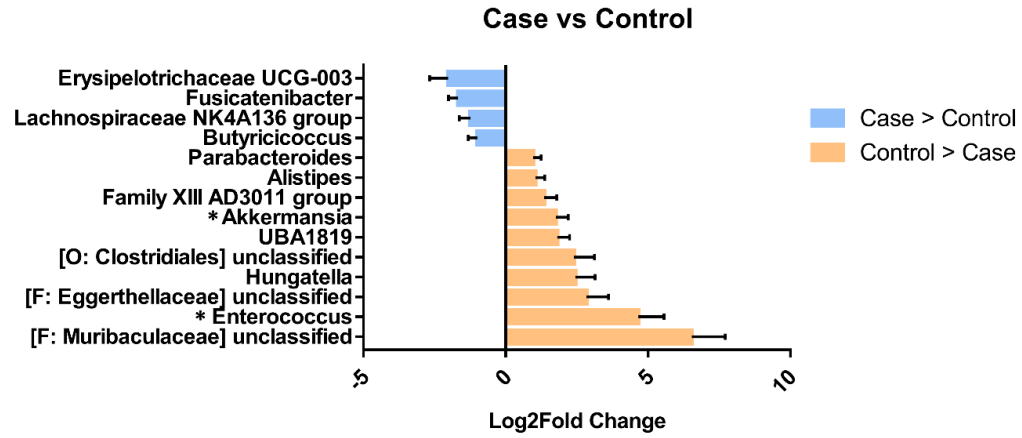
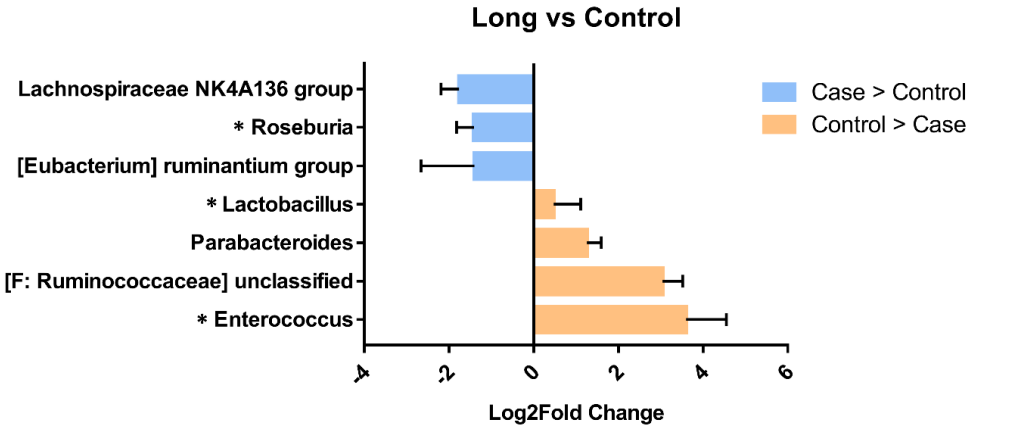


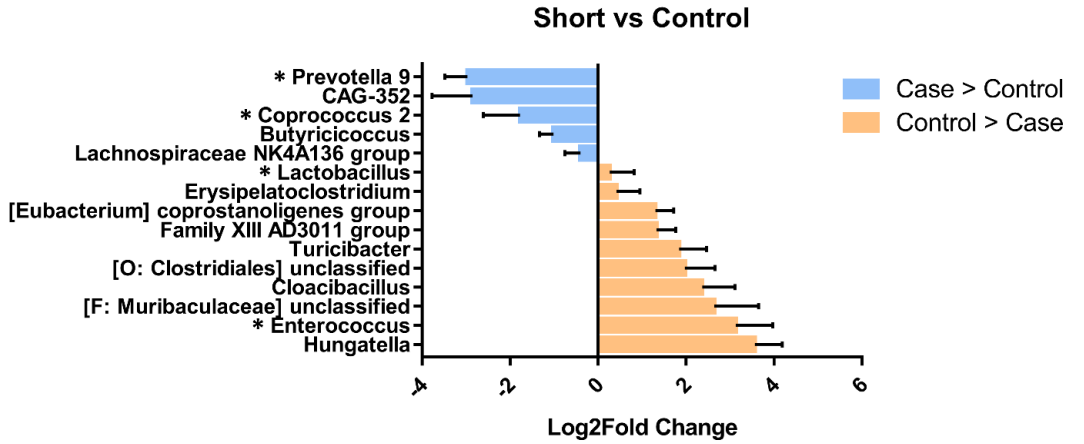
Figure 2. Visualizations of microbial composition differences via non-metric multidimensional scaling (NMDS) plot and principal coordinate (PCoA) plots. (Up-left: Bray-Curtis distance, up-right: Weighted UniFrac, down-left: Canberra distance, down-right: Unweighted-UniFrac; Red: PD patients, Green: Household controls, Blue: Population controls).



(A) Case vs control



(B) Long disease duration case vs control



(C) Short disease duration case vs control

Figure 3. Shifts in microbial abundances (genus) in PD patients vs household controls. (A) Comparison of PD patients (N=81) and controls (N=56). (B) Comparison of PD duration 10 years or longer patients (N=29) and household controls (N=56). (C) Comparison of PD duration shorter than 10 years patients (N=52) and household controls (N=56). Difference were determined at the genus level using a negative binomial distribution (B-H adjusted p-value < 0.05). Horizontal axis shows log2-fold difference in abundance between two groups. ∗ indicates consistent result from previous studies[8,10,12,28,29].

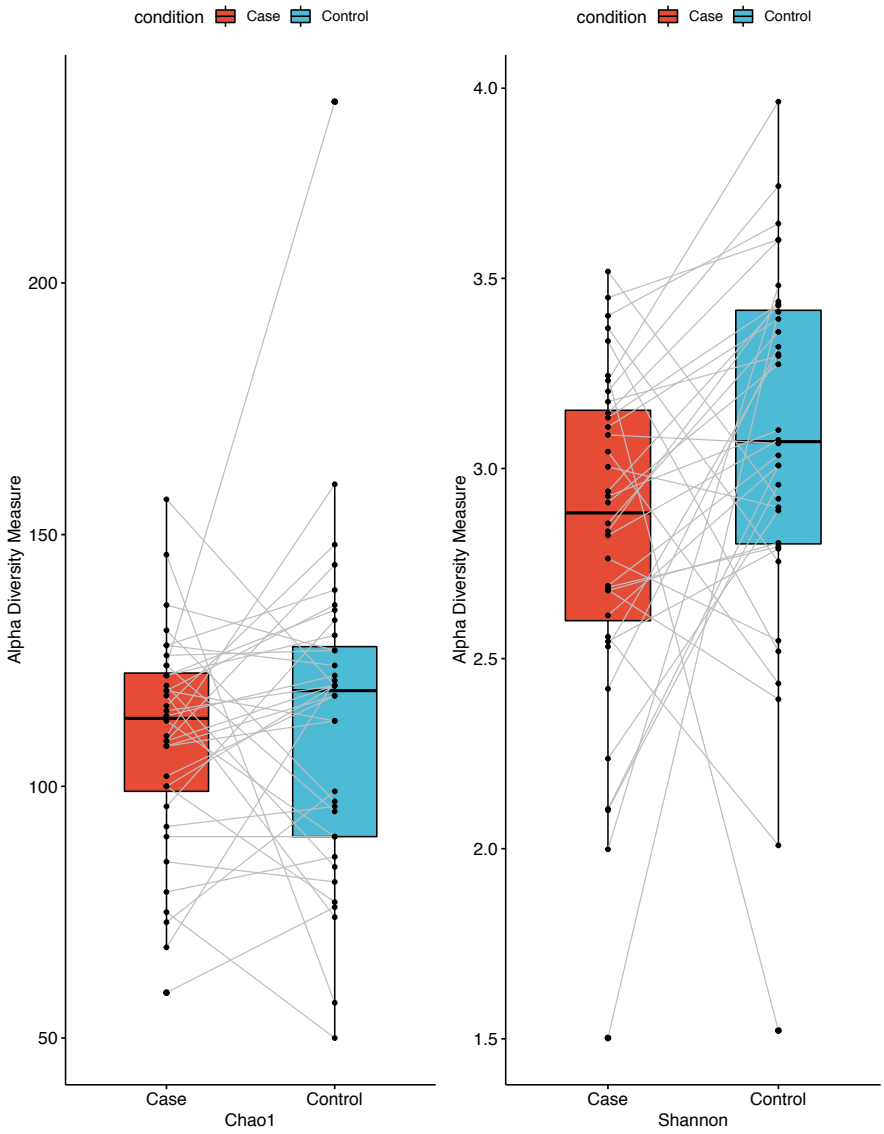
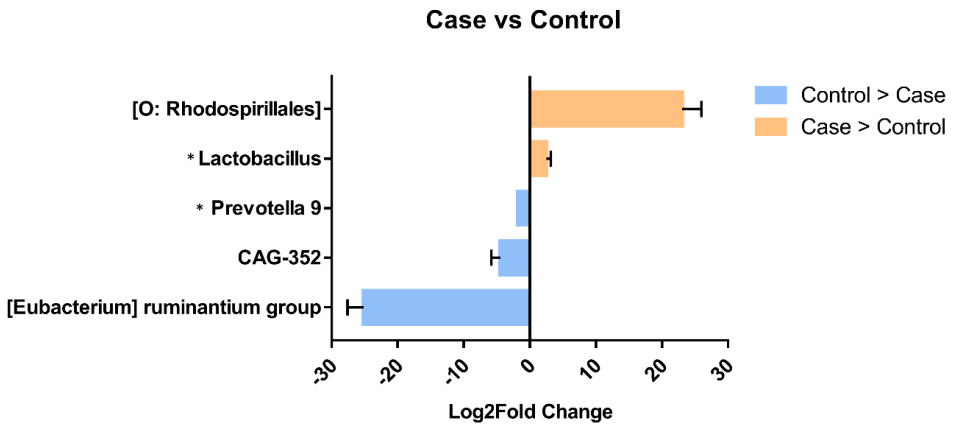
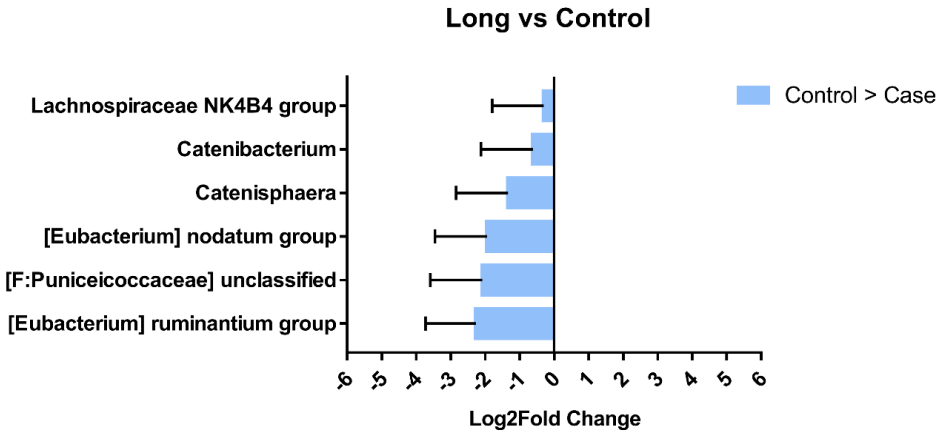


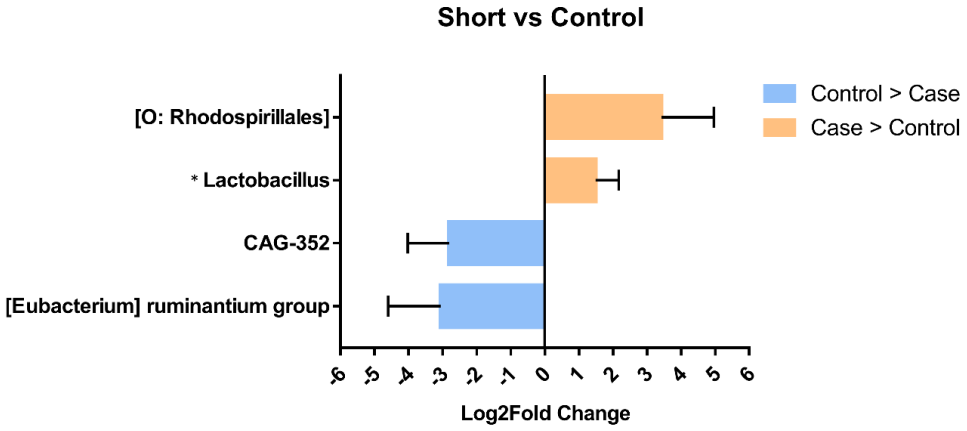
Figure 4. Analysis of alpha diversity using Chao1 (left) and Shannon index (right). Total of 36 PD patients and 36 paired household controls. Grey lines are the paired case and control. Greater richness/alpha diversity shown in Control group vs PD case, which reflects reduction of gut in PD patients.



(A) Case vs household control



(B) Long disease duration case vs household control



(C) Short disease duration case vs household control

Figure 5. Shifts in microbial abundances (genus) in PD patients vs household controls. (A) Comparison of PD patients (N=36) and controls (N=36). (B) Comparison of PD duration 10 years or longer patients (N=10) and household controls (N=36). (C) Comparison of PD duration shorter than 10 years patients (N=26) and household controls (N=36). Difference were determined at the genus level using a negative binomial distribution (B-H adjusted p-value < 0.05). Horizontal axis shows log2-fold difference in abundance between two groups. ∗ indicates consistent result from previous studies[8,10,12,28,29].

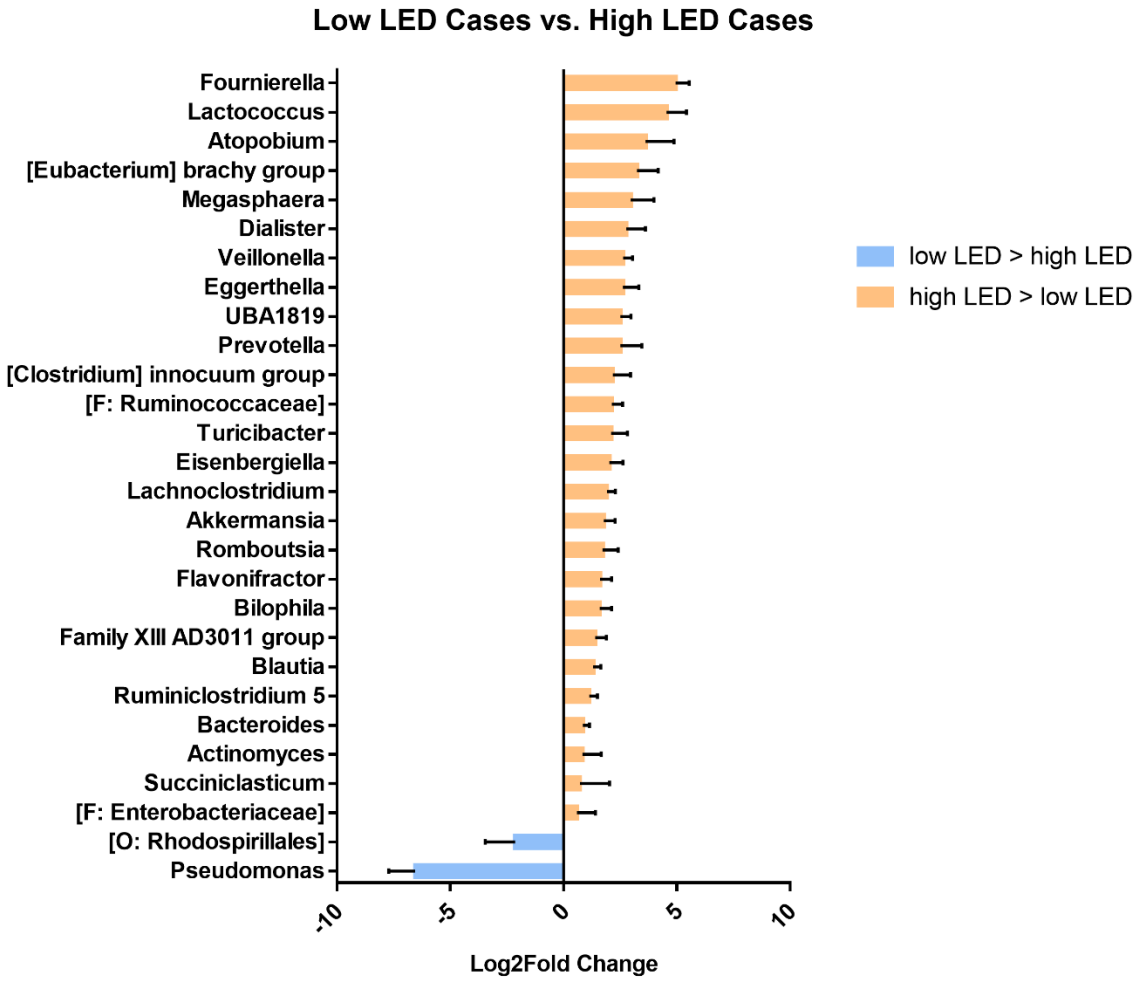


Figure 6. Shifts in microbial abundances (genus) in high LED PD patients vs low LED PD patients. Comparison of higher LED (LED > median, 690 mg) and lower LED (LED ≤ median, 690 mg). Difference were determined at the genus level using a negative binomial distribution (B-H adjusted p-value < 0.05). Horizontal axis shows log2-fold difference in abundance between two groups.

4. Discussion

In recent years, researchers started to investigate the relationship between the gut microbiome and Parkinson's disease. However, few studies were conducted with populations living in the United States and none in a rural environment. Our population-based case-control study provides first data describing the gut microbiota composition of PD patients and two types of controls who live in three agricultural counties of California. Although no significant differences in alpha diversity measures (species richness of a group) were detected, we found 10 bacterial taxa at the genus level with statistically significant abundance increases in PD patients and 4 bacterial taxa with statistically significant abundance decrease in PD patients compared with healthy controls (B-H adjusted p-value < 0.05). Also, 3 bacterial taxa remained statistically significant among the shorter disease duration PD patients (< 10 years) and the longer disease duration PD patients (10 + years). Interestingly, we found although *[O: Rhodospirillales]* showed statistically significant increase in longer disease duration PD patients (10 + years) vs control, it showed statistically significant increase in low LED vs high LED.

In our study, we found a reduction of *Prevotellaceae* (family) and lower *Prevotella* (genus) levels in feces of PD patients with shorter disease duration compared with controls, which is consistent with several previous reports[8,10,12,28]. Decreased *Prevotellaceae* abundance may also correspond to observations of an increased gut permeability in PD patients, which might be due to decreased mucin synthesis caused by low *Prevotella* levels, which in turn increase gut permeability[9]. *Lactobacillaceae* were more abundant in our PD patients, which is concordant with the results from a German study[29] and several other studies[8,10,13]. *Akkermansia* (genus) were also increased in PD patients in our study, in concordance with another German study[30]. This later study compared PD patients in early stages who were L-DOPA drug naïve with age-matched

controls and reported that *Eubacterium* (genus) were remarkably lower in PD patients. We however found that this bacterium, *Eubacterium [ruminantium group]* was statistically significantly reduced only in our longer disease duration PD patients compared with controls. Interestingly, *Eubacterium [coprostanoligenes group]*, a known cholesterol-reducing anaerobe[31], was found to be increased in our PD patients with shorter disease duration. Further investigations regarding cholesterol levels in our PD cases may help understand the differences we see concerning these bacteria.

A Chicago study of 38 PD subjects (12 drug naive/26 treated with PD meds) and 34 healthy controls found some evidence that the anti-inflammatory butyrate-producing bacteria *Blautia*, *Roseburia*, and *Coprococcus* are statistically significantly less abundant in PD cases than controls; low abundance of SCFA butyrate-producing bacteria in PD patients might also contribute to gut leakiness[14]. In our study, we also found that the genera of *Roseburia* and *Coprococcus* were significantly less abundant in PD cases than controls, but not *Blautia*. Additionally, a Finnish study[8] had reported higher abundance of the family *Enterobacteriaceae* among patients with a postural instability and gait difficulty (PIGD) phenotype compared to patients with a tremor dominant (TD) phenotype, suggesting not only that the intestinal microbiome is altered in PD subjects but may also play a role in the onset and/or severity of motor symptoms in PD subjects. In our study, although we did not identify this phenotype specific association, we found *Enterobacteriaceae* to be increased in our case group.

For correlations between taxa and clinical disease status, the same study from Chicago mentioned above observed that PD duration was highly positively associated with the phylum *Bacteroidetes* and negatively associated with the phylum *Firmicutes*[14]. We found that these two phyla were associated with PD duration in our cohort in the same direction; however, our results

were not statistically significant. We found that compared with controls, PD patients had higher constipation scores. Based on the Wexner score for constipation, we found that the families of *Coriobacteriaceae*, *Eggerthellaceae,* and *Tannerellaceae* were associated with a higher score, and *Pasteurellaceae* were associated with a lower score rather than the previously reported family *Ruminococcaceae* associated with a higher score in the Finnish study[8]. Interestingly, among controls, 11 different family level taxa were associated with higher Wexner scores. These results suggest that intestinal symptoms such as constipation are associated with different microbiota composition in PD patients compared with controls. Further investigation may be needed to assess whether this is disease specific or not.

We also investigated gut microbiome composition differences according to patients' current PD medication dose represented by LED. We found that LED was significantly associated with microbiome composition represented by beta-diversity analyses for three metrics (Bray-Curtis: P = 0.02, Canberra: P = 0.003, Unweighted UniFrac: P = 0.001; Table 2 (E)). When comparing patients treat at high LED (> median, 690 mg) or low LED (≤ median, 690 mg), 28 bacterial genera showed statistically significant differences in abundance. To our knowledge, this is one of the first reports in the field that investigated gut microbiome composition and PD medication doses represented by LED. Further exploration is needed to verify these bacterial targets and analyze the potential relationship between these bacteria and PD medications.

Other factors including dietary habits, major infections, immune diseases, host genetics, and antibiotics use history may also alter the stability and the composition of the gut microbiota[16,17,32,33]. Diet is thought to be one of the most dominating factors for microbiome composition as a study showed that diet changes explained 57% of the total structural variation in the gut microbiota[34], suggesting profound influences of diet on gut microbiota. In one of the largest

studies of gut microbiota in PD, 327 participants (212 PD cases, 136 controls including 54 spouses) from the Neuro Genetics Research Consortium study, fruit and vegetable intake were reported to have influenced the participants' microbiome[13]. In our study, however, we found no between group differences in macronutrients (total fat, protein, and carbohydrate), dietary fiber, and calorie intake that influenced microbiome composition. We acknowledge the potential limitation of our cohort size and the limitations of self-reported dietary information.

In our study, 36 PD patients were paired with controls living in the same household and we were able to conduct pair-wise microbiota composition analyses and compare these with all other controls. Interestingly, even though no statistically significant alpha diversity was detected, pair-wise comparison of gut microbiome composition for the household control group showed a greater richness of control microbiota compared with PD patients. Previous reports have demonstrated that there is an undergoing latent inflammatory process in the intestine of PD patients, which was considered a trigger factor for α-synuclein misfolding in gut neurons[8,14]. Our observation of a reduction in gut microbiota diversity in PD patients compared with their household controls might also be related to disease specific inflammatory processes[12,35]. In pair-wise PERMANOVA tests, we identified differences in microbial composition, and we found statistically significant between-case-control group differences. Compared with household controls only, we also identified 2 genera (*[O: Rhodospirillales] unclassified* and *Lactobacillus*) with statistically significant abundance increase and 3 genera (*Prevotella 9, CAG-352,* and *[Eubacterium] ruminantium group*) with abundance decrease in PD patients. Specifically, *Prevotella* showed concordant association in our study (81 cases vs. 56 controls) and previous studies[8-10,12,28].

There are limitations of our current study and findings because there may be other potential confounders such as other medication or lifestyle and environmental impacts that we did not adjust for in our statistical model. Although we see some differences in gut microbiome composition between PD cases and controls, with our relatively moderate sample size (N = 137), our results may be more representative of our cohort than the general PD population due to the uniqueness of the gut microbiota composition in each individual. Moreover, the differences in methodologic approaches (e.g. sequencing platform, QC criteria, analysis methods, etc.) and geographical background of the investigated subjects make it difficult to compare our results with the previous studies.

In summary, our study confirms that PD patients have differences in abundance of certain gut microbiota compared with controls. Our results largely overlapped with previously reported findings in terms of several taxa having shifted in abundance in PD patients relative to controls. The household control pair-wise results suggested differences in the gut microbiome of PD patients controlling for similar living environments. While current studies provided evidence that environmental factors influence neurodegeneration, our future goals are to elucidate whether an altered gut microbiome and its function contributes to the onset and/or progression of neurodegeneration in PD, and whether the microbiome is affected by chronic environmental factors and exposures, specifically the exposure of pesticides since the agricultural central California counties are highly exposed. Research to uncover potentially novel etiologies in the aspect of gut microbiome for PD may ultimately advance the long-term goal of developing new treatment options for PD and neurodegeneration.

5. Reference

1. Khoo TK, Yarnall AJ, Duncan GW, et al. The spectrum of nonmotor symptoms in early Parkinson disease. Neurology 2013;80:276-81.

2. Adler CH, Beach TG. Neuropathological basis of nonmotor manifestations of Parkinson's disease. Movement disorders : official journal of the Movement Disorder Society 2016;31:1114-9.

3. Stokholm MG, Danielsen EH, Hamilton-Dutoit SJ, Borghammer P. Pathological α-synuclein in gastrointestinal tissues from prodromal Parkinson disease patients. Annals of Neurology 2016;79:940-9.

4. Shannon KM, Keshavarzian A, Mutlu E, et al. Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. Movement Disorders 2012;27:709-15.

5. Sampson TR, Debelius JW, Thron T, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 2016;167:1469-80.e12.

6. Malkki H. Could gut microbiota influence severity of Parkinson disease? Nature Reviews Neurology 2016;13:66.

7. Tremlett H, Bauer KC, Appel-Cresswell S, Finlay BB, Waubant E. The gut microbiome in human neurological disease: A review. Annals of Neurology 2017;81:369-82.

8. Scheperjans F, Aho V, Pereira PAB, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. Movement Disorders 2015;30:350-8.

9. Forsyth CB, Shannon KM, Kordower JH, et al. Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. PloS one 2011;6:e28032-e.

10. Hasegawa S, Goto S, Tsuji H, et al. Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease. PloS one 2015;10:e0142164-e.

11. Suzuki A, Ito M, Hamaguchi T, et al. Quantification of hydrogen production by intestinal bacteria that are specifically dysregulated in Parkinson's disease. PLOS ONE 2018;13:e0208313.

12. Petrov VA, Saltykova IV, Zhukova IA, et al. Analysis of Gut Microbiota in Patients with Parkinson’s Disease. Bulletin of Experimental Biology and Medicine 2017;162:734-7.

13. Hill-Burns EM, Debelius JW, Morton JT, et al. Parkinson's disease and Parkinson's disease medications have distinct signatures of the gut microbiome. Movement disorders : official journal of the Movement Disorder Society 2017;32:739-49.

14. Keshavarzian A, Green SJ, Engen PA, et al. Colonic bacterial composition in Parkinson's disease. Movement Disorders 2015;30:1351-60.

15. Bedarf JR, Hildebrand F, Coelho LP, et al. Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naïve Parkinson’s disease patients. Genome Medicine 2017;9:39.

16. Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. Nature 2016;535:56-64.

17. Modi SR, Collins JJ, Relman DA. Antibiotics and the gut microbiota. The Journal of clinical investigation 2014;124:4212-8.

18. Forslund K, Hildebrand F, Nielsen T, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature 2015;528:262.

19. Costello S, Cockburn M, Bronstein J, Zhang X, Ritz B. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. American journal of epidemiology 2009;169:919-26.

20. Wang A, Costello S, Cockburn M, Zhang X, Bronstein J, Ritz B. Parkinson's disease risk from ambient exposure to pesticides. European journal of epidemiology 2011;26:547-55.

21. Alegre ML, Mannon RB, Mannon PJ. The microbiota, the immune system and the allograft. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2014;14:1236-48.

22. Agachan F, Chen T, Pfeifer J, Reissman P, Wexner S. A Constipation Scoring System to simplify evaluation and management of constipated patients1996.

23. Song SJ, Amir A, Metcalf JL, et al. Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. mSystems 2016;1:e00021-16.

24. Tong M, Jacobs JP, McHardy IH, Braun J. Sampling of intestinal microbiota and targeted amplification of bacterial 16S rRNA genes for microbial ecologic analysis. Current protocols in immunology 2014;107:7.41.1-7..11.

25. H. McArdle B, Anderson M. Fitting Multivariate Models to Community Data: A Comment on Distance-Based Redundancy Analysis2001.

26. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one 2013;8:e61217-e.

27. Tomlinson CL, Stowe R, Patel S, Rick C, Gray R, Clarke CE. Systematic review of levodopa dose equivalency reporting in Parkinson's disease. Movement Disorders 2010;25:2649-53.

28. Unger MM, Spiegel J, Dillmann K-U, et al. Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. Parkinsonism & Related Disorders 2016;32:66-72.

29. Hopfner F, Künstner A, Müller SH, et al. Gut microbiota in Parkinson disease in a northern German cohort. Brain Research 2017;1667:41-5.

30. Bedarf JR, Hildebrand F, Coelho LP, et al. Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naïve Parkinson's disease patients. Genome medicine 2017;9:39-.

31. Freier TA, Beitz D, Li L, Hartman PD. Characterization of Eubacterium coprostanoligenes sp. nov., a cholesterol-reducing anaerobe. International journal of systematic bacteriology 1994;44:137-42.

32. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nature Reviews Neuroscience 2012;13:701.

33. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology 2014;146:1489-99.

34. Zhang C, Zhang M, Wang S, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. The Isme Journal 2009;4:232.

35. Lapthorne S, Pereira-Fantini PM, Fouhy F, et al. Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome. Gut microbes 2013;4:212-21.