

Structural changes of gut microbiota in Parkinson's disease and its correlation with clinical features

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The aim of this study was to compare the structure of gut microbiota in Parkinson's disease (PD) patients and healthy controls; and to explore correlations between gut microbiota and PD clinical features. We analyzed fecal bacterial composition of 24 PD patients and 14 healthy volunteers by using 16S rRNA sequencing. There were significant differences between PD and healthy controls, as well as among different PD stages. The putative cellulose degrading bacteria from the genera *Blautia* ($P=0.018$), *Faecalibacterium* ($P=0.048$) and *Ruminococcus* ($P=0.019$) were significantly decreased in PD compared to healthy controls. The putative pathobionts from the genera *Escherichia-Shigella* ($P=0.038$), *Streptococcus* ($P=0.01$), *Proteus* ($P=0.022$), and *Enterococcus* ($P=0.006$) were significantly increased in PD subjects. Correlation analysis indicated that disease severity and PD duration negatively correlated with the putative cellulose degraders, and positively correlated with the putative pathobionts. The results suggest that structural changes of gut microbiota in PD are characterized by the decreases of putative cellulose degraders and the increases of putative pathobionts, which may potentially reduce the production of short chain fatty acids, and produce more endotoxins and neurotoxins; and these changes is potentially associated with the development of PD pathology.

microbiome, α -synuclein, gastrointestinal dysfunction, gut-brain-axis, 16S rRNA sequencing, short chain fatty acids

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INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive, disabling neurodegenerative disorder that begins in mid to late life. The etiology of PD is not well understood but likely to be the interactive effects of environmental and genetic factors (de Lau and Breteler, 2006); whereas the potential contributions of gut microbiota and the enteric nervous system to aging and PD are becoming increasingly recognized (Fasano et al.,

2015; Hu et al., 2016; Keshavarzian et al., 2015; Scheperjans et al., 2015). Braak et al. demonstrated that the appearance of α -synuclein-positive Lewy pathology initially occurred in both the enteric nervous system and the dorsal motor nucleus of the vagus at the presymptomatic stage of PD; however, when pathological changes were observed in the substantia nigra, most patients had entered symptomatic stages of PD (Braak et al., 2003; Braak et al., 2004). Recent studies reported that gastrointestinal dysfunction occurred with relatively high frequency in PD patients: 70%–100% of PD patients reported gastric motility disorders, 80%–90% of patients reported constipation, and 25%–67% of patients

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reported small intestinal bacterial overgrowth (Dobbs et al., 2012; Fasano et al., 2013; Fasano et al., 2015; Heetun and Quigley, 2012; Tan et al., 2014). These gastrointestinal symptoms and disorders are frequently reported years before the onset of parkinsonian motor symptoms (Cersosimo et al., 2013; Lang, 2011; Rayner and Horowitz, 2013).

The gut microbiota contributes to regulation of gastrointestinal motility, facilitating nutrient absorption and metabolism, modulating mucosal immune system and maintaining intestinal health (Collins and Bercik, 2009; Macpherson and Harris, 2004). Furthermore, accumulating evidence indicates an intense bidirectional interaction between gut microbiota and the central nervous system (Bercik et al., 2011; Cryan and Dinan, 2012; Felice et al., 2016; Mulak and Bonaz, 2015; Scheperjans et al., 2015). Recent studies suggest a role for gut microbiota in metabolic, neoplastic, and immunologic diseases (Brugman et al., 2006; Qin et al., 2014; Scher et al., 2013; Sjögren et al., 2009), and even some neurological disorders, such as autism and major depressive disorders (Adams et al., 2011; Jiang et al., 2015; Keshavarzian et al., 2015; Parracho et al., 2005; Scheperjans et al., 2015). Thus, we assume that the gut microbiota composition of PD patients may differ from healthy controls, and this difference may be correlated with PD clinical features.

RESULTS

Subjects

Characteristics of the participants are shown in Table 1. There was no statistically significant differences between patient and control groups in terms of age, gender and body mass index (BMI). All but two PD patients were taking anti-parkinsonian medication. No study subjects had diabetes, infectious diseases, or required special diets.

Characteristics of sequencing data

From 14 fecal samples of healthy controls (HC), 1,413,984 reads were obtained (average 100,999 reads per sample). From 24 fecal samples of PD patients, 2,735,933 reads were obtained (average 113,997 reads per sample). Comparisons of community richness (Chao) and diversity (Shannon) were performed after equalizing library sizes to the minimum library size by random subtraction. There were no statistically significant differences of community richness or diversity between PD patients and controls. Summary information for sequencing data is shown in Table 2. The Shannon rarefaction curves approached plateau indicating that no more Operational taxonomic units (OTUs) would likely be detected by increasing the number of sequences (Figure S1 in Supporting Information). Good's coverage for each sample was more than 98%, indicating that the OTUs identified in each sample represented the majority of bacteria species identified in all samples.

Intestinal microbial compositions of PD and HC groups differed significantly

Phylum-level comparison of PD and HC. 22 phyla in PD group and 20 phyla in HC group were identified respectively. The top four dominant phyla of both groups were Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria, which were 98.8% of total reads, and this agreed with previous studies (Ottman et al., 2012). Despite the highly diverse bacterial communities among individuals, the fecal microbial compositions of the PD group were distinctively different from controls at the phylum level (Figure 1A). Compared with controls, the relative abundance of Actinobacteria were highly enriched in patients with PD (1.42% vs. 0.22%, $P<0.001$), and a similar trend was seen with Proteobacteria

Table 1 Demographic characteristics of the participants^{a)}

	Healthy control	PD patients	P-value
<i>n</i>	14	24	N/A
Gender (Male/Female)	6/8	16/8	0.152*
Age (mean±SD)	74.64±5.57	73.75±6.26	0.662**
BMI*** (mean±SD)	24.00±2.66	22.96±3.64	0.357**

a) *, Chi-squared test; **, Student's *t*-test; ***, BMI=kg m⁻²

Table 2 Summary of sequencing data^{a)}

	Healthy control	PD patients	P-value
Sequences (mean±SD)	100,998.9±21,022.25	113,997.2±28,341.41	0.145*
OTUs (mean±SD)	3,252.79±995.37	2,925.96±934.5	0.317*
Chao (mean±SD)	5,392.49±1,384.9	4,681.49±1,407.94	0.140*
Shannon (mean±SD)	4.87±0.50	4.65±0.59	0.248*
Good's coverage	98.43%	98.75%	N/A

a) *, Student's *t*-test.

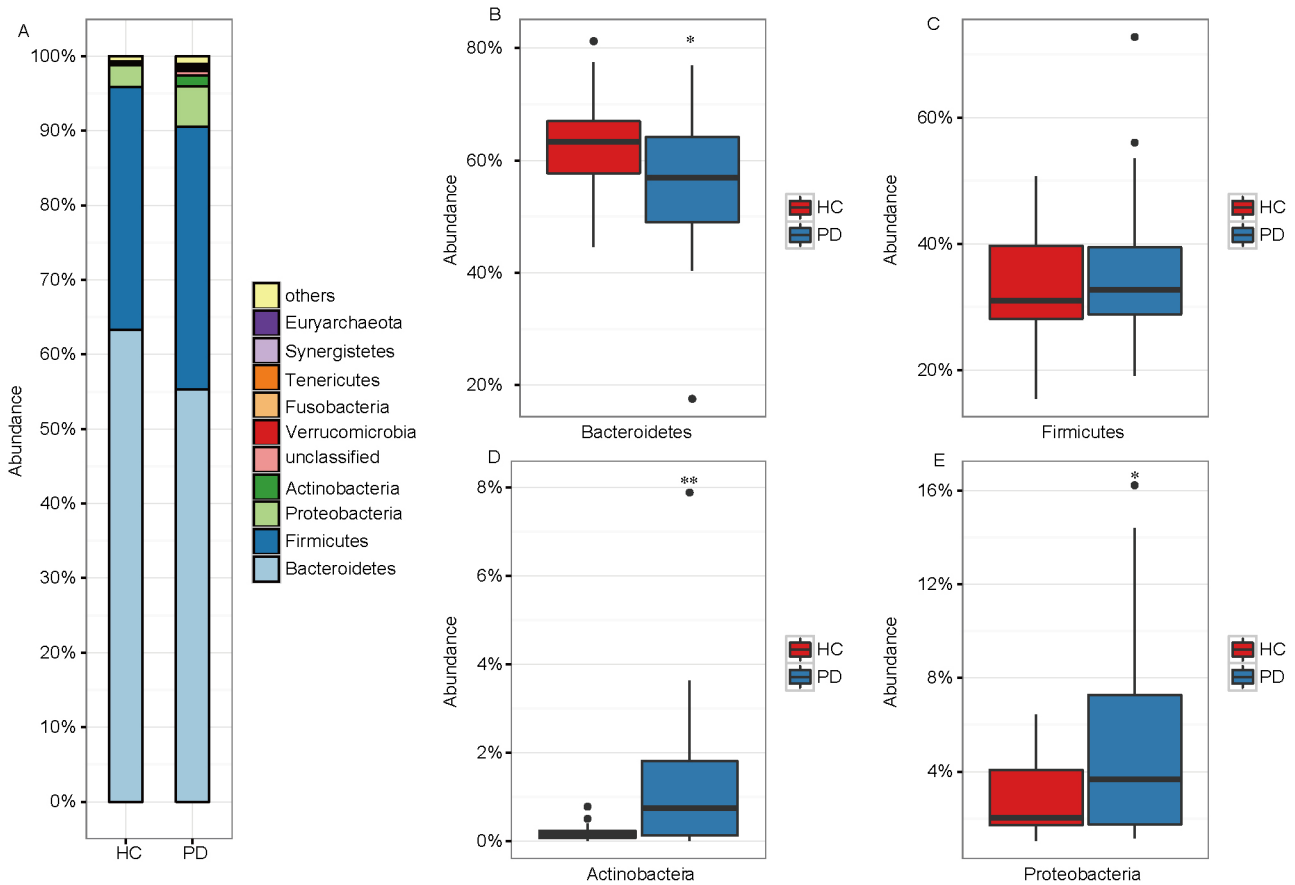


Figure 1 Phylum level comparison. A, Comparison of the relative abundance of the ten most abundant bacterial phyla in HC and PD groups; B–E, Comparison of the relative abundance of the top four dominant bacterial phyla: Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria. Statistical analysis was performed by Metastats method. *, $P < 0.05$; **, $P < 0.01$.

(5.51% vs. 2.92%, $P = 0.029$) (Figure 1D and E). Whereas, Bacteroidetes were significantly decreased in the PD group (55.27% vs. 63.26%, $P = 0.045$) (Figure 1B). Although the phylum Firmicutes did not significantly change (Figure 1C), two classes in this phylum: Bacilli (1.19% vs. 0.12%, $P < 0.001$) and Negativicutes (9.16% vs. 3.22%, $P = 0.005$) were both significantly enriched in the PD group.

There were 26 statistically significant differences between PD and HC groups at the family level. The relative abundance of Enterobacteriaceae, Veillonellaceae, Erysipelotrichaceae, Coriobacteriaceae, Streptococcaceae, Moraxellaceae and Enterococcaceae were significantly higher in PD group compared to the HC group. The family of Prevotellaceae, Ruminococcus and Lachnospiraceae were substantially decreased in PD group compared with controls, although they did not achieved statistically significance (Figure S2 in Supporting Information).

Genus-level comparison of PD and HC. The microbial composition was also distinctly different at the genus level. Among the top 50 abundant genera, *Acidaminococcus*, *Acinetobacter*, *Enterococcus*, *Escherichia-Shigella*, *Megamonas*, *Megasphaera*, *Proteus*, *Streptococcus* were significantly more abundant in PD compared to HC. *Blautia*, *Faecal-*

ibacterium, *Ruminococcus* were significantly more abundant in HC (Table S1 in Supporting Information). Interestingly, among these genera, *Enterococcus*, *Escherichia-Shigella* and *Proteus*, putative pathobionts that could produce endotoxins and promote inflammation in human intestines, were enriched significantly in the PD group (Figure 2D–F) (Chen et al., 2011; Huycke et al., 2002; Schaffer and Pearson, 2015). *Blautia*, *Faecalibacterium*, and *Ruminococcus*, putative cellulose degraders that facilitate fermentation of resistant starch and cellulose in the colon and produce short chain fatty acids (SCFAs), were decreased significantly in PD group (Figure 2A–C) (Abell et al., 2008; Leitch et al., 2007; Takahashi et al., 2016; Walker et al., 2011).

Structural differentiation between PD and HC. According to the unweighted UniFrac principal co-ordinates analysis (PCoA) analysis, the gut microbiota of the HC and PD patients are separated according to PC1 and PC2 (8.03% and 6.32%) (Figure 3). Hierarchical cluster analysis based on the relative abundance of genera demonstrated that the HC and PD groups were different according to the Spearman's correlation distance matrix, and samples from each group were clustered with relatively high similarity (Figure 4). The heatmap based on the genus-level data agreed with this out-

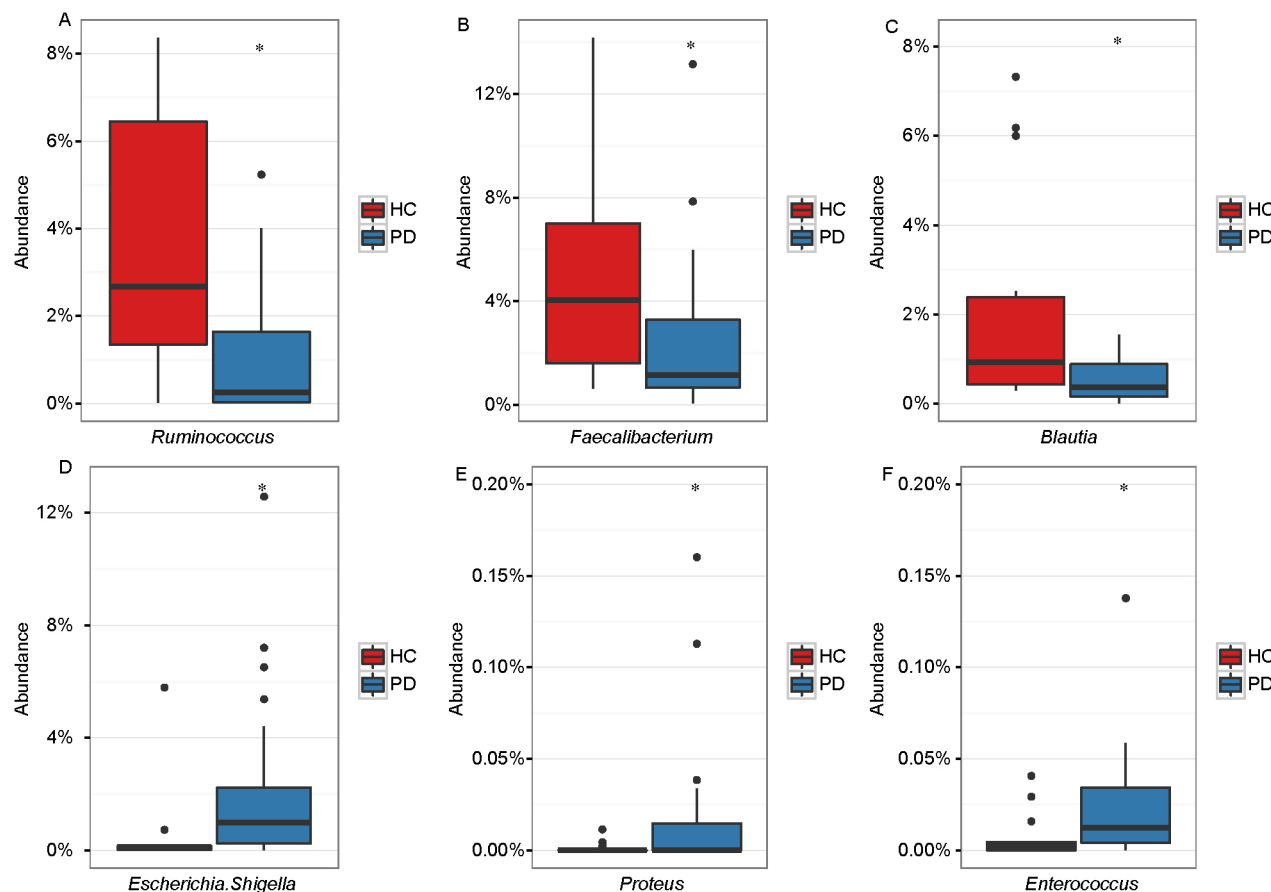


Figure 2 Relative abundance of putative cellulose degraders and pathobionts. A–C, Relative abundance of putative cellulose degraders, *Ruminococcus*, *Faecalibacterium* and *Blautia* in HC and PD groups. D–F, Relative abundance of putative pathobionts, *Escherichia-Shigella*, *Proteus* and *Enterococcus* in HC and PD groups. Statistical analysis was performed by Metastats method. *, $P < 0.05$.

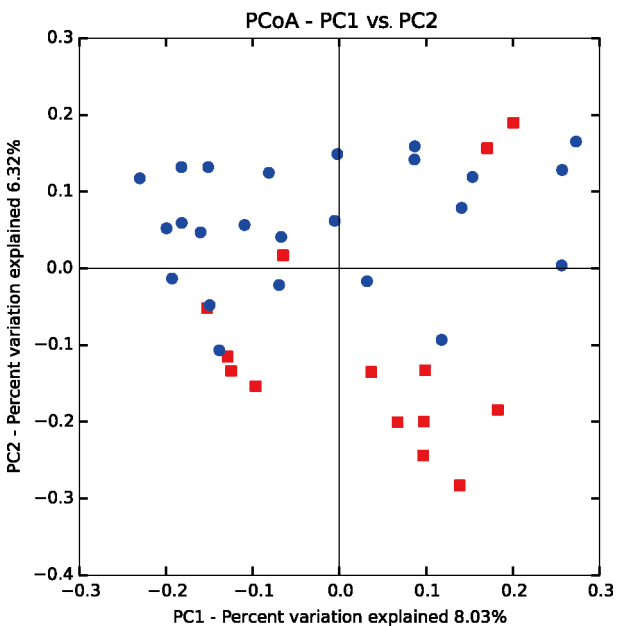


Figure 3 Principal co-ordinates analysis (PCoA). PCoA plots based on unweighted UniFrac distance metrics using the relative abundance of OTUs. Red points stand for HC samples, blue squares stand for PD samples. The PCoA plots separate the PD subjects from HC.

come (Figure S3 in Supporting Information).

Intestinal microbial compositions of different PD stages differed significantly

We divided patient group into two subgroups according to disease stages, to learn if there were bacterial indicators that changed consistently with the development of PD pathology. Patients with Hoehn and Yahr (H&Y) stage ranging from 1–2.5 were included in the mild PD (MPD) group, while patients with H&Y stage ranging from 3–5 were included in the severe PD (SPD) group. There were no statistically significant differences among MPD, SPD and control groups in terms of age, gender and BMI, however, the unified Parkinson's disease rating scale (UPDRS) scores of the MPD group were significantly lower than that of the SPD group, which proved our assumption (Table S2 in Supporting Information). At the genus level, only two bacterial genera changed consistently with the development of PD pathology. The relative abundance of *Faecalibacterium* was 5.14% in control group, reduced to 3.17% in MPD group, and remarkably decreased to 1.22% in SPD group ($P = 0.048$). The relative abundance of *Megasphaera* was 0.04% in control group, increased to

2.52% in MPD group, and substantially increased to 5.50% in SPD group ($P=0.003$) (Figure 5). When the two untreated PD samples were removed from the analysis, the significant differences were maintained for genera *Faecalibacterium* ($P=0.038$) and *Megasphaera* ($P=0.004$), suggesting that though medication was likely having an effect, disease stage was still significantly impacting gut microbiota in PD. These data indicate that gut microbiota of different disease stages may differ as PD pathology progresses.

Intestinal microbiota correlates with PD clinical features

Clinical PD features such as PD duration, disease severity (UPDRS and H&Y stage), constipation (Wexner), non-motor symptoms (NMSS), and anxiety and depression symptoms (HAMA & HAMD) were quantified and associated with intestinal microbial composition. Clinical details of all subjects were summarized in Table S3 in Supporting Information.

The correlation matrix based on the Spearman's correlation distance confirmed correlations between the relative abun-

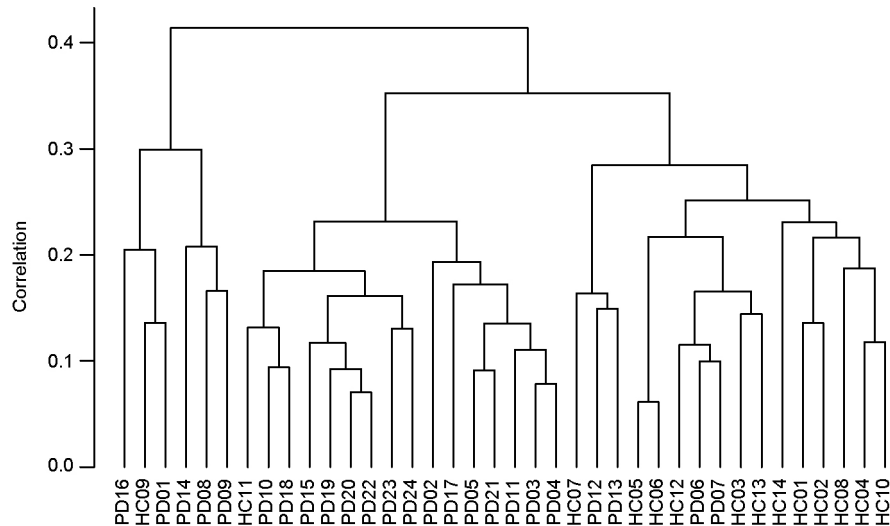


Figure 4 Hierarchical clustering analysis. Hierarchical clustering based on Spearman's correlation distance matrix using the relative abundance of genus, and ward. D method is used to cluster the samples using R stats package. The clustering shows grouping of PD subjects toward the left part of the dendrogram, and grouping of HC subjects toward the right part of the dendrogram. This pattern indicates a differentiation between PD and HC groups.

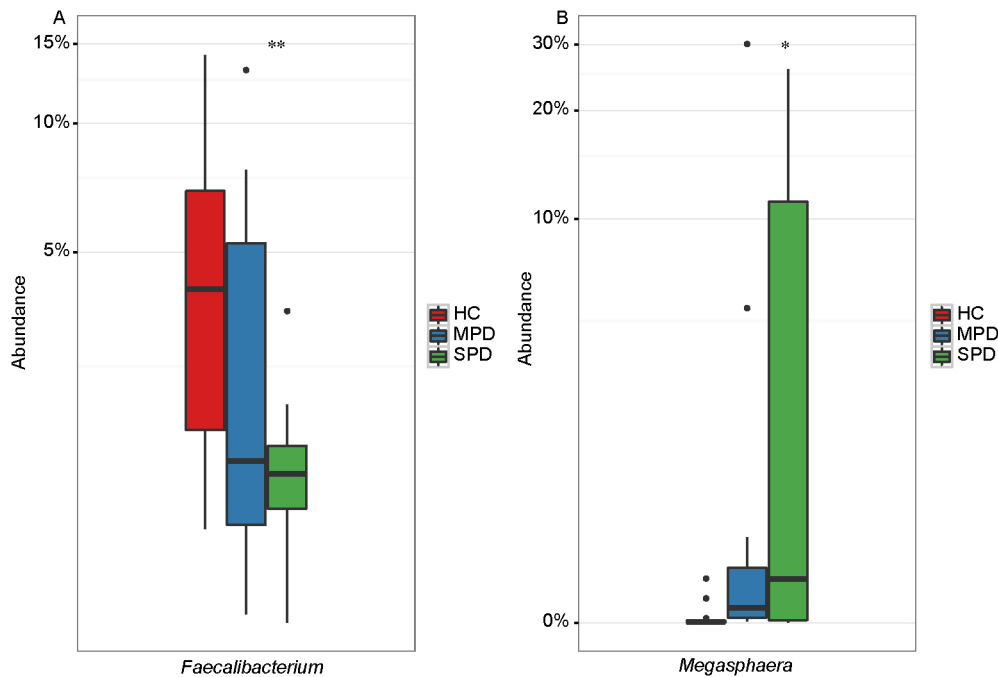


Figure 5 Genus level comparison among groups of different disease stages. Comparison of relative abundance of *Faecalibacterium* and *Megasphaera* in groups of different disease stages. Statistical analysis was performed by Kruskal-Wallis test. *, $P<0.05$; **, $P<0.01$.

dance of the top 65 abundant genera and clinical features (Figure 6). At the genus level, UPDRS scores positively correlated with *Enterococcus* ($r=0.336$, $P=0.039$), *Proteus* ($R=0.45$, $P=0.005$) and *Escherichia-Shigella* ($R=0.383$, $P=0.018$), and negatively correlated with *Blautia* ($r=-0.50$, $P=0.002$), *Faecalibacterium* ($R=-0.357$, $P=0.028$), *Ruminococcus* ($r=-0.51$, $P=0.001$), *Haemophilus* ($r=-0.46$, $P=0.004$) and *Odoribacter* ($r=-0.41$, $P=0.011$). PD duration positively correlated with *Proteus* ($r=0.46$, $P=0.003$), *Enterococcus* ($r=0.56$, $P<0.001$), *Escherichia-Shigella* ($r=0.52$, $P=0.001$), *Megasphaera* ($r=0.41$, $P=0.012$), and negatively correlated with *Blautia* ($r=-0.40$, $P=0.013$), *Ruminococcus* ($r=-0.58$, $P<0.001$), *Sporobacter* ($r=-0.40$, $P=0.013$), *Haemophilus* ($r=-0.47$, $P=0.003$). Interestingly, we noticed that putative pathobionts *Enterococcus*, *Escherichia-Shigella* and *Proteus* were significantly increased in the PD group, and positively correlated with disease severity (UPDRS) and PD duration (Figure 7A–C); whereas, putative cellulose degraders *Blautia*, *Faecalibacterium*, and *Ruminococcus* were significantly decreased in the PD group, and correlated negatively with disease severity (UPDRS) and PD duration (Figure 7D–F).

DISCUSSION

Gut microbiota interact extensively with the host through metabolic exchange and co-metabolism of substrates to maintain normal functions and health of the intestinal tract

and the whole body (Nicholson et al., 2005). In this study, we found that the intestinal microbiota composition of PD patients and HC differed significantly at all taxonomic levels. One characteristic of this structural differentiation is fewer putative cellulose degraders. Previous studies indicate that *Ruminococcus* is a key cellulose degrader colonized in the large bowel, cecum or rumen of the host, which facilitates the fermentation of carbohydrates such as cellulose, pectin and starch (Abell et al., 2008; Leitch et al., 2007; Wang et al., 1997). *Blautia* and *Faecalibacterium* are butyrate -producing bacteria that facilitate the degradation of cellulose and starch (Takahashi et al., 2016). Fermentation of resistant starch and dietary fibers in the large bowel by bacteria produce volatile SCFAs, including acetate, propionate and butyrate (Topping and Clifton, 2001). Acetate and propionate could affect cholesterol synthesis; reduction of acetate propionate proportion could reduce the risk of cardiovascular disease (Erkkilä et al., 2008). Butyrate is the major trophic factor of enterocytes, which is beneficial for intestinal mucosa and for preventing colonic cancer (Cho et al., 2014; Nakano et al., 1997). In addition, SCFAs have been recognized as potential mediators involved in the effects of gut microbiota on intestinal immune function; SCFAs regulate the inflammatory process by acting on leukocytes and endothelial cells (Vinolo et al., 2011). Because *Ruminococcus*, *Blautia* and *Faecalibacterium* are closely related to the production of SCFAs, significant decreases of these genera in PD subjects compared to HC is likely to reduce SCFAs in the host intes-

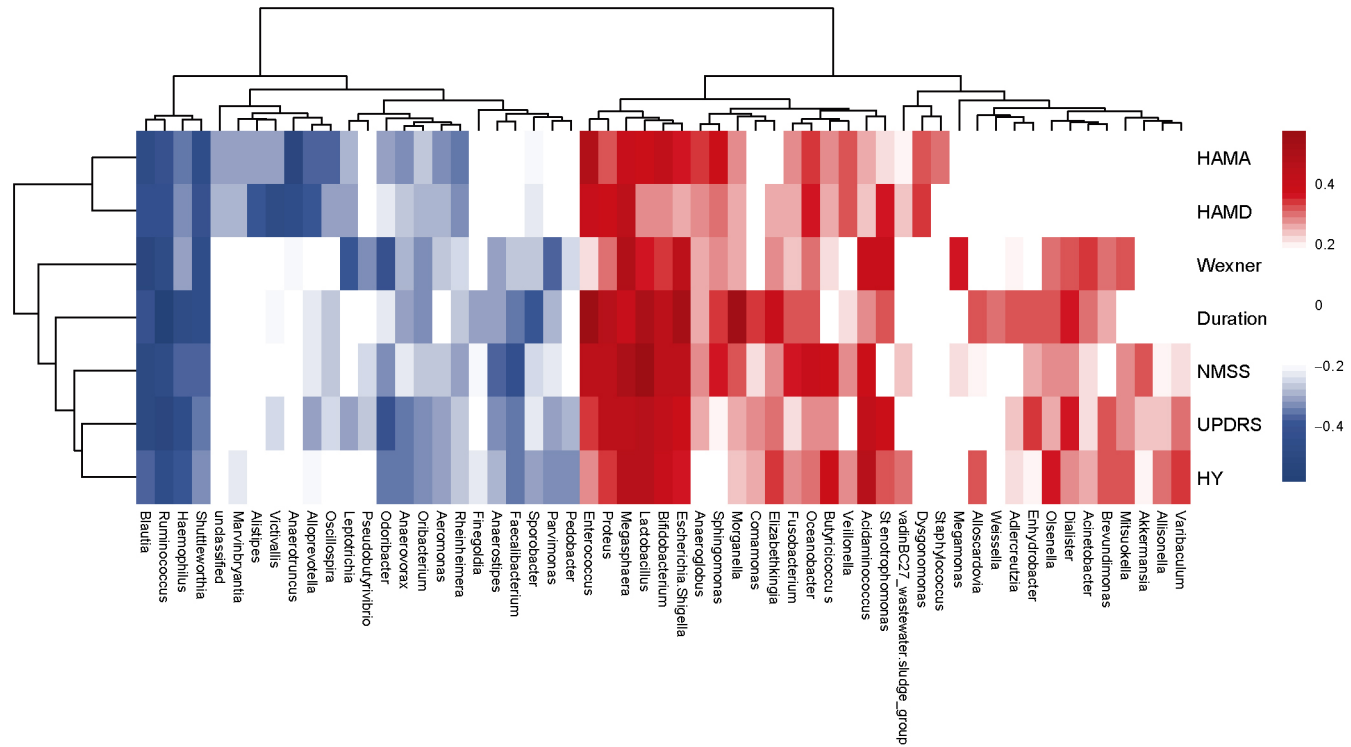


Figure 6 Correlation matrix. The correlation matrix based on the Spearman's correlation distance confirmed correlations between the relative abundance of the top 65 abundant genera and clinical features. Color blue indicate negative correlation, color red indicate positive correlation.

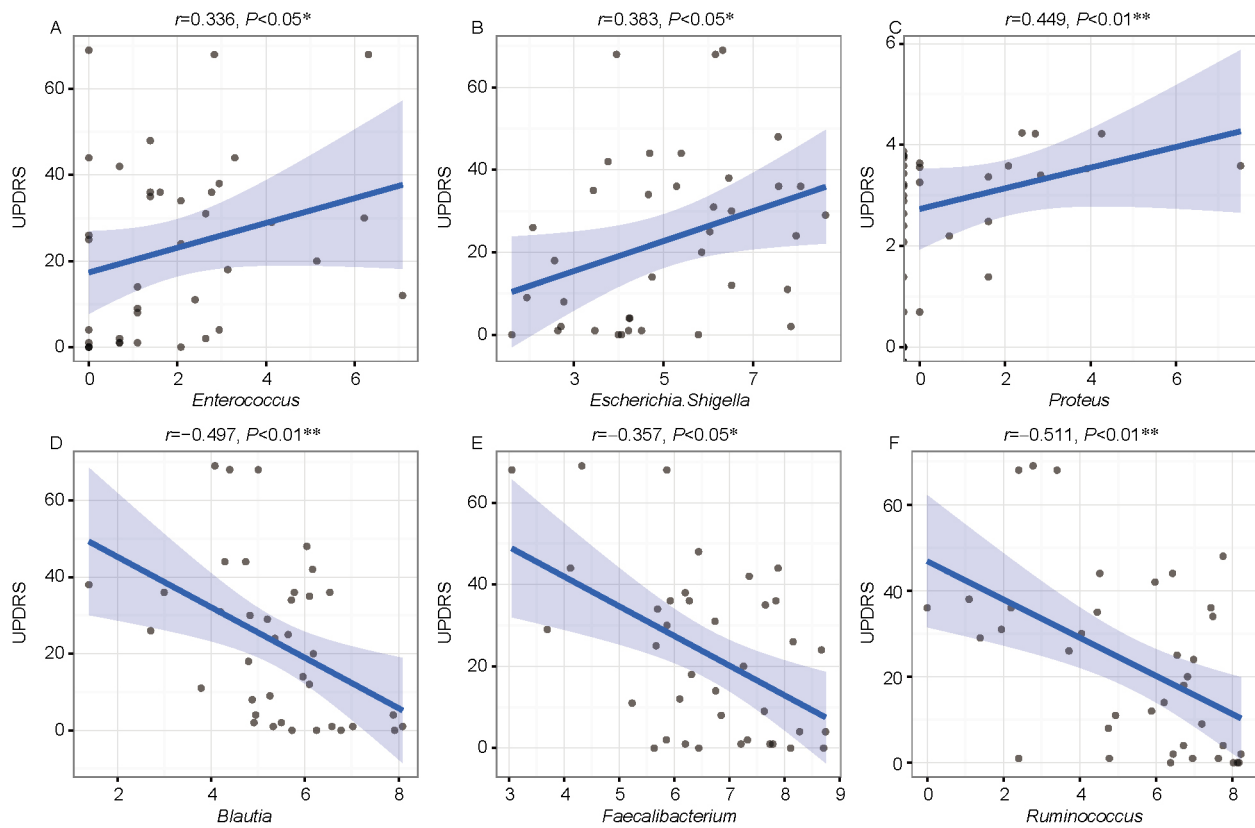


Figure 7 Correlation scatter plots with fitting line. A–C, UPDRS score positively correlated with the relative abundance of putative pathobionts, *Enterococcus*, *Escherichia-Shigella* and *Proteus*. D–F, UPDRS score negatively correlated with the relative abundance of putative cellulose degraders, *Blautia*, *Faecalibacterium*, and *Ruminococcus*.

time, which might promote gut inflammation and increase the risk of α -synuclein deposition in the gastrointestinal tract. A recent study indicated that fecal SCFAs concentrations were significantly reduced in PD, which agreed with our assumption (Pfeiffer, 2011). In a previous study of the gut microbiome in PD, Keshavarzian et al. reported that *Blautia* was significantly reduced in fecal samples of PD subjects, and that *Faecalibacterium* was significantly reduced in colonic mucosal samples of PD subjects (Keshavarzian et al., 2015).

Another important characteristic of this structural change of the gut microbiome subjects is a significant increase in putative pathobionts in PD. *Escherichia-Shigella* could cause diarrhea and produce Shiga toxin, which could cause functional lesions in the central nervous system of rabbits and rodents (Bridgewater et al., 1955; Cavanagh et al., 1956). *Streptococcus* could also produce neurotoxins such as streptomycin, streptodornase and streptokinase, which might lead to permanent neurological damages. Clinical research indicates that *Escherichia* and *Streptococcus* might be responsible for the bacterial infection in liver cirrhosis (Qin et al., 2014; Riordan and Williams, 2006). *Proteus* are commonly responsible for urinary and septic infection, which produce endotoxin and hematoxin in the gut (Schaffer and Pearson, 2015; Endimiani et al., 2005). *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that

damages colonic epithelial cell DNA (Huycke et al., 2002); and the increase in *Enterococcus* was reported in colorectal cancer patients (Wang et al., 2012). In our study, we found that the abundance of *Escherichia-Shigella*, *Streptococcus*, *Proteus* and *Enterococcus* were significantly increased in PD subjects compared to HC. The Abnormal increase of these putative pathobionts could produce endotoxins and neurotoxins and elevate inflammation; and evidence suggests that there are systemic immuno-inflammatory processes in PD (Dobbs et al., 1999; Dobbs et al., 2016). Significantly overgrowth of these putative pathobionts in PD could cause or be the result of elevated inflammation, which might provide an internal environment for emergence and development of PD pathology.

In an earlier study of the gut microbiota in PD of European population, Scheperjans et al. reported that the abundance of family Prevotellaceae in feces of PD patients was reduced by 77.6% compared with controls, which was considered to be a biomarker of PD (Scheperjans et al., 2015). We reported that family Prevotellaceae was markedly reduced in PD (4.56% vs. 10.35%), as well as the genus *Prevotella* (4.4% vs. 9.79%), but these reductions did not reach statistical significance. This difference may be attributed to differences in race and dietary patterns of Asian and European populations, or it may be due to the smaller sample size of our study.

With correlation analysis, we found that the abundances of those putative cellulose degraders (*Ruminococcus*, *Blautia* and *Faecalibacterium*) were negatively correlated with UPDRS score and PD duration, but not correlated with age, gender or BMI index, which indicated that reductions in putative cellulose degraders might be related to PD pathology. Second, we found that abundances of putative pathobionts (*Enterococcus*, *Escherichia-Shigella* and *Proteus*) positively correlated with UPDRS score and PD duration, indicating that abnormal increases in these putative pathobionts might be related with PD pathology. Keshavarzian et al. reported that *Blautia* and *Faecalibacterium* negatively correlated with PD duration, but not with UPDRS (Keshavarzian et al., 2015). Research also reported that *Enterococcus* and *Escherichia-Shigella* significantly increased in colorectal cancer patients (Wang et al., 2012); and *Proteus mirabilis* significantly increased in children with autism disorders (Adams et al., 2011), but these putative pathobionts have not been correlated with PD clinical features.

With cross-sectional case-control study, it is not possible to deduce causal relationships between gut microbiota alterations and PD; a large cohort study is needed to give more definitive conclusions. We could not strictly control for medication and diet of our subjects and this may have confounding effects on the results. We did rule out some medication and dietary habits that could strongly affect the gut microbiome. As samples were collected from patients already diagnosed with PD, changes in gut microbiota identified in this work might be just a consequence of PD. But, the structural changes might contribute to progression of PD via mechanisms discussed above. A function study based on metagenomics sequencing would better identify functional pathways of gut microbiota on PD. In the future, clinical interventions for PD such as specific probiotic treatment or fecal transplantation from healthy donors may be considered for PD patients; and the mechanism of the gut microbiota acting on PD may be elucidated by separating specific strains of gut microbiota and examining their effects on PD pathology in an animal model.

In summary, our study suggests that structural changes of gut microbiota in PD are characterized by the decreases of putative cellulose degraders and the increases of putative pathobionts, which may potentially reduce the production of SCFAs, and produce more endotoxins and neurotoxins; and these changes is potentially associated with the development of PD pathology. In the future, structural changes of gut microbiota may become sensitive biomarkers for predicting and evaluating the risk for PD, and this may provide a potential solution for early diagnosis and treatment for PD.

MATERIALS AND METHODS

Patients and controls

PD subjects ($n=24$) were recruited from Beijing Hospital

(Beijing), whereas healthy volunteers ($n=14$) were recruited by research advertisements in communities. All subjects consented to use of their samples for research, and signed written informed consent. The study protocol was approved by the Ethics Committee of the Institute of Psychology, Chinese Academy of Sciences, and performed according to the declaration of Helsinki.

PD was diagnosed according to the UK Brain Bank Criteria (Hughes et al., 1992) by experienced neurologists. Exclusion criteria for PD subjects were: (i) atypical or secondary Parkinsonism, (ii) regular use of probiotics or antibiotics within three months prior to sample collection, (iii) active or persistent primary gastrointestinal diseases, or (iv) unstable medical, neurological, or psychiatric illness.

HC were citizens who matched the PD group by age and gender. Exclusion criteria for HC subjects were: (i) active or persistent primary gastrointestinal disease or neurodegenerative diseases, (ii) unstable medical, neurological, or psychiatric illness, or (iii) regular use of probiotics or antibiotics within 3 months before sample collection (Keshavarzian et al., 2015).

Parkinsonian symptoms were measured using the Unified Parkinson's Disease Rating Scale and the Hoehn and Yahr scale (Goetz et al., 2004; Movement Disorder Society Task Force on Rating Scales for Parkinson's, 2003). Non-motor symptoms were assessed using the Non-Motor Symptoms Scale (Chaudhuri et al., 2006). Constipation severity was quantified using the Wexner Constipation Scoring System (Agachan et al., 1996). Anxiety and depression symptoms were rated using the Hamilton Anxiety Scale (HAMA) and Hamilton Depression Scale (HAMD) (Hamilton, 1959; Hamilton, 1960).

Sample collection and DNA extraction

Subjects collected fecal samples into sterile disposable containers at the hospital or at home. Fecal samples were frozen and stored at -80°C for further use. Bacterial DNA was extracted using a TIANamp stool DNA kit (Tiangen Biotech Co. Ltd., Beijing) according to the manufacturer's instructions. DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, USA) and DNA quality was confirmed using 0.8% agarose gel electrophoresis.

16S rRNA gene amplification

Isolated fecal DNA was used as a template for the amplification of the V3–V5 region of 16S rRNA gene. The V3 and V4 regions were amplified using forward primers containing the sequence 5'-CCTACGGRBGCASCAGKVRVGAAT-3' and reverse primers containing the sequence 5'-GGACTAC-NVGGGTWCTAATCC-3'. The V4 and V5 regions were amplified using forward primers containing the sequence 5'-GTGYCAGCMGCCGCGGTAA-3' and reverse primers

containing the sequence 5'-CTTGTGCGGKCCCCGY-CAATTC-3'. Besides the 16S target-specific sequence, primers also contained adaptor sequences allowing uniform amplification of a highly complex library ready for downstream NGS sequencing on Illumina MiSeq.

DNA libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA), and quantified by Qubit and real time PCR (Applied Biosystems, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, USA). Sequencing was performed using a 2×250 or 2×300 paired-end (PE) configuration; image analysis and base calling were conducted with MiSeq Control Software on the MiSeq instrument.

Bioinformatics and statistical data analyses

The barcodes and primers were trimmed from the sequences, and the bases below quality score of 20 were cut off at the start and the end of reads (Trimmomatic v0.30) (Bolger et al., 2014). Sequences that contained ambiguous base calls and short sequences <400 bp were removed from the raw data. Then, chimeras were removed using USEARCH (v8.0). OTUs were identified clustering at 97% similarity (3% divergence) using Mothur (version 1.34.4) (Schloss et al., 2009). Taxonomical assignments of OTUs were performed in accordance with SILVA 16S rRNA database v119 at 80% confidence level (Pruesse et al., 2007). The library size of each sample was normalized based on the smallest library size of samples by random subtraction following the Mothur MiSeq SOP (Kozich et al., 2013). Bacterial diversity and richness were analyzed by calculating the Chao1 index, the Shannon index and Good's coverage, and Rarefaction curves were plotted based on the Shannon index.

The Metastats method was used to evaluate abundance features between PD and control groups (<http://metastats.cbcb.umd.edu/detection.html>) (White et al., 2009). Heatmaps and hierarchical clustering was generated by using R package "heatmap" and "stats" respectively. Unweighted UniFrac distance metrics analysis was performed using OTUs, and PCoA was conducted according to the matrix of distance. Correlation between variables was computed using Spearman rank correlation, and a scatter plot was generated using R package "ggplot2". Kruskal-Wallis test, Student's *t*-test, Chi-squared test, and Spearman's rank correlation were conducted using SPSS version 20.0 for Windows.

Compliance and ethics The author(s) declare that they have no conflict of interest.

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Abell, G.C.J., Cooke, C.M., Bennett, C.N., Conlon, M.A., and McOrist, A.L.

(2008). Phylotypes related to *Ruminococcus bromii* are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. *FEMS Microbiol Ecol* 66, 505–515.

Adams, J.B., Johansen, L.J., Powell, L.D., Quig, D., and Rubin, R.A. (2011). Gastrointestinal flora and gastrointestinal status in children with autism-comparisons to typical children and correlation with autism severity. *BMC Gastroenterol* 11, 22.

Agachan, F., Chen, T., Pfeifer, J., Reissman, P., and Wexner, S.D. (1996). A constipation scoring system to simplify evaluation and management of constipated patients. *Dis Colon Rectum* 39, 681–685.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett, P., Macri, J., McCoy, K.D., Verdu, E.F., and Collins, S.M. (2011). The intestinal microbiota affect central levels of brain-derived neurotrophic factor and behavior in mice. *Gastroenterology* 141, 599–609.e3.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120.

Braak, H., Ghebremedhin, E., Rüb, U., Bratzke, H., and Del Tredici, K. (2004). Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res* 318, 121–134.

Braak, H., Rüb, U., Gai, W.P., and Del Tredici, K. (2003). Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm (Vienna)* 110, 517–536.

Bridgewater, F.A., Morgan, R.S., Rowson, K.E., and Wright, G.P. (1955). The Neurotoxin of *Shigella shigae*: morphological and functional lesions produced in the central nervous system of rabbits. *Br J Exp Pathol* 36, 447–453.

Brugman, S., Klatte, F.A., Visser, J.T.J., Wildeboer-Veloo, A.C.M., Harmsen, H.J.M., Rozing, J., and Bos, N.A. (2006). Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? *Diabetologia* 49, 2105–2108.

Cavanagh, J., Howard, J., and Whitby, J. (1956). The neurotoxin of *Shigella shigae*. A comparative study of the effects produced in various laboratory animals. *Br J Exp Pathol* 37, 272.

Cersosimo, M.G., Raina, G.B., Pecci, C., Pellene, A., Calandra, C.R., Gutiérrez, C., Micheli, F.E., and Benarroch, E.E. (2013). Gastrointestinal manifestations in Parkinson's disease: prevalence and occurrence before motor symptoms. *J Neurol* 260, 1332–1338.

Chaudhuri, K.R., Healy, D.G., and Schapira, A.H. (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. *Lancet Neurol* 5, 235–245.

Chen, Y., Yang, F., Lu, H., Wang, B., Chen, Y., Lei, D., Wang, Y., Zhu, B., and Li, L. (2011). Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 54, 562–572.

Cho, Y., Turner, N.D., Davidson, L.A., Chapkin, R.S., Carroll, R.J., and Lupton, J.R. (2014). Colon cancer cell apoptosis is induced by combined exposure to the n-3 fatty acid docosahexaenoic acid and butyrate through promoter methylation. *Exp Biol Med* 239, 302–310.

Collins, S.M., and Bercik, P. (2009). The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. *Gastroenterology* 136, 2003–2014.

Cryan, J.F., and Dinan, T.G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 13, 701–712.

de Lau, L.M., and Breteler, M.M. (2006). Epidemiology of Parkinson's disease. *Lancet Neurol* 5, 525–535.

Dobbs, R.J., Charlett, A., Dobbs, S.M., Weller, C., A Ibrahim, M.A., Iguodala, O., Smee, C., Plant, J.M., Lawson, A.J., Taylor, D., and Bjarnason, I. (2012). Leukocyte-subset counts in idiopathic parkinsonism provide clues to a pathogenic pathway involving small intestinal bacterial overgrowth. A surveillance study. *Gut Pathog* 4, 12.

Dobbs, R.J., Charlett, A., Purkiss, A.G., Dobbs, S.M., Weller, C., and Peterson, D.W. (1999). Association of circulating TNF- α and IL-6 with ageing and parkinsonism. *Acta Neurol Scand* 100, 34–41.

- Dobbs, S.M., Dobbs, R.J., Weller, C., Charlett, A., Augustin, A., Taylor, D., Ibrahim, M.A.A., and Bjarnason, I. (2016). Peripheral aetiopathogenic drivers and mediators of Parkinson's disease and co-morbidities: role of gastrointestinal microbiota. *J Neurovirol* 22, 22–32.
- Endimiani, A., Luzzaro, F., Brigante, G., Perilli, M., Lombardi, G., Amicosante, G., Rossolini, G.M., and Toniolo, A. (2005). *Proteus mirabilis* bloodstream infections: risk factors and treatment outcome related to the expression of extended-spectrum β -lactamases. *Antimicrob Agents Chemother* 49, 2598–2605.
- Erkkilä, A., de Mello, V.D.F., Riserus, U., and Laaksonen, D.E. (2008). Dietary fatty acids and cardiovascular disease: an epidemiological approach. *Prog Lipid Res* 47, 172–187.
- Fasano, A., Bove, F., Gabrielli, M., Petracca, M., Zocco, M.A., Ragazzoni, E., Barbaro, F., Piano, C., Fortuna, S., Tortora, A., Di Giacopo, R., Campanale, M., Gigante, G., Lauritano, E.C., Navarra, P., Marconi, S., Gasbarrini, A., and Bentivoglio, A.R. (2013). The role of small intestinal bacterial overgrowth in Parkinson's disease. *Mov Disord* 28, 1241–1249.
- Fasano, A., Visanji, N.P., Liu, L.W.C., Lang, A.E., and Pfeiffer, R.F. (2015). Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol* 14, 625–639.
- Felice, V.D., Quigley, E.M., Sullivan, A.M., O'Keefe, G.W., and O'Mahony, S.M. (2016). Microbiota-gut-brain signalling in Parkinson's disease: implications for non-motor symptoms. *Parkinsonism Relat Disord* 27, 1–8.
- Goetz, C.G., Poewe, W., Rascol, O., Sampaio, C., Stebbins, G.T., Counsell, C., Giladi, N., Holloway, R.G., Moore, C.G., Wenning, G.K., Yahr, M.D., Seidl, L., and Seidl, L. (2004). *Movement Disorder Society Task Force report on the Hoehn and Yahr staging scale: status and recommendations* The *Movement Disorder Society Task Force on rating scales for Parkinson's disease*. *Mov Disord* 19, 1020–1028.
- Hamilton, M. (1959). The assessment of anxiety states by rating. *Brit J Med Psychol* 32, 50–55.
- Hamilton, M. (1960). A rating scale for depression. *J Neurol Neurosurg Psychiatry* 23, 56–62.
- Heetun, Z.S., and Quigley, E.M.M. (2012). Gastroparesis and Parkinson's disease: a systematic review. *Parkinsonism Relat Disord* 18, 433–440.
- Hu, X., Wang, T., and Jin, F. (2016). Alzheimer's disease and gut microbiota. *Sci China Life Sci* 59, 1006–1023.
- Hughes, A.J., Daniel, S.E., Kilford, L., and Lees, A.J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* 55, 181–184.
- Huycke, M.M., Abrams, V., and Moore, D.R. (2002). *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 23, 529–536.
- Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., Wang, W., Tang, W., Tan, Z., Shi, J., Li, L., and Ruan, B. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav Immun* 48, 186–194.
- Keshavarzian, A., Green, S.J., Engen, P.A., Voigt, R.M., Naqib, A., Forsyth, C.B., Mutlu, E., and Shannon, K.M. (2015). Colonic bacterial composition in Parkinson's disease. *Mov Disord* 30, 1351–1360.
- Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 79, 5112–5120.
- Lang, A.E. (2011). A critical appraisal of the premotor symptoms of Parkinson's disease: potential usefulness in early diagnosis and design of neuroprotective trials. *Mov Disord* 26, 775–783.
- Leitch, E.C.M.W., Walker, A.W., Duncan, S.H., Holtrop, G., and Flint, H.J. (2007). Selective colonization of insoluble substrates by human faecal bacteria. *Environ Microbiol* 9, 667–679.
- Macpherson, A.J., and Harris, N.L. (2004). Opinion: interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 4, 478–485.
- Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease. (2003). The Unified Parkinson's Disease Rating Scale (UPDRS): status and recommendations. *Mov Disord* 18, 738–750.
- Mulak, A., and Bonaz, B. (2015). Brain-gut-microbiota axis in Parkinson's disease. *World J Gastroenterol* 21, 10609–10620.
- Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H., and Sakai, T. (1997). Butyrate activates the *WAF1/Cip1* gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J Biol Chem* 272, 22199–22206.
- Nicholson, J.K., Holmes, E., and Wilson, I.D. (2005). Opinion: gut microorganisms, mammalian metabolism and personalized health care. *Nat Rev Micro* 3, 431–438.
- Ottman, N., Smidt, H., de Vos, W.M., and Belzer, C. (2012). The function of our microbiota: who is out there and what do they do? *Front Cell Inf Microbiol* 2, 104.
- Parracho, H.M.R.T., Bingham, M.O., Gibson, G.R., and McCartney, A.L. (2005). Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol* 54, 987–991.
- Pfeiffer, R.F. (2011). Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat Disord* 17, 10–15.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glöckner, F.O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 35, 7188–7196.
- Qin, N., Yang, F., Li, A., Prifti, E., Chen, Y., Shao, L., Guo, J., Le Chatelier, E., Yao, J., Wu, L., Zhou, J., Ni, S., Liu, L., Pons, N., Batto, J.M., Kennedy, S.P., Leonard, P., Yuan, C., Ding, W., Chen, Y., Hu, X., Zheng, B., Qian, G., Xu, W., Ehrlich, S.D., Zheng, S., and Li, L. (2014). Alterations of the human gut microbiome in liver cirrhosis. *Nature* 513, 59–64.
- Rayner, C.K., and Horowitz, M. (2013). Physiology of the ageing gut. *Curr Opin Clin Nutr Metab Care* 16, 33–38.
- Riordan, S.M., and Williams, R. (2006). The intestinal flora and bacterial infection in cirrhosis. *J Hepatol* 45, 744–757.
- Schaffer, J.N., and Pearson, M.M. (2015). *Proteus mirabilis* and Urinary Tract Infections. *Microbiol Spectr* in press doi: 10.1128/microbiolspec.UTI-0017-2013.
- Scheperjans, F., Aho, V., Pereira, P.A.B., Koskinen, K., Paulin, L., Pekkonen, E., Haapaniemi, E., Kaakkola, S., Eerola-Rautio, J., Pohja, M., Kinnunen, E., Murros, K., and Auvinen, P. (2015). Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov Disord* 30, 350–358.
- Scher, J.U., Szczesnak, A., Longman, R.S., Segata, N., Ubeda, C., Bielski, C., Rostrom, T., Cerundolo, V., Pamer, E.G., Abramson, S.B., Huttenhower, C., and Littman, D.R. (2013). Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *eLife* 2, e01202.
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., and Weber, C.F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75, 7537–7541.
- Sjögren, Y.M., Jenmalm, M.C., Böttcher, M.F., Björkstén, B., and Sverremark-Ekström, E. (2009). Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy* 39, 518–526.
- Takahashi, K., Nishida, A., Fujimoto, T., Fujii, M., Shioya, M., Imaeda, H., Inatomi, O., Bamba, S., Sugimoto, M., and Andoh, A. (2016). Reduced abundance of butyrate-producing bacteria species in the fecal microbial community in Crohn's disease. *Digestion* 93, 59–65.
- Tan, A.H., Mahadeva, S., Thalha, A.M., Gibson, P.R., Kiew, C.K., Yeat, C.M., Ng, S.W., Ang, S.P., Chow, S.K., Tan, C.T., Yong, H.S., Maras, C., Fox, S.H., and Lim, S.Y. (2014). Small intestinal bacterial overgrowth in Parkinson's disease. *Parkinsonism Relat Disord* 20, 535–540.

- Topping, D.L., and Clifton, P.M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev* 81, 1031-1064.
- Vinolo, M.A.R., Rodrigues, H.G., Nachbar, R.T., and Curi, R. (2011). Regulation of inflammation by short chain fatty acids. *Nutrients* 3, 858–876.
- Walker, A.W., Ince, J., Duncan, S.H., Webster, L.M., Holtrop, G., Ze, X., Brown, D., Stares, M.D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A.M., Lobley, G.E., Parkhill, J., and Flint, H.J. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 5, 220–230.
- Wang, R.F., Cao, W.W., and Cerniglia, C.E. (1997). PCR detection of *Ruminococcus* spp. in human and animal faecal samples. *Mol Cell Probes* 11, 259–265.
- Wang, T., Cai, G., Qiu, Y., Fei, N., Zhang, M., Pang, X., Jia, W., Cai, S., and Zhao, L. (2012). Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* 6, 320–329.
- White, J.R., Nagarajan, N., and Pop, M. (2009). Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Comput Biol* 5, e1000352.

SUPPORTING INFORMATION

Figure S1 Rarefaction curves of Shannon index.

Figure S2 Family level comparison.

Figure S3 The heatmap based on the relative abundance of top 65 abundant genera.

Table S1 Significantly different genera between PD and HC groups

Table S2 Demographic characteristics of three groups

Table S3 Summary of clinical information between PD and HC groups

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