Alterations and Association of Fecal Microbiota and Amino Acids in Parkinson's Patients

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Research

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Abstract

Background: Intestinal microbiota and amino acids that are one of their metabolites play important roles in the mechanism of pathology of Parkinson's disease (PD). It has been reported that the level of amino acids in vivo participate in neurodegeneration by regulating adaptive immune response, while the current researches on alteration of amino acids in intestinal microbiota are still insufficient. Here, we evaluate the correlation between gut microbiota alterations and clinical parameters of PD, amino acid concentrations.

Methods: Stool samples from PD and healthy controls were collected for microbiome and targeted metabolome analyses.

Results: At the genus level, there was a greater abundance of Rikenellaceae_RC9_gut_group in PD patients with more severe motor symptoms. Metabonomics analysis showed that multiple fecal amino acid concentrations in PD patients were decreased. Moreover, the findings that by spearman analysis Rikenellaceae_RC9_gut_group associated with PD had a significantly negative correlation with phenylalanine ($r = -0.488$, $P < 0.01$), tyrosine ($r = -0.541$, $P < 0.01$) and isoleucine ($r = -0.434$, $P < 0.01$).

Conclusions: Our results not only find Rikenellaceae_RC9_gut_group, a new pro-inflammatory genus of intestinal microbiota in PD, but also reveal that it's related to amino acids. These findings are beneficial to identifying microbial therapeutic targets for PD.

Introduction

The pathological characteristics of PD include that the loss of dopaminergic neurons in the dense part of the substantia nigra and Lewy bodies containing α-synuclein in surviving neurons in different regions [1]. But so far, the etiology and pathogenesis of Parkinson's disease is not particularly distinct. Nowly, PD is considered as multifactorial and sporadic diseases mainly related to susceptibility genes, environmental exposures and age factors.

The accumulated data suggested that intestinal microbiota and their metabolites were regarded as the important environmental factors to promote the occurrence of neurodegenerative diseases. On the one hand, it has been found that the abundant of proinflammatory bacteria Akkermansia, Oscillospira, and Bacteroides were significant in PD fecal samples, and the induced-inflammatory response can promote the formation of pathological α-synuclein [2]. On the other hand, the metabolite of intestinal microbiota can also trigger neuroinflammation and then result in neuronal damage [3]. Metabonomics approaches were employed to study the amino acid concentrations, an common metabolite of intestinal microbiota, in different samples of patients and animal models with PD, such as brain tissue, cerebrospinal fluid, serum and urine [4, 5, 6]. The findings were that not only the concentrations of branched chain amino acids (BCAAs) and phenylalanine were significantly increased but also the balances of BCAAs in vivo play a crucial role to maintain normal brain physiology [7].

And since fecal samples can be collected easily and noninvasively, they were selected as a sample for our study to determine the relevance of shifts in gut microbiota with targeted fecal metabolites. So, the 16S ribosomal RNA (16S rRNA) gene sequencing and gas chromatography-mass spectrometry (GC-MS) platform were applied to analyzed and compared the microbiota communities and the concentration of fecal amino acids respectively.

Methods

Subjects

The study was approved by the Ethics Board at the First Affiliated Hospital of Harbin Medical University and all the procedures were conducted in accordance with the Declaration of Helsinki. All participants agreed to use their stool samples and signed written informed consent.

All participants did not intake antibiotic drugs or probiotic during the last 3 months, and had no history of active or persistent primary gastrointestinal disorder. PD patients (n = 20) was diagnosed by experienced neurologists on the basis of the diagnostic criteria proposed by the International Parkinson Disease and Movement Disorder Society in 2015 [8]. All PD patients were using antiparkinsonian medications. The all individual data of PD and healthy groups were shown in the Table 1.

Table 1
Demographic characteristics of all the participants.

	PD(n = 20)	HC(n = 20)	P-value
Gender(Male/Female)	10/10	10/10	1[b]
Age(years)[a]	63.65 ± 5.64	61.95 ± 4.73	0.3082[c]
BMI (kg/m2)[a]	23.355 ± 2.33	23.425 ± 2.61	0.9292[c]
PD duration (years)[a]	3.63 ± 2.10	N.A.	N.A.
Age of onset (years)[a]	59.95 ± 5.79	N.A.	N.A.
Hoehn-Yahr stage (off)[a]	1.775 ± 0.70	N.A.	N.A.
UPDRS part III (off)[a]	16.30 ± 10.60	N.A.	N.A.
NMS scores[a]	8.65 ± 5.72	N.A.	N.A.
HAMD scores[a]	10.00 ± 7.58	N.A.	N.A.
MOCA scores[a]	21.45 ± 4.03	N.A.	N.A.
RBDSQ scores[a]	4.60 ± 2.85	N.A.	N.A.

[a], Data are shown as mean ± SD; [b], Chi-squared test; [c], Student's t-test; PD, Parkinson' disease; HC, Healthy controls; SD, standard deviation; BMI, body mass index;UPDRS, Unified Parkinson's Disease Rating Scale; NMS, Non-Motor Symptoms; HAMD, Hamilton Depression Scale; MoCA, Montreal Cognitive Assessment;RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire;N.A., not available.

Sample collection

Stool samples were collected by subjects in the hospital according to our instructions. The feces were immediately frozen in liquid nitrogen and then stored at − 80 ° C.

DNA extraction, amplicon, and sequencing analysis

Following the manufacturer's instructions, the total genomic DNA was extracted using DNeasy PowerSoil Kit (Qiagen), and then used as a template for PCR amplification with the barcoded primers and Tks Gflex DNA Polymerase (Takara). The V3 and V4 variable regions of the bacterial 16S rRNA gene were amplified using forward primers containing the sequence 343F-5 '- TACGGRAGGCAGCAG-3' and reverse primers

containing the sequence 798R-5 '- AGGGTATCTAATCCT-3', respectively. Ultimately, the amplicons were on an Illumina Miseq platform for subsequent sequencing.

In order to trim low quality sequences with average quality score below 20, Paired-end reads firstly were preprocessed withTrimmomatic software [9], and then assembled with FLASH software [10]. The 10 bp of minimal overlapping, 200 bp of maximum overlapping and 20% of maximum mismatch rate were as assembly parameters. QIIME software [11] (version 1.8.0) was applied to further denoising the sequences including reads with ambiguous, homologous sequences or below 200 bp abandoned, and reads with 75% of bases above Q20 retained, and then reads with chimera detected and removed. Clean reads were subjected to primer sequences removal and clustering to generate operational taxonomic units (OTUs) using Vsearch software [12] with 97% similarity cutoff. The representative read of each OTU was selected using QIIME package. All representative reads of 16 s rDNA were annotated and blasted against Silva database Version 123 using RDP classifier (confidence threshold was 70%) [13].

Fecal metabolite identification of amino acids

Fecal samples were analyzed for amino acids. We added 400 μL methanol-water (4:1, v/v) to 30 mg samples,and then grind the sample using the grinder (60 Hz, 2 min). After a series of homogenization, ultrasonication and centrifugation treatment, the supernatant was collected for GC-MS metabonomics analysis. The TSQ 9000 GC system (TSQ 9000, Thermo Scientific) determined qualitatively and quantitatively amino acids. The chromatographic conditions: DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA, USA), Helium with purity not less than 99.999% was used as carrier gas, and then the required flow rate and injection temperature were 1.2 ml/min and 300 ℃ respectively. Injection requirements: the sample was injected was 1 μ L without split flow, and then put in the solvent after 4 minutes. Temperature programming: the initial temperature of column temperature box is 50 ℃ for 0.5 min, then programing temperature from 15 ℃ / min to 125 ℃ for 2 min, 8 ℃ / min to 210 ℃ for 2 min, 11 ℃ / min to 270 ℃ for 1 min, 25 ℃ / min to 305 ℃ for 3 min. The mass spectrum conditions: the scanning mode was selected reaction detection scanning (SRM), which ensures the scanning range of m / z: 40–600.

Statistical analysis

Student’s t-test and Chi-squared test were used to evaluate the significant difference of clinical characteristics. Species diversity analysis includes α diversity that was evaluated by chao1, observed species, PD whole tree, Shannon and Simpson indexes, and β diversity that was assessed with principal coordinates analysis (PCoA) on the Bray-Curtis dissimilarity and unweighted Unifrac distances. We applied the Wilcoxon rank-sum test and linear discriminant analysis effect size (LEfSe) analysis to to discriminate the communities or species. In order to examine the relationship between microbiota and PD clinical parameters, targeted metabolites(amino acids) respectively, Spearman’s rank-correlation analysis

was applied. And then the false-discovery rate (FDR) was necessary to modify the P-value. $P < 0.05$ was considered to be statistically significant. All analyses were performed using R software.

Results

Alpha and beta diversity of intestinal microbiota of PD patients

The rarefaction curves of richness based on the Shannon index showed that the sequencing depth meeted the experimental requirements(Fig. 1a). Then in order to determine whether the sample size of the study can be used for effective analysis, we also plotted the species cumulation boxplot (Fig. 1b).

Chao1 and Shannon represented community richness and diversity respectively. We found that the gut microbiota alpha diversity (richness and diversity) in PD patients was higher than that in controls, however, there were no statistically significant differences by calculating these diversity indices ($p > 0.05$) (Fig. 1c,d). We were informed of the β-diversity values by calculating the Bray-Curtis dissimilarity and unweighted Unifrac distances to compare the structural differentiation of different cohorts. The PCoA showed the different bacterial components in the gut microbiota significantly between PD patients and controls ($P = 0.005$)(Fig. 1e, f).

Different taxa of gut microbiota between PD patients and controls

The dominant phylum of intestinal microbiota includes Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria, which account for the majority of the total reads(Fig. 2a). Whereas, we only found that there was more abundant Actinobacteria in patients with PD(Fig. 2b). Genus-level comparison between PD and healthy controls showed that Alistipes, Rikenellaceae_RC9_gut_group, Bifidobacterium, Parabacteroides, Sphingomonas were found more in PD patients, while Faecalibacterium was significantly more abundant in controls(Fig. 2c). There were also some differences in the top 10 abundances of intestinal microbiota between PD and control groups at the species level(Fig. 2d).

As a supervised comparison method, LEfSe analysis is used to identify the specific microbial communities associated with PD. When we set a logarithmic linear discriminant analysis (LDA) score cutoff of 3.6, we identified different abundances of intestinal microbiota between PD and controls at one phylum, two classes, two orders, three families, and five genera levels (Fig. 3a,b).

3.3 Gut microbiota correlates with clinical parameters and fecal amino acid concentrations

According to the Modified Hoehn and Yahr rating scale, the PD patients were divided into early (stage 1–1.5) and advanced stages (stage 2–3) in our study. We found the similar abundance of Rikenellaceae_RC9_gut_group in different Hoehn and Yahr staging of PD patients(Fig. 4a). However, it is associated with the severity of motor symptoms defined by the UPDRS Part III motor score (Fig. 4b).

The metabolism of amino acids in feces was employed using a targeted metabolomics technique. We screened a total of 19 amino acids and found that the concentrations of these amino acids differed

substantially between PD patients and controls (Table 2). Comparing PD patients to controls, a significant decrease was found in Leucine, Isoleucine, Phenylalanine and Tyrosine (P<0.01). It suggested that there may be a strong correlation between these amino acids and disease occurrence.

Table 2
Wilcoxon rank-sum test analysing alterations of amino acids concentrations.

ID	Compound	HC(n = 20) mean(ng/mL)	PD(n = 20) mean(ng/mL)	p-value
1	Alanine	662164.104173565	246287.764790203	0.000052182493800912
2	Asparagine	73266.1165980816	37543.3516272435	0.0516940592445507
3	Aspartic acid	133967.483039702	46303.7076804287	0.000110247582789961
4	Cysteine	40403.5546060898	22270.494355365	0.000767472711126502
5	Glutamic acid	1434178.89363143	424823.710307797	0.00120486898606024
6	Glutamine	200110.114830363	32081.3285598769	0.0125989494064329
7	Glycine	195517.000170878	74530.7822476359	0.000118539201727784
8	Isoleucine	618813.761796962	85248.0004036877	0.0000307236733904794
9	L−4-Hydroxyproline	7565.69078536681	3250.77557352768	0.00291392724601169
10	Leucine	392546.918338564	261830.23539528	0.0103124280860234
11	Lysine	602995.477315084	139921.829240755	0.000156318043930851
12	Methionine	217304.579743607	51758.300375273	0.000046414975356988
13	Phenylalanine	315508.641259868	62185.8393193591	0.0000857891320616695
14	proline	1308024.28548493	28086.9899406816	0.00179096701496249
15	Serine	295276.412075522	25792.9504317877	0.000108227628563053
16	Threonine	181814.82860289	37227.7902617905	0.0000560310283744711
17	Tryptophan	241614.810228436	12161.6774330516	0.00173806552224935
18	Tyrosine	764042.747648738	134904.154640686	0.0000270980773388775
19	Valine	336522.465729918	132620.092480144	0.000132035020257514

We next investigated correlations between fecal microbiota and 19 amino acids at the genus and species levels, respectively. Firstly, we confirmed correlations between the relative abundance of the top 20 abundant genera and amino acids by the Spearman's rank-correlation analysis (Fig. 5a). We found Rikenellaceae_RC9_gut_ group were negatively associated with the concentrations of tyrosine (r=-0.541,

P<0.01), isoleucine (r=-0.434, P<0.01) and phenylalanine in feces(r=-0.488, P<0.01). However, Sphingomonas was only correlated with tyrosine (r=-0.406, P<0.05). Secondly, the heatmap also described correlations between the top 20 abundant species and specific amino acids (Fig. 5b). Therefore, the changes in amino acid concentrations originating from the gut microbiota dysbioses, which may contribution to Parkinson's disease.

Discussion

The present study has demonstrated that there was a obvious alteration of gut microbiome composition in PD patients. The relative abundance of Rikenellaceae_RC9_gut_group, which was increased in both early and advanced PD patients, associated with both the motor symptoms and concentration of amino acids. Therefore, our results not only identify the fresh genera of gut microbiota associated with the severity of PD, but also may help us to explore the potential pathogenesis mediated by amino acids in PD.

The proposed microbiota-gut-brain axis suggests that there are some ways facilitating communication between the microbiota and the brain, which include the neuroendocrine, neuroimmune, the vagus nerve, the enteric nervous system and microbial metabolites [14]. For example, Braak's staging thought that α -synuclein can be retrogradely transported from the gut to the brain via the vagus nerve [15]. All these evidences show that PD may be the intestinal onset, which is regulated by intestinal microbiota. Therefore, it is necessary to conduct a comprehensive analysis of intestinal microbiota and identify microbial therapeutic targets for PD.

We performed 16S rRNA gene sequencing to investigate the intestinal microbiota composition of PD patients. our findings showed the β-diversity index (structure) of PD patients significantly different from that of healthy controls qualitatively, indicating that the intestinal microbiota composition altered in PD patients. At the phylum, genus and species levels, we observed significant differences of fecal microbiota in patients with PD compared with healthy controls in our study. We observed an increased abundance in Sphingomonas, in a correspondence with the results by Qian et al [16]. Moreover, we also found that the abundance of Rikenellaceae_RC9_gut_group, known as the putative ‘pro-inflammatory' bacterial genus, was increased at the genus level. We speculate that the differences may be related to the different diet, geographical background and disease duration and other factors.

The targeted metabolomics method was applied to detect the change of amino acid content in feces. We found that there was a significant decrease in both absolute and relative concentrations for phenylalanine, tyrosine and isoleucine by quantitative analysis of amino acid concentrations in PD patient's fecal samples. However, the alteration of amino acid concentration in feces is inconsistent with that in the serum, which may be related to gastrointestinal symptoms in PD. PD patients are susceptible to delayed gastric emptying and slow digestion of the small intestine [17], and ultimately may lead to impaired absorption of amino acids.

Meanwhile, most intestinal microbes affect the host through their metabolites. For example, it has been reported that phenylalanine, tyrosine, leucine, isoleucine and valine were inversely correlated with

Bacteroides that includes B. thetaiotaomicron, B. intestinalis, B. ovatus and B. uniformis [18]. In addition, a study by Fan et al reported that the high concentration of dietary protein can increase the abundance of Rikenellaceae_RC9_gut_group [19]. And then we found that Rikenellaceae_ RC9_gut_group in PD patients was negatively correlated with phenylalanine, tyrosine and isoleucine in feces, indicating that Rikenellaceae_ RC9_ gut_group may be a microorganism closely related to PD by affecting the metabolism of amino acids. However, Rikenellaceae_RC9_ gut_ group whether affects PD through phenylalanine, tyrosine and isoleucine remain to be experimentally verified.

It is worth noting that amino acids can participate in the pathogenesis of PD via a variety of mechanisms. BCAAs, especially leucine, can regulate the activity of mTORC1 to promote protein synthesis, and then accelerate the growth and proliferation of proinflammatory immune cells, such as Th1 and Th17 [20]. Superoxide anion and nitric oxide (NO) free radicals are produced by cells of the immune system in inflammatory processes, which then form the nitrogen radical peroxynitrite ($ONOO^-$) that leads to mitochondrial damage and dopaminergic neuron death. And it is reported that the proportion of Th1 cells in peripheral blood of patients with PD was increased [21]. Aromatic amino acids (AAAs) are precursors of serotonin, dopamine and norepinephrine neurotransmitters. As previously described, serum tryptophan levels may be associated with depression and gastrointestinal regulation disorder [22]. Taken together, various kinds of amino acids play different roles in PD, which may lead to different symptoms of PD patients.

There are still some limitations of the present study. Firstly, in order to reflect overall body metabolism, different biological samples (feces, blood, cerebrospinal fluid and et al.) from the same patients should be analyzed in the same study. Secondly, although it was proven that levodopa and large neutral amino acids (LNAAs) use the same transporter when crossing the blood-brain barrier [23], whether levodopa interferes with the plasma levels of BCAAs and AAAs in patients with PD is still unclear. Since all PD patients in our study were taking anti-parkinsonian medication, the medications were unlikely to be the main confounders. Finally, in order to obtain more detailed information about the microbiota, it is necessary to conduct metagenome and transcriptome analysis, which are conducive to the identification of BCAAs and AAAs specific strains.

Conclusion

We found that alterations of gut microbiota were accompanied by the change of amino acids in PD patients, which may potentially result in the development of PD pathology. Therefore, the current study allows a better understanding on the relationship between intestinal microbiota and host metabolism, and points out it may be necessary to intervene therapy of PD by metabolites related to intestinal microbiota in the future.

Abbreviations

PD: Parkinson's disease; BCAA: branched chain amino acid; 16S rRNA: 16S ribosomal RNA; GC-MS: gas chromatography-mass spectrometry; OTU: operational taxonomic unit; PCoA: principal coordinates analysis; LEfSe: linear discriminant analysis effect size; FDR: false-discovery rate; LDA: linear discriminant analysis; AAA: Aromatic amino acid; LNAA: levodopa and large neutral amino acid

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Board at the First Affiliated Hospital of Harbin Medical University (permission 201985).

Consent for publication

The study has been agreed to publish by all contributing authors

Availability of data and materials

The datasets used or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

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

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Authors' contributions

ZY, LY proposeed the conception and design of the study. ZY, FY, JC, WD, SY, WS, LY, SW acquired the data.ZY, FY, JC, WD, SY analyzed and interpreted the data and contributed to the writing of the manuscript.FY, LY,SW critically reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

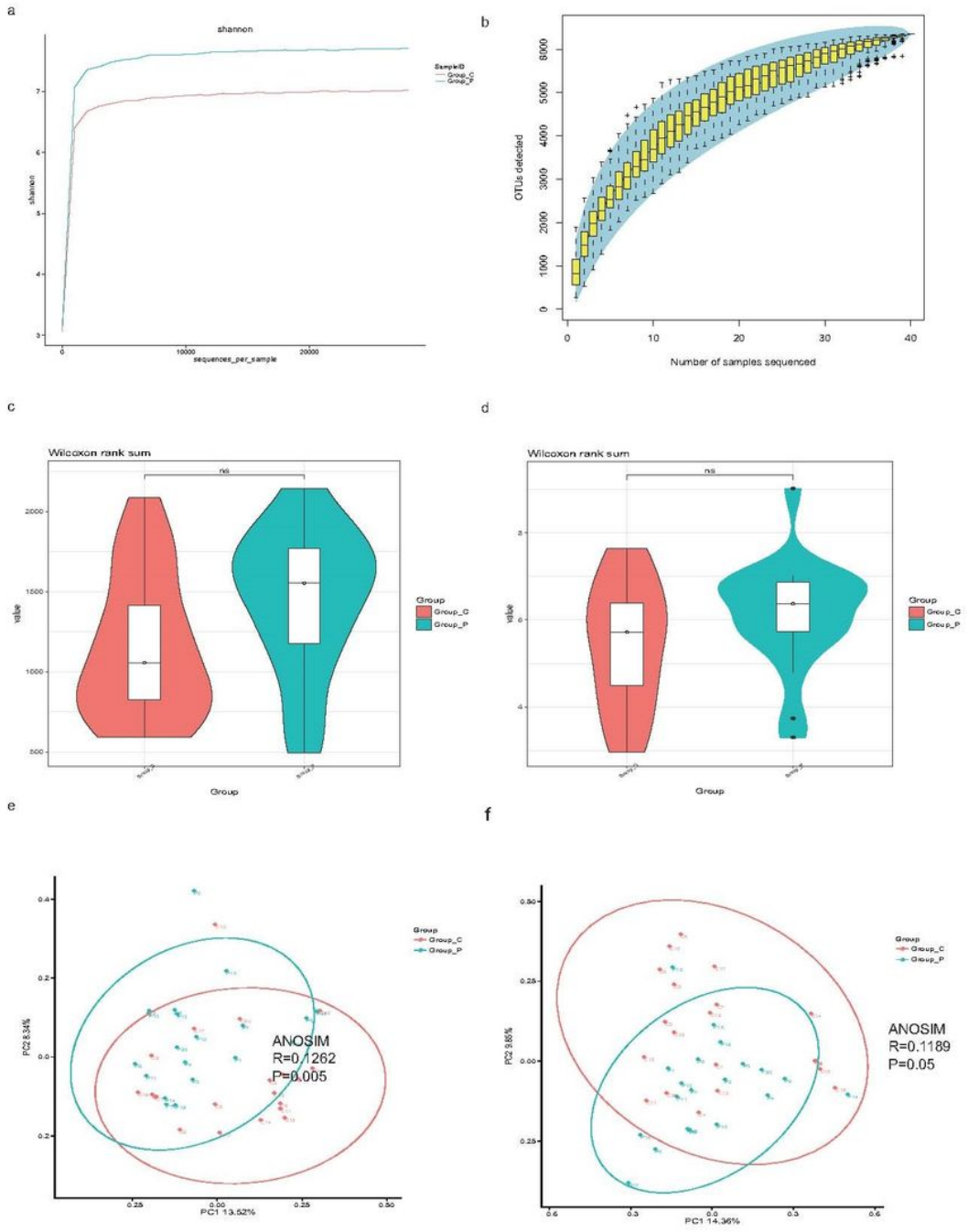
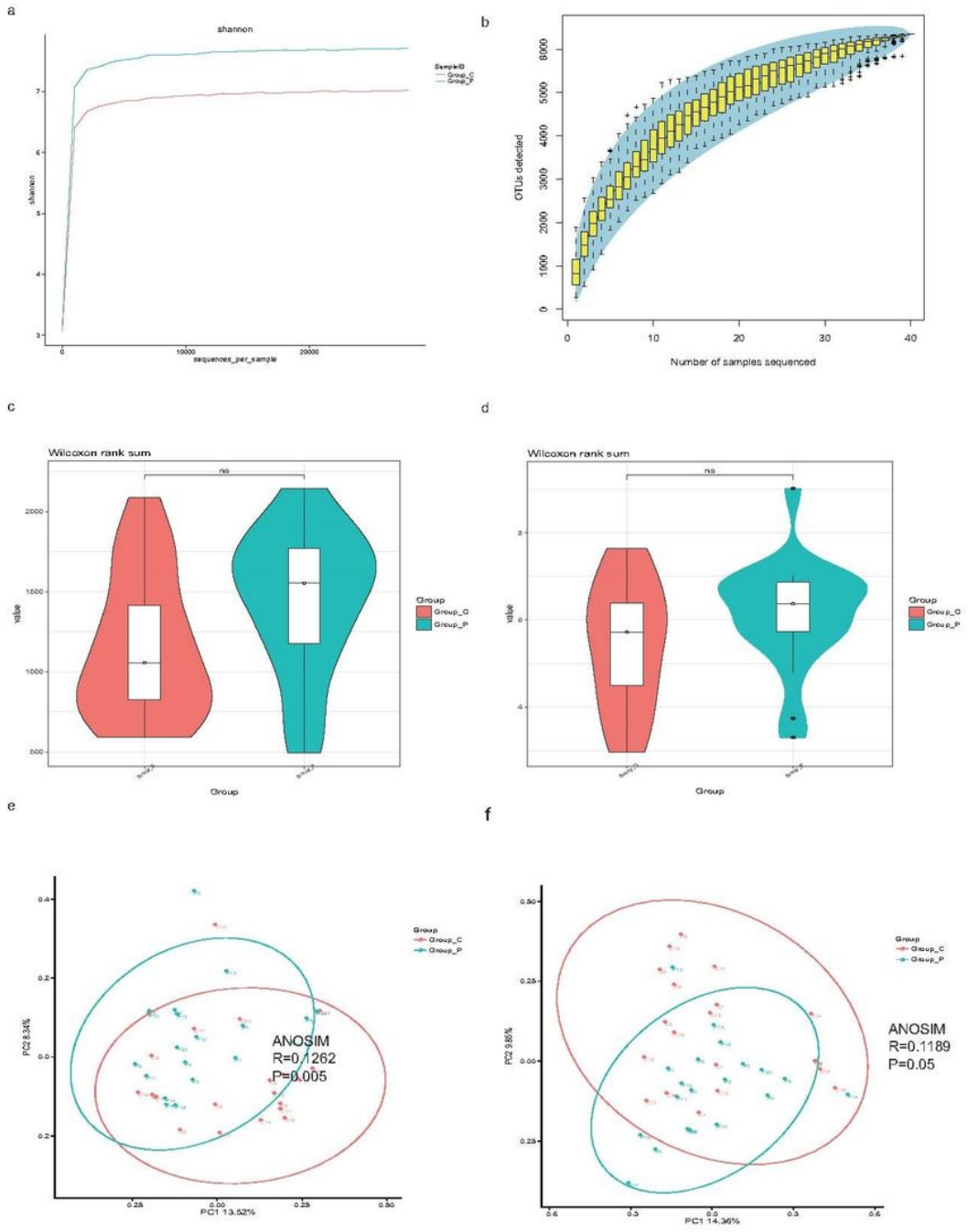


Figure 1

The alpha-diversity and β-diversity of the fecal microbiome alteration. a Rarefaction curves based on the Shannon index showed the adequate the adequate sequencing depth. b Species accumulation curve of Speccacum showed the adequate sample size. c,d Chao indices (c) and Shannon indices (d) were used to estimate gut microbiota richness and diversity (ns:P□0.05). e,f β-diversity analyses using PCoA plotted with unweighted Unifrac (e) (ANOSIM R=0.1262, P=0.005) and Bray-Curtis (f) (ANOSIM R=0.1189, P=0.005). P, PD patients (blue); C, controls (red); PCoA, principal coordinates analysis; PC, principal coordinate; ANOSIM, analyses of similarities.



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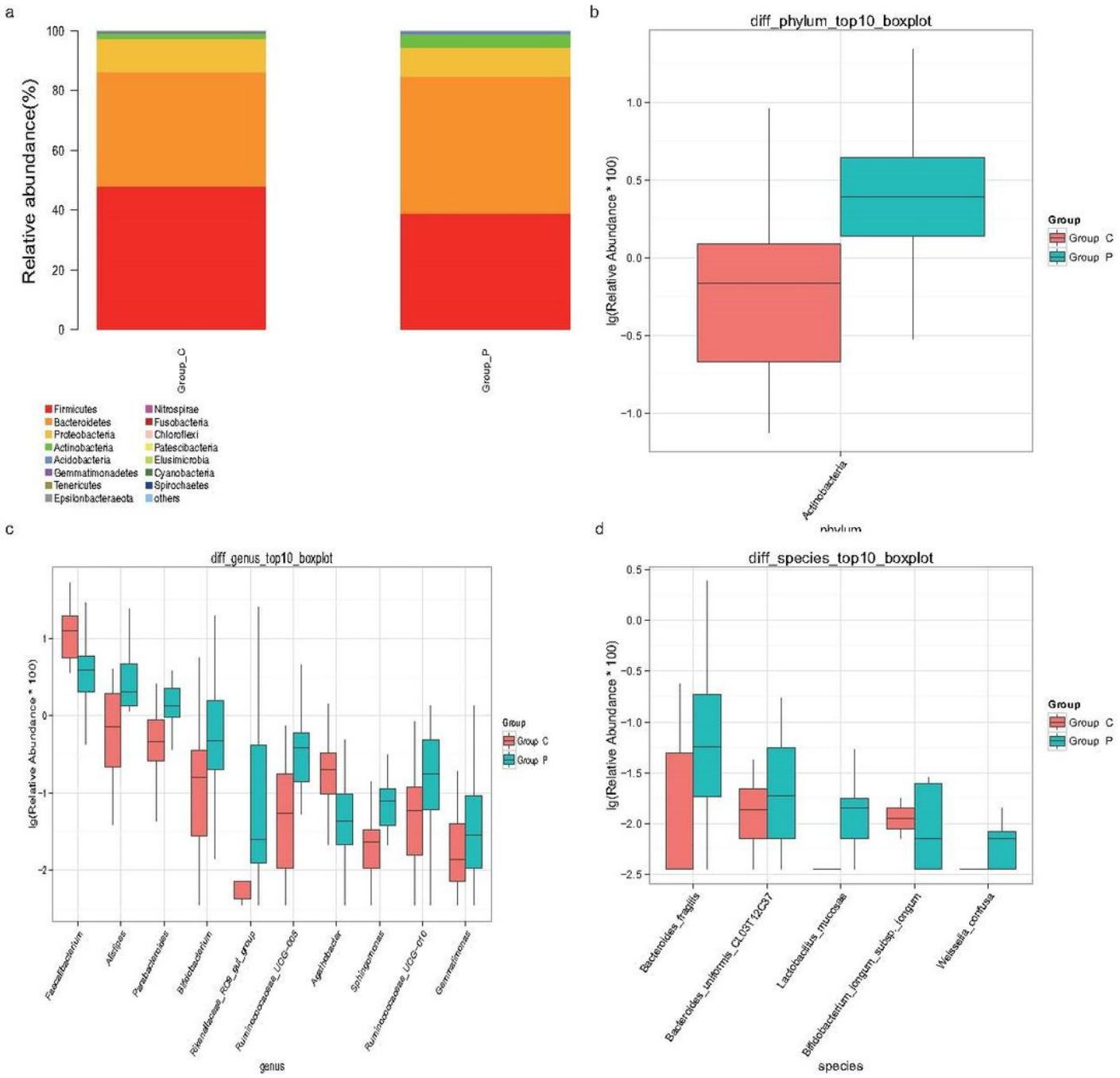
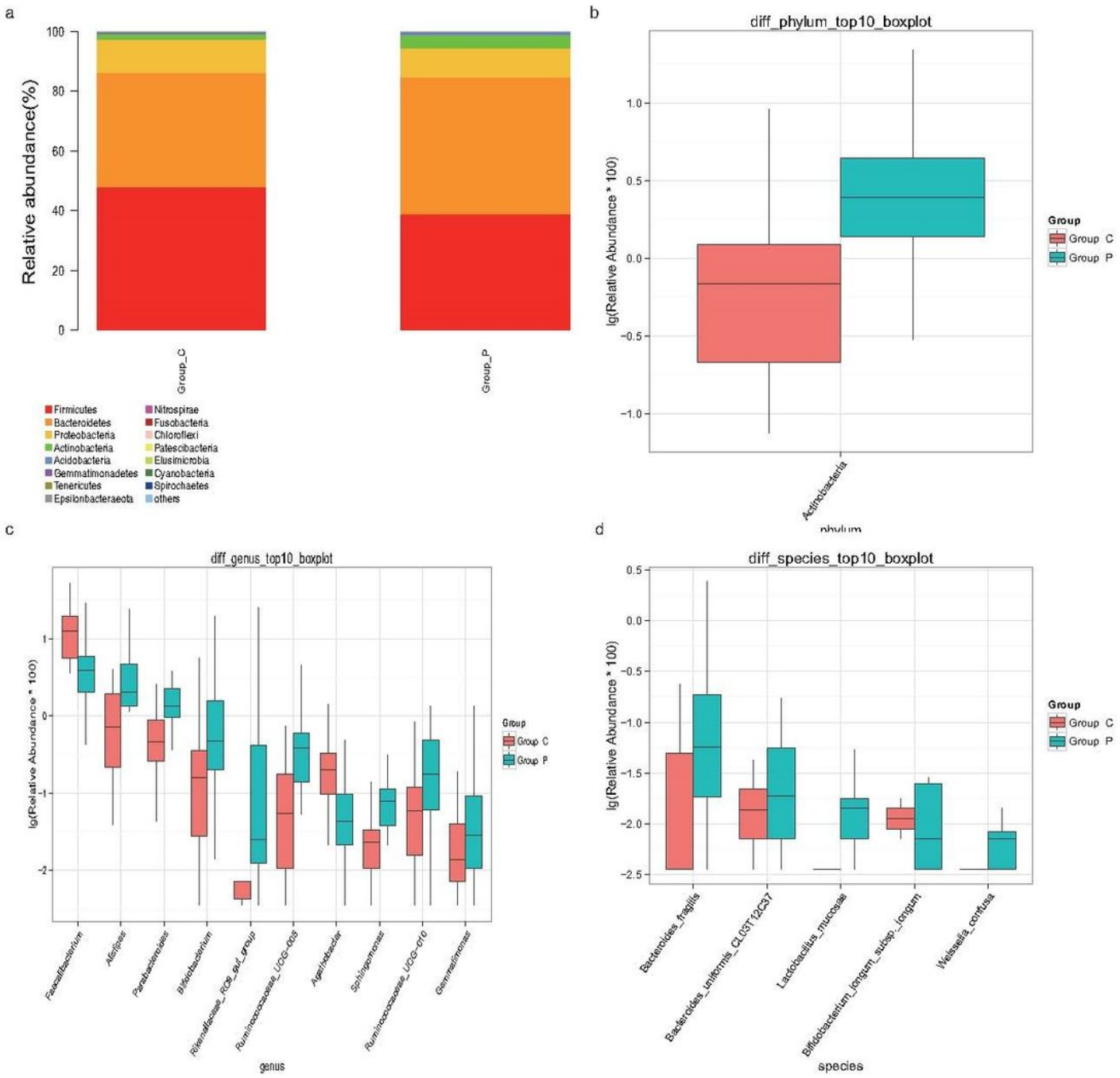


Figure 2

The differences of gut microbiota analyzed by Wilcoxon rank-sum test. a-d The different abundance of dominant intestinal microbiota at phylum (a), phylum (b), genus (c), and species (d) levels.



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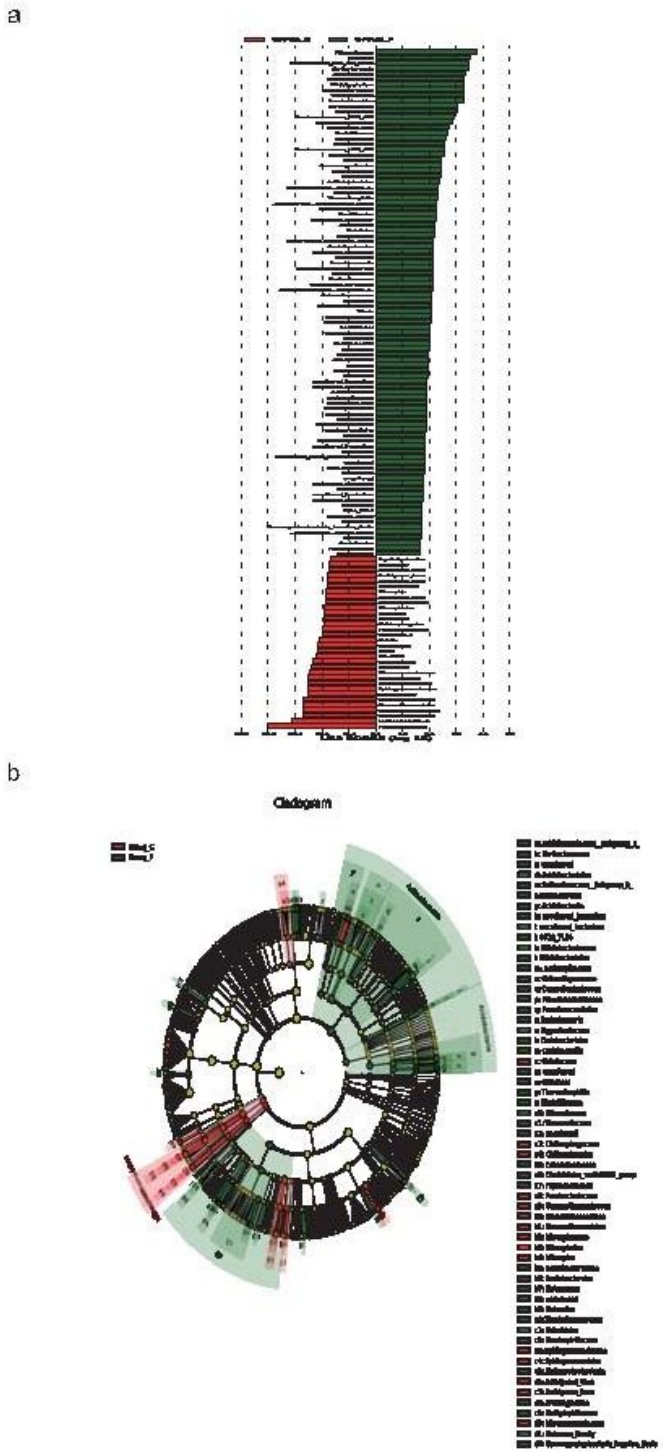
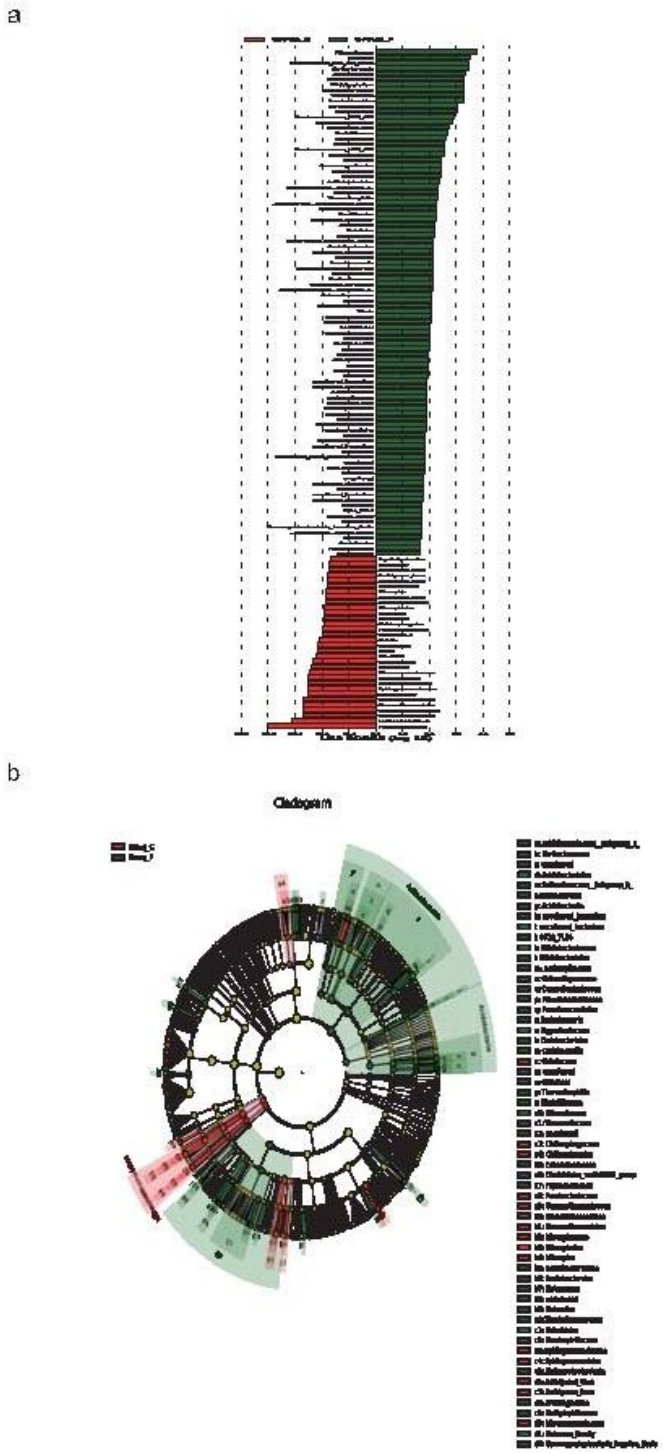


Figure 3

Taxonomic differences of gut microbiota detected by LEfSe analysis. a A bar graph of linear discriminant analysis (LDA) score indicated the differences in abundance between the P and C groups. b Cladogram indicated the phylogenetic distribution of fecal microbiota. P, PD (red); C, controls (green)



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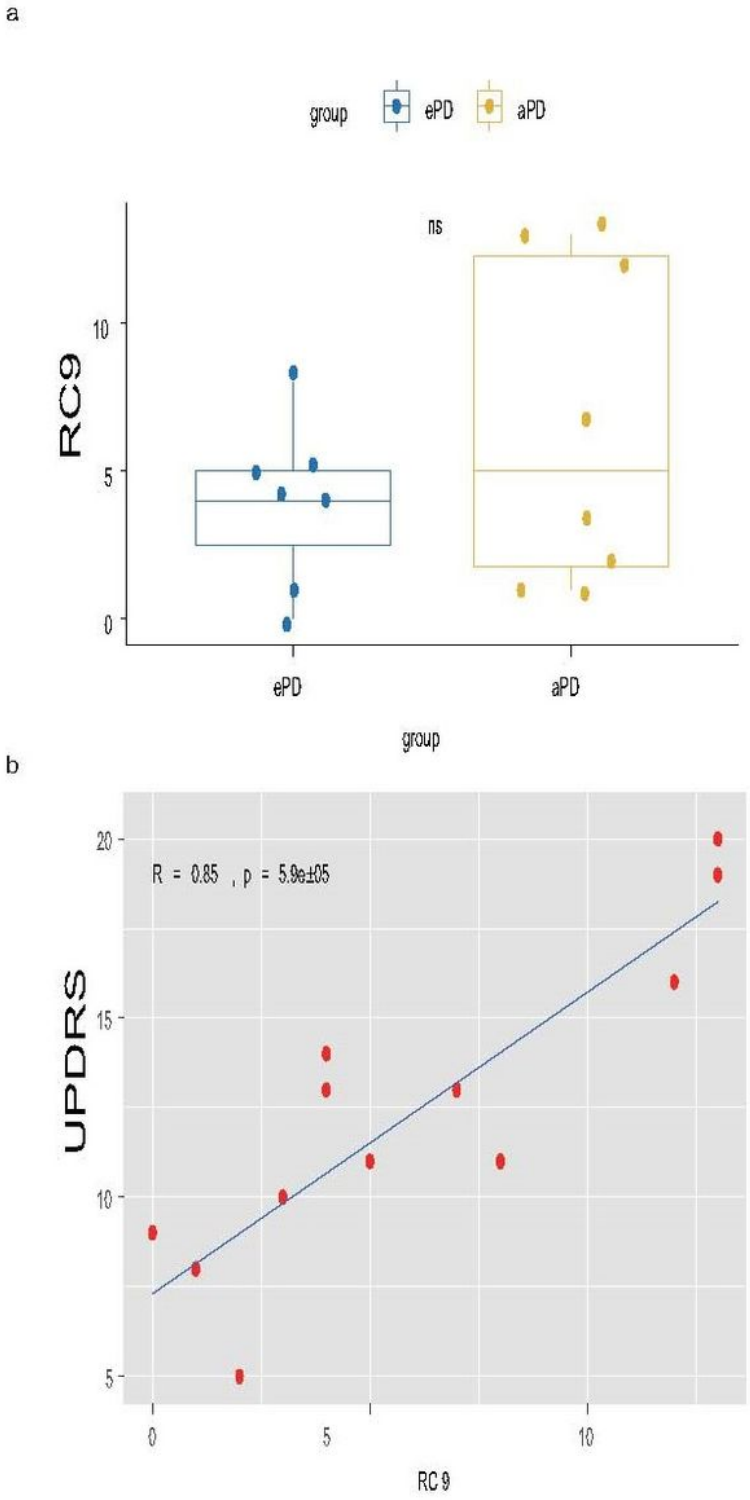
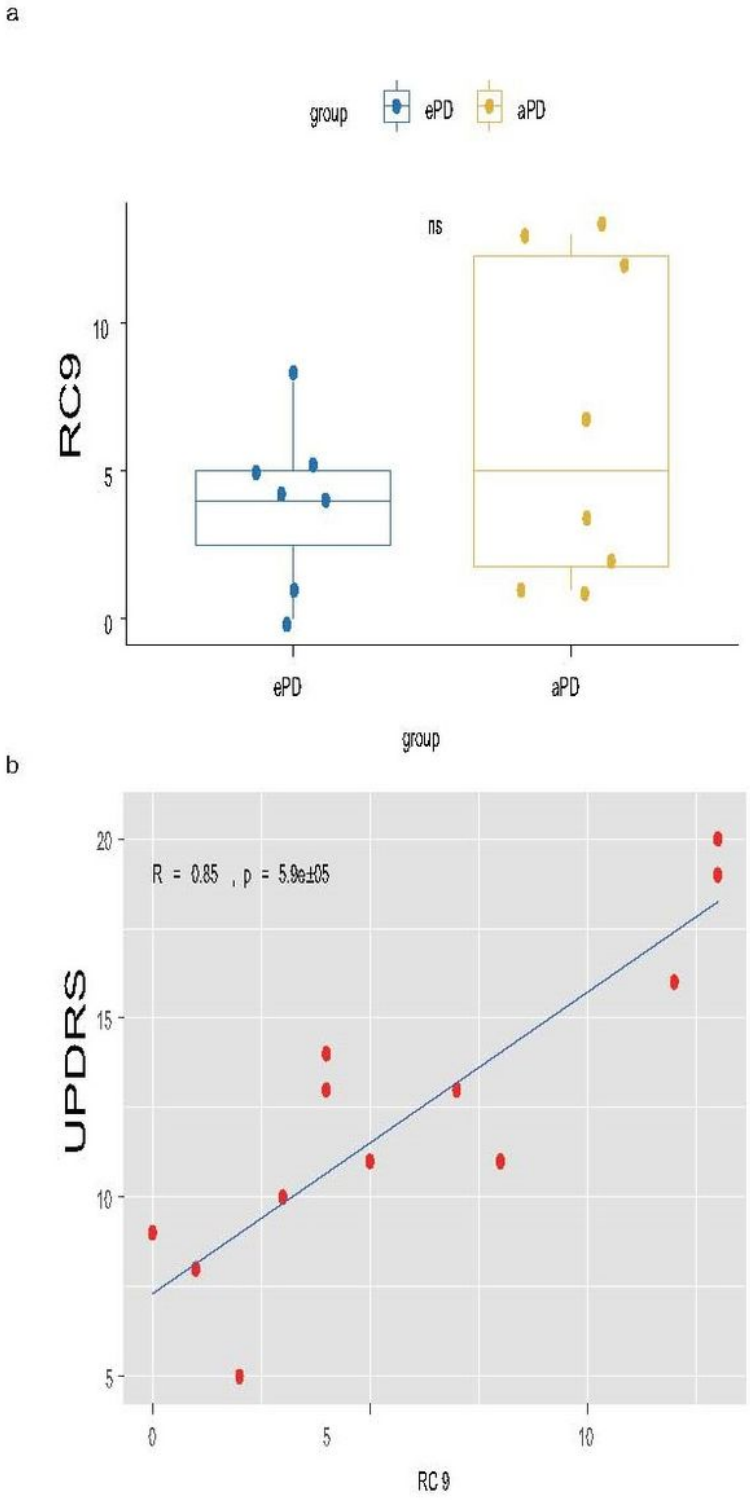


Figure 4

The relationship between fecal microbiota and clinical characteristics in PD patients. a The difference of Rikenellaceae_ RC9_ gut_ group abundance in the early (blue) and advanced stages (yellow) of PD patients by T test (P=0.25).b The abundance of Rikenellaceae_ RC9_ gut_ group correlated with UPDRS part III motor scores (r = 0.93, P < 0.01 by Spearman correlation analysis). RC9Rikenellaceae_ RC9_ gut_ group.



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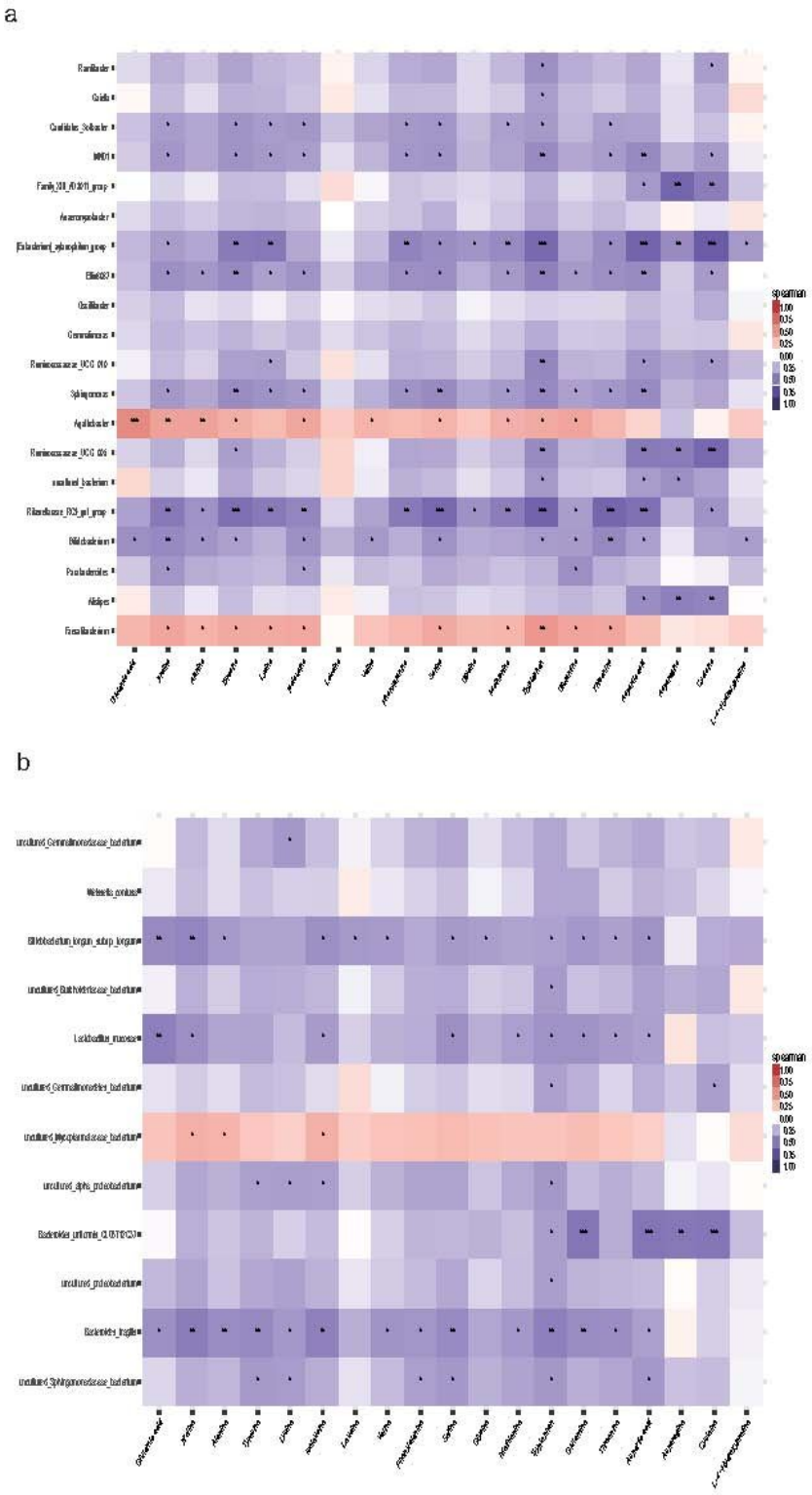
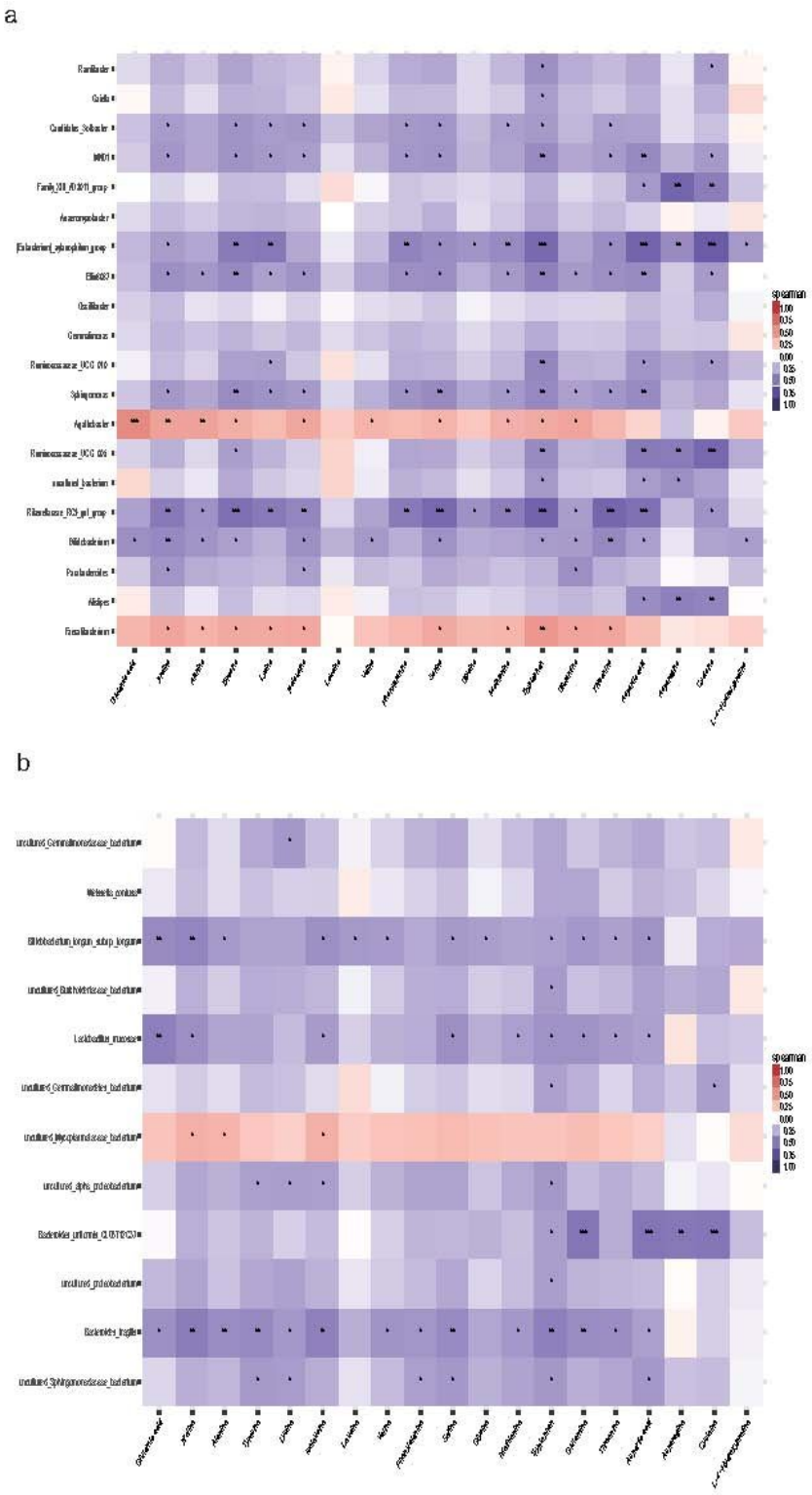


Figure 5

Spearman correlation analysis of fecal microbiota and 19 amino acids. a The correlation at genus level. b The correlation at species level. * P < 0.05, ** P < 0.01, *** P < 0.001.



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