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RESEARCH ARTICLE

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Parkinson's disease alters the composition of subgingival microbiome

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ABSTRACT

Aim: The current study aimed to test the hypothesis that Parkinson's disease exacerbates periodontitis by altering its microbiome.

Materials and Methods: Clinical periodontal parameters were recorded. Subgingival samples from healthy controls, periodontitis patients (PD), and Parkinson's patients with periodontitis (PA+PD) were analyzed using the checkerboard DNA-DNA hybridization technique for targeting 40 bacterial species typically associated with periodontal disease and health. Next-generation sequencing (NGS) of the 16S ribosomal RNA gene (V1-V3 regions) was performed to analyze the microbiome comprehensively.

Results: Parkinson's patients had mild-to-moderate motor dysfunctions. Bleeding on probing was significantly increased in the PA+PD group compared to PD ($p < 0.05$). With checkerboard analysis, PA was associated with increased *Treponema socranskii* ($p = 0.0062$), *Peptostreptococcaceae_[G-6] [Eubacterium]_nodatum* ($p = 0.0439$), *Parvimona micra* ($p < 0.0001$), *Prevotella melaninogenica* ($p = 0.0002$), *Lachnoanaerobaculum saburreum* ($p < 0.0001$), and *Streptococcus anginosus* ($p = 0.0020$). *Streptococcus intermedia* ($p = 0.0042$), *P. nodatum* ($p = 0.0022$), *P. micra* ($p = 0.0002$), *Treponema denticola* ($p = 0.0045$), *L.saburreum* ($p = 0.0267$), *P.melaninogenica* ($p = 0.0017$), *Campylobacter rectus* ($p = 0.0020$), and *T.socranskii* ($p = 0.0002$) were higher; *Aggregatibacter actinomycetemcomitans* ($p = 0.0072$) was lower in deep pockets in the PA+PD compared to PD. *Schaalia odontolytica* ($p = 0.0351$) and *A.actinomycetemcomitans* ($p = 0.002$) were lower; *C.rectus* ($p = 0.0002$), *P. micra* ($p = 0065$), *Streptococcus constellatus* ($p = 0.0151$), *T.denticola* ($p = 0.0141$), *P.melaninogenica* ($p = 0.0057$), and *T.socranskii* ($p = 0.0316$) were higher in shallow pockets in the PA+PD. Diversity decreased in PD ($p = 0.001$) and PA+PD ($p = 0.026$) compared to control, with minimal differences in alpha and beta diversities among PD and PA+PD based on NGS results.

Conclusion: These data demonstrated that Parkinson's disease modifies PD-associated subgingival microbiome.

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Checkerboard; microbiota; Parkinson's disease; pathogenesis; periodontitis; microbiome

Introduction

Parkinson's Disease (PA) is a progressive neurodegenerative disorder driven by hereditary and environmental risk factors such as age, male gender, family history of PA, cigarette smoking, alcohol, and vitamin D deficiency [1,2]. The presence of fibrillar aggregates, Lewy bodies that contain misfolded α-synuclein, is the histopathological hallmark of PA [3]. The clinical findings include resting tremors, bradykinesia, postural instability, and rigidity. PA also demonstrates non-motor symptoms such as cognitive decline, anxiety, and gastrointestinal complaints, particularly constipation. These non-motor features often precede motor disability onset by many years [4], and its progression may interfere with daily habits, including oral hygiene techniques, compromising oral health.

The oral cavity harbors a complex microbial community, of which a shift towards dysbiosis often leads to periodontitis (PD) [5]. The complex interaction between periodontal pathogens and the host defense mechanisms, affected by predisposing factors, results in chronic, polymicrobial, and inflammatory disease [6]. PD induces low-grade systemic inflammation via the pro-inflammatory cytokine release and periodontal bacterial invasion (e.g. *Porphyromonas gingivalis*) and their virulence factors, such as lipopolysaccharides (LPS), into the blood circulation [7]. The oral microbiome creates distinctive microbial communities in saliva and on different intraoral structures, such as the gingival sulcus and tongue dorsum [8,9]. *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola* are strongly associated with PD [10] and 17 species/phylotypes recently associated with disease severity [11].

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PD is strongly associated with several systemic diseases, such as diabetes, cardiovascular diseases, rheumatic diseases, and Alzheimer's disease. Most of these diseases are related to PD by its chronic inflammatory burden and systemic bacterial dissemination [12]. In addition to contributing to systemic inflammation, PD is associated with neuroinflammation and activating brain immune cells such as microglia [7]. Although there are limited studies on the link between PD and PA, poor oral health, worsening of periodontal status, and severe tooth loss in PA patients have been reported [13,14]. These findings concur with a study that found that routine scaling for five years drastically lowered the incidence of PA in people without PD [15]. However, the mechanistic link between PA and PD is unclear. While impaired manual dexterity is an apparent confounding factor, the PA-induced neuroinflammatory process could impact the periodontal disease pathogenesis altering the microbiome.

Microbial link to PA pathogenesis has been mainly focused on gut microbiota, suggesting a bidirectional connection between the gastrointestinal system and brain through the brain-gut axis. Indeed, PA patients showed diverse gut microbiota with significant taxonomic diversities [16–18]. However, studies on oral microbiota are limited. Since the oral microbiota has the most comprehensive and highest bacterial diversity in the human body after the gut microbiome [19] and has a fundamental impact on systemic diseases, we hypothesized that PA affects the microbial composition of the subgingival microbiome and analyzed the impact of PA on the microbiological contents of subgingival plaque in PD patients.

Materials and methods

Study population

This study was approved by the human subject ethics board of Istanbul Medipol University's Faculty of Dentistry (date: 12 November 2020; Number: 854) for use and access of human subjects in research and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. The study was conducted between (11/2020) and (1/2022) at the Department of Periodontology, School of Dentistry, Istanbul Medipol University, Istanbul, Turkey. We recruited patients with Stage III, Grade B periodontitis (PD) with and without Parkinson's disease (PA) and healthy participants. PA patients were recruited from the Parkinson's Disease and Movement Disorders Center, Istanbul Medipol University, Istanbul, Turkey. The criteria of the United Kingdom Parkinson's Disease Society Brain Bank were used to diagnose idiopathic PA [20]. All PA patients were examined by a movement disorder-experienced neurologist (A.Z.) [20]. To minimize the impact of PA on manual dexterity and oral hygiene of patients, PA patients included in this study were recruited among those not diagnosed with Parkinson's Plus Syndrome and received deep brain stimulation therapy at least 4 months ago. The duration and pharmacologic management of PA were recorded.

The exclusion criteria were as follows: use of antibiotics and/or anti-inflammatory, nonsteroidal anti-inflammatory drugs, steroids, immunosuppressants, beta-blockers, calcium channel blockers (except for PA patients), anticoagulants, and hormonal contraceptives within 6 months preceding the study; nonsurgical periodontal treatment (previous 6 months); surgical periodontal treatment (previous 12 months); having less than 20 natural teeth excluding third molars; diabetes diagnosis; rheumatoid arthritis diagnosis; or pregnancy, lactating, or smoking; and systemic conditions, including human immunodeficiency virus infection and acquired immunodeficiency syndrome, cardiovascular disorders, epilepsy, renal disorders, and hepatic disorders. Among 586 individuals, those who did not meet the inclusion criteria were excluded. In addition, 45 patients who had other systemic diseases and 80 patients with PA who had additional systemic diseases other than Parkinson's disease and hypertension; and were not diagnosed with Idiopathic Parkinson's disease or Parkinson Plus Syndrome were excluded. In total, 20 systemically and periodontally healthy individuals were included as the healthy control group, 20 patients with periodontitis and 20 patients with PA+PD were included.

Assessment of periodontal disease and parkinson's disease

The clinical diagnosis of periodontal disease was made according to the '2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions' [21]. The periodontal examination of periodontitis and healthy subjects was based on clinical and radiographic criteria, as detailed previously [22]. Periodontally healthy participants had probing pocket depth (PPD) ≤ 3 mm and no signs of inflammation. Patients with a clinical attachment level (CAL) of ≥2 mm in nonadjacent teeth were defined as periodontitis. For each tooth, the highest interdental CAL was recorded. CAL ≥5 mm was defined as stage III PD. The PD group consisted of patients with Stage III grade B disease. These patients were graded according to the bone loss/age index (Grade B, 0.25–1.00). The clinical periodontal parameters of the plaque index (PI), PPD, gingival recession (GR), CAL, and bleeding on probing (BOP) were recorded. Measurements were taken at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual). Average scores for whole-mouth PPD,

CAL, GR, and the percentage of sites with BOP were calculated for each subject. All measurements were performed using a periodontal probe with William's markings by two calibrated periodontists (EY, MY). Before the study, two examiners were calibrated on 10 non-study volunteers [23]. The probing depth values demonstrated good reproducibility, assessed by inter-examiner analysis ($\kappa = 0.892$). In addition to the kappa agreement, the reproducibility assessment showed that, for 90% of the sites, the mean of repeated probing measurements was within 1 mm. To assess the severity of motor functions of PA patients, the Unified Parkinson's Disease Rating Scale (UPDRS) – part III was used to assess the severity of the disease [24]. The stage of the disease was assessed using the Hoehn and Yahr scale (H&Y) [25]. UPDRS and H&Y scores were evaluated by an experienced neurologist (B.B.K.). Current pharmacologic treatments and periods were also recorded.

Subgingival plaque sampling

Subgingival plaque samples were collected from the single rooted teeth: 1) with the deepest pocket and 2) with the shallowest pocket. Before subgingival plaque sampling, the area was isolated with sterile gauze and gently air-dried [26]. The supragingival plaque was removed carefully using sterile curettes. Each plaque sample was immediately placed in Eppendorf tubes containing 150 µL of sterilized Tris-EDTA buffer solution (10 mM Tris HCl +1 mM EDTA). Samples were sonicated for 10 seconds. Then 100 µL of 0.5 M NaOH were added, and samples were stored at −80°C until further use.

Targeted microbiological detection of subgingival microbiome

We used the targeted DNA-DNA checkerboard hybridization technique to quantify the 40 bacterial species involved in periodontal disease and health [10,27]. Briefly, samples were boiled in a water bath for 10 min. Then, 800 µL of fresh-made 5 M ammonium acetate was added to neutralize the basic pH of the patient samples. The released DNA was laid onto a nylon membrane (Roche, Basel, Switzerland) (Roche catalog #11417240001) using 28 lanes of the Mini-Slot device (Immunetics, Cambridge, MA). After DNA was fixed, the membrane was placed in a Miniblotter 45 (Immunetics, Cambridge, MA) with the lanes of DNA at a 90° angle to the channels of the device. Digoxigenin-labeled whole chromosomal DNA probes of 40 species and hybridization buffer (45% formamide, 5 X saline-sodium citrate, 1 X Denhardt's reagent, 2 mM Na phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 20 ng/ml of labeled probe, 10% dextran sulfate, and 1% casein) were placed in each lane of the Miniblotter 45. Membranes were hybridized overnight at 42°C. After hybridization, the membranes were washed at high stringency, and hybrids were detected by applying an anti-digoxigenin antibody conjugated with alkaline phosphatase. Detection of the chemiluminescent signals was performed by G:BOX (SynGene, Cambridge, UK), and pictures of each membrane were captured with the GeneSnap program (SynGene, Frederick, MD, USA). The density of the signals was quantified using the Phoretix™ Array v10 (TotalLab, Newcastle upon Tyne, UK). The signals were quantified by comparing the obtained signals with the standards of the 40 species.

Comprehensive next-generation sequencing of subgingival microbiome

To characterize the entire subgingival microbiome in patients and healthy controls, DNA was isolated in accordance with the manufacturer's instructions (MasterPure™ DNA Purification Kit, Epicentre, Madison, WI, USA). Briefly, 1 µL of Ready-Lyse Lysozyme solution was added to each sample and incubated overnight. Then, samples mixed with 1 µL Proteinase K were incubated for 30 minutes to complete the lysis process. After the DNA wash up, residual DNA was suspended with 25 µL TE Buffer. DNA purity and concentrations were estimated using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The nucleic acids were stored at −80°C until further use. The 16S ribosomal RNA gene (16S rRNA) was targeted for metagenomic analysis. DNA isolated from subgingival plaque samples was sequenced using Next-generation sequencing of the V1V3 region of the 16S rRNA gene (ZymoBIOMICS® targeted metagenomic sequencing-Zymo Research, Irvine, CA). Briefly, after the sample quality was evaluated, the samples were prepared for sequencing. Next-Gen sequencing was performed, and then sequences were checked for quality. Absolute abundances were recorded, and bioinformatics analyses were made.

Statistical analyses

Probe counts for checkerboard DNA-DNA hybridization results in each study group were measured as means. The Kolmogorov-Smirnov test was used to analyze the data distribution. Normally distributed data were analyzed with one-way ANOVA. The Kruskal – Wallis test was used for data that was not normally distributed. All analyses were performed using GraphPad Prism 9.4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Bioinformatics analyses were performed for NGS results. Differences were tested at the operational taxonomic units (OTUs), genus, and family levels. Alpha and beta diversity analyses were used to illustrate the

differences/similarities between groups. Differential abundance was compared between three groups with ANCOM-BC (Analysis of Compositions of Microbiomes with Bias Correction) and LefSe (Linear Discriminant Analysis Effect Size) analysis. The criterion for statistical significance was $p < 0.05$.

Results

Demographic and clinical findings

In the PA+PD group, there were 4 females and 16 males, the age range was 32–65 years, and the mean age was 54.8 ± 10.4 years. In the PD group, there were 9 females and 11 males, aged 25–56 years, and the mean age was 39.8 ± 9.4 years. In the healthy group, there were 12 females and 8 males, the age range was 24–47 years, and the mean age was 32.7 ± 7.4 years (Table 1). Clinical periodontal parameters (PPD, BOP, GR, CAL, and PI) of all sites, deep and shallow pockets, are presented in Table 2. Probing depth and clinical attachment levels were higher in P and PA+PD groups than in the healthy control group ($p < 0.0001$). BOP showed a statistically significant increase in the PA+PD group than HC and P groups. According to the Hoehn and Yahr scale, 7 patients (35%) were diagnosed with Stage 1, whereas 13 patients (65%) were with Stage 2. The mean UPDRS Part III score was 18.6 ± 6.3. Antiparkinsonian drugs used by PA patients were as follows: levodopa ($n = 19$), rasagiline ($n = 10$), pramipexole ($n = 6$), amantadine ($n = 4$), pribedil ($n = 2$), and apomorphine ($n = 1$). In addition to antiparkinsonians, PA patients were using antidepressants (sertraline, olanzapine, mirtazapine,

Table 1. Demographic and clinical data of healthy control (HC), periodontitis (PD), and periodontitis-Parkinson's disease (PA+PD) groups. Data are shown as mean ± standard deviation. All data were checked for normality using the Kolmogorov-Smirnov test and analyzed with one-way ANOVA and Kruskal-Wallis tests to determine whether the distribution was normal, respectively.

	HC n = 20	PD n = 20	PA+PD n = 20	*p*-value
Age (year)[*,#,†]	32.7 ± 7.4	39.8 ± 9.5	54.8 ± 10.4	<0.0001
Gender (F/M[†])	12/8	11/9	4/16	0.0215
PPD (mm)[*,#]	1.59 ± 0.2	2.84 ± 0.62	2.63 ± 0.45	<0.0001
BOP (%)[*,#,†]	7.31 ± 4.85	47.72 ± 18.08	63.24 ± 23.98	<0.0001
CAL (mm)[*,#]	1.68 ± 0.39	3.11 ± 0.66	3 ± 1.07	<0.0001
PI[*,#]	0.56 ± 0.3	1.53 ± 0.39	1.74 ± 0.44	<0.0001
GR (mm)[#]	0.08 ± 0.33	0.27 ± 0.2	0.48 ± 0.55	0.0001

Note: *Statistically significant difference between HC and PD ($p < 0.05$), [#]Statistically significant difference between HC and PA+PD ($p < 0.05$), [†]Statistically significant difference between PD and PA+PD ($p < 0.05$).
PPD: probing pocket depth, BOP: bleeding on probing, CAL: clinical attachment loss, PI: plaque index, GR: gingival recession.

Table 2. Clinical data of healthy control (HC), periodontitis (PD), and periodontitis-Parkinson's disease (PA+PD) groups. Data are shown as mean ± standard deviation. All data were checked for normality using the Kolmogorov-Smirnov test and analyzed with one-way ANOVA and Kruskal-Wallis tests to determine whether the distribution was normal, respectively.

All Sites	HC n = 40	PD n = 39	PA+PD n = 35	*p*-value
PPD (mm)[*,#]	1.65 ± 0.66	3.54 ± 1.31	3.23 ± 1.26	<0.0001
BOP (%)[*,#,†]	8 ± 26	79 ± 40	91 ± 28	<0.0001
CAL (mm)[*,#]	1.68 ± 0.66	3.90 ± 1.55	3.51 ± 1.48	<0.0001
PI[*,#]	0.43 ± 0.64	1.51 ± 0.94	1.46 ± 0.66	<0.0001
GR (mm)	0.03 ± 0.16	0.36 ± 0.74	0.29 ± 0.62	ns
Deep Pockets	n = 20	n = 20	n = 18	
PPD (mm)[*,#]	2 ± 0.65	4.4 ± 1.23	3.94 ± 1.35	<0.0001
BOP (%)[*,#,†]	15 ± 36.6	85 ± 36.6	100	<0.0001
CAL (mm)[*,#]	2 ± 0.65	4.75 ± 1.45	4.28 ± 1.6	<0.0001
PI[*,#]	0.45 ± 0.6	1.45 ± 1.05	1.56 ± 0.7	<0.0001
GR (mm)	0	0.35 ± 0.75	0.33 ± 0.69	ns
Shallow Sites	n = 20	n = 19	n = 17	
PPD (mm)[*,#]	1.3 ± 0.47	2.63 ± 0.6	2.47 ± 0.51	<0.0001
BOP (%)[*,#,†]	0	74 ± 45.2	82.3 ± 39.2	<0.0001
CAL (mm)[*,#]	1.35 ± 0.49	3 ± 1.11	2.71 ± 0.77	<0.0001
PI[*,#]	0.4 ± 0.68	1.58 ± 0.84	1.35 ± 0.61	<0.0001
GR (mm)	0.05 ± 0.22	0.37 ± 0.76	0.24 ± 0.56	ns

Note: *Statistically significant difference between HC and PD ($p < 0.05$), [#]Statistically significant difference between HC and PA+PD ($p < 0.05$), [†]Statistically significant difference between PD and PA+PD ($p < 0.05$).
PPD: probing pocket depth, BOP: bleeding on probing, CAL: clinical attachment loss, PI: plaque index, GR: gingival recession.

quetiapine, and escitalopram, $n = 1$ of each) and calcium channel blockers (amlodipine, $n = 2$).

Quantification of periodontal subgingival microbiome species

Figure 1 and Figure 2 present mean Checkerboard DNA probe counts of bacteria in all sites where subgingival plaque samples were collected. *Streptococcus gordonii, Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Peptostreptococcaceae_[G-6][Eubacterium]_ nodatum, Parvimona micra, Lachnoanaerobaculum saburreum, Prevotella melaninogenica, Streptococcus anginosus, and Treponema socranskii* were significantly higher in PA+PD group than HC and PD groups in all sites ($p < 0.05$). In deep pockets, *Streptococcus intermedia* ($p = 0.0002$, $p = 0.0042$), *C. rectus* ($p = 0.0049$, $p = 0.0020$), *P. nodatum* ($p < 0.0001$, $p = 0.0022$), *P. micra* ($p < 0.0001$, $p = 0.0002$), *T. denticola* ($p < 0.0001$, $p = 0.0045$), *L. saburreum* ($p = 0.0178$, $p = 0.0267$), *P. melaninogenica* ($p < 0.0001$, $p = 0.0017$), and *T. socranskii* ($p < 0.0001$, $p = 0.0002$) counts were significantly higher in PA+PD group compared to both HC and PD groups, respectively (Figure 3 and Figure 4). *A. actinomycetemcomitans* was significantly lower in PA+PD than the PD group ($p = 0.0072$). In shallow pockets, *Schaalia odontolytica* ($p = 0.0351$) and *A. actinomycetemcomitans* ($p = 0.002$) were significantly lower in the PA+PD group than in the PD group (Figure 5 and Figure 6). *C. rectus* ($p = 0.0010$, $p = 0.0002$), *P. micra* ($p = 0.0026$, $p = 0065$), *Streptococcus constellatus* ($p < 0.0001$, $p = 0.0151$), *T. denticola* ($p = 0.0001$, $p = 0.0141$), *P. melaninogenica* ($p < 0.0001$, $p = 0.0057$), and *T. socranskii* ($p < 0.0001$, $p = 0.0316$) were significantly higher in the PA+PD group than the HC and PD groups, respectively.

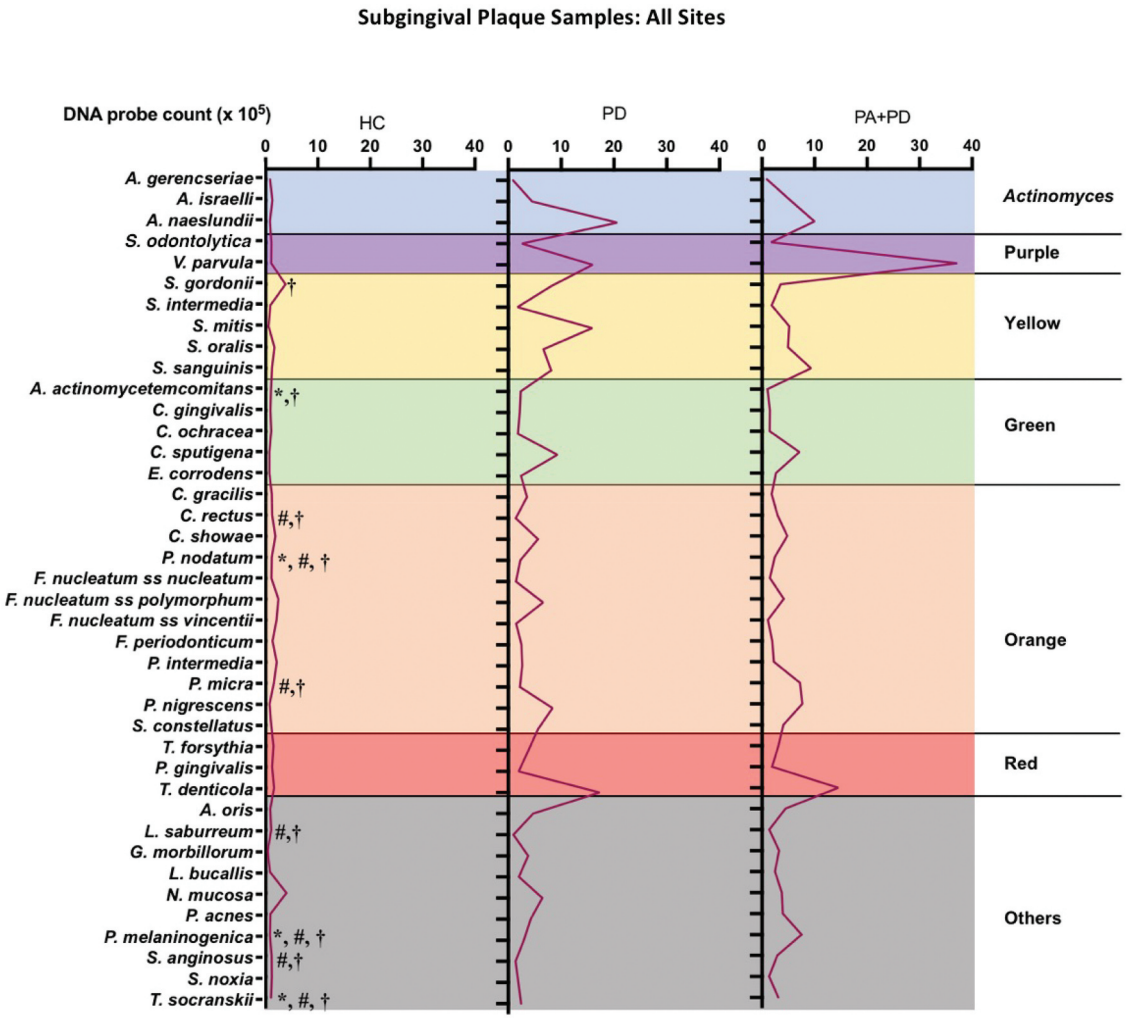

Figure 1. Mean DNA probe counts of 40 bacterial species in all sites from subgingival plaque samples of HC, PD, and PA+PD groups (x10) [5]. Values present results of 40, 39, and 35 samples from control, periodontitis, and periodontitis-Parkinson's disease groups, respectively, analyzed with the checkerboard DNA-DNA hybridization technique. Normality was tested with the Kolmogorov-Smirnov test. The Kruskal-Wallis test was used for data not distributed normally, and one-way ANOVA was used for normally distributed data. Bacterial species were arranged by the microbial complexes described by Socransky et al. [10] *Statistically significant difference between HC and PD ($p < 0.05$), #Statistically significant difference between HC and PA+PD ($p < 0.05$), †Statistically significant difference between PD and PA+PD ($p < 0.05$).

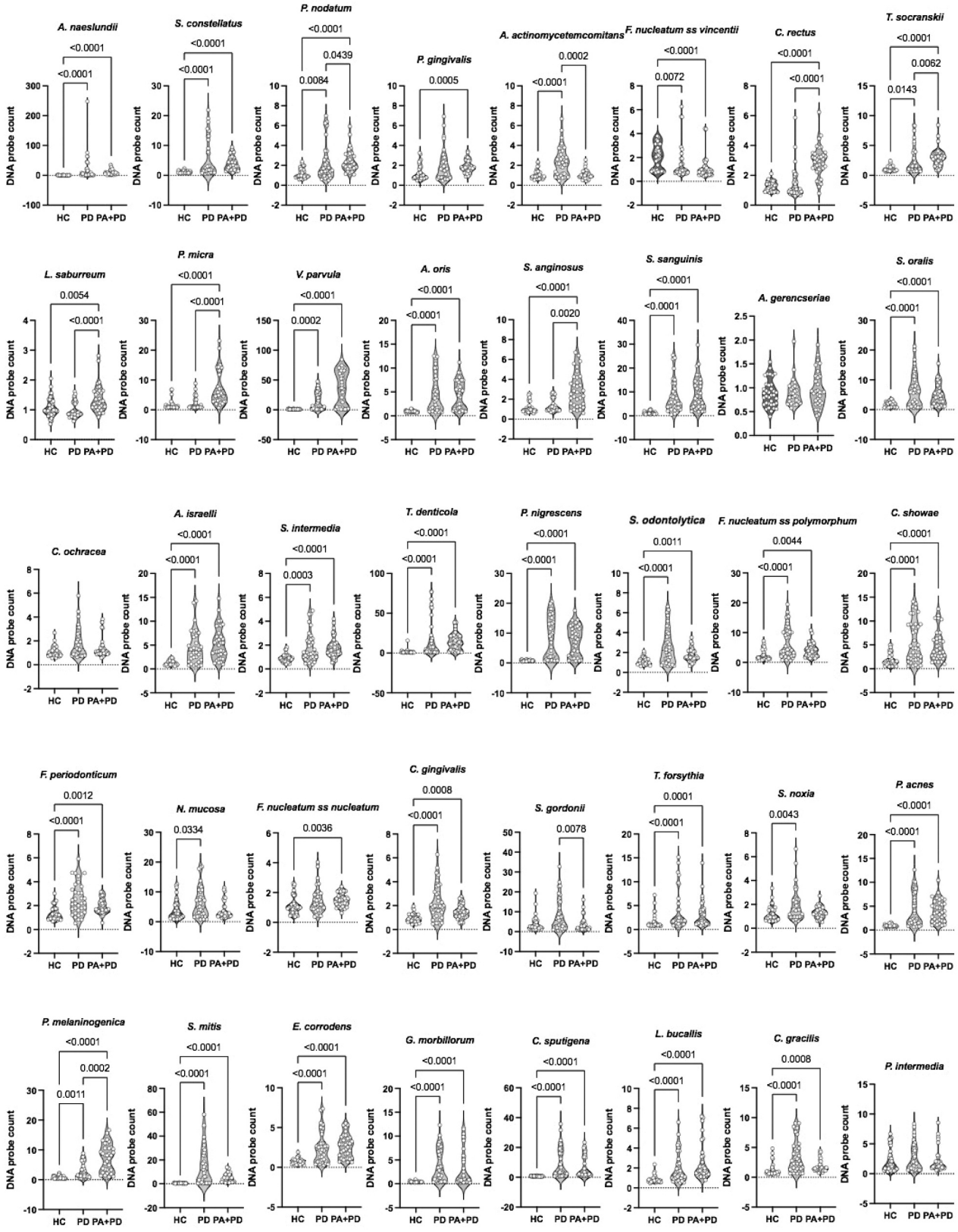

Figure 2. Group comparisons of subgingival samples from all sites. Mean values of DNA probe counts (x10) [5] of subgingival samples from HC ($n = 40$), PD ($n = 39$), and PA+PD ($n = 35$) patients are represented. Normality was tested with the Kolmogorov-Smirnov test. Kruskal-Wallis test was used for data not distributed normally, and one-way ANOVA was used for normally distributed data. $p < 0.05$ values are shown on the graphs.

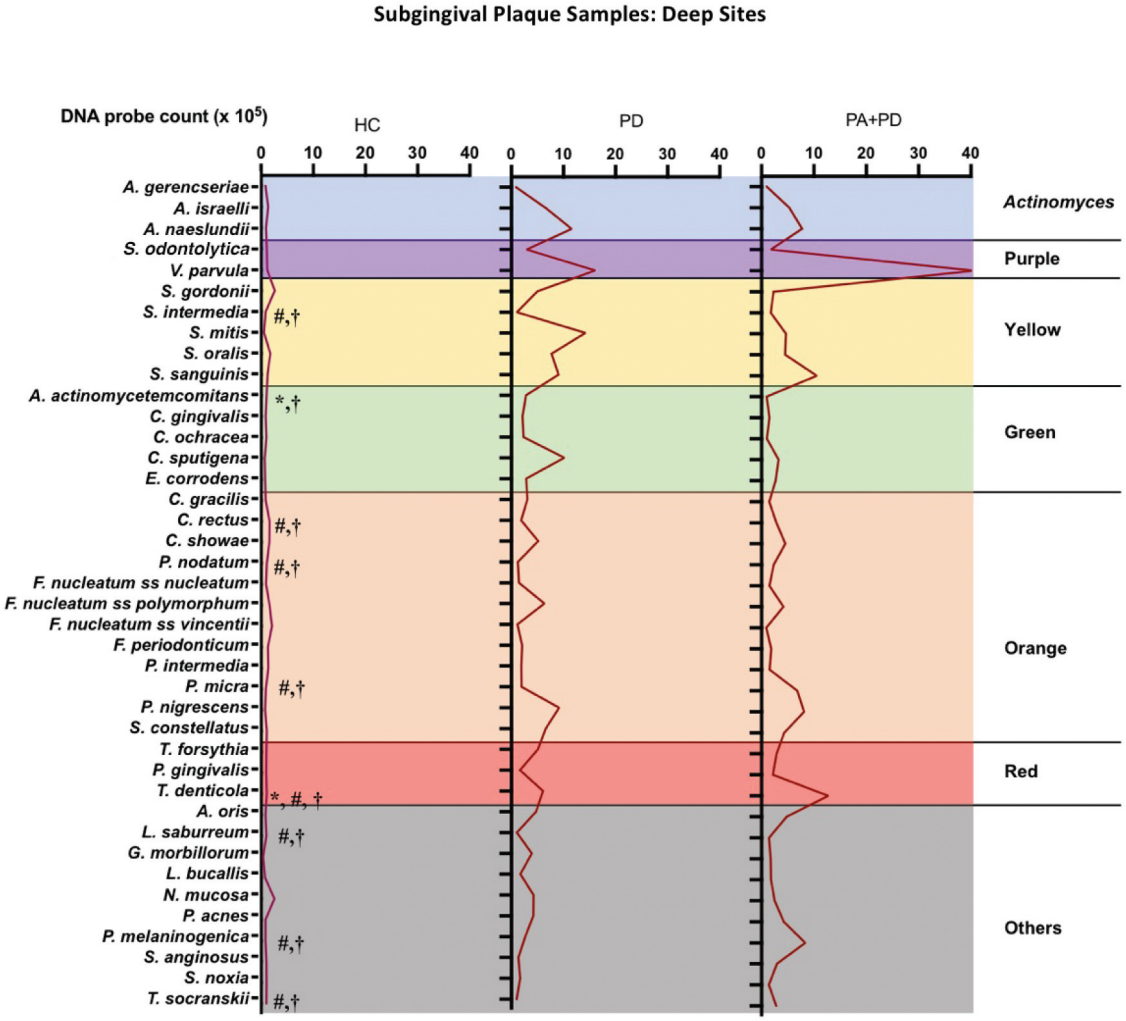

Figure 3. Mean DNA probe counts of 40 bacterial species in deep pockets from subgingival plaque samples of HC, PD, and PA +PD groups (x10) [5]. Values present results of 20, 20, and 18 samples from control, periodontitis, and periodontitis-Parkinson's disease groups, respectively, analyzed with the checkerboard DNA-DNA hybridization technique. Normality was tested with the Kolmogorov-Smirnov test. The Kruskal-Wallis test was used for data not distributed normally, and one-way ANOVA was used for normally distributed data. Bacterial species were arranged in accordance with the microbial complexes described by Socransky et al. [10], *Statistically significant difference between HC and PD ($p < 0.05$), #Statistically significant difference between HC and PA+PD ($p < 0.05$), †Statistically significant difference between PD and PA+PD ($p < 0.05$).

Next generation sequencing

To characterize the entire subgingival microbiome, we analyzed the samples from PA+PD, PD, and H groups by NGS. A total of 382 taxa were detected in all groups (Supplemental Table 1). Alpha diversity significantly differed between HC and PD or PA+PD groups (Figure 7). The control group contained diverse microbiome profiles compared to PD ($p = 0.001$) and PA+PD ($p = 0.026$) groups. There were little or no differences in alpha and beta diversities comparing PD with the PA+PD groups. PD and PA +PD microbiomes contained similar periodontal pathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella* spp, *Eubacterium* spp, *Selenomonas* spp., *Campylobacter*, *Synergistales* (e.g. Tm7), *Fusobacterium* spp, and *Treponema* spp. Notably, there was a high prevalence (more than 20% in some subjects) of *Treponema* spp, including *T. denticola*, in both groups (Figure 8) Meanwhile, there were also differences between PD and PA+PD microbiomes (Figure 9) many species of *Prevotella* were more prevalent in PA+PD, *Eubacterium infirmum*, *E. brachy*, *Dialister*, and species of *Selenomonas*, all often associated with PD, were more prevalent in PA+PD. On the other hand, *Prevotella nigrescens*, *Aggregatibacter* spp, and *Johnsonella* spp were more prevalent in PD than PA+PD, demonstrating a unique PA-associated subgingival microbiome in PD.

Discussion

We evaluated the impact of Parkinson's disease on the subgingival microbiome in patients with periodontitis by two strategies. We combined a targeted quantification of 40 species associated with

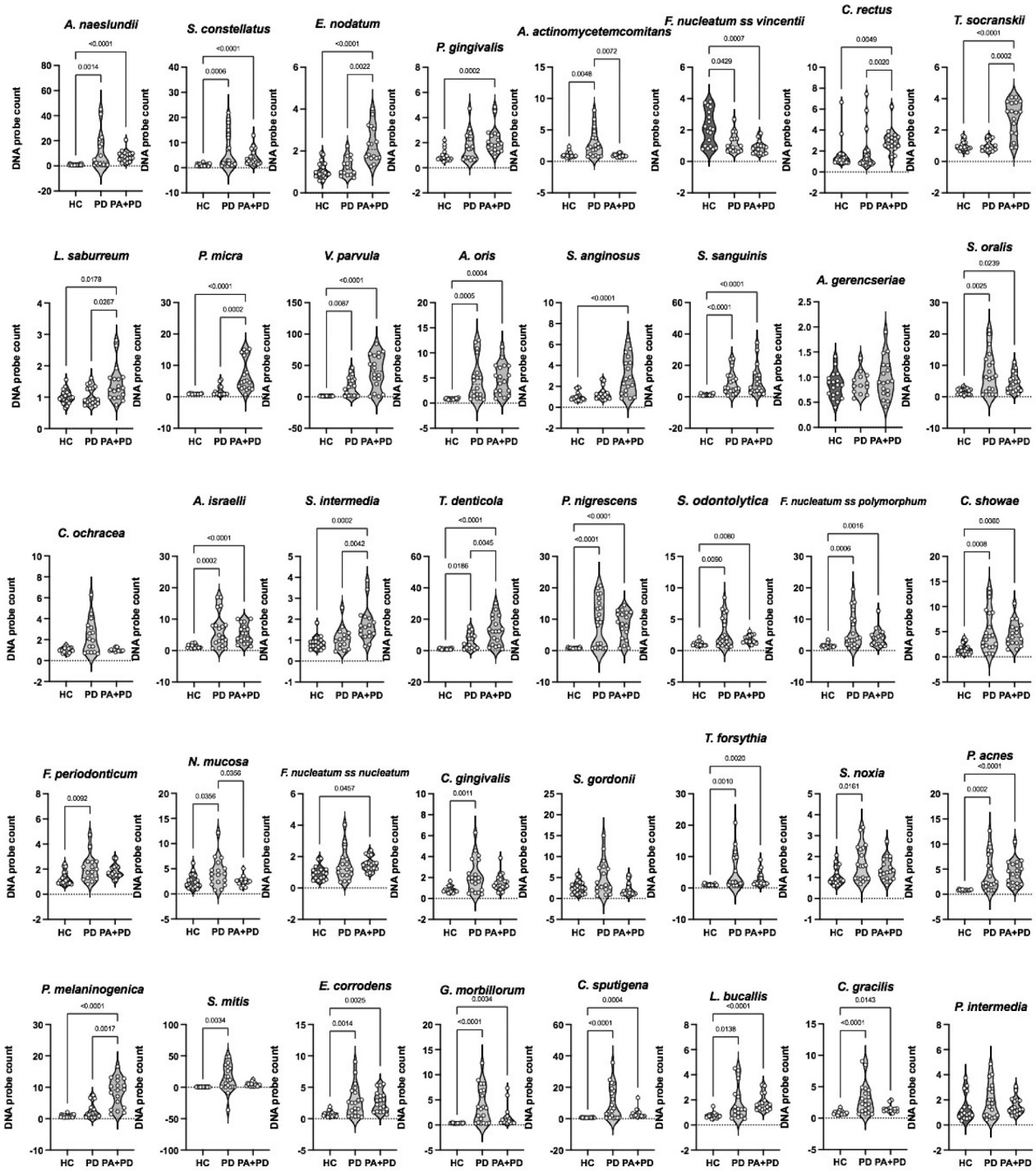

Figure 4. Group comparisons of subgingival samples from the deep pockets. Mean values of DNA probe counts (x10) [5] of subgingival samples from HC ($n = 20$), PD ($n = 20$), and PA+PD ($n = 18$) patients are represented. Normality was tested with the Kolmogorov-Smirnov test. Kruskal-Wallis test was used for data not distributed normally, and one-way ANOVA was used for normally distributed data. $p < 0.05$ values are shown on the graphs.

periodontal disease and health and a comprehensive approach to characterizing the relative abundance of subgingival species in the same pockets. The data demonstrated that distinct species were associated with PA, suggesting a direct impact of PA on the periodontal microbiome. Since our patients performed oral hygiene efficiently and did not show any exacerbated periodontal tissue destruction compared to PD-alone patients, the data showed the microbial changes associated with PD. Taken together with the increased gingival inflammation induced by PA, the findings suggested that PA is an independent risk factor for modifying the PD-associated subgingival microbiome.

Demographic findings showed statistically higher mean age in the PA+PD group, in accordance with studies indicating that PA prevalence increased with age [4,28]. Similarly, males were higher in the PA

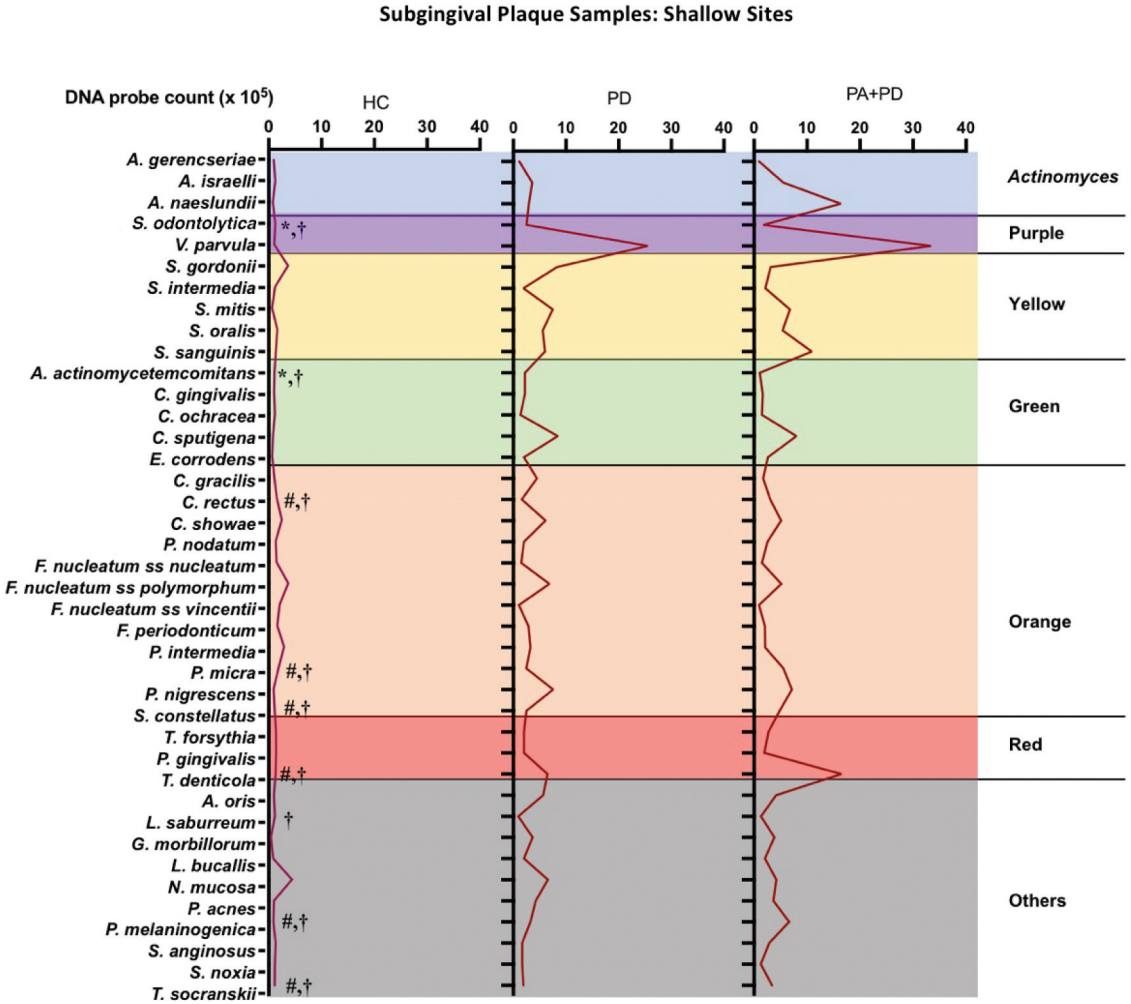

Figure 5. Mean DNA probe counts of 40 bacterial species in shallow pockets from subgingival plaque samples of C, P, and PA +PD groups (x10) [5]. Values present results of 20, 19, and 17 samples from control, periodontitis, and periodontitis-Parkinson's disease groups, respectively, analyzed with the checkerboard DNA-DNA hybridization technique. Normality was tested with the Kolmogorov-Smirnov test. The Kruskal-Wallis test was used for data not distributed normally, and one-way ANOVA was used for normally distributed data. Bacterial species were arranged in accordance with the microbial complexes described by Socransky et al. [10], *Statistically significant difference between HC and PD ($p < 0.05$), $^{\#}$Statistically significant difference between HC and PA+PD ($p < 0.05$), †Statistically significant difference between PD and PA+PD ($p < 0.05$).

group, which correlates with a study concluding that PA occurs later in females. In animal studies, it was reported that estrogen has a neuroprotective effect, which may be the reason for the late onset of the disease in females [29]. Since PD is also a condition that is more common in men, the male gender may be a common risk factor for these two diseases. Age and gender could be confounding factors on the oral cavity's microbial composition, irrespective of the PA's contribution. While our study was not designed and therefore not powered to address these two critical factors, it is important to acknowledge the potential involvement of age- and gender-associated perturbations in the oral microbiome. To this end, the data about the impact of gender on the oral microbiome is scarce. An earlier study found no differences between genders in the subgingival microbiota [30]. On the other hand, recent work has suggested that age could impact oral microbial changes where *Lactobacillales, Gemellaceae, Bacteroides*, and *Fusobacterium* decreased as the age increased in both males and females [31]. These species differed from those we identified in this study, suggesting that more extensive and focused analyses are required to determine the impact of aging on oral microbial species.

UPDRS and H&Y were used to measure PA severity [24,32]. UPDRS Part III scores motor features with 18 items, with the highest score being 72. PA patients in this study had a minimum of 9, and a maximum of 30 scores in UPDRS Part III which showed that they had early-stage PA and their motor disabilities were not severe. According to the daily oral hygiene questionnaire, 50% of the PA+PD group brushed regularly, while it was 10% in the PD group. Interdental cleaning frequency was 65% in the control group and 5% in both PD and PA+PD groups. Thus, while motor abnormalities may potentially hinder the ability to maintain good oral hygiene and

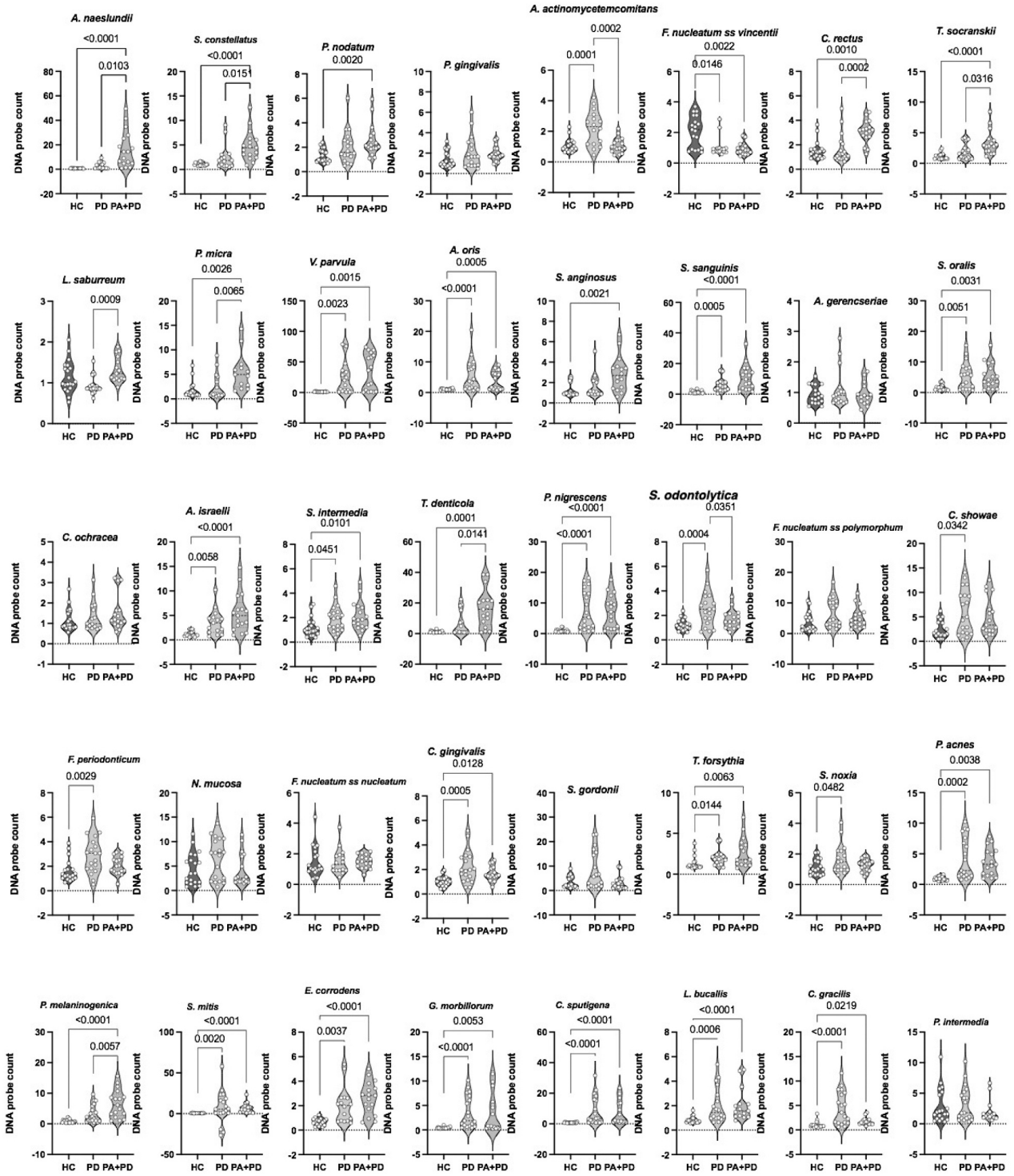

Figure 6. Group comparisons of subgingival samples from the shallow pockets. Mean values of DNA probe counts (x10) [5] of subgingival samples from HC ($n = 20$), PD ($n = 19$), and PA+PD ($n = 17$) patients are represented. Normality was tested with the Kolmogorov-Smirnov test. Kruskal-Wallis test was used for data not distributed normally, and one-way ANOVA was used for normally distributed data. $p < 0.05$ values are shown on the graphs.

decrease oral health in PA, leading to an elevated risk of developing periodontitis [14,33,34], PA was not a confounding factor in PD severity in this study. These data suggested that our results reflected the direct impact of PA on the periodontal microbiome since the study groups had equivalent oral hygiene status. On the other hand, gingival inflammation, as assessed by bleeding on probing, was significantly increased in PA patients, demonstrating the impact of PA as an independent variable on gingival health. In addition to the impact of PA on the oral microbiome, PA-induced host response and inflammation could also act as a modifier of the local microbiota. Indeed, PA led to elevated levels of BOP in deep and shallow pockets in the PA+PD group (Table 1), indicative of gingival inflammation, which may manifest

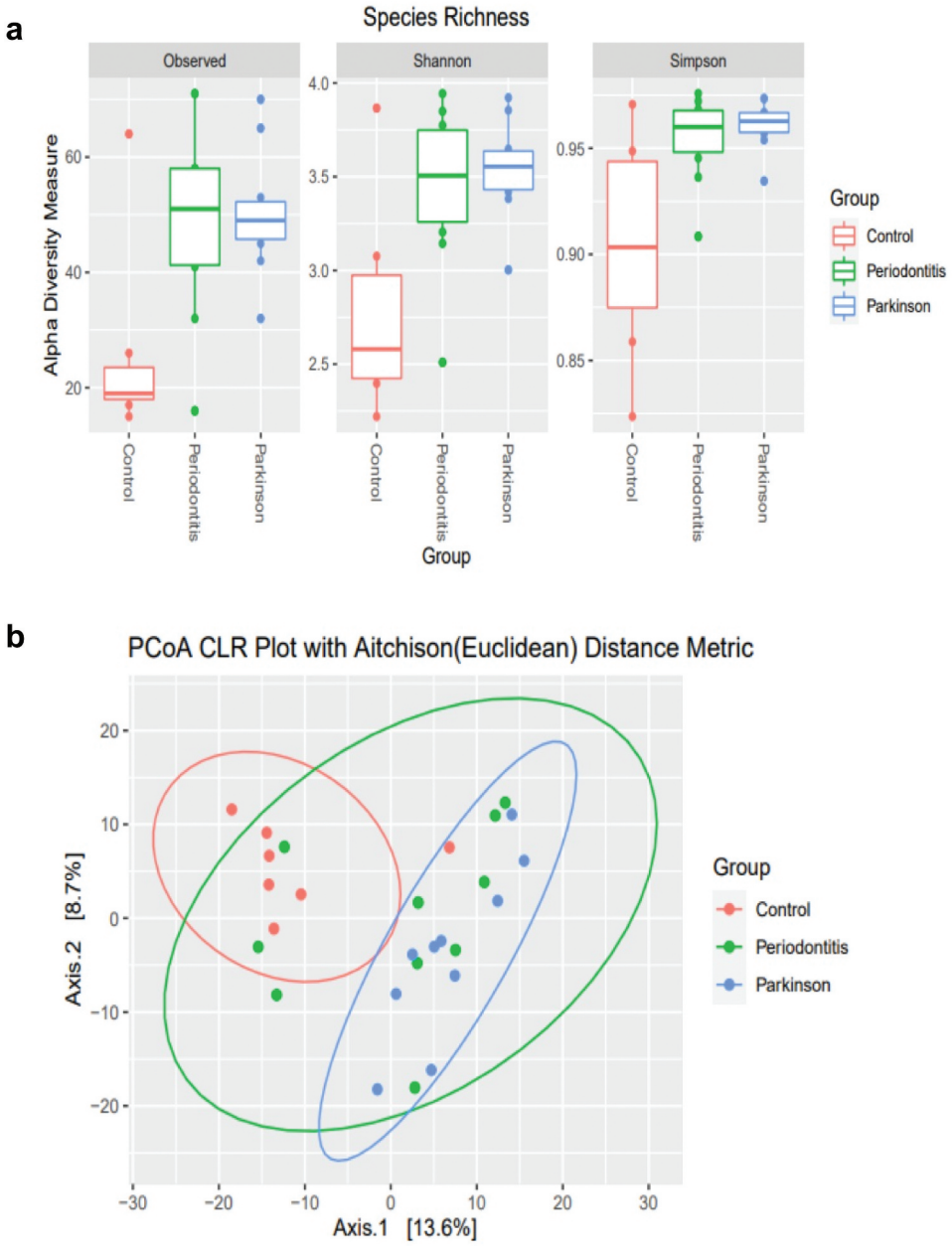

Figure 7. Alpha and beta diversity indices of the subgingival samples from HC, PD, and PA+PD groups. **Panel A**. observed species index, Shannon index, and Simpson's diversity index illustrate differences between three groups based on OTU counts. **Panel B**. PCoA plots of bacterial beta-diversity depict the statistically significant difference in PD and P.A+PD groups compared to HC.

as a change in the community structure of an altered environment for bacterial growth.

Of note, antiparkinsonian drugs are agonists of the dopamine hormone and are often prescribed with levodopa to reduce the motor symptoms of PA patients [35]. These drugs have anti-inflammatory effects, especially when combined with levodopa [36], decreasing TNF-α, prostaglandin E2, and nitric oxide [35]. However, our patients with PA had increased gingival inflammation, further demonstrating that the Parkinsonian medications did not affect the local immune response to gingival inflammation of PA. These findings may suggest that the oral microbiome shifts in PA patients with PD may increase gingival inflammation that is exacerbated by PA.

While we only studied the impact of PA on patients with PD, the association between these two diseases could also be bidirectional, where the PD-associated microbiome can impact the PA-associated neurodegeneration. Considering that a severe PD patient swallows 10 [12–]10 [13] *P. gingivalis* per day introduces a microbial risk for systemic diseases [37]. Animal and human studies have shown that *P. gingivalis* may be responsible for the systemic inflammation, abnormal α-synuclein deposition in intestinal neurons, and the dysbiosis of the gut microbiome, as well as that oral bacteria,

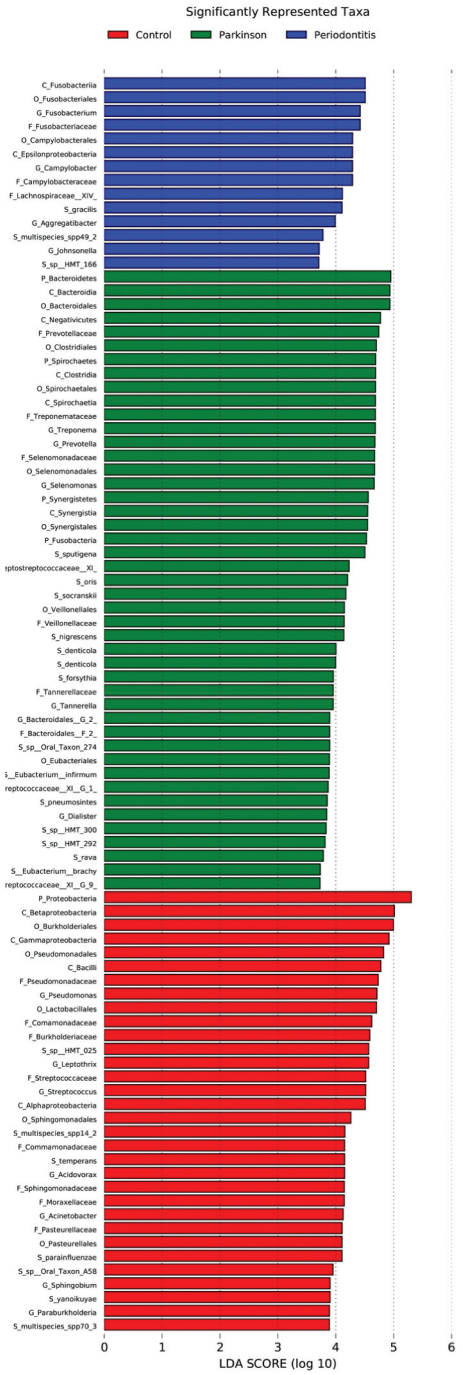

Figure 8. LEfSe analysis representing the similarities between HC, PD, and PA+PD samples. LDA scores of (log 10) > 2 and $p < 0.05$ are listed.

may translocate to extraoral areas, suggesting a microbial link between PA and PD [38–41]. As in the dysbiotic gut, which stimulates intestinal inflammation, increases intestinal permeability (leaky gut), and transfers signals to the central nervous system (CNS) due to the endotoxins of pathogens [42], induces or sustains excessive phos-α-synuclein expression and misfolding, whereby aggregated phos-α-synuclein may translocate to the CNS through trans-synaptic transmission, leading to PA degeneration [43], a similar mechanism could be associated with PD-dysbiosis.

The oral cavity harbors a variety of surfaces with diverse surface characteristics, food sources, and mechanical features where microorganisms can exist. These areas offer favorable conditions for colonizing yeast, bacteria, and viruses [44]. Working with plaque samples from subgingival areas has several advantages. Additionally, being highly related to local and general health, analyzing oral microbiota provides aspects of diseases with local and systemic impacts. To examine the microbial content of the microbial dental plaque, which changes with the increase in pocket depth, subgingival plaque samples were examined in two separate groups: deep and shallow. By examining the change in the number of periodontopathogens and the relative abundance of microbial species in the samples taken from pockets of different depths, we aimed to clarify the effect of PA on PD. This approach demonstrated important clues for different microbial species colonizing different depths of pockets.

Our results showed that *S. anginosus* and *S. sanguinis* were abundant in the PA+PD group compared to HC and PD groups in all sites. Furthermore, there was an increase in *S. intermedius* in the PA+PD group in deep pockets. The increase of *Streptococcus* species, which can sometimes cause permanent neurological damage by secreting neurotoxins, has been associated with immunoinflammatory pathways in PA [45]. *Streptococcus* species are higher in stool samples of PA patients [46]. One recent study found increased Streptococcus mutans abundance in PA patients' subgingival dental plaque compared to healthy controls [43]. In addition to being found in healthy oral flora, these bacterial species increase in number in periodontal disease [10]. Although they do not initiate periodontal disease, they participate in dysbiosis, leading to the disease. Our results indicated that PA might lead to an environment that eventually induces periodontal disease by prompting the increase of *Streptococcus* species in the subgingival area.

The spirochete *T. socranskii* is known to interact directly with target cells using their endotoxins and degradation products to invade tissues [47]. This is one of the most abundant spirochetes, found in sites with severe periodontal destruction and associated with periodontal disease [48]. This species was found in the trigeminal ganglions of patients with Alzheimer's disease, indicating that *T. socranskii* may have access to neuronal axons. *T. socranskii* induces osteoclastogenesis through a RANKL (nuclear factor-κ B (RANK) ligand)-dependent pathway in periodontal tissues by up-regulating RANKL and down-regulating osteoprotegerin, which suggests an inflammatory role of this pathogen in periodontitis [49]. Thus, in higher numbers in the PA+PD group, *T. socranskii* could be a specific pathogen to exacerbate periodontitis in PA patients.

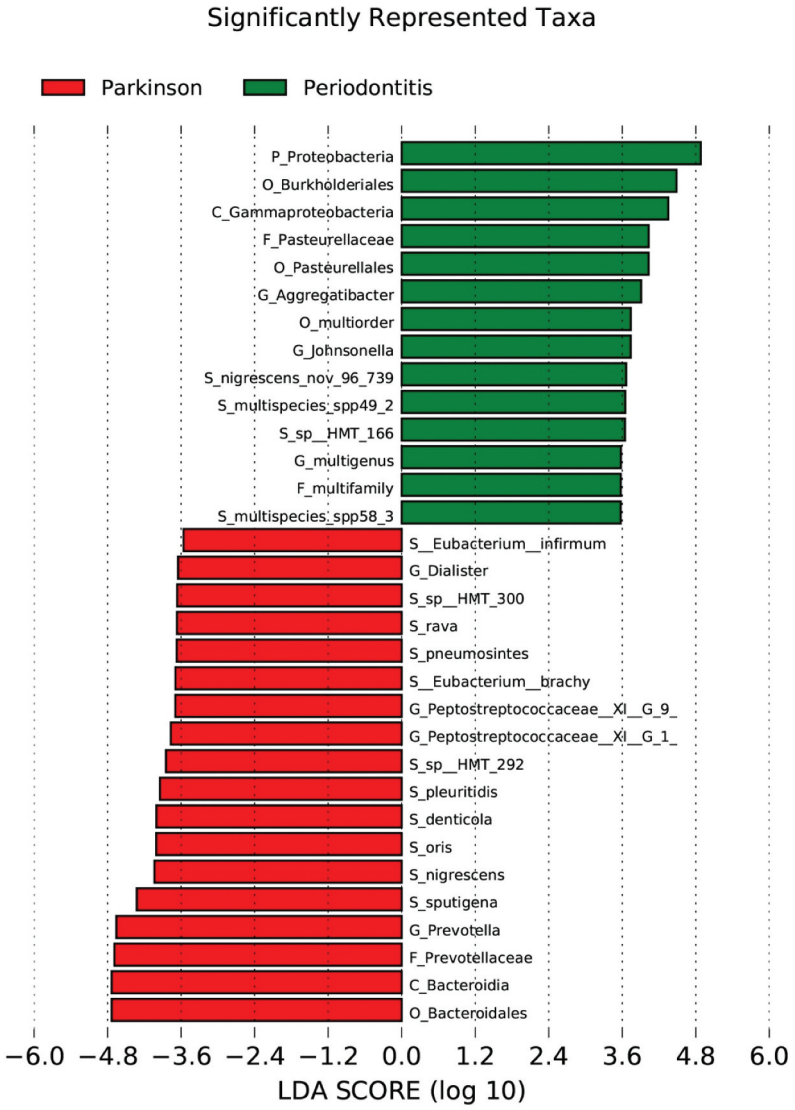

Figure 9. LEfSe analysis results of direct comparison between PD and PA+PD groups show differential abundances among the groups.

Specific forms of severe periodontitis are linked to the pathogen *A. actinomycetemcomitans* [50]. This Gram-negative, capnophilic, facultative anaerobic bacillus promotes colonization, causes tissue destruction in the host, and inhibits the host's repair mechanisms by its virulence factors. It has strong adhesion and invasion capacity [51] with low abundance in the oral microbiome [52]. We found a statistically significant decrease in *A. actinomycetemcomitans* levels in the PA+PD group compared to the P group in all pockets, a decrease more evident in deeper pockets. This finding may result from relatively older patients recruited in this study, as a higher prevalence of *A. actinomycetemcomitans* was reported in younger patients [53–55]. On the other hand, it does not explain why patients with PA had lower numbers, as the PA+PD and PD groups were similar ages. Thus, a more plausible explanation is that PA may modify the subgingival microbiome in favor of specific species while others are less detectable.

Another important species is the motile *C. rectus*, an orange complex species characterized by its role in transitioning from periodontal health to disease [10]. In a microbiological study, *C. rectus* has been associated with the symptoms of Alzheimer's disease along with *P. gingivalis* and *P. melaninogenica* [56]. We found a statistically significant increase in *C. rectus* as well as *P. gingivalis* and *P. melaninogenica* in the PA +PD group. This finding has significant repercussions. *C. rectus* can induce interleukin-1 alpha production from monocytes, while *P. gingivalis* can suppress 72% of interleukin-1 alpha production [57], thus suggesting an immunomodulatory impact of periodontopathogens in the subgingival environment.

Our study examined the microbiota of the subgingival region in PA patients with targeted and unbiased approaches. Only one previous study used DNA

sequencing for subgingival samples [43]. Other studies focused on the oral microbiome on buccal and sublingual mucosa profiles in PA patients [58]. Both alpha ($p < 0.05$) and beta ($p = 0.026$) diversities showed statistical differences due to the PD, with no major differences between PA+PD and PD groups. *Prevotella nigrescens* were consistently detected in checkerboard DNA-DNA hybridization and next-generation sequencing, while *Prevotella, Bacteriodales*, and *Eubacterium* species were more abundant in the PA+PD group compared to PD. Methodologically, combining the two techniques provided a more comprehensive understanding of the subgingival microbiome of PD in PA patients. The study included two different methods, each with its own strengths and limitations. Since we cannot make a direct comparison, several species were detected by both methods, indicating both similarity and dissimilarity levels of these methods in genus and species levels. Comparing the two methods, we observed increasing levels of *P. melaninogenica* in all sites in the PA+PD group, in accordance with NGS results that detected increased *Prevotella* species in the same group. Compared to sequencing, the checkerboard DNA-DNA hybridization method has the advantages of being cost-effective and quantifying bacterial DNA. This technique allowed us to detect the counts of specific periodontopathogens in the study group. We observed the relative abundances of microbial species among groups using next-generation sequencing. Thus, it was possible to have a perspective of the microbial profile of the subgingival area. The primary limitation of this study is that it was cross-sectional; thus, we cannot make any causal inferences. The study sample size was limited because of our strict inclusion and matching criteria between PA+PD and PD groups.

Conclusions

PA and PD are complex diseases that might be linked through several variables. PA has a potential etiology impact on PD-associated inflammation and bacterial dysbiosis. In this study, we identified several changes in specific bacterial species indicating that PA might be a risk factor for microbial shifts in the PD microbiome.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The next-generation sequencing data for this study are available at https://microbiome.forsyth.org/ftp/fomc/FOMC7142_full/REPORT.html.

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