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Original Article

Effects of Nonsurgical Periodontal Treatment on Bacterial and Clinical Parameters in Down Syndrome Patients Based on 16S rRNA Gene Amplicon Sequencing

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Individuals with Down syndrome (DS) are more susceptible to periodontal disease; however, microbial changes following treatment remain insufficiently understood. This study evaluated the effects of nonsurgical periodontal therapy on clinical outcomes and oral microbiome dynamics in 6 patients with DS using 16S rRNA gene amplicon sequencing. Bacterial diversity, composition, network structure, and predicted functional pathways were analyzed using dental plaque samples. Bleeding on probing decreased significantly ($p = 0.047$) after treatment, with a trend toward reduction in periodontal inflamed surface area ($p = 0.05$). The abundance of *Fusobacteria* at the class level decreased significantly after treatment. The abundance of *Mogibacterium timidum* was higher in the pretreatment group than in the posttreatment group. *M. timidum* was positively correlated with *Treponema denticola* and associated with multiple bacterial taxa in the network during pretreatment. Predicted functional pathways related to aromatic compound degradation were more abundant in posttreatment samples than in pretreatment samples. An increase in the abundance of *Fusobacterium* and the positive correlation between *T. denticola* and *M. timidum*, together with their associations with other periodontal pathogens before treatment, may contribute to the development of periodontitis in individuals with DS. Nonsurgical periodontal therapy produces measurable clinical improvement and promotes microbial shifts in patients with DS.

Key words: Down Syndrome, 16S rRNA Gene Amplicon Sequencing, periodontitis, nonsurgical periodontal treatment, oral microbiome

Down syndrome (DS), a genetic disorder caused by full or partial trisomy 21, is the most common genetic cause of intellectual disability. In addition to cognitive impairment, individuals with DS frequently present with systemic complications, including

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congenital heart defects, Alzheimer's disease, leukemia, and immune dysfunction [1]. Individuals with DS commonly exhibit distinctive oral features, such as hypodontia, microdontia, delayed tooth eruption, and a fissured tongue [2,3]. Poor manual dexterity often complicates oral hygiene maintenance, increasing the risk of periodontal disease. However, the prevalence of dental caries remains relatively low, likely owing to differences in dietary habits and oral care behaviors, as well as in the frequency of dental visits [4].

Periodontal disease is a chronic inflammatory disease triggered by dysbiotic plaque biofilms and host immune-inflammatory responses, leading to the destruction of periodontal supporting tissues [5]. Periodontal bacteria present in dental plaque possess various virulence factors, including lipopolysaccharides, fimbriae, and enzymes, which elicit inflammatory responses in periodontal tissues [6]. Periodontal disease affects systemic health by increasing systemic inflammatory mediators as a consequence of local periodontal infection [7]. Therefore, periodontal infection has long been associated with an increased risk of various diseases, such as cardiovascular disease [8], nonalcoholic fatty liver disease [9], and chronic obstructive pulmonary disease [10].

Young adults with DS have an increased prevalence of periodontal disease [11]. A meta-analysis concluded that periodontal treatment is less effective in reducing pocket depths in individuals with DS than in those without DS [12]. In contrast to the many reports evaluating clinical parameters in patients with DS, few studies have examined microbial characteristics in this population. The abundances of *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*, which are major periodontal pathogens, were reported to be significantly higher in patients with DS than in healthy children [13], although another study found no significant difference in the abundances of these bacteria between adults with and without DS [14].

Owing to advances in next-generation sequencing, the microbiome comprising many bacterial species can now be analyzed comprehensively from a single sample. However, few studies have performed oral microbial analyses using next-generation sequencing in patients with DS. One such analysis reported lower microbial diversity, decreased relative abundances of *Alloprevotella* and *Atopobium*, and higher abundances of *Kingella, Staphylococcus, Gemella, Cardiobacterium, Rothia*, and *Actinobacillus* [15]. However, no study has compared the oral microbiome of patients with DS before and after periodontal treatment using next-generation sequencing. Thus, the purpose of this study was to evaluate the efficacy of nonsurgical periodontal treatment in patients with DS through comprehensive 16S rRNA gene sequencing-based microbiome analysis, regardless of baseline inflammation status.

Materials and Methods

Ethical statement. This study was conducted in accordance with the Ethical Guidelines for Clinical Studies (2008 Notification Number 415 of the Ministry of Health, Labour, and Welfare) and received approval from the Ethics Committee of Okayama University (2006-044). Patients visiting the Center for Special Needs Dentistry at Okayama University Hospital were recruited from June 1, 2020, to September 30, 2021, and samples were collected. All participants provided written informed consent before participating. The study adhered to the principles of the Declaration of Helsinki, as revised in 2013.

Participants and clinical assessment. Six participants with DS were recruited for the study. The inclusion criteria were as follows: patients (or their legal guardians) able to provide written informed consent and aged 20 years at the time of consent enrollment. Exclusion criteria included pregnancy or possible pregnancy and inability to attend regular follow-up visits. The treatment regimen consisted of monthly visits primarily for scaling, root debridement, and tooth brushing instructions (TBI) without flap surgery. The following clinical parameters were assessed before treatment to evaluate the periodontal condition: probing pocket depth (PPD), bleeding on probing (BOP), plaque control record (PCR), periodontal inflamed surface area (PISA), and periodontal epithelial surface area (PESA).

Sample collection and preparation for sequencing analysis. This study included samples from both periodontally healthy and diseased sites. Teeth were isolated using sterile cotton rolls, and both saliva and gingival crevicular fluid were air-dried. Subgingival plaque samples were carefully collected using Hu-Friedy (Chicago, IL, USA) Gracie curette scalers #11/12 and 13/14. Sampling was preferentially performed on teeth exhibiting PPDs of ≥ 4 mm. In the absence of such

pockets in the molar region, samples were collected from anterior teeth with PPDs of ≥4 mm. When no sites with pockets ≥4 mm were present, the most posterior molar was selected for sampling. In cases where the most posterior molars on both sides of the mandible were of the same tooth type, the specimen was taken from the right molar. These samples were thoroughly washed with sterile saline, placed in sterile 1.5 ml tubes, and stored at −20°C until further analysis. The samples were freeze-dried using the VD-250R Freeze Dryer (TAITEC, Saitama, Japan). The frozen samples were then pulverized using the Multi Beads Shocker (Yasui Kikai, Osaka, Japan) at 1,500 rpm for 2 min. Lysis Solution F (Nippon Gene, Tokyo, Japan) was added to the pulverized samples, which were then left to stand at 65°C for 10 min. The samples were centrifuged at 12,000×g for 1 min, and the supernatant was collected. DNA was purified from the collected supernatant using the MPure-12 system and the MPure Bacterial DNA Extraction Kit (MP Biomedicals, Santa Ana, CA, USA).

The DNA concentration was measured using the Synergy LX (BioTek, Winooski, VT, USA) and the QuantiFluor dsDNA System (Promega, Madison, WI, USA). Libraries were constructed using the two-step tailed PCR method. The first PCR, targeting the V3–V4 region of the 16S rRNA gene, was conducted with the primers 1st-341f_MIX ACACTCTTTCCCTACACG ACGCTCTTCCGATCT- NNNNN- CCTACGGGNGG CWGCAG and 1st-805r_MIX GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT- NNNNN-GACTAC HVGGGTATCTAATCC (95°C for 2 min; 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s; and a final extension at 72°C for 5 min) using ExTaq HS (TaKaRa, Shiga, Japan). The purified PCR product was used as a template in the second PCR using primers 2ndF AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC and 2ndR CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG. After initial denaturation and enzyme activation at 94°C for 2 min, amplification was performed for 10 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 30s, followed by a final extension at 72°C for 5 min, using ExTaq HS. AMPure XP (Beckman Coulter, Brea, CA, USA) was added at 0.7× the PCR reaction volume for both the first and second reactions to purify the PCR products. Synergy H1 (BioTek) and the QuantiFluor dsDNA System were used to measure the DNA concentration. The MiSeq platform (Illumina, San Diego, CA, USA) was used to generate 2×300 bp paired-end reads with the MiSeq Reagent Kit v3 (Illumina). Sequence data are available at the DNA Data Bank of Japan (DDBJ) under accession number PRJDB20322.

Data analysis. The 16S rRNA gene amplicon sequencing data were processed and analyzed using QIIME2 (version 2022.2) as in our previous research [16]. The parameters were –p-trim-left-f=17, –p-trim-left-r=21, –p-trunc-len-f=260, and –p-trunc-len-r=230. After quality filtering and denoising, the number of amplicon sequence variants (ASVs) and α-diversity metrics (Chao1 and Shannon indices) were calculated using QIIME2. Rarefaction curves for each group were generated using the rarefaction. single command in mothur software (version 1.33.3) based on the number of ASVs [17]. The assignment of bacterial composition was performed with the HOMD database (version 15.22); diversity analyses, bar plots, and principal component analysis (PCA) were conducted as described previously. In addition, differentially abundant taxa (DAT) were determined using the ANCOM-BC2 method, following the previously described procedure [16]. A sensitivity analysis was performed in ANCOM-BC2, prioritizing the assessment of the robustness and reliability of the findings rather than solely emphasizing *p*-values.

The functional abundances were predicted from 16S rRNA gene amplicon sequencing data using the PICRUSt2 plugin for QIIME2 (q2-picrust2 ver. 2021.2). The functional composition of the dataset was inferred based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology database. Network analysis was conducted by calculating positive and negative correlation coefficients between bacterial taxa using the SparCC method. To prioritize high-specificity edges and enhance reliability, we applied a deliberately stringent cutoff (SparCC ≥0.93 or ≤−0.93), which is stricter than in typical microbiome network analyses [18], and retained only taxon pairs meeting this criterion. Only statistically significant pairs ($q<0.1$), as determined by the Benjamini–Hochberg method, were extracted and visualized using Cytoscape (version 3.10.2) as described previously [19].

Statistical analysis. Data normality was evaluated using the Shapiro–Wilk test. Paired *t*-tests (for parametric data) or Wilcoxon signed-rank tests (for

nonparametric data) were applied to clinical parameters and to evaluate α-diversity metrics, including the number of ASVs, Chao1, and Shannon indices. Bacterial community dissimilarities between groups were tested using PERMANOVA with the vegan::adonis2 function (https://cran.r-project.org/web/packages/vegan/index.html) and 9,999 permutations, thereby employing a permutation-based inference to enhance robustness under small-sample conditions. Differentially abundant taxa were analyzed using ANCOM-BC2, with sensitivity analyses conducted to assess the effect of pseudocount addition on zero-inflated data. Putative functional pathways were analyzed using paired *t*-tests. In this network analysis, statistical significance was defined as $p<0.05$ and $q<0.1$ using PseudoPvals in SparCC and the Benjamini–Hochberg procedure, respectively.

Results

Clinical data. The demographic and clinical parameters of the study participants are shown in Table 1. Five male participants and one female participant were enrolled in this study. Initial periodontal treatment, including oral hygiene instructions, significantly decreased BOP from 27.0 ± 23.1% to 18.9 ± 18.9% ($p=0.047$). Additionally, the percentage of sites with PPD ≥ 4 mm ($p=0.07$) and PISA ($p=0.05$) decreased after periodontal treatment. However, the percentage of sites with PPD ≥ 6 mm, PCR, and PESA did not differ significantly between pre- and posttreatment assessments (Table 1).

Sequencing reads. Sequencing reads were obtained from each sample to assess bacterial composition. The final dataset yielded an average of 57,489 ± 5,970.6 reads per sample, with a range of 44,743 to 64,660 reads.

Alpha and beta diversity analyses. Analyses of α-diversity based on the observed features, Chao1, and Shannon indices at the ASV level showed no significant differences between pre- and posttreatment samples ($p=0.16$, 0.16, and 0.07, respectively) (Fig. 1A-C). However, all α-diversity values showed an increasing trend after treatment compared with those before treatment. PCA indicated differing tendencies in bacterial composition before and after treatment within the same subjects, although the difference was not significant ($F=0.8155$, $R^2=0.0754$, $p=0.656$) (Fig. 1D).

Comparison of bacterial composition analyses.

Table 1 Characteristics of the study subjects and clinical parameters before and after non-surgical periodontal treatment

No	Age	Sex	BMI	present teeth	before treatment						after treatment					
					PPD≥4 mm (%)	PPD≥6 mm (%)	BOP (%)	PCR (%)	PISA	PESA	PPD≥4 mm (%)	PPD≥6 mm (%)	BOP (%)	PCR (%)	PISA	PESA
1	47	M	22.0	23	19.6	0	26.1	56.5	562.2	1,483.3	13	0	7.2	26.1	111.4	1,345.8
2	25	M	24.1	28	23.8	0	17.3	68.8	453.1	1,727.2	0	0	11.9	14.3	165.6	1,149.2
3	46	M	27.6	26	21.2	5.8	42.3	24	1,035.6	1,715.2	23	5.1	33.3	23.1	896.4	1,553.6
4	38	F	26.9	27	0	0	0	14.8	0	993.2	0	0	0	8.3	0	1,086.5
5	42	M	27.9	13	67.9	3.8	64.1	100	637.6	958.5	50	3.8	50	53.8	546.1	909.6
6	39	M	31.5	21	8.7	0	11.9	42.9	187.6	871	0	0	11.1	78.6	118.9	877.5
mean ± SD	39.5 ± 8.0		26.7 ± 3.3	23.0 ± 5.5	23.5 ± 23.5	1.6 ± 2.6	27.0 ± 23.1	51.2 ± 31.2	479.4 ± 362.5	1,291.4 ± 395.7	14.3 ± 19.8	1.5 ± 2.3	18.9 ± 18.9*	34.0 ± 26.9	306.4 ± 344.2	1,153.7 ± 259.7

PPD, probing pocket depth; BOP, bleeding on probing; PCR, plaque control record; PISA, periodontal inflamed surface area; PESA, periodontal epithelial surface area.
*: $p<0.05$ $p=0.07$ $p=0.047$ $p=0.05$

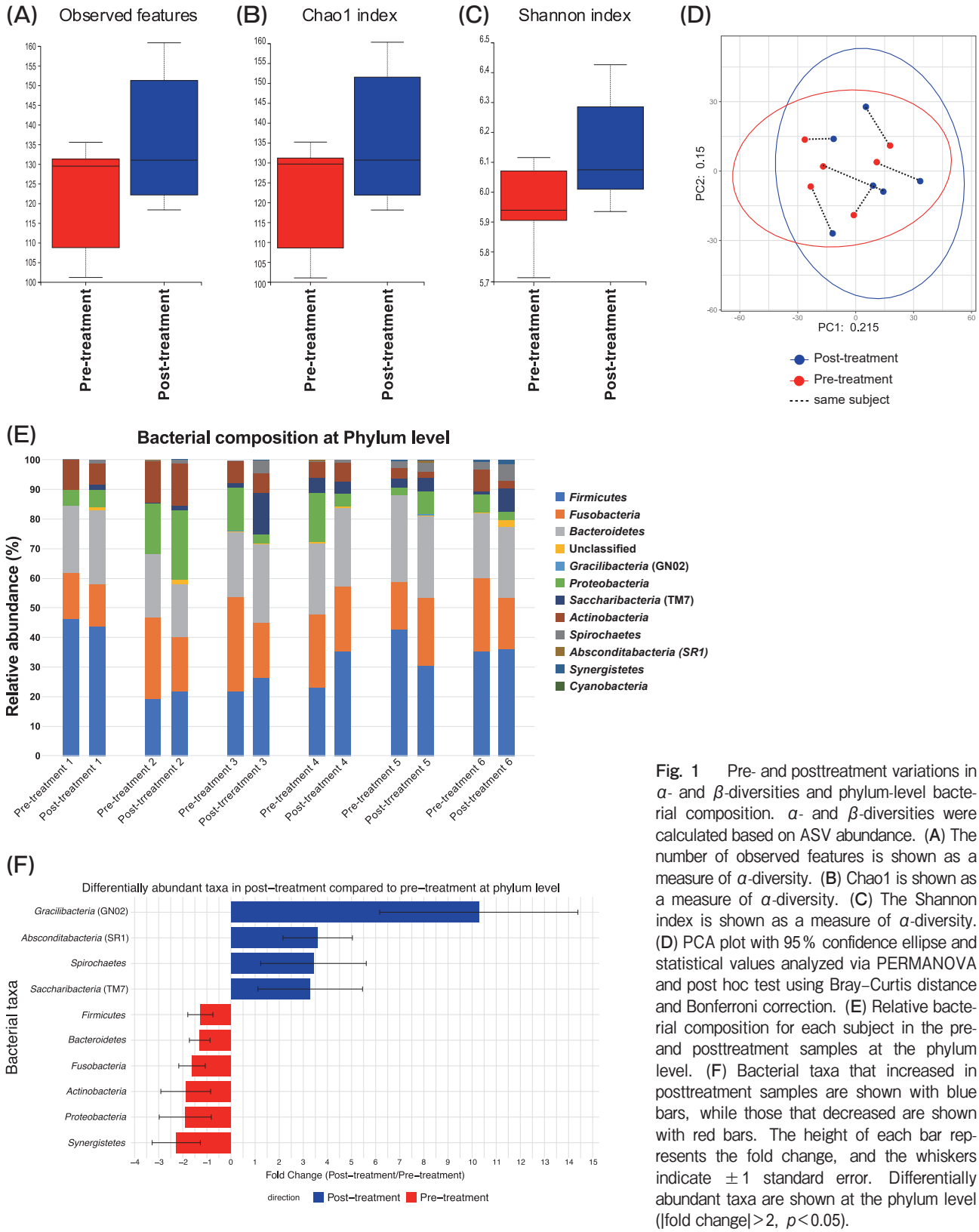

Fig. 1 Pre- and posttreatment variations in α- and β-diversities and phylum-level bacterial composition. α- and β-diversities were calculated based on ASV abundance. (**A**) The number of observed features is shown as a measure of α-diversity. (**B**) Chao1 is shown as a measure of α-diversity. (**C**) The Shannon index is shown as a measure of α-diversity. (**D**) PCA plot with 95% confidence ellipse and statistical values analyzed via PERMANOVA and post hoc test using Bray–Curtis distance and Bonferroni correction. (**E**) Relative bacterial composition for each subject in the pre- and posttreatment samples at the phylum level. (**F**) Bacterial taxa that increased in posttreatment samples are shown with blue bars, while those that decreased are shown with red bars. The height of each bar represents the fold change, and the whiskers indicate ± 1 standard error. Differentially abundant taxa are shown at the phylum level (|fold change| > 2, $p < 0.05$).

At the phylum level, analysis of the bacterial composition before and after treatment revealed that the three most abundant phyla were consistently *Firmicutes*, *Bacteroidetes*, and *Fusobacteria*. *Fusobacteria* abundance decreased after treatment compared with pretreatment levels (from an average of 23.4% to 19.1%). In contrast, *Saccharibacteria* (TM7) abundance increased after treatment compared with pretreatment (from an average of 1.92% to 5.70%). Additionally, *Gracilibacteria* (GN02) abundance increased after treatment compared with pretreatment, although the overall abundance remained low (from an average of 0.011% to 0.138%; $p < 0.05$; |fold change| = 10.3) (Fig. 1E and F).

In comparisons of pre- and posttreatment abundances at the class, order, and family levels with *p*-values < 0.05, the abundances of *Spirochaetales* at the order and family levels and of *Spirochaetaceae* and *Saccharibacteria* (TM7) [F-1] at the family level decreased (|fold change| = 5.42, 5.50, and 5.00, respectively), while that of *Fusobacteria* at the class level also decreased (|fold change| = 2.12) after treatment (Fig. 2A, B, and C).

At the genus level, in descending order of abundance, the top five genera before treatment were *Leptotrichia*, *Fusobacterium*, *Streptococcus*, *Prevotella*, and *Porphyromonas* (mean abundances of 11.9%, 11.4%, 10.8%, 10.1%, and 5.21%, respectively) (Fig. 3A), whereas the top five genera after treatment were *Prevotella*, *Fusobacterium*, *Streptococcus*, *Leptotrichia*, and *Neisseria* (mean abundances of 13.7%, 10.6%, 9.41%, 8.44%, and 4.46%, respectively) (Fig. 3B). The abundances of the bacterial genera *Treponema*, *Peptostreptococcaceae* [XI] [G-7], *Catonella*, and *Veillonellaceae* [G-1] increased after treatment ($p < 0.05$, |fold change| = 5.20, 4.22, 3.38, and 3.06, respectively) (Fig. 3C).

At the species level, in descending order of abundance, the top five bacterial species before treatment were *Streptococcus* sp., *Fusobacterium* sp., *Fusobacterium nucleatum* subsp. *vincentii*, *Leptotrichia buccalis*, and *Prevotella* sp. HMT 317 (mean abundances of 8.86%, 7.97%, 2.93%, 2.69%, and 2.56%, respectively) (Fig. 4A), whereas the top five bacterial species after treatment were *Streptococcus* sp., *Fusobacterium* sp., *Neisseria* sp., *Fusobacterium nucleatum* subsp. *vincentii*, and *Prevotella intermedia* (mean abundances of 7.77%, 6.20%, 3.25%, 3.13%, and 3.03%, respectively) (Fig. 4B). Increases in the abundances of the bacterial species *Actinomyces israelii*, *Saccharibacteria* (TM7) [G-1] sp. HMT 952, *Prevotella* sp. HMT 300, *Catonella morbi*, and *Peptostreptococcaceae* [XI] [G-7] [Eubacterium] *yurii* subsp. *yurii* and *margaretiae* were observed after treatment compared with pretreatment ($p < 0.05$, |fold change| = 5.42, 5.26, 4.44, 4.06, and 3.67, respectively) (Fig. 4C). *Peptostreptococcaceae* [XI] [G-7] [Eubacterium] *yurii* subsp. *yurii* and *margaretiae* are not shown in Fig. 4B because they were not among the top 15 most abundant species. In contrast, the abundances of the bacterial species *Capnocytophaga haemolytica*, *Arachnia propionica*, *Tannerella* sp. HMT 286, and *Kingella oralis* were significantly decreased after treatment compared with pretreatment ($p < 0.05$, |fold change| = 11.3, 6.05, 3.85, and 2.69, respectively) (Fig. 4C).

Metagenome prediction of the oral microbiome. PICRUSt2 analysis was performed to estimate the abundance of gene functions within the microbiome. In total, 10,315 and 10,311 KO numbers were predicted from pretreatment samples and posttreatment samples, respectively. There were eight enriched KO numbers in posttreatment samples ($p < 0.01$; |fold change| > 2). These enriched pathways were associated with microbial metabolic pathways involved in the degradation of toluene, benzoate, and aminobenzoate (Fig. 5).

Network analysis. From pretreatment to posttreatment, increases were observed in the number of nodes (from 79 to 81), number of edges (from 69 to 74), clustering coefficient (from 0.22 to 0.263), network density (from 0.267 to 0.286), network heterogeneity (from 0.464 to 0.612), and network centralization (from 0.351 to 0.572). The number of subnetworks with three or more nodes showed little variation, with 14 and 13 in pre- and posttreatment groups, respectively (Fig. 6A and B). In the pretreatment network, the red-complex bacteria *Treponema denticola* and *Tannerella forsythia*, which are highly pathogenic and strongly associated with severe periodontal disease, were included as the bacterial taxa forming the network. *Treponema denticola* formed edges with *Mogibacterium timidum*, *Peptostreptococcaceae* [XI] [G-6] [Eubacterium] *nodatum*, and *Leptotrichia shahii*, whereas *Tannerella forsythia* formed edges with *Peptostreptococcaceae* [XI] [G-4] sp. HMT 369; both bacteria established their respective clusters within the network. However, these taxa were absent in the posttreatment network.

In pre- and posttreatment networks, the maximum number of edges connected to a single node was five. In the pretreatment network, this maximum connectivity

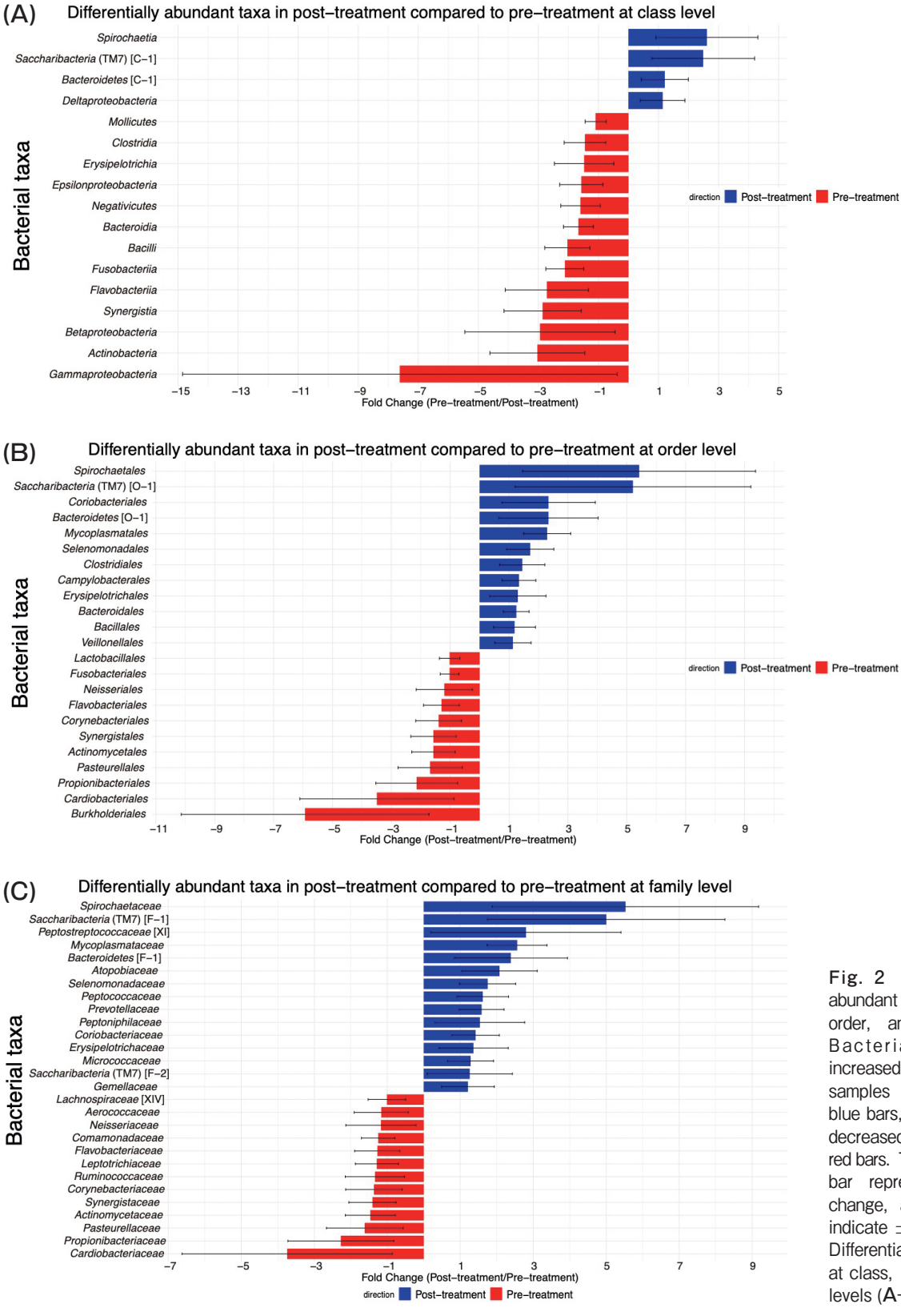

Fig. 2 Differentially abundant taxa at class, order, and family levels. Bacterial taxa that increased in posttreatment samples are shown with blue bars, while those that decreased are shown with red bars. The height of each bar represents the fold change, and the whiskers indicate ±1 standard error. Differentially abundant taxa at class, order, and family levels (**A**–**C**).

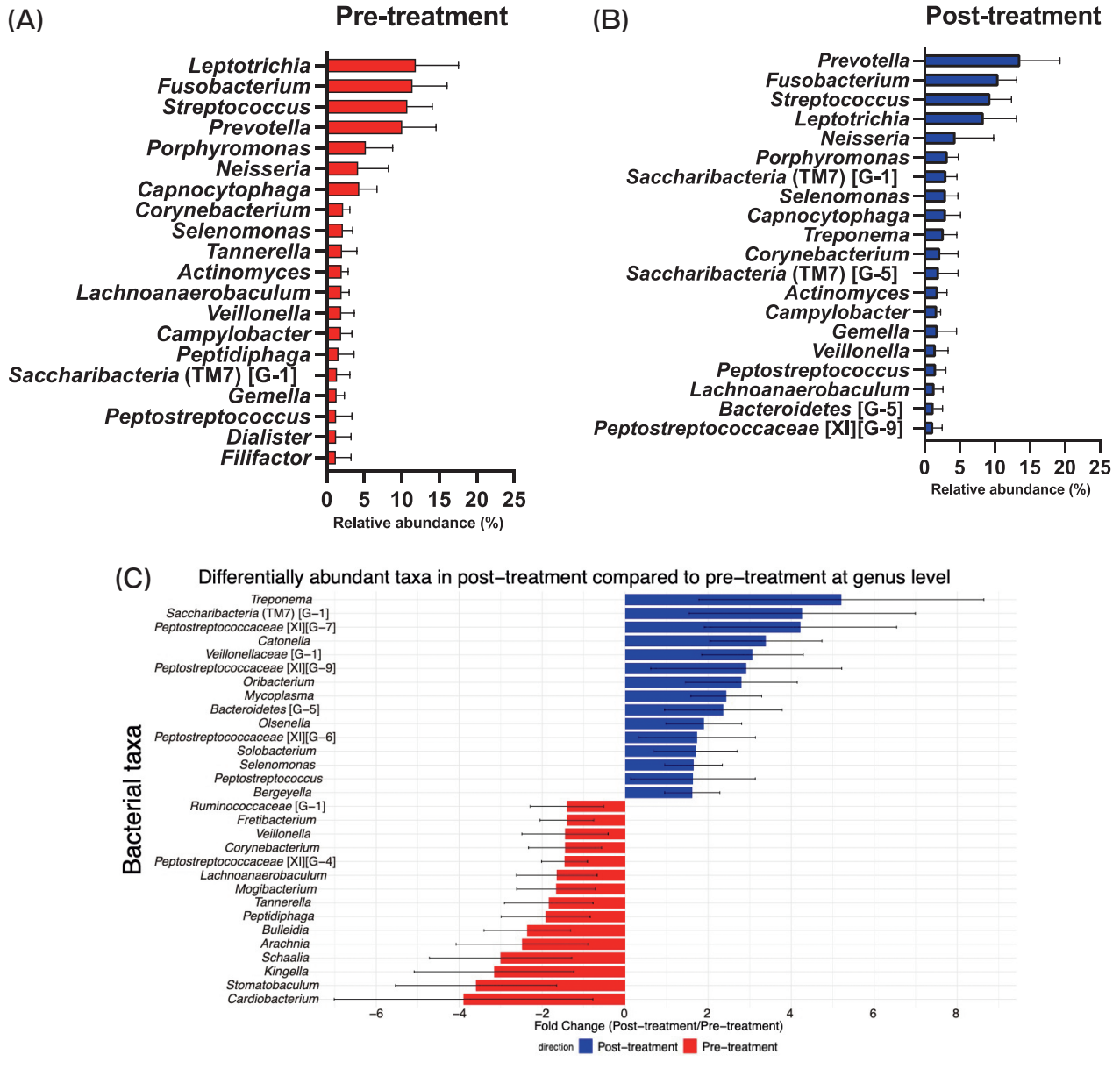

Fig. 3 Bacterial composition and differentially abundant taxa at the genus level. Relative bacterial composition of pretreatment samples at the genus level. (B) Relative bacterial composition of posttreatment samples at the genus level. (C) Differentially abundant taxa at the genus level, as described in Fig. 1.

was observed for two bacterial taxa: *Mogibacterium timidum* and *Capnocytophaga sputigena*. In the post-treatment network, this maximum connectivity was observed for four bacterial taxa: *Filifactor alocis*, *Capnocytophaga* sp., *Streptococcus constellatus*, and *Prevotella* sp. HMT 300.

Discussion

Patients with DS are more susceptible to progressive periodontitis compared to healthy individuals, intellectually disabled individuals without DS, or those with cerebral palsy [11, 20]. A meta-analysis of patients with DS and periodontitis highlighted the difficulty in reducing PPD in this population compared with healthy con-

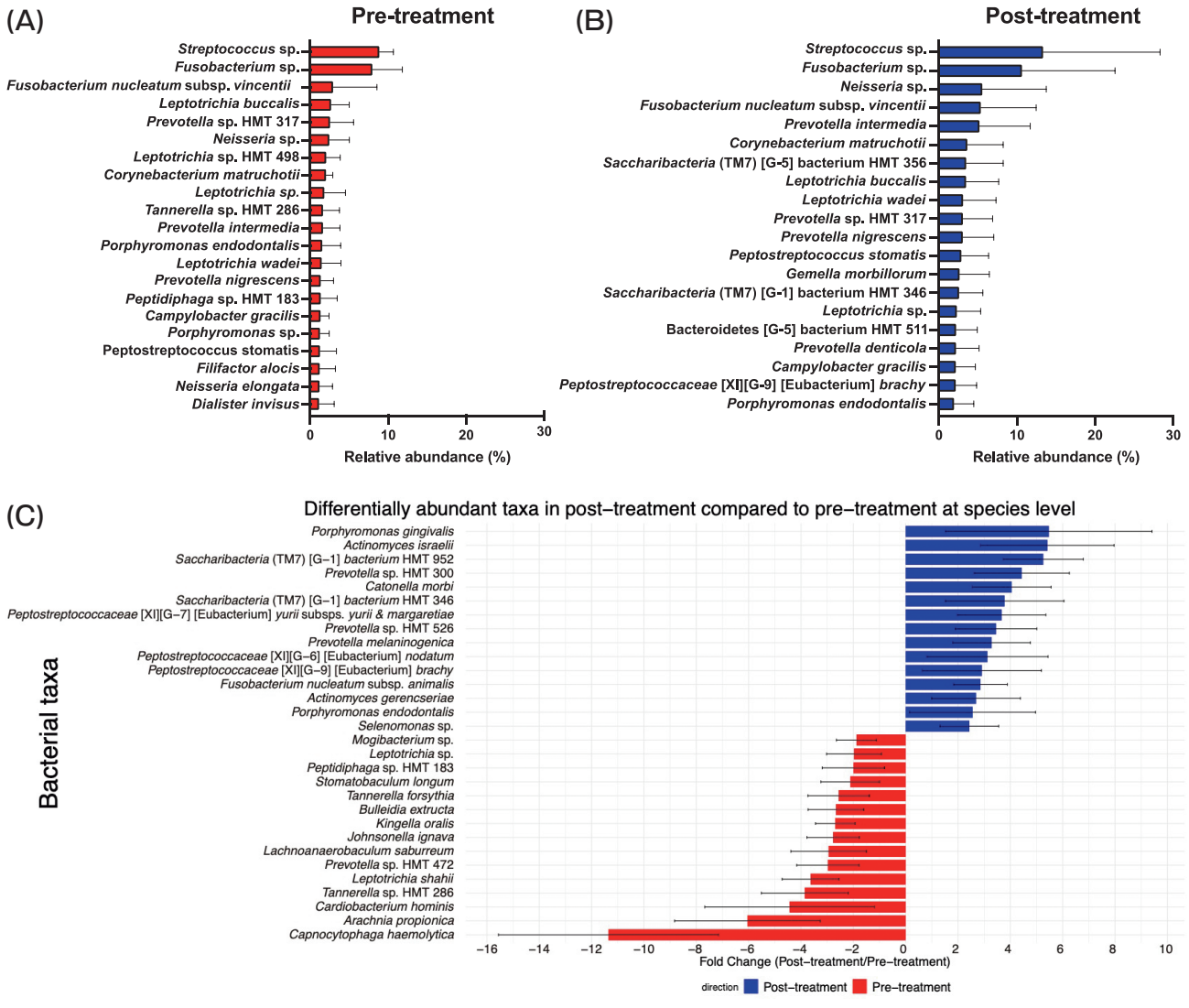

Fig. 4 Bacterial composition and differentially abundant taxa at the species level. Relative bacterial composition of pretreatment samples at the species level. (**B**) Relative bacterial composition of posttreatment samples at the species level. (**C**) Differentially abundant taxa at the species level, as described in Fig. 1.

trols [12]. Despite the small sample size in the present study, significant reductions in BOP and trends toward improvement in PPD and PISA were observed in untreated patients presenting for their initial dental visit. These findings suggest that facilitating access to dental care in patients with untreated DS is a critical strategy for managing periodontitis in this population. These interventions demonstrated potential efficacy even in previously untreated patients with DS. However, the improvement in PPD was not significant, indicating that the effects of TBI on self-care are limited. Furthermore, deep PPD (≥6 mm) showed minimal improvement after 6 months, consistent with previous findings [21]. Although BOP showed significant improvement, the limited improvements in PPD and PISA underscore the challenges of treating periodontitis in this group.

A slight increasing trend in α diversity was observed from pre- to posttreatment. This finding contrasts with previous findings describing decreased diversity and simplified microbial communities after treatment in non-DS periodontitis patients [22]. In the present study, major periodontal pathogens such as

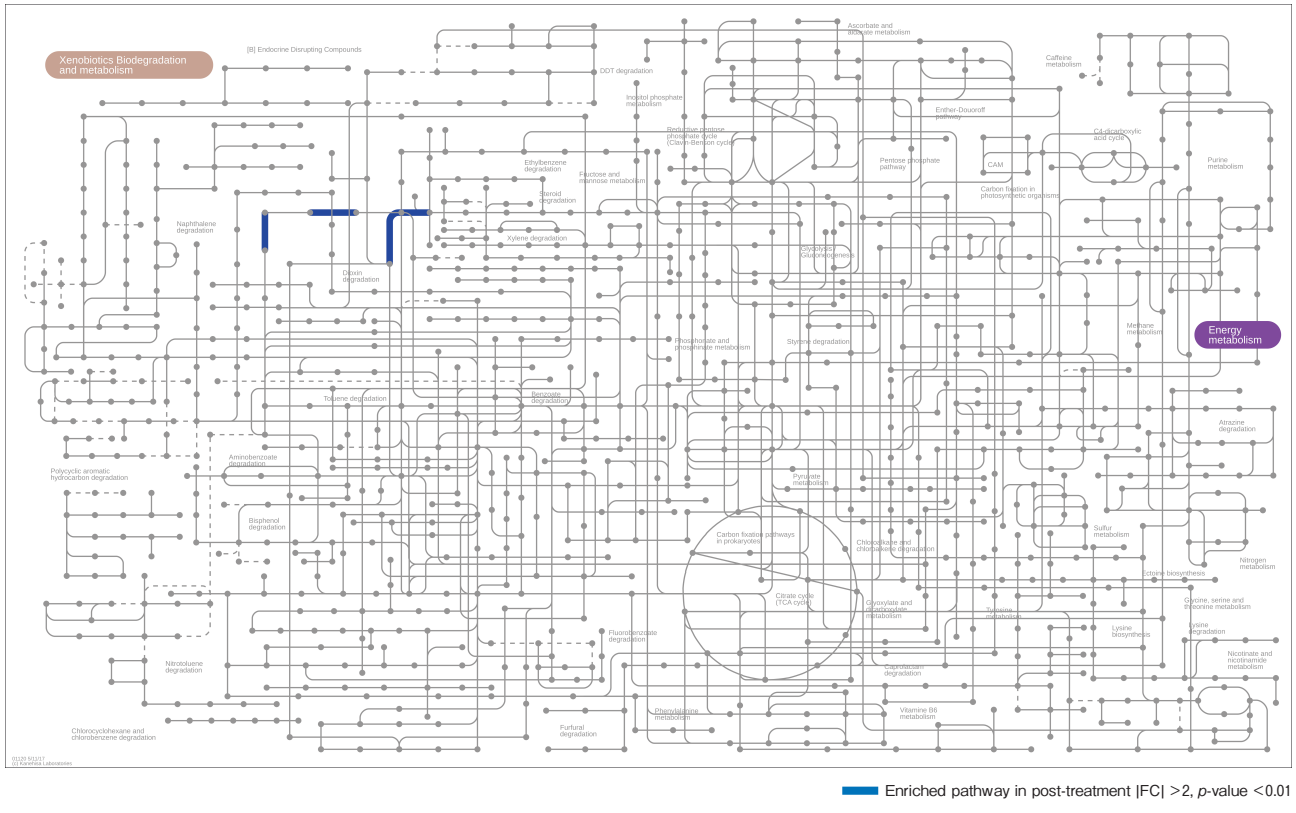

Fig. 5 Differentially enriched pathways in posttreatment samples compared with pretreatment samples. Enriched pathways in posttreatment samples with |FC| >2 and $p<0.01$ are shown with blue lines.

Porphyromonas and *Fusobacterium* remained dominant even after treatment, suggesting that the healing trajectory of the microbial community in DS may differ from that observed in general periodontitis. Although no significant difference in β-diversity was detected, individual shifts in microbial profiles were evident. Regarding microbial composition, decreases in *Fusobacteria* and *Tannerella forsythia* suggest a partial therapeutic effect. Conversely, unexpected increases in *Treponema* and *Peptostreptococcaceae* [XI] [G-7] in posttreatment samples may reflect residual deep pockets or difficulty in plaque control, particularly in mandibular lingual molars. Additionally, the observed increase in *Gracilibacteria* (GN02), which has been associated with caries [23], may reflect a microbial shift toward a more cariogenic flora during the course of periodontal therapy [24]. While these changes provide insight into the complexity of microbial responses, they also highlight the need for careful interpretation, especially given the small sample size. These microbial changes may partly explain the clinical findings: reductions in *Fusobacteria* and *T. forsythia* are consistent with the observed improvement in BOP, whereas the persistence of major pathogens such as *Porphyromonas* and *Fusobacterium* may account for the limited resolution of deep PPD. This divergence between BOP and PPD outcomes suggests that different bacterial taxa may be associated with distinct clinical parameters. Such interpretations remain tentative given the exploratory nature of the study and the limited statistical power resulting from its small sample size.

Network analysis revealed distinct differences in bacterial interactions before and after treatment. *Capnocytophaga sputigena* and *Mogibacterium timidum* played central roles in the pretreatment network, showing multiple positive correlations with other periodontopathogens. Additionally, *M. timidum* demonstrated positive correlations with *Treponema denticola*, a periodontal pathogen classified within the red complex. Furthermore, *Tannerella forsythia*, a member of the red

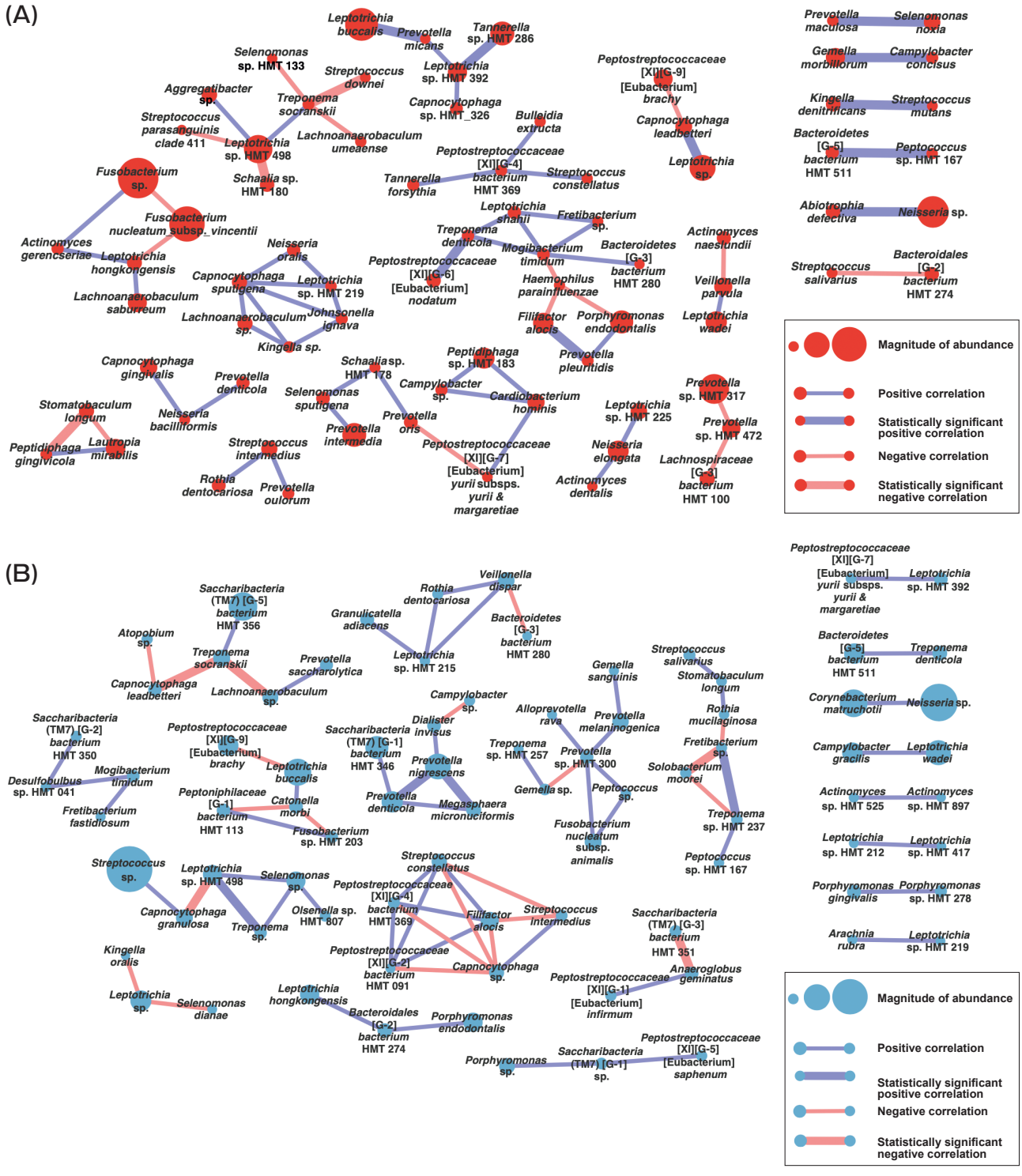

Fig. 6 Bacterial network in the microbiome at pre-and posttreatment. All networks are shown with each species represented by a node, and positive and negative relationships are indicated by connecting edges. Interactions with statistically significant co-occurrence networks are indicated by bold lines ($p < 0.05$; $q < 0.1$). Positive and negative correlations are shown in blue and red, respectively. The size of each node represents the magnitude of its abundance. (A) Bacterial network at pretreatment. (B) Bacterial network at posttreatment.

complex, was present in the pretreatment network and showed a positive correlation with *Peptostreptococcaceae* [XI][G-4] sp. HMT 369; however, both of these bacteria disappeared from the network after treatment. In addition, new central nodes such as *Filifactor alocis* and *Prevotella* sp. HMT 300 emerged. This shift suggests that nonsurgical therapy not only reduces pathogenic taxa but also restructures the microbial network, possibly enhancing resilience and reducing the risk of recurrence [25]. Indeed, previous work has shown that healthy or stable ecosystems are characterized by higher edge density and balanced positive–negative interactions, which confer robustness to perturbations, including disturbances caused by invading bacteria [26]. The posttreatment increases in network complexity observed here may therefore indicate a shift toward a more stable state.

Functional prediction using PICRUSt2 demonstrated increased abundance of metabolic pathways associated with the degradation of aromatic compounds, such as benzoate and toluene, in the posttreatment samples [27, 28]. These pathways may support an anti-inflammatory environment in periodontitis by metabolizing substrates that favor the growth of spirochetes [29, 30]. However, the biological impact of such metabolites can be context- and dose-dependent, with some studies reporting both pro- and anti-inflammatory effects [31]. Therefore, our findings should be interpreted as reflecting a functional tendency linked to microbial changes after treatment. The concurrent increase in network complexity and heterogeneity after treatment suggests that the microbial ecosystem may have become more stable and functionally resilient against perturbations. Nevertheless, whether such network complexity can serve as a reliable long-term marker of treatment success remains uncertain and requires validation in longitudinal studies.

This study has several limitations. First, although we used 16S rRNA gene amplicon sequencing, more detailed information can be obtained through metagenomic or metatranscriptomic analyses. These methods would enable more reliable species-level phylogenetic analyses and provide insights into the presence of pathogenic genes and their associated metabolic pathways [18, 25]. Second, the limited sample size may have affected its statistical power. Future studies with larger sample sizes are needed to confirm these results and provide more robust conclusions. Third, only nonsurgical periodontal therapy was administered; incorporating surgical treatment could have achieved better therapeutic outcomes, particularly in addressing persistent deep pockets and severe inflammation.

In conclusion, the presence of *Fusobacterium* and the positive correlation between *T. denticola* and *M. timidum* within networks involving other periodontal pathogens prior to treatment may contribute to the progression of periodontitis in individuals with DS. Nonsurgical periodontal therapy produced moderate clinical improvements and significant microbial alterations in patients with DS, underscoring the important role of primary care providers, including general dentists, in managing periodontal health in this population.

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