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Original Article

Potential short-term shift in oral microbiota of patients with stage III-IV periodontitis and type 2 diabetes treated by non-surgical periodontal therapy

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Microbiota;
Non-surgical periodontal treatment

Abstract *Background/purpose:* Adjunctive use of systemic antibiotics has more clinical improvement than scaling and root planing alone in stage III-IV periodontitis patients with type 2 diabetes mellitus (T2DM). There is still no study that concentrate on dynamic changes in oral microbiota by high throughput sequencing technique with the treatment regimen including adjunctive antibiotics.

Materials and methods: Thirty-two periodontitis patients with T2DM who received non-surgical periodontal treatment (NSPT) in the previously published randomized trial were selected for microbiological analysis. Seventeen subjects in the test group received scaling and root planing (SRP) and antibiotics (500 mg of amoxicillin [AMX], and 200 mg of metronidazole [MTZ], three times daily for seven days). Fifteen subjects in the control group received SRP only. Examination of periodontal and hematological parameters, cytokines in serum and gingival crevicular fluid, and collection of subgingival plaque was taken at baseline and three months after treatment. The V3–V4 region of 16S DNA was sequenced, and taxonomic assignment was based on the Human Oral Microbiome database.

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Results: Both the test and the control group showed lower richness and diversity for subgingival microbiota after treatment. The distribution of subgingival microbial composition was different between the baseline and 3 months in both groups. The subgingival microbial dysbiosis index decreased significantly in both groups at 3 months, and 8 out of 12 dysbiotic discriminatory genera decreased significantly in the test group. The relative abundance of the red complex, *Porphyromonas gingivalis* and *T. forsythus* decreased more in the test group than that in the control group. The decrease of clinical periodontal parameters was positively correlated with the decline of *Treponema*, *Porphyromonas*, *Capnocytophaga* and *PeptostreptococcaceaeXIG-6*, and negatively correlated with the increase of *Neisseria* and *Pseudomonas*. HbA1c level decrease was positively related to the changes of *Leptotrichia*, *Veillonella*, *Saccharibacteria TM7 G-5* and *Actinomyces*.

Conclusion: NSPT could significantly change the oral microbiome towards healthy status in patients with stage III-IV periodontitis and diabetes. AMX + MTZ had more advantages in the decrease of periodontal pathogens. Periodontists should cautiously use the antibiotics to treat such patients.

Introduction

Periodontitis is characterized by a destruction of the tooth-supporting apparatus with disordered microorganisms. The two-way relationship between periodontitis and type 2 diabetes mellitus (T2DM) has been deeply studied.[1—3] Patients with diabetes have a roughly threefold increased risk of developing periodontitis, and their systemic and local periodontal inflammation is more severe than that of those without diabetes.[4] In contrast to previous techniques that culture the specific microorganisms or detect DNA at the molecular level, high-throughput sequencing can provide a picture of the composition and function of the entire microbiome. With the new technique, studies showed that T2DM could decrease the richness and diversity of oral microbiota,[5—7] which would further decrease in patients with inadequate glycemic control (hemoglobin A1c $\geq$ 8 %).[8] At the genus level, *Saccharibacteria (TM7), Aggregatibacter, Neisseria, Gemella, Eikenella, Fusobacterium and Actinobacteria* were more abundant in patients with T2DM.[6,9] While alterations in the oral microbiota composition are more complicated by glycemic status and different stages of periodontal disease, there is still not much consensus about the specific bacteria taxa or bacteria groups under such context. Individuals with periodontitis showed a less prominent microbiome shift in T2DM than in nondiabetic subjects after initial periodontal therapy.[10] The long-term maintenance of periodontal health depends on the healthy and stable state of subgingival microbiome. Therefore, considering these deleterious effects T2DM has on periodontal condition, dentists need to be aware of the connection between the two conditions, and there is an additional challenge to make clinical decisions in periodontal treatment and prevention of more intensive infections in T2DM periodontitis patients.

Previous studies showed more clinical improvement with adjunctive use of systemic antibiotics than with scaling and root planing (SRP) alone. For chronic periodontitis patients, adjunctive use of systemic antibiotics could reduce pocket depth by an extra 0.62 mm $\pm$ 0.17 mm when PD was more than 6 mm, and extra 0.27 mm $\pm$ 0.09 mm in pockets with 4 to 6 mm depth.[11] As to patients with periodontitis and T2DM, PD decreased by only an additional 0.19 mm when treated with SRP and systemic antibiotics.[12] We recently reported that adjunctive use of amoxicillin and metronidazole (AMX + MTZ) would improve periodontal parameters in patients with generalized severe periodontitis and T2DM.[13] As the initiating cause of the periodontitis, the microbiome is more directly affected by periodontal treatment, whether mechanical or antibiotic. Lu et al. reported that chronic periodontitis patients treated with adjunctive use of AMX + MTZ had significantly lower microbial richness, lower microbial diversity, and less abundant *Porphyromonas* than patients treated by SRP-only at 3 months for both subgingival microbiomes.[14] Considering the different clinical responses between patients with or without diabetes, we suppose the microbiome changes in T2DM patients treated by adjunctive antibiotics would also differ from those of general healthy ones. One randomized controlled study examined the presence of 7 periodontal pathogens in subgingival biofilm by qPCR technique.[15] The results showed that the antibiotic-treated patients with T2DM and periodontitis presented reduced levels and greater decreases of the three red complex species, *Eubacterium nodatum* and *Prevotella intermedia*, compared to the SRP-only group at 1 year.[15] However, there are still no studies that concentrate on the complete picture and the dynamic changes in oral microbiota by high-throughput sequencing techniques with the treatment regimen including adjunctive antibiotics for T2DM patients with periodontitis. Therefore, the aim of this study is to explore whether the adjunctive use of AMX + MTZ can benefit more to the short-term changes in oral microbiota in stage III-IV periodontitis patients with T2DM by next-generation 16S rDNA gene sequencing.

Materials and methods

Study design

This was a second analysis of a single-center, examiner-blind, randomized clinical study with two parallel arms. The principles in the Declaration of Helsinki and Consolidated Standards of Reporting Trials (CONSORT) guidelines were adhered to throughout the trial. The study was registered at the Chinese Clinical Trial Registry (approval no. ChiCTR-TRC-1900027377) and the protocol was approved by the Peking University School of Stomatology Institution Review Board (approval no. PKUSSIRB-201627026).

Inclusion and exclusion criteria

Inclusion and exclusion criteria were as described previously.[13] Briefly, patients diagnosed as type 2 diabetes (based on the WHO 1999 criteria and had stable medication regimens for at least 6 months before and after recruitment) and generalized severe chronic periodontitis (based on 1999 classification),[16] which were classified as stage III-IV periodontitis (based on 2018 classification) were enrolled.[17] And patients with other infectious diseases, severe diabetic complications, pregnancy, lactation, allergic to amoxicillin or metronidazole, previous periodontal treatment or antibiotic use within 3 months, smoking or alcohol abuse were excluded.

Experimental design and group allocation

Thirty-two stage III-IV periodontitis patients with type 2 diabetes were enrolled and allocated to the test group (SRP with administration of antibiotics) and the control group (SRP only). All subjects underwent examination and sample collection before periodontal treatment. Two half-mouth subgingival SRP procedures were performed under local anesthesia using a subgingival ultrasonic scaler and Gracey curettes. Patients in the test group received 500 mg of amoxicillin and 200 mg of metronidazole three times daily for 1 week at the beginning of subgingival SRP. During the experiment, any adverse events related to medication was assessed and recorded.

Clinical examinations and hematological tests

Periodontal clinical examinations were performed before (baseline) and 3 months after SRP. Clinical parameters were recorded, including the probing depth (PD), attachment loss (AL), bleeding index (BI), and plaque index (PI).

Fasting venous blood was collected before and 3 months after SRP using anticoagulant and coagulation-promoting tubes. Serum was separated by centrifugation at 3500 rpm for 15 min after setting for 30 min at 4 °C. HbA1c levels were measured using an automatic biochemical analyzer (TOSOH, Yamaguchi, Japan). Glucose levels were measured using a chemical analyzer (Olympus, Brentwood, NH). Complete blood count analyses (e.g., white blood cell [WBC] counts) were performed using a calibrated Sysmex XS-1000 automated hematology analyzer (Sysmex, Kobe, Japan). Systemic immune inflammation (SII) index was calculated as platelet count * neutrophil count/lymphocyte count, and the neutrophil-to-lymphocyte ratio (NLR) was calculated as the total neutrophil count/lymphocyte count.

Sample collection

Gingival crevicular fluid (GCF) sample and subgingival plaque samples were pooled of six non-adjacent bucco-mesial sites from 4 quadrants and baseline PD $\geq$ 5 mm. A sterilized 3 MM CHR filter paper strip (Whatman, Maidstone, England) was gently placed into the pocket until resistance for 30s to collect GCF sample. After that subgingival plaque sample was collected at the same sites with sterilized curets. All samples were stored in Eppendorf tubes at −80 °C until tested.

Cytokines examination

Both serum and GCF samples were setting at room temperature before tested. GCF in filter paper strip was eluted by centrifugation at 13000 rpm for 10 min with 100 ml PBS after 40 min vibration at 20 °C. Interleukin (IL) −17, receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) and osteoprotegerin (OPG) levels in serum and GCF were assayed using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the instructions of the manufacturer. And OPG/RANKL ratio in serum and GCF was calculated.

DNA extraction

Soil genomic DNA was extracted using E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) following the manual. Concentration and quality of the genomic DNA were checked by NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). DNA samples were stored at - 20 °C for subsequent experiments.

High-throughput sequencing and bioinformatic analysis

The V3-4 hypervariable region of bacterial 16S rDNA gene were amplified with the universal primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′- GGACTACNNGGGTATCTAAT-3′). For each sample, 8-digit barcode sequence was added to the 5′ end of the forward and reverse primers (Allwegene Company, Beijing, China). The PCR was carried out on a Mastercycler Gradient (Eppendorf, Hamburg, Germany) using 25 μL reaction volumes, containing 12.5 μL 2 × Taq PCR MasterMix (Vazyme Biotech Co.,Ltd, Nanjing, China), 3 μL BSA(2 ng/μl), 1 μL Forward Primer(5 μM)、1 μL Reverse Primer(5 μM), 2 μL template DNA, and 5.5 μL ddH2O. Cycling parameters were 95 °C for 5 min, followed by 28 cycles of 95 °C for 45 s, 55 °C for 50 s and 72 °C for 45 s with a final extension at 72 °C for 10 min. The PCR products were purified using a Agencourt AMPure XP Kit (Beckman Coulter, Inc., Pasadena, CA, USA). Sequencing libraries were generated using NEB Next Ultra II DNA Library Prep Kit (New England Biolabs, Inc., Ipswich, MA, USA) following the

manufacturer's recommendations. The library quality was assessed by Nanodrop 2000 (Thermo Scientific Inc.), Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Pasadena, CA, USA), and ABI StepOnePlus Real Time PCR System (Thermo Scientific Inc.), successively.

Deep sequencing was performed on Illumina Miseq/Novaseq (Illumina, Inc., San Diego, CA, USA) platform at Beijing Allwegene Technology Co., Ltd.. After the run, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline Version 2.6 (Illumina, Inc.).

Statistics analysis

The BLAST tool was used to classify all operational taxonomic units (OTUs) representative sequences into different taxonomic groups against Human Oral Microbiome Database (HOMD), and e-value threshold was set to 10^{-5}. QIIME (v1.8.0) was used to generate rarefaction curves and to calculate the richness and diversity indices based on the OTU information, and use R (v3.6.0) software to plot. Based on the results of taxonomic annotation and relative abundance, R (v3.6.0) software was used for bar–plot diagram analysis. To describing the dissimilarity between multiple samples, PCA was analyzed by R (v3.6.0) based on the OTU information from each sample. The β-Diversity distance matrix between samples were calculated using the Bray Curtis algorithms and plotted principal coordinates analysis (PCoA).

Subgingival microbial dysbiosis index (SMDI) was calculated at the genus level.[18] Nineteen discriminating genus-level taxa from this publication were used to determine the SMDI (Table S1). For this analysis, the OTUs were agglomerated and annotated at the genus level, and the genus reads were transformed to centred-log ratios using the microbiome package in R (version 1.14.0; http://microbiome.github.com/microbiome).

Inter-groups analysis of periodontal parameters and microbiota was conducted using Student t-test or Mann–Whitney U test, and intra-group analyses were conducted by paired t-test or Wilcoxon signed-rank test. The correlation between the changes of top 20 bacterial genera in subgingival microbiota, clinical parameters, hematological parameters and cytokines was tested using Spearman correlation coefficients and presented as heatmaps.

Results

Demographic and clinical analysis

Demographic and clinical characteristics of the subjects included in the study are showed in Tables 1 and 2. General information at baseline of test and control group was comparable. Significant differences ($P < 0.05$) after treatment were found for all clinical parameters in both groups. WBC, SII and NLR decreased after treatment but without statistical significance. IL-17 levels in serum and GCF were significantly decreased and the ratio of OPG/RANKL in serum were increased in the test group ($P < 0.05$). The control group showed significant changes of serum IL-17 and OPG/RANKL ratio ($P < 0.05$). The increase of serum OPG/RANKL ratio was significantly higher in the test group than that in the control group (−21.32 [−44.65, −7.61] vs −2.37 [−32.67, 18.78], $P = 0.045$).

Table 1 General information.

		Test (n = 17)	Control (n = 15)
Age (Years)	(Mean ± SD)	55.82 ± 2.72	54.8 ± 2.19
	(Range)	32–75	39–72
Gender	Male	9 (52.9 %)	7 (46.7 %)
	Female	8 (47.1 %)	8 (53.3 %)
BMI	kg/m^2	24.82 ± 0.5	25.07 ± 0.71
Baseline HbA1c	>7.5 %	7 (41.2 %)	8 (53.3 %)
	≤7.5 %	10 (58.8 %)	7 (46.7 %)

Abbreviations: SD, standard deviation; BMI, body-mass index; HbA1c, hemoglobin A1c.

Diversity and structure of bacterial communities

The analysis of the bacterial richness and diversity of subgingival bacterial in the test and control group at baseline and 3month showed significant differences according to the chao1, observed species and PD whole tree indices (Fig. 1). Subgingival microbiota in the test group showed a significant lower richness and diversity at 3month compared to baseline.

The distribution of microbial composition was assessed by PCoA plots of Bray–Curtis distance as showed in Figure S1. For subgingival microbiota, both test and control group showed significant differences between baseline and 3month (test group: $P = 0.001$, $R^2 = 0.13$; control group: $P = 0.001$, $R^2 = 0.09$). No significant difference was showed between the test and the control group neither at baseline nor at 3month ($P > 0.05$).

The top 20 genera in each group with more relative abundance were showed in Fig. 2. These 20 genera, including *Fusobacterium*, *Streptococcus*, *Prevotella*, *Porphyromonas*, *Capnocytophaga*, *Neisseria* and others, accounted for more than 75 % of the sequences detected in all samples.

The SMDI decreased significanlty in both groups at 3month (Fig. 3). In the test group, the SMDI was below the control group level but without statistical difference. The relative abundance of the 19 discriminating genus-level taxa determing the SMDI was compared between groups (Fig. 4). For dysbiotic discriminatory genera, 8 out of 12 genera showed significantly decrease in the test group, including *Desulfobulbus, Filifactor, Fretibacterium, Mogibacterium, Peptostreptococcaceae [XI][G-4], Peptostreptococcaceae [XI][G-6], Tannerella and Treponema,* and only 5 genera decreased significantly in the control group. While 3 out of 7 normobiotic discriminatory genera increased significantly in the control group, including *Actinomyces, Rothia, Granulicatella*, which showed the increase tendency in the test group. The relative abundance of red complex and its composition, including *Porphyromonas gingivalis, T. denticola, T. forsythus*, showed significant decrease after treatment in both groups, except for *T. denticola* in the control group (Fig. 4). Besides, the

Table 2 Clinical, hematological and inflammatory indicators.

		Test (n = 17)		Control (n = 15)	
		T1	T2	C1	C2
HbA1c	%	7.65 ± 0.28	7.11 ± 0.22*	7.48 ± 0.18	7.11 ± 0.17*
Glu	mmol/L	8.71 ± 0.40	8.00 ± 0.45*	8.24 ± 0.34	7.38 ± 0.29*
WBC	*10^9/L	7.07 ± 1.48	6.52 ± 1.62	7.33 ± 1.46	6.39 ± 1.40
SII		610.36 ± 410.48	439.82 ± 192.23	544.42 ± 334.40	381.75 ± 214.21
NLR		2.50 ± 1.12	1.99 ± 0.50	2.02 ± 1.12	1.52 ± 0.56
Serum IL-17	pg/ml	74.97 ± 42.34	53.25 ± 37.62*	99.73 ± 53.8	76.61 ± 43.75*
Serum OPG/RANKL		47.64 ± 44.7	75.09 ± 45.64*	70.21 ± 58.69	87.12 ± 54.82*
Whole mouth		T1	T2	C1	C2
PD	mm	4.94 ± 0.25	3.47 ± 0.22*	5.04 ± 0.27	3.69 ± 0.27*
PD ≥ 6	Sites %	34.6 ± 5.44	7.66 ± 2.85*	35.86 ± 5.83	10.93 ± 5.08*
AL	mm	4.94 ± 0.22	3.8 ± 0.26*	4.84 ± 0.26	3.77 ± 0.31*
BI		3.19 ± 0.15	1.86 ± 0.16*	3.20 ± 0.17	2.13 ± 0.22*
PI		2.54 ± 0.12	1.78 ± 0.23*	2.58 ± 0.11	2.03 ± 0.21*
Sample sites		T1	T2	C1	C2
PD	mm	5.04 ± 1.02	3.52 ± 0.82*	4.95 ± 0.95	3.58 ± 1.04*
AL	mm	4.97 ± 0.86	3.89 ± 0.96*	4.8 ± 0.98	3.61 ± 1.17*
BI		3.28 ± 0.61	1.89 ± 0.65*	3.2 ± 0.62	2.07 ± 0.81*
PI		2.62 ± 0.5	1.92 ± 1.04*	2.51 ± 0.37	1.88 ± 0.62*
GCF IL-17	pg/ml	37.34 ± 11.05	28.78 ± 6.25*	33.31 ± 13.24	28.66 (8.13, 30.22)
GCF OPG/RANKL		39.98 (25.49, 113.54)	66.14 (33.27, 137.51)	67.85 (35.84, 198.17)	97.39 (65.58, 169.34)

Note: Significant differences ($P < 0.05$) compared to baseline of each index in the same group are noted with *.
Abbreviations: T1, test group at baseline; T2, test group at 3month; C1, control group at baseline; C2, control group at 3month; SD, standard deviation; BMI, body-mass index; HbA1c, hemoglobin A1c; Glu, glucose level; WBC, white blood cells; SII, systemic immune inflammation index, platelet * neutrophil/lymphocyte; NLR, neutrophil-lymphocyte ratio; PD, probing depth; AL, attachment loss; BI, bleeding index; PI, plaque index; GCF, gingival crevicular fluid.

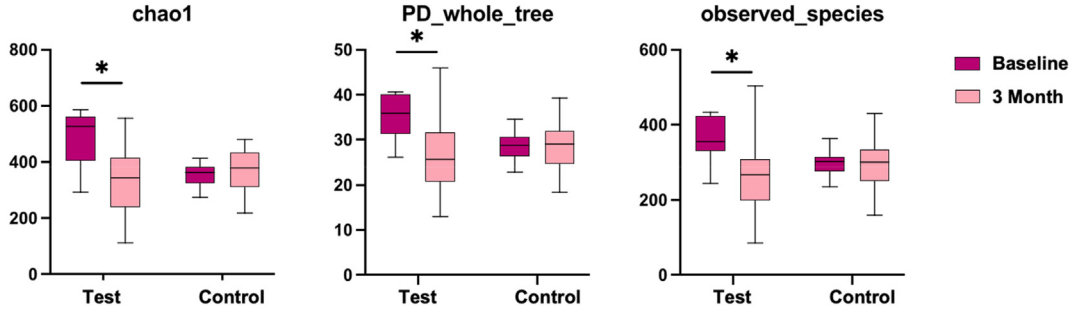

Figure 1 Richness (chao1 and observed species) and diversity (PD whole tree) of the subgingival microbiota in test and control groups. *$P < 0.05$.

relative abundance of red complex, *P. gingivalis* and *T. forsythus* decreased more in the test group than that in the control group ($P < 0.01$) (Table S1).

Microbiome associated with the periodontal condition and systemic indicators

The relationship between the changes of clinical parameters and top 20 general in subgingival microbiota were tested (Fig. 5). The decrease of PD and BI was positively correlated with the decline of *Treponema, Porphyromonas, Capnocytophaga* and *PeptostreptococcaceaeXIG-6*, and negatively correlated with the increase of *Neisseria* and *Pseudomonas*. HbA1c level decrease was positively related to the changes of *Leptotrichia, Veillonella, Saccharibacteria TM7 G-5* and *Actinomyces*. Besides, *Filifactor* was positively correlated with the change of PD and NLR. IL-17 decrease in serum was positively correlated with the decline of *Treponema*, and negatively correlated with the increase of *Capnocytophaga* and *Campylobacter*.

Side effects of the medication

None of the patients in the test group reported antibiotic-related adverse event at any point.

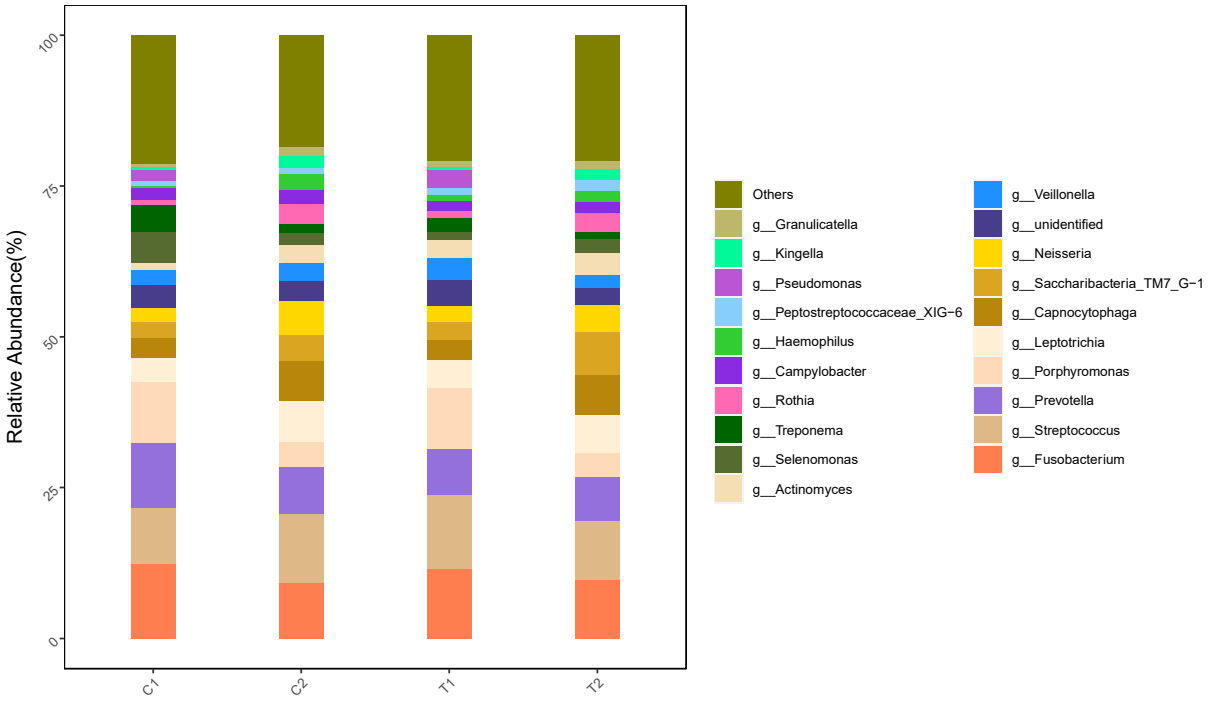

Figure 2 Relative abundance of the top 20 bacterial genera identified in the study subjects. T1, test group at baseline; T2, test group at 3month; C1, control group at baseline; C2, control group at 3month.

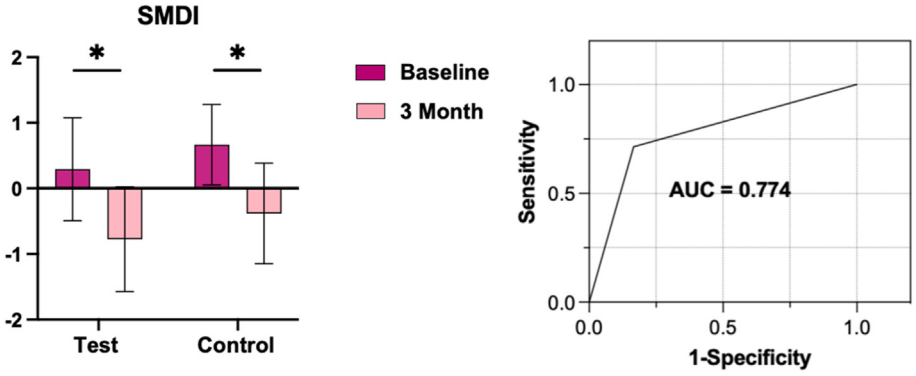

Figure 3 Subgingival microbial dysbiosis index (SMDI) and receiver operating characteristic (ROC) curves. AUC: area under the curve; *$P < 0.05$.

Discussion

This study showed that, for patients with stage III-IV periodontitis and T2DM, both adjunctive antibiotics and mechanical therapy alone made the subgingival microbiome less diverse and changed its composition. While compared to SRP alone, adjunctive antibiotics had a more prominent impact on subgingival microbiota in short term by reducing periodontitis-associated genera and decreasing subgingival dysbiosis more effectively than mechanical therapy.

Previous studies showed that periodontal mechanical therapy combined with adjunctive antibiotics caused microbiome shifts towards healthy status in periodontitis patients.[4,19–21] While to date, no such work has been done in periodontitis patients with T2DM. One RCT study compared the microbiological effect of adjunctive antibiotics to SRP alone, analyzing the presence of seven periodontal pathogens by qPCR.[15] Subgingival microbiome shifts took place with a decrease in pocket depths, relief in periodontal inflammatory status after periodontal therapy, and then the environment of subgingival microbiota changes.[22] This shift would be investigated more clearly by high-throughput sequencing, providing a more comprehensive picture of microbiome dysbiosis and dynamic changes than other techniques used in previous studies, which was also one of the strengths of the present study.

Subgingival microbial dysbiosis index (SMDI) comprises data from samples of 96 healthy patients and 123 patients with moderate to severe periodontitis and is based on 19 discriminating genera.[18] It is a dysbiosis index that can be used as a quantitative and objective tool to measure microbiome modulation. Hagenfeld et al. used SMDI to

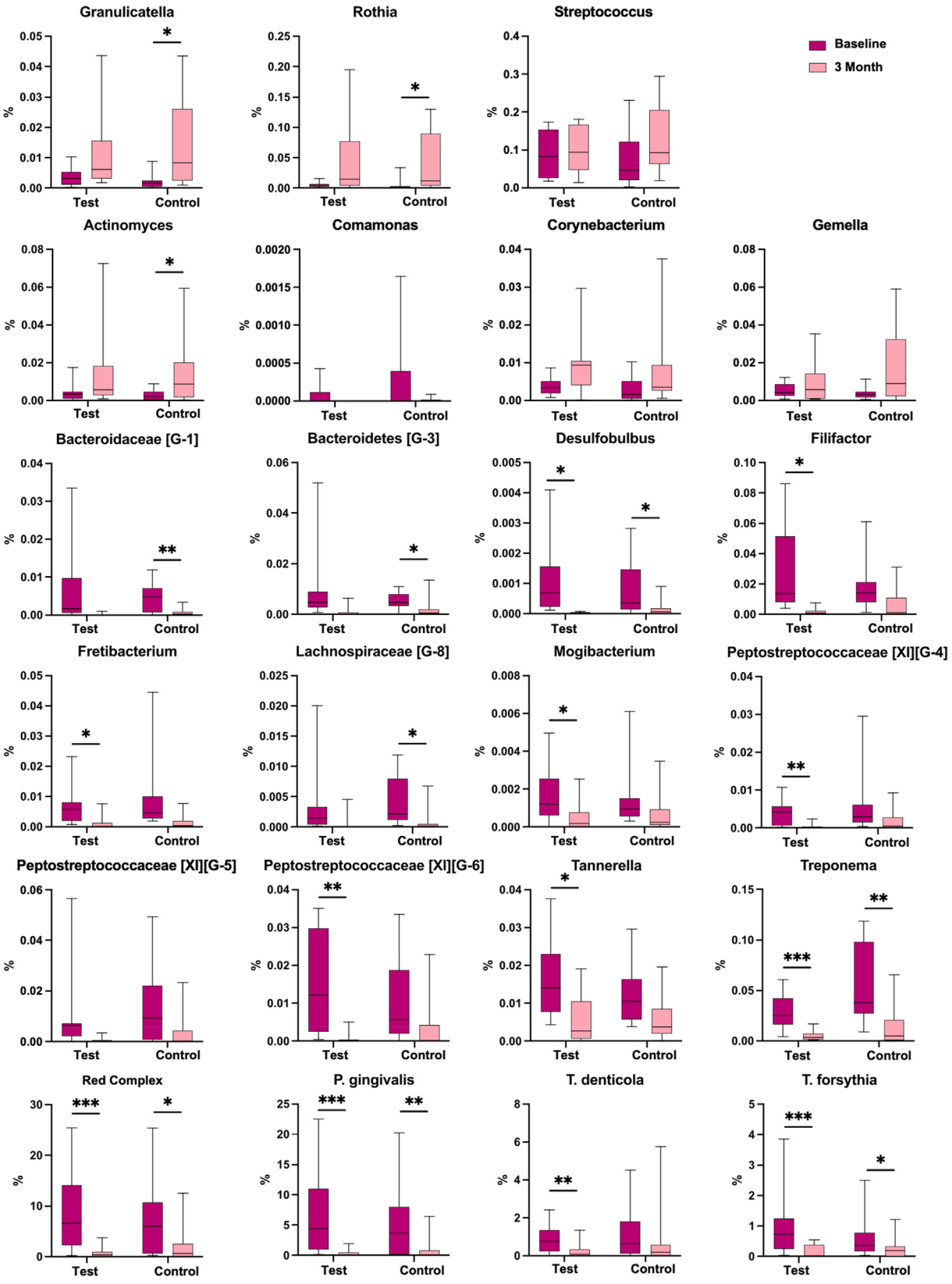

Figure 4 The relative abundance of the 19 genera included in the subgingival microbial dysbiosis index (SMDI) and red complex in the test and the control group at baseline and 3month. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

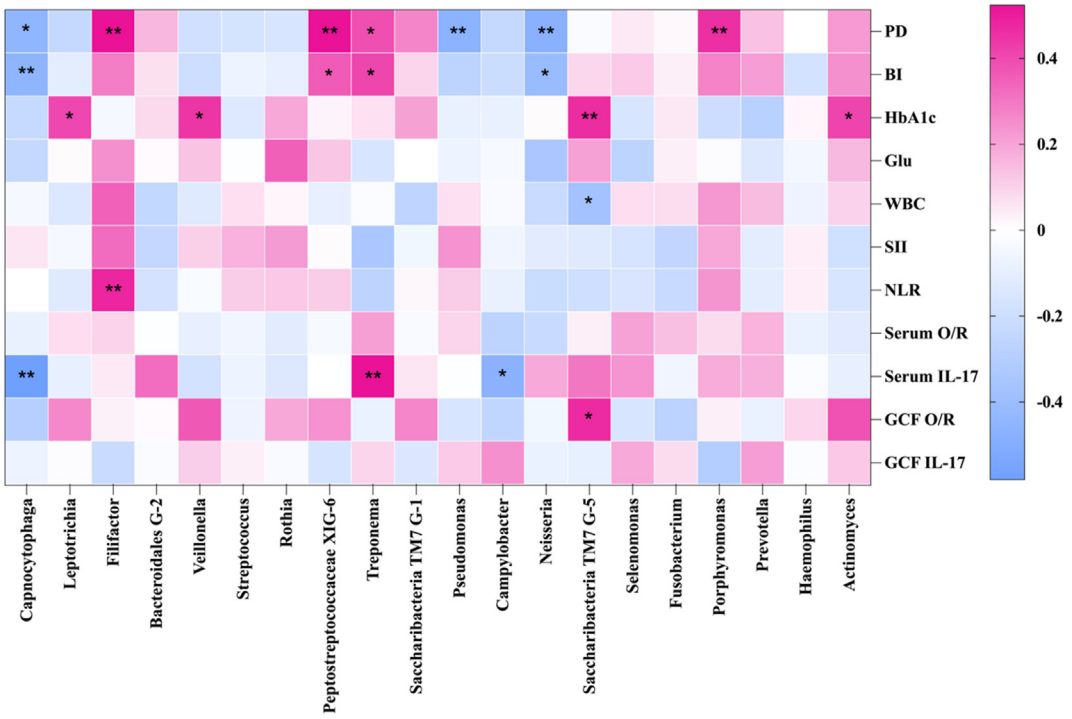

Figure 5 Heatmap of relative analysis between the changes of clinical parameters and top 20 bacterial genera in subgingival microbiota. PD, probing depth; BI, bleeding index; HbA1c, hemoglobin A1c; Glu, glucose level; WBC, white blood cells; SII, systemic immune inflammation index; NLR, neutrophil-lymphocyte ratio; Serum O/R, serum OPG-RANKL ratio; Serum IL-17, serum interleukin 17; GCF O/R, OPG-RANKL ratio in gingival crevicular fluid; GCF IL-17, interleukin 17 concentration in gingival crevicular fluid; pink represents positive and blue represents negative; $^{*}P < 0.05$, $^{**}P < 0.01$.

analyze the subgingival dysbiosis of patients with stage III–IV periodontitis treated by mechanical therapy and adjunctive systemic antibiotics, and the results showed that SMDI could correlate with clinical results and be specific for subgingival microbiota changes.[21] In the present study, SMDI in both groups decreased after treatment, consistent with the changes of clinical periodontal parameters. To further detect the differences between the effect of adjunctive antibiotics and SPR on microbiota, we compared the relative abundance of 19 discriminating genera separately. For dysbiotic discriminatory genera, 8 out of 12 genera decreased significantly in the test group, and 7 of them decreased just in the test group. In addition, for typical periodontal pathogens, the red complex, all decreased in both groups, while the trend was more significant in the adjunctive antibiotics group. These results could support that adjunctive antibiotics have benefits on the subgingival microbiome to some extent.

Patients with periodontitis and diabetes show more inflamed gingiva and have more destruction in periodontal supportive tissue than those without diabetes.[23] Diabetes could elevate the inflammatory cytokines such as IL-17, RANKL and CRP in GCF as well as in circulation.[24–28] Adjunctive antibiotics could additionally decrease clinical parameters, making periodontal inflammation relieved in patients with severe periodontitis and T2DM.[13] Our results showed that both SRP alone and combined with systemic antibiotics could decrease HbA1c and serum IL-17 levels and increase OPG-RANKL ratios, and antibiotics could further degrade this inflammatory status in circulation. Inflammatory status in periodontal tissue was also degraded with improved clinical parameters and lower GCF IL-17 levels.

Oral microbiota also has changes under diabetic conditions, with a decreased richness and diversity in subgingival microbiota[6] and the diversity would be even more decreased when patients have poorly controlled diabetes (HbA1c $\geq$ 8.0 %).[8] Besides, the microbial shift in patients with periodontitis and diabetes is less prominent than those without diabetes.[10] These adverse impacts of diabetes on periodontitis make the condition complicated; therefore, the treatment strategies should be well-directed for such patients. Our results demonstrated that SRP combined with antibiotics could have a more prominent microbial shift, lowering the pathogenicity of microbiota in the oral environment. The red complex, consisting of *P. gingivalis, T. denticola, T. forsythus* decreased significantly after mechanical treatment with adjunctive antibiotics or SRP alone. Furthermore, patients treated with antibiotics presented greater decreases of *P. gingivalis* and *T. forsythus*, which was similar to Miranda's study.[15] This benefit that adjunctive antibiotics reduce periodontal pathogens was coordinated with their extra effect on periodontal inflammation as well as systemic inflammation. The microbiota change was consistent with the change in clinical periodontal parameters, as shown in the heatmap that the change of clinical parameters was positively correlated to some periodontitis-related genera and negatively related to some health and neutral genera. Serum OPG/RANKL level increased and GCF IL-17 level decreased were also more

prominent in the test group. Therefore, adjunctive antibiotics should be an option for patients with poorly controlled diabetes and severe periodontitis.

The two-way relationship between diabetes and periodontitis has been widely discussed. Animal study showed that diabetes alters the oral microbiota and renders it more pathogenic.[29] and in reverse, insulin resistance is enhanced by pathogen-induced periodontitis in the high-fat fed mouse.[30] Clinical study also found proof that alteration of oral microbiota has an impact on gut microbiota in patients with T2DM.[31,32] Our result showed a positive correlation between the reduction of HbA1c level after periodontal therapy and the decline in the abundance of certain disease-related genera, which included *Leptotrichia, Veillonella, Saccharibacteria TM7 G-5* and *Actinomyces*. Higher GCF glucose levels were observed in patients with diabetes,[33] leading to abundant carbohydrates with hypoxia in pockets, which might explain the anaerobic bacteria decreased along with the HbA1c level decrease. Inflammatory indicators like NLR and IL-17 were also associated with subgingival microbiota; their decreases were positively correlated with the decrease of periodontal pathogens, and negatively correlated with the increase of neutral genera. These close interactions between systemic indicators and microbiome implied the effect that diabetes might have on periodontal microbiota. Microbiome dysbiosis and inflammation reducing immune response efficacy link periodontitis and T2DM, which was partly confirmed in the present study. Treatment towards patients with this comorbidity should take the complexity as a whole to gain overall health.

However, we also noticed that the level of some health-associated genera, such as *Actinomyces*, *Rothia* and *Granulicatella*, were only significantly increased in the control group but not in the test group. This is a hint that periodontists should be aware of the possible risk of antibiotics abuse, decreasing both periodontitis- and health-associated bacteria, even leading to antibiotic resistance. Our study used 200 mg of metronidazole, which was less than the doses used in other studies[15,34] and had a lower risk of side effects, while it was still able to improve periodontal inflammation and the microbial environment.

This study still has a few limitations. The 16S rDNA analysis was only taken in parts of the patients from our previous RCT study. The sample size would be small, and the follow-up period was limited to three months, considering other studies prolonged to two to five years.[35,36] SMDI was developed from periodontitis subjects, and we took an attempt to utilize it in patients with diabetes. The results seemed reasonable, while the AUC of ROC in this study was 0.774, lower than an AUC of 0.919 in Chen et al.'s study.[18] Therefore, a modified index that more specific for periodontitis and diabetes patients is needed. Besides, with the continuous development of detection technology, genomics has also been applied in periodontology. In the future, it is also worth using more cutting-edge technology to conduct research on the oral microbiome of patients with diabetes and periodontitis.

In conclusion, our study showed that SRP only and adjunctive use of AMX + MTZ could significantly change the oral microbiome towards healthy status in patients with stage III-IV periodontitis and diabetes, and AMX + MTZ had more advantages in the decrease of periodontal pathogens and in oral microbiota stabilization. Clinicians may take systemic antibiotic use into consideration cautiously when treating such complicated conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2025.01.014.

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