Received: 30 August 2019 | Revised: 6 February 2020 | Accepted: 26 April 2020

DOI: 10.1002/JPER.19-0498

JOURNAL OF Periodontology

ORIGINAL ARTICLE

Well-maintained patients with a history of periodontitis still harbor a more disbiotic microbiome than health

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Funding information
Natural Science Foundation of China, Grant/Award Numbers: 81870773, 81570980; Program for New Clinical Techniques and Therapies of Peking University School and Hospital of Stomatology, Grant/Award Number: PKUSSNCT-11B07

Abstract

Background: It remains unclear whether well-maintained subjects, with periodontitis in the past, effectively treated, and maintained for a long time, have the same subgingival microbiome as healthy subjects. Therefore, the objective of this study was to investigate the characteristics of the subgingival microbiome in well-maintained patients with a history of periodontitis compared with healthy subjects.

Materials and methods: We recruited in 17 well-maintained individuals (no evidence of clinical inflammation and progress of periodontitis) and 21 healthy individuals. Periodontal clinical parameters, consisting of missing teeth, plaque index (PLI), periodontal depth (PD), and bleeding index (BI), were recorded and analyzed. The pooled subgingival samples from mesiobuccal sites of two maxillary first molars were collected. The V3-V4 region of 16S rRNA gene from 38 subgingival samples was sequenced and analyzed. Alpha diversity, microbial composition, types of bacteria, functional pathways between well-maintained group and health group were compared using Mann-Whitney *U* test. Spearman correlation was used in analyzing the symbiotic relationship among taxa. A classification model was constructed to distinguish two ecological types.

Results: The maintained individuals demonstrated a different microbiome from healthy subjects, with higher diversity, more disordered structure, more pathogenic microbiota, and more host-destructive metabolism pathways. The genera *Actinomyces*, *Streptococcus*, *Leptotrichia*, *Capnocytophaga*, *Lautropia*, and *Fusobacterium* were predominant components with relative abundance >5% in the subgingival microbiome of well-maintained patients. The classification model by microbiota got a remarkable accuracy of 83.33%.

Conclusions: Individuals with well-maintained periodontitis showed a more dysbiotic microbial community than healthy individuals. Therefore, close monitoring and scheduled maintenance treatment are necessary for them to maintain a healthy periodontal condition.

KEYWORDS
classification model, microbial risk, microbiology, well-maintained periodontitis

1 | INTRODUCTION

Periodontitis is caused by bacteria deposit and microbiological dysbiosis,[1] and characterized by the destruction of supporting tissue[2] and eventual tooth loss.[3] Affecting almost 50% of the population in the world,[4,5] periodontitis becomes the sixth most prevalent disease worldwide,[6] which causes great trouble to people's life and brings a huge burden to public health service.[7] The expected outcome of periodontitis is to eliminate inflammatory response by active periodontal treatment and maintain long-term periodontal stability with healthy condition.[8] Supportive periodontal treatment could effectively remove dental plaque and keep a relatively balanced microbial community.[9]

However, many epidemiological studies demonstrated that maintained patients still had a higher risk of recurrence as compared with periodontally healthy individuals.[10,11] It is generally accepted that the challenge of bacteria and host susceptibility are the two main factors accounting for the progress of periodontitis.[12] Bacteria initiate and perpetuate inflammatory responses that develop in the periodontal tissues. The well-maintained individuals are patients, with periodontitis in the past, having received successful periodontal treatment, and being maintained clinical health (probing depth [PD] $\leq$ 5 mm and bleeding on probing [BOP%] $\leq$ 25%) for a long time (at least 2 years), which may be the evidence of periodontal homeostasis. The existing question is whether well-maintained patients with a history of periodontitis have the same subgingival microbiome as healthy subjects. Haffajee et al.[13] detected 40 subgingival species in 35 maintained elder subjects and 30 periodontally healthy subjects using checkerboard DNA-DNA hybridization and did not find any significant difference. Conversely, another study by Teles et al.,[10] using the same method, demonstrated that pathogenic bacteria in the maintenance subjects remained significantly higher when compared with healthy subjects. Therefore, the subgingival microbial community of maintained subjects still needs to be investigated.

In recent years, high-throughput sequencing throws light on microbiological research. It gives us a global view of the subgingival microbial community and provides deep insight into the composition of the oral microbiome, which could help us understand the pathogenesis of subgingival bacteria.[14] A considerable amount of literature has reached an agreement on that subgingival microbiome is a key factor in the maintenance of periodontal condition.[9,15] Balanced community could keep periodontal health; whereas dysbiotic community could trigger periodontitis.[16,17] Well-maintained patients have kept long-term periodontal stability. Whether they harbor similar subgingival microbiome to healthy individuals has long been a question of great interest.

Identifying the microbiological profile of well-maintained periodontitis could establish essential baseline data for future applications in evaluating the effect of periodontal treatment. It is also of great significance to assess the microbiological risk of maintained patients. Therefore, the objective of this present investigation is to examine the subgingival microbial profile of well-maintained patients with a history of periodontitis compared with healthy subjects.

2 | MATERIALS AND METHODS

This observational clinical study was approved by Peking University Institutional Review Board (approval number: IRB00001052-05106, PKUSSIRB-201627026), and it was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All participating patients had read study-related information and signed the informed consent before the inclusion.

2.1 | Participants

Well-maintained subjects with a history of periodontitis were from a longitudinal trial[9] performed at the Department of Periodontology, Peking University School and Hospital of Stomatology. The inclusion criteria for well-maintained subjects were as follows:

1. More than 20 residual teeth;
2. No periodontal pocket deeper than 5 mm (wisdom teeth and distal aspects of the second molar were not included) for at least 2 years;
3. Sites with probing depth (PD) = 5 mm should have been <10% at least 2 years;
4. No sites with attachment loss $\geq$ 2 mm within a 2-year observation.
5. Bleeding on probing (BOP, %) $\leq$ 25%;
6. Periapical X-rays show clear and continuous cortical bone of crevicular bone and no sites with bone resorption $\geq$ 2 mm within a 2-year observation.

Periodontally healthy subjects were recruited from subjects who had matched age, gender, PD, and BOP. The inclusion criteria for healthy subjects were as follows:

1. Complete dentition;
2. No periodontal pocket deeper than 3 mm;
3. No attachment loss or clinically detectable inflammation;

4. BOP (%) ≤ 25%;
5. Periapical X-rays show no bone loss.

For both groups, subjects with systemic diseases, who were pregnant or breastfeeding, who were smokers, who had periodontal treatment within the previous 3 months, or who accepted antibiotic therapy within the previous 3 months were excluded. The subjects were recruited in after the initial evaluation between August 2011 and March 2013. Information on body mass index (BMI), education, income, smoking, alcohol drinking was collected.

2.2 | Sample collection and clinical monitoring

Sample collection and clinical examination were performed 1 week after recruitment. The sample collection was carried out before the clinical examination to avoid disturbing subgingival dental plaque. After removing the supragingival plaque, subgingival plaque samples at the mesiobuccal sites of two mandibular first molars (if the first molar was lost, the mandibular second molar would be investigated) were collected separately by sterile curettes. The samples were pools of subgingival plaque from the mesiobuccal sites of two mandibular first molars. Clinical periodontal examinations including plaque index (PLI), PD, and bleeding index (BI), clinical attachment loss (CAL) were performed after sample collection. All the clinical examinations were carried out by a single experienced periodontist with high self-consistency (kappa = 0.91).

2.3 | DNA extraction and sequencing

Samples were dealt with lysozyme (20 mg/mL*180 mL, at 37°C overnight). Then, DNA was extracted using the Microscale genomic DNA extraction kit*, following the manufacturer's instructions. 16S rRNA gene V3-V4 region was amplified using 338F/806R universal primer. Library preparation and sequencing was performed on the Illumina MiSep PE300 platform.†

2.4 | Data processing and statistical analysis

The sequencing data were processed using QIIME v 1.9.1 pipeline.[18] After raw sequences being trimmed and filtered, the remaining high-quality sequences were normalized to the minimum number of sequences obtained. Clean reads with a similarity threshold of 97% were clustered as an operational taxonomic unit (OTU) by UPARSE 7.0.[19] Then sequences were annotated separately against the Human Oral Microbiome Database (HOMD 15.1).[20] Predictive function analysis was performed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[21] according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) classification.[22]

The comparisons of clinical parameters, alpha diversity, taxa, and predictive function pathways were estimated using Mann-Whitney *U* test. Partial least squares discrimination analysis (PLS-DA) was performed to examine the robustness of intra-group clustering based on OTU.[23] Spearman correlation was performed to evaluate the genus-level co-occurrence of the network. Discriminant analysis based on the Fisher method was used to construct a classification model with microbiota.[24] Statistical comparisons and visualization were performed using R 3.5.0.

3 | RESULTS

This study recruited 17 maintained patients and 21 healthy subjects. The well-maintained patients recruited were originally moderate to severe periodontitis and had received regular periodontal maintenance care every 3 to 6 months for 2.5 to 24.5 years (mean 14.2 years). Species Accumulation Curve (Specaccum, see Figure S1 in online Journal of Periodontology) suggested that the sample sizes are sufficient for microbiological analysis in this study. The demographic and clinical characteristics of participants are presented in Table 1. The healthy group was homogenous with the well-maintained group in demographic characteristics, socio-economic factors, and clinical condition.

A total of 2,380,216 raw reads were generated from 38 samples, with an average of 62,637.26 sequences per sample (ranging from 28,049 to 94,025). After data trimming and quality filtering, there were 2,210,380 clean reads, with an average of 58,167.89 sequences per sample (ranging from 26,543 to 85,942). Clustering of all high-quality sequences at 97% identify resulted in 329 OTUs. In total, 14 phyla, 28 classes, 47 orders, 82 families, 160 genera, and 317 species were detected in the subgingival microbiome.

3.1 | Well-maintained individuals harbored distinct subgingival microbial assemblages from healthy individuals

The comparisons of subgingival microbiota between the maintained periodontitis group and the healthy

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TABLE 1 Demographic characteristics and clinical parameters of subjects in the health group and the well-maintained group

	Health group ($N = 21$)	Well-maintained group ($N = 17$)
Age (years ± SD)	40.33 ± 6.51	47.70 ± 14.88
Gender (male%)	47.62%	41.18%
BMI (mean ± SD)	21.21 ± 2.0	22.77 ± 1.49
Education (proportion of college, %)		
High/vocational school	0%	35.29%
College	100%	64.71%
Annual household income (Yuan)		
< 100 000	23.81%	11.76%
100 000-500 000	52.38%	58.83%
> 1000 000	23.81%	29.41%
Smoking (%)	0%	0%
Alcohol drinking (%)	14.29%	11.76%
No. of missing teeth (mean ± SD)	0	1.88 ± 1.74
Plaque index (median [Q1, Q3])	1 (0, 1)	0 (0, 1)
Probing depth (mm ± SD)	2.43 ± 0.23	2.51 ± 1.14
Percentage of sites with PD = 4 mm	0	5.44% ± 4.05%
Percentage of sites with PD = 5 mm	0	2.39% ± 1.69%
Clinical attachment loss (mm ± SD)	0	1.60 ± 0.74
Bleeding index (mean ± SD)	0.79 ± 0.47	1.04 ± 0.35

Tested by Mann-Whitney *U* test. No significant differences were found with 95% confidence interval.

group showed significant differences (Figure 1). Microbial richness, presented with Chao 1, was significantly lower in the well-maintained group as compared with that in the healthy group ($P < 0.05$, Figure 1A). Microbial diversity, presented by the Shannon index, was significantly higher in the well-maintained group than the health group ($P < 0.05$, Figure 1B). The PLS-DA showed that the communities in the well-maintained group clustered separately from the communities in healthy group ($P < 0.05$, Figure 1C).

3.2 | Well-maintained individuals harbored a more pathogenic composition than healthy individuals

At phylum level, the well-maintained group harbored more abundant phyla consisting of *Spirochaetes*, *Bacteroidetes* compared with the health group ($P < 0.05$, Figure 2A). At class to family level, some taxa, such as class of *Spirochaetia*, were more abundant in the well-maintained group, whereas some taxa, such as class of *Bacilli*, were less abundant in the well-maintained group compared with health group ($P < 0.05$, Figure 2B–D). At genus level, compared with the health group, the well-maintained group showed significantly more abundant periodontitis-associated genera, such as *Treponema*, *Leptotrichia*, and less health-associated genera, such as *Streptococcus*, *Granulicatella* ($P < 0.05$, Figure 2E). At species level, some species such as *Streptococcus* 058, *Neisseria mucosa*, *Neisseria flavescens*, *Granulicatella adiacens*, *Gemella morbillorum*, *Neisseria oralis*, were significantly lower in the well-maintained group than healthy group; whereas species, such as *Leptotrichia hongkongensis*, *Capnocytophaga granulosa*, *Cardiobacterium hominis*, *Capnocytophaga 336*, *Capnocytophaga leadbetteri*, *Capnocytophaga sputigena*, *Selenomonas noxia*, *Capnocytophaga 326*, *Prevotella saccharolytica*, *Treponema socranskii* were significantly higher in the well-maintained group than healthy group ($P < 0.05$, Figure 2F, Figure S2 in online Journal of Periodontology).

3.3 | The comparisons of bacterial types and functional analysis

The comparisons of bacterial types according to staining characteristics and oxygen requirements between the well-maintained group and the health group did not show a significant difference (Figure 3A, B). However,

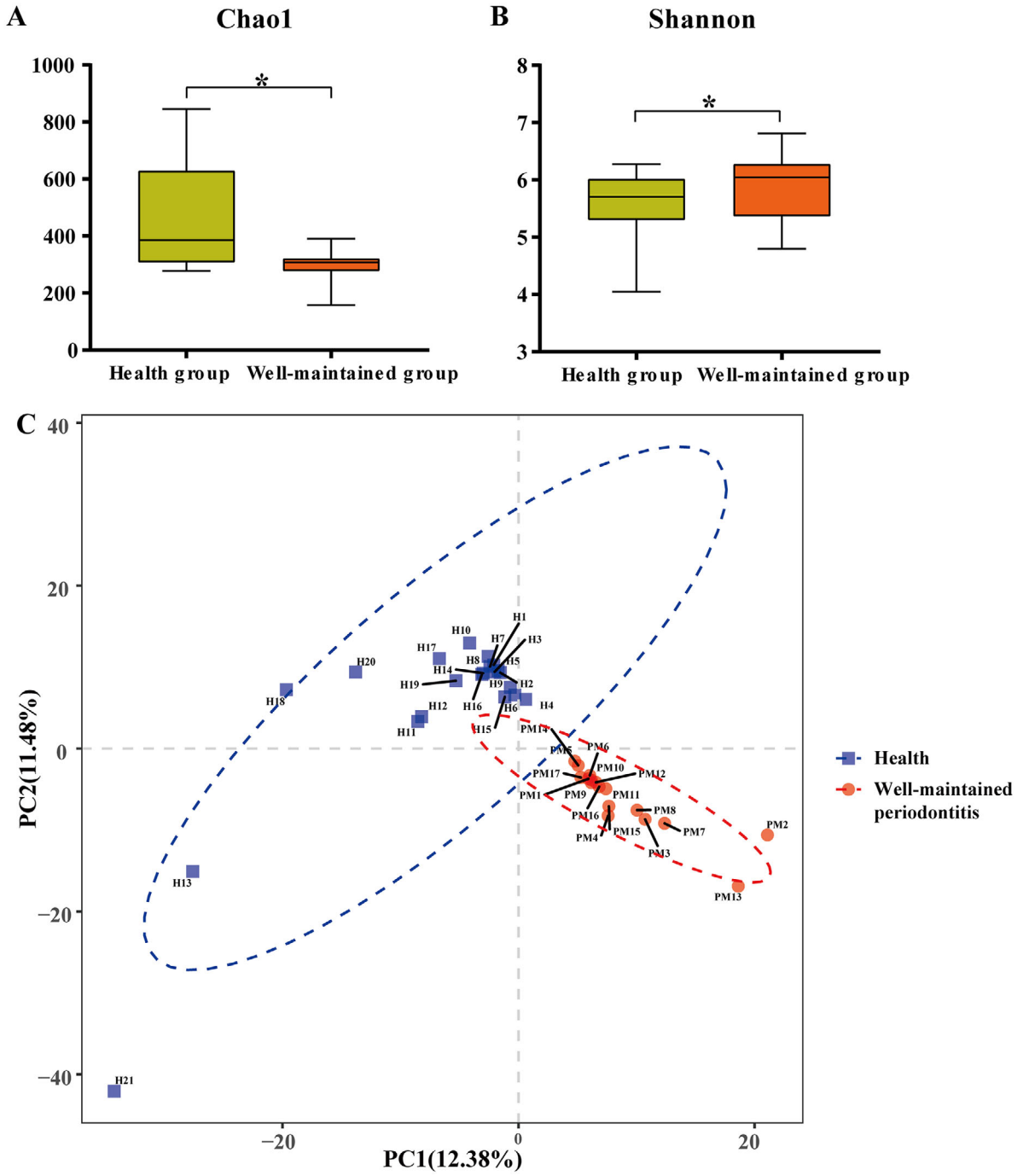

FIGURE 1 Comparisons of alpha diversity and beta diversity between well-maintained group and health group. (**A**) Microbial richness presented by Chao1. (**B**) Microbial diversity presented by Shannon. $^{*}P < 0.05$ by Mann-Whitney *U* test. (**C**) Partial least squares discrimination analysis (PLS-DA) between well-maintained patients and health

the well-maintained group showed significantly more abundant rod-shaped microbiota and less abundant coccus-shaped microbiota compared with the health group ($P < 0.05$, Figure 3C). Predictive function by PICRUSt based on KEGG database showed that well-maintained group presented a significantly higher abundance of pathogenic bacterial metabolisms, such as *Bacterial invasion of epithelial cells*, metabolism, and transcription related pathways, whereas the relative proportion of some pathways, such as *Apoptosis*, was significantly lower when compared with health group ($P < 0.05$, Figure 3D).

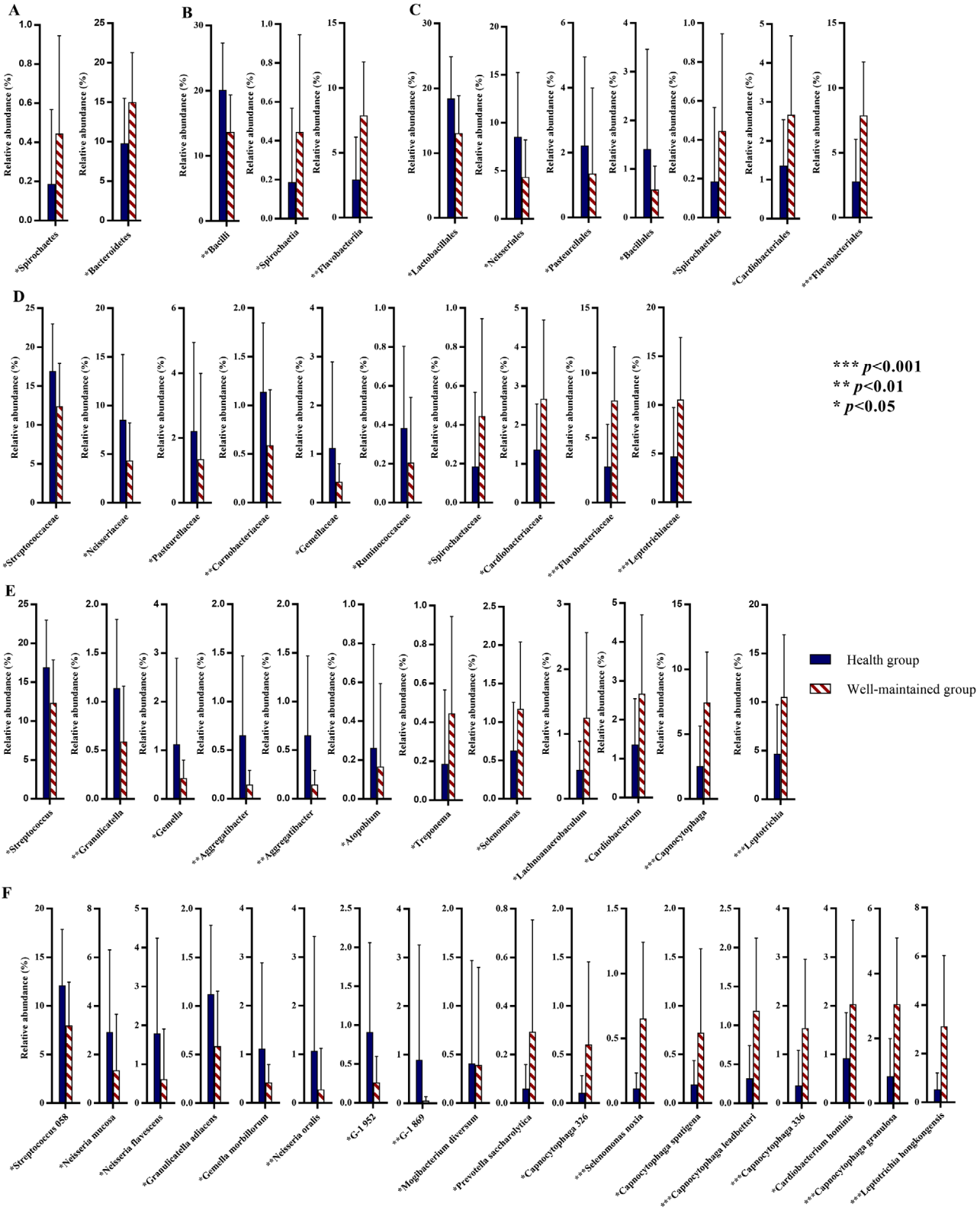

FIGURE 2 Comparisons of sequences (average relative abundance >0.2%) between well-maintained group and health group. (**A**) phylum level, (**B**) class level, (**C**) order level, (**D**) family level, (**E**) genus level, (**F**) species level. Error bars represent standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, tested by Mann-Whitney *U* test

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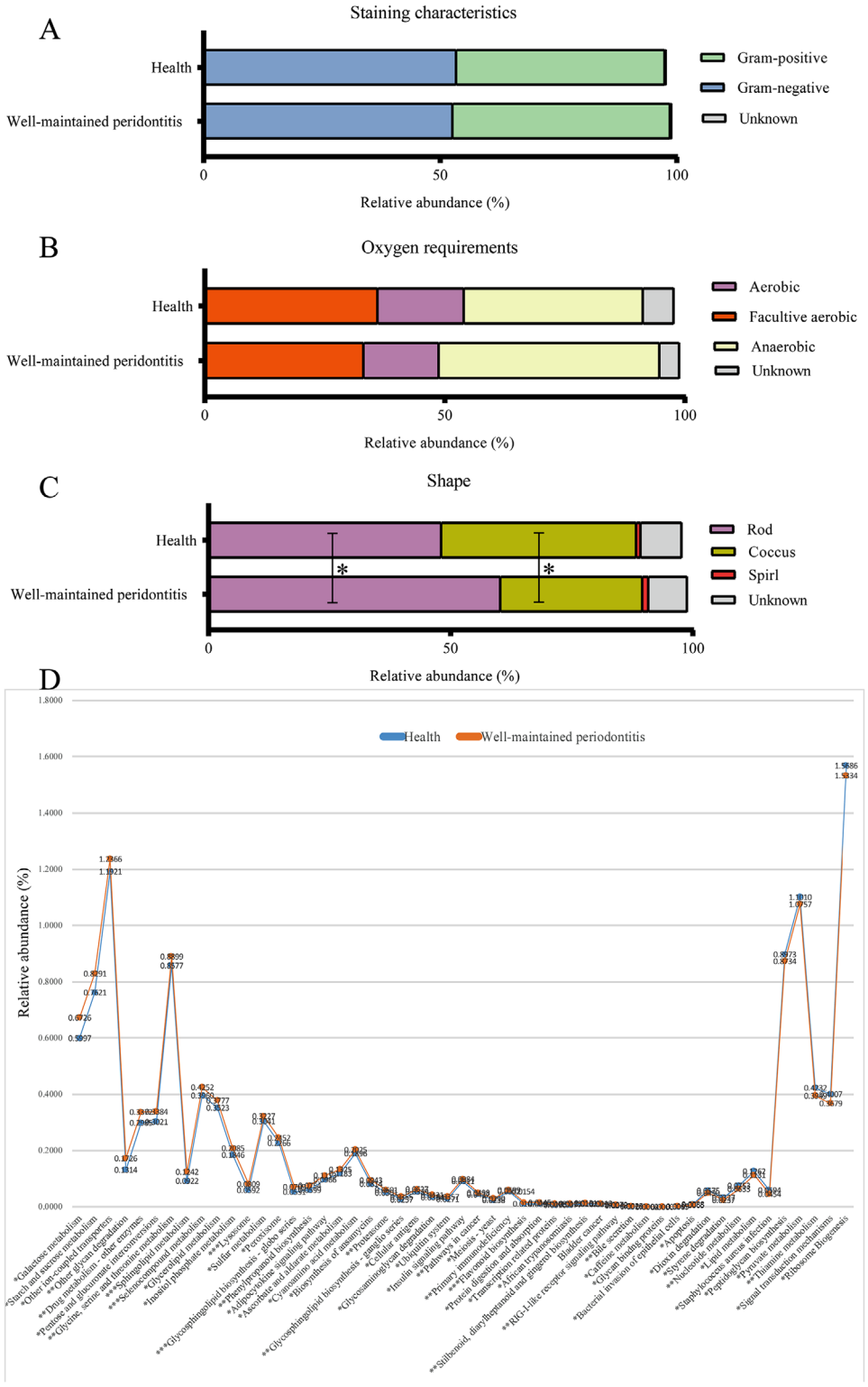

FIGURE 3 The comparisons of bacterial types and functional analysis between well-maintained group and health group. (**A**) Bacterial types by staining characteristics. (**B**) Bacterial types by shapes. (**C**) Bacterial types by oxygen requirements. (**D**) Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) referring to KEGG. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, tested by Mann-Whitney *U* test

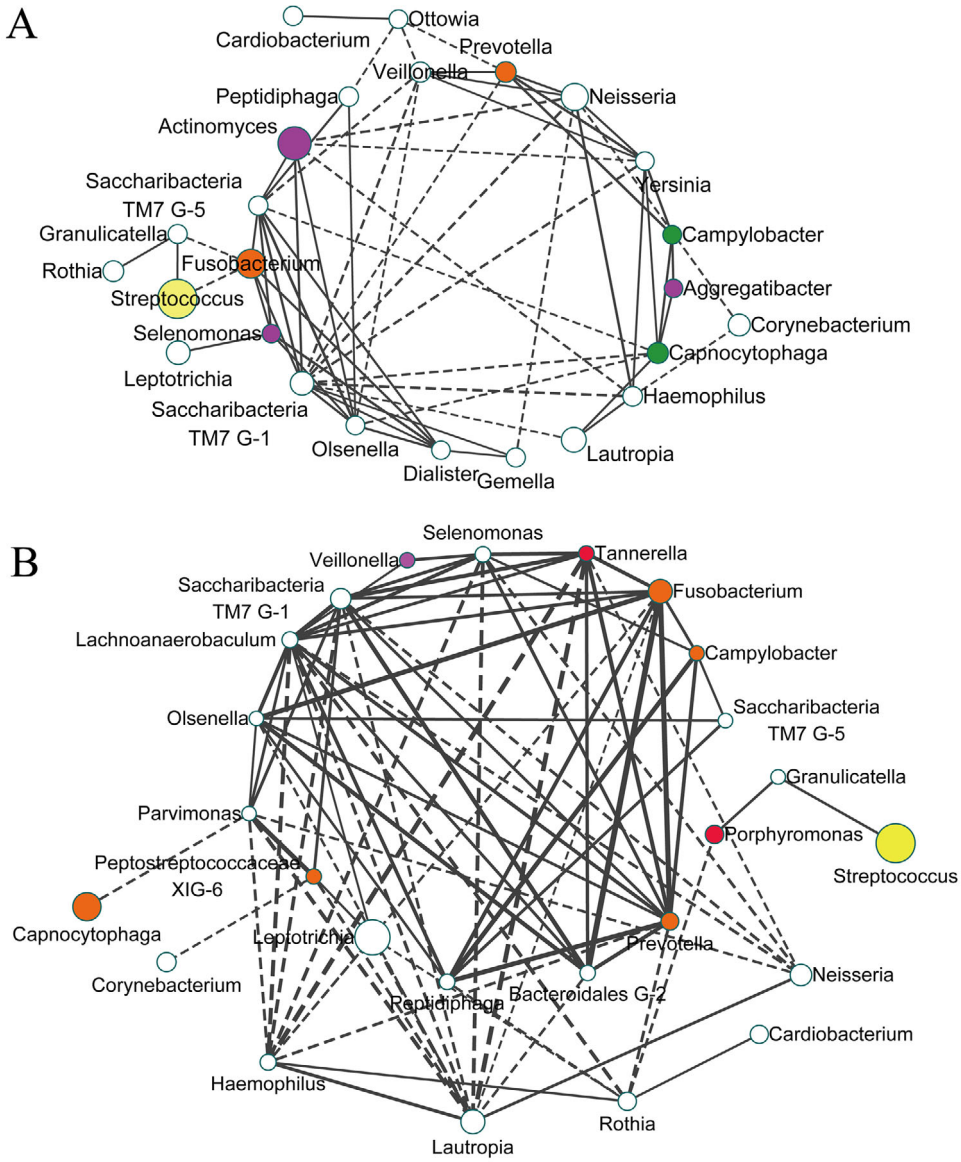

FIGURE 4 The co-occurrence network of core genera. The co-occurrence network of core genera (relative abundance > 0.5%) tested by Spearman with $P < 0.05$. (**A**) Health group (**B**)Well-maintained group. The size of the nodes was determined by relative abundance. The thickness of the connecting lines was determined by the correlation coefficient. The colour of the nodes was determined by the complexes to which they belonged , as described by Socransky.[27] Dash lines represent the negative relationship between two genera, and solid lines represent the positive relationship between two genera

3.4 | Potential interactions and niche-sharing among oral taxa

Network analysis was performed with core genera (with relative abundance > 0.5%, prevalence > 70%) to show the interactions among genera and microbial structure ($P < 0.05$, Figure 4). The network of the health group was sparse and balanced, whereas the network of the well-maintained group showed much more pathogenic composition more complex and robust interactions than the health group. Several periodontitis-associated genera, such as the genus of *Porphyromonas*, *Tannerella*, *Prevotella, Fusobacterium*,[25,26] had multiple robust interactions with other microbiota and formed an intertwined symbiotic network.

3.5 | Classification model by microbiota

Discriminant analysis based on the Fisher method was conducted to construct a classification model with microbiota as previously reported.[24] Firstly, the data weres

randomly split into training set (70%) and testing set (30%) in each group. we constructed the classification model with the training set and validated it with the testing set. It screened out eight genera using the stepwise method to build discriminant functions as follows:

$$\begin{aligned}\text{Well-maintained group:}\\ &= -1364.03\,G1 - 3302.97\,G2 + 4681.23\,G3\\ &\quad - 107642.93\,G4 - 195285.08\,G5 + 7198.17\,G6\\ &\quad + 475.90\,G7 - 27.82\,G8 - 21.24\end{aligned}$$

$$\begin{aligned}\text{Health group:} &= 220.56\,G1 + 620.66\,G2 - 233.23\,G3\\ &\quad +18561.10\,G4 + 35624.02\,G5 - 274.36\,G6\\ &\quad - 3.85\,G7 + 42.19\,G8 - 3.61\end{aligned}$$

(G1, *Aggregatibacter*. G2, *Gracilibacteria* GN02 G-1. G3, *Megasphaera*. G4, *Mycoplasma*. G5, *Agrobacterium*. G6, *Veillonellaceae* G-1. G7, *Capnocytophaga*. G8, *Fusobacterium*).

Then, the classification model was validated with the testing set. The samples in the testing set should be unlabeled, the relative abundance of eight genera (biomarkers) included should be calculated with the formulas and get a value. The sample would belong to the group of which got the larger value when tested by the formulas. The classification model got a remarkable performance with an accuracy of 83.33%.

4 | DISCUSSION

The periodontal microbiome of maintained individuals rebuilds a balance to maintain a relatively healthy condition. However, this balance is not equal to the that in healthy individuals. For patients with a history of periodontitis, even though the subgingival microenvironment previously appropriate for dysbiotic microbiome disappeared after periodontal treatment, they still harbored a more dysbiotic microbiome than healthy subjects. Therefore, the subgingival microbiome in well-maintained patients is dynamically balanced, which needs close monitoring and regular maintenance treatment, and if left untreated, the microbiome will be dysbiotic again. This present study provides powerful evidence to understand the high susceptibility of recurrence for well-maintained patients with a history of periodontitis.

Global differences between healthy and well-maintained communities were visualized by the separation of groups in PLS-DA. Higher microbial diversity in the well-maintained group indicated a more complex composition. Besides, well-maintained patients presented a more robust relationship and complex structure in the co-occurrence network. The intricated network contained several periodontitis-associated genera, such as *Porphyromonas*, *Prevotella*, *Tannerella*, *Fusobacterium*,[25,26] showing interactions with other microbiota, which might indicate a more dysbiotic microbiome in well-maintained patients. In this study, increased microbial diversity and robust interactions together revealed a more disordered microbiome in the well-maintained group.

Maintained communities demonstrated a more pathogenic composition. They harbored more periodontitis-associated taxa, such as genera of *Leptotrichia*, and *Treponema*, and less beneficial taxa, such as Genus of *Streptococcus* and *Granulicatella*.[16] Many studies confirmed that species of *Treponema* had a strong ability to stimulate immune response[28] and to invade periodontal tissue.[29] The previous study by checkboard DNA-DNA hybridization technique also showed elevated pathogenic bacteria in maintenance subjects than prophylaxis subjects,[10] which was consent with our study. The subgingival microbiome of maintained patients was dominated by genera *Actinomyces*, *Streptococcus*, *Leptotrichia*, *Capnocytophaga*, *Fusobacterium*, and *Lautropia* with relative abundance more than 5%. Among them genera of *Fusobacterium* and *Leptotrichia* have been reported to be associated with periodontitis in several studies.[30,31] Whereas genera of *Actinomyces*, *Streptococcus*, and *Lautropia*, as commensal components,[16,31–33] served as a counterpart to keep a comparatively balanced community in well-maintained patients. This study might be helpful to understand the microbial risk of well-maintained patients.

PICRUSt analysis also showed significant differences. Some functional pathways such as *Bacterial invasion of epithelial cells* were significantly higher in the well-maintained group than the health group. The ability to adhere to and invade epithelial cells is one of the most important features that allow bacteria to cross the epithelial barrier and infect tissues,[34] it was confirmed to a host-destructive pathway in previous studies.[35] Besides, well-maintained patients harbored more pathways involved in active life pathways, such as *Protein digestion and absorption*, and *Transcription related proteins*. However, some pathways, such as *Apoptosis*, were lower in the microbiome of well-maintained patients.

The classification model was built using eight genera as biomarkers, which could distinguish subgingival microbiome from well-maintained patients and healthy subjects with a high accuracy of 83.33%. It demonstrated that there were two different ecological types in well-maintained patients and healthy individuals. Each individual harbors

an ecological type of subgingival microbiome which has the capability to maintain health or elicit disease. It could help to understand the difference between maintained patients and healthy subjects. Also, stratifying periodontal conditions based on microbiota may pave the way to assess the susceptibility of periodontitis by etiology and apply personalized therapy. Indeed, the classifier would require validation in a larger population of different regions and races before application on a larger scale.

One of the strengths of this study is that it provides a preliminary global-scale framework of subgingival communities in maintained periodontitis. And it could help us to understand the high susceptibility of recurrence for patients with a history of periodontitis. Another strength lays in the strict inclusion criteria ensuring the patients recruited in were well-maintained for a long time. The potential weakness of this study is the limited sample size. Although the subgingival microbiome of well-maintained patients with a history of periodontitis and health showed significant differences, it should be confirmed in a larger population from different regions and races. Another limitation might be the slight difference in criteria for well-maintained patients and healthy subjects. The residual 4 mm or 5 mm pockets in the well-maintained patients might have a potential influence on the subgingival microbiome.

The most obvious finding emerging from this study is that subgingival microflora in well-maintenance patients is more pathogenic than healthy individuals. Even patients with periodontitis have been treated with good efficiency and maintained for a long time, they still harbored a more disbiotic microbial community. Therefore, close monitoring and effective maintenance treatment are necessary to reduce the risk of recurrence of periodontitis. Furthermore, it enlightens us to investigate why patients with a history of periodontitis are more likely to suffer from peri-implant diseases. The residual pathogenicity of oral microbiology, even after effective treatment, should be taken into consideration. In addition, this present study could provide essential baseline data of the subgingival microbiome for future applications in accessing the effect of periodontal treatment. Further studies should consider including the subgingival microbiome of well-maintained patients as the outcome in the prediction model of periodontal treatment. In addition, for infectious diseases, even after successful treatment, microflora during the maintenance period should be paid more attention.

ACKNOWLEDGMENTS

We especially thank Dr. Zhibin Chen for her generous help. The authors have stated explicitly that there are no conflicts of interest in connection with this article. Hongye Lu and Lu He contributed equally to this work.

FUNDING INFORMATION

Natural Science Foundation of China (81870773 and 81570980). Peking University School and Hospital of Stomatology New Technology and New Therapy (PKUSSNCT-11B07).

AUTHORS CONTRIBUTION

All authors have made substantial contributions to the conception and design of the study. Hongye Lu, Yibing Zhao, Lu He, Jingling Xu, and Wenli Song have been involved in sample collection and processing. Hongye Lu, Huanxin Meng, Yibing Zhao, Lu He, Jingling Xu, and Xianghui Feng have been involved in data interpretation, drafting the manuscript and revising it critically.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Lu H, He L, Xu J, et al. Well-maintained patients with a history of periodontitis still harbor a more disbiotic microbiome than health. *J Periodontol*. 2020;1–11. https://doi.org/10.1002/JPER.19-0498