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ORIGINAL ARTICLE

Peri-implant mucositis sites with suppuration have higher microbial risk than sites without suppuration

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Abstract

Objective: The aims of the present study were to compare the microbial differences between peri-implant mucositis sites with or without suppuration, and to construct a classification model with microbiota.

Methods: Twenty-four implants with peri-implant mucositis were divided into suppuration (SUP) group and non-suppuration (Non-SUP) group. Clinical assessments of bleeding index, probing depth, suppuration following probing (SUP) were recorded. Submucosal samples were collected from mesiobuccal sites and distobuccal sites, and analyzed by 16S rRNA gene sequencing. Generalized linear mixed model was used to adjust age, gender, location of implants, and intraindividual correlation.

Results: It was demonstrated that the microbial richness was lower in SUP group. The relative abundance of some pathogenic taxa, such as genera of *Fusobacterium*, *Tannerella*, and *Peptostreptococcus*, were significantly higher in SUP group than Non-SUP group. In addition, SUP group had less Gram-positive bacteria, aerobic bacteria, and more metabolic pathway related to life activity. The classification model constructed with 12 genera got a 100% accuracy in identifying sites with or without suppuration.

Conclusions: The results from this study demonstrate a higher pathogenicity of microbiome at peri-implant mucositis sites with suppuration than without suppuration, which supports suppuration as a clinical indicator for higher microbial risk.

KEYWORDS

16S, dental implants, microbiology, mucositis, ribosomal, RNA, suppuration

1 | INTRODUCTION

Osseo-integrated dental implants have become a favorable treatment option for partially or fully edentulous patients in the last three decades. Although a high long-term success rate ranging from 90% to 95% over 20 years has been reported,[1–3] pathological conditions may occur in hard and soft peri-implant tissues. Biological complications associated with dental implants are mostly inflammatory conditions which include peri-implant mucositis and peri-implantitis.[4–6] Peri-implant mucositis is the first clinical sign in response to plaque accumulation, which may develop into peri-implantitis if left untreated.[7]

The consensus report of the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions proposed that peri-implant mucositis is characterized by bleeding upon probing and visual signs of inflammation, which can be reversed with measures aimed

at controlling peri-implant biofilms.[8] The main characteristic of peri-implant mucositis is bleeding upon gentle probing, whereas additional signs may include erythema, swelling, and/or suppuration.[8] Dental sites with suppuration upon probing might be an indication of active periodontal breakdown.[9] Similarly, implant sites with suppuration might also be a sign of active progression of inflammation. Suppuration is a common clinical sign of peri-implant diseases.[6,7] Epidemiological studies with large population identified that presence of pus was associated with bone loss, which might increase the risk of peri-implantitis.[10,11] However, studies concerning the presence of suppuration in peri-implant mucositis is very limited.

A consensus has been reached that microbiota is a key etiological factor for peri-implant diseases.[7,12] Studies focus on the microbial difference at healthy, peri-implant mucositis and peri-implantitis sites demonstrated that sites with peri-implant diseases harbored more pathogenic microbiota than healthy sites.[13–19] Besides, it is claimed that peri-implant mucositis might be an important early transitional phase during the development of peri-implantitis.[20] Although the microbiological features of peri-implant mucositis have been well studied, researches concerning the microbiome at peri-implant mucositis sites with or without suppuration is very limited.

Recently, the use of 16S rRNA gene sequence analysis has provided new insights into the diversity of oral microbiota associated with peri-implant diseases.[20–25] The aim of this study was to evaluate the microbiota at peri-implant mucositis sites with or without clinical signs of suppuration using 16S rRNA gene sequence analysis. Although it is easy to identify suppuration in clinical practice, to explore the underlining mechanism is of great significance. Investigating the microbiome at suppuration sites could help us understand the mechanism of suppuration and find potential approaches to hinder the progress of peri-implant diseases.

2 | MATERIALS AND METHODS

2.1 | Patient and implant recruitment

The present study is a retrospective analysis of the clinical and microbiological data extracted from a 2-year prospective study. This study was approved by the human subjects ethics board of Peking University Health Science Center (Approval No. IRB00001052-10047) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All the patients recruited in this study had signed an informed consent form before their inclusion and the extracted data in the present study was anonymized.

Patients recruited in this study were selected from a population of patients who had completed dental implant therapy and received routine maintenance visits at 1 month, 1 year and 2 years after crown rehabilitation. The inclusion criteria were patients with at least one implant diagnosed as peri-implant mucositis at 2 years after crown rehabilitation and did not take antibiotics for the previous 6 months. All patients included in the current study were systemically healthy and non-smokers. The diagnosis of peri-implant mucositis required the presence of bleeding and/or suppuration upon gentle probing with or without increased probing depth (PD) compared with previous examinations and the absence of bone loss beyond crest bone level changes resulting from initial bone remodeling.[6–8]

2.2 | Sample collection and clinical examination

Before the clinical examinations, peri-implant sulcular fluid samples were collected from the buccomesial and buccodistal aspects of the peri-implant sulcus and pooled for each implant. The sampling sites were isolated with cotton rolls, and after removing supramucosal plaques, filter strips (2 mm × 10 mm)* were inserted into the bottom of the peri-implant sulcus with mild resistance for 30 seconds. All strips were transferred into a sterile Eppendorf tube† and then stored at −80°C for further processing.

PD and bleeding index (BI) were assessed carefully using light forces, and the presence of suppuration (SUP) was also analyzed. Intraoral periapical radiographs were obtained to evaluate the bone levels at the mesial and distal aspects of each dental implant by measuring the distance from the implant shoulder to the first visible bone-to-implant contact. The images acquired at baseline (1 month after crown rehabilitation) were used as a reference for the future assessment of the bone loss for each implant.

Based on the record of the presence of SUP, recruited implants with clinical signs of SUP were allocated to group SUP, whereas implants without clinical signs of SUP were allocated to group non-SUP.

2.3 | DNA extraction and sequencing

Bacterial genomic DNA in samples was extracted following the manufacturer's guidelines using a TIANamp Micro DNA Kit.‡

The V3-4 hypervariable region of the bacterial 16S rRNA gene was amplified with universal primers (338F 5′-GTACT CCTACGGGAGGCAGCA-3′ and 806R 5′-GTGGACTAC HVGGGTWTCTAAT-3′). For each sample, a 10-digit barcode sequence was added to the 5′-end of the primers.§ PCR

* Whatman Grade 3MM Chr; Whatman International Ltd., Maidstone, UK.

† Eppendorf, Hamburg, Germany.

‡ TIANGEN BIOTECH, Beijing, China.

§ Auwigene Company, Beijing, China.

was carried out on a Mastercycler Gradient† using 50 μl reaction volumes that contained 5 μl 10 × Ex Taq buffer (Mg^{2+} plus), 4 μl 12.5 mM dNTP mix (each), 1.25 U Ex *Taq* DNA polymerase, 2 μl template DNA, 200 nM 967F and 1406R barcoded primers (each) and 36.75 μl ddH_2O. The following cycling parameters were used: initial temperature of 94°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 10 minutes. Three PCR products per sample were pooled to mitigate reaction-level PCR bias. The PCR products were purified using a QIAquick Gel Extraction Kit,* quantified using real-time PCR, and sequenced on an Illumina MiSep PE300 platform.§ Image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

2.4 | Data analysis and statistical analysis

Sequences were removed if they were shorter than 200 bp, had an inferior quality score (≤20), contained ambiguous bases or did not exactly match with the primer sequences and barcode tags. Then, qualified reads were separated using the sample-specific barcode sequences and trimmed with Illumina Analysis Pipeline Version 2.6. The dataset was analyzed using QIIME. Sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97%. All sequences were classified into different taxonomic groups based on the Human Oral Microbiome Database (HOMD).[26]

Clinical parameters, including PD and BI at implant sites with and without suppuration, were compared using Student *t* test. Generalized linear mixed model (GLMM) was used to compare the alpha diversity and relative abundance of taxa in different groups after the correction of age, gender, location of implants and multiple implants per patient. Alpha diversity was presented with Chao1 and Shannon. Chao1 is used to estimate microbial richness, that is, total number of species.[27,28] Shannon is an index of microbial diversity demonstrating variability among microbiota, which is a composite metric that combines richness and evenness.[29] Principal component analysis (PCA) based on UniFrac distance measurements was performed to examine the similarity of microbial composition among different samples.[30] Analysis of similarities (ANOSIM) was performed to compare intra- and inter-group similarities.[30] If the intergroup difference was higher than intragroup difference with $P < 0.05$ and $R > 0$, the two groups were significantly different. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) based on Kyoto Encyclopedia of Genes and Genomes[31] was performed using the PICRUSt algorithm.[32] The co-occurrence network was generated with symbiotic relationships of core genera (relative abundance > 1%) by Spearman, and the color of the nodes was determined by the complexes as described by Socransky.[33] Discriminant Analysis based on the Fisher method was applied to build the classifier, and it was verified by receiver operating characteristic (ROC). Statistical analyses were performed using SPSS 20.0 with a 95% confidence interval. R 3.3.2 and Cytoscape 3.5.1 were used to make the results visualized.

* Qiagen, Duesseldorf, Germany.

3 | RESULTS

A total of 24 conical locking-taper implants (Integra-CP™; Bicon Dental Implants, Boston, MA, USA) in 12 Chinese patients with a mean age of 48 years were recruited in this study. Information regarding the number of studied implants and residual teeth on each subject can be seen at Supplementary Table 1 in online Journal of Periodontology. Species Accumulation Curve (Specaccum) suggested that the sample sizes are sufficient for microbiological analysis in this study. The patients recruited in this study initially belonged to stage IV, grade C periodontitis based on the new classification of periodontitis proposed by the 2017 World Workshop.[34] All implants included in the present study were diagnosed with peri-implant mucositis. Twenty-one out of 24 implants were implant-supported single crowns whereas the remaining three implants in the anterior region were abutments for implant-supported cantilever fixed prostheses. The distribution of these implants was as follows: 10 implants were distributed in the maxillary anterior region, four implants were distributed in the mandibular anterior region, eight implants were distributed in the maxillary posterior region and two implants were distributed in the mandibular posterior region. Based on the clinical records of the presence of suppuration, 12 implants with suppuration were observed. The demographics of all patients and the comparison of clinical parameters in implants with and without suppuration are shown in Table 1. No statistically significant differences were observed between SUP group and Non-SUP group for the PD and BI ($P > 0.05$).

A mean value of 37,547 raw tags (range from 14,299 to 102,050) and a mean value of 36,195 clean tags (range from 14,067 to 98,920) were generated. We finally observed an average of 251 OTUs (range from 145 to 389) using a 97% similarity cut-off.

3.1 | Submucosal microbiome was distinct at peri-implant mucositis sites with or without suppuration

The comparison of microbial richness and diversity between implants with and without suppuration was analyzed by the

TABLE 1 The demographics of all subjects and a comparison of the clinical parameters in implants with or without suppuration

Parameters	Non-SUP group ($N = 12$ implants)	SUP group ($N = 12$ implants)	*P*-value
Age (years)	48.00 ± 8.92		
Gender (% males)	41.7		
PD of implants (mm)	4.21 ± 1.80	3.49 ± 1.12	0.25
BI of implants	2.05 ± 1.07	2.13 ± 1.27	0.84
PD of residual teeth (mm)	3.06 ± 0.37	3.11 ± 0.53	0.54
BI of residual teeth	1.79 ± 0.45	1.78 ± 0.62	0.78

BI, bleeding index; Non-SUP, Non-suppuration group; PD, probing depth; SUP, suppuration group.

Chao1 (Figure 1A) and Shannon (Figure 1B), respectively. Microbial diversity by Shannon did not show a significant difference between implants with and without clinical signs of suppuration ($P > 0.05$), whereas microbial richness by Chao1 were significantly higher in group non-SUP compared with group SUP ($P < 0.05$).

PCA based on UniFrac distance measurements was used to analyze the difference in microbial composition between implants with and without clinical signs of suppuration. The result demonstrated that peri-implant sites with suppuration harbored distinct submucosal microbiome from sites without suppuration ($P < 0.05$) (Figure 1C). ANOSIM analysis showed that intergroup difference was significantly higher than intragroup difference ($P = 0.044$, $R = 0.113$, Figure 1D), which confirmed the significant differences in microbial composition between SUP group and Non-SUP group.

3.2 | Peri-implant mucositis sites with suppuration harbor more pathogenic taxa than sites without suppuration

In the barplot, the phylum of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Fusobacteria* accounted for the main part of submucosal microbiome with relative abundances of 85.57% (SUP group) and 82.16% (Non-SUP group) (Figure 2A). In the heatmap, the genus of *Fusobacterium*, *Yersinia*, and *Treponema* were predominant in submucosal microbiome with relative abundance > 5%, followed by genus of *Streptococcus*, *Porphyromonas*, *Prevotella*, *Leptotrichia*, and *Fretibacterium* with relative abundance > 3% (Figure 2B).

GLMM was used to compare the abundance of taxa at the phylum, class, order, family, and genus levels between SUP

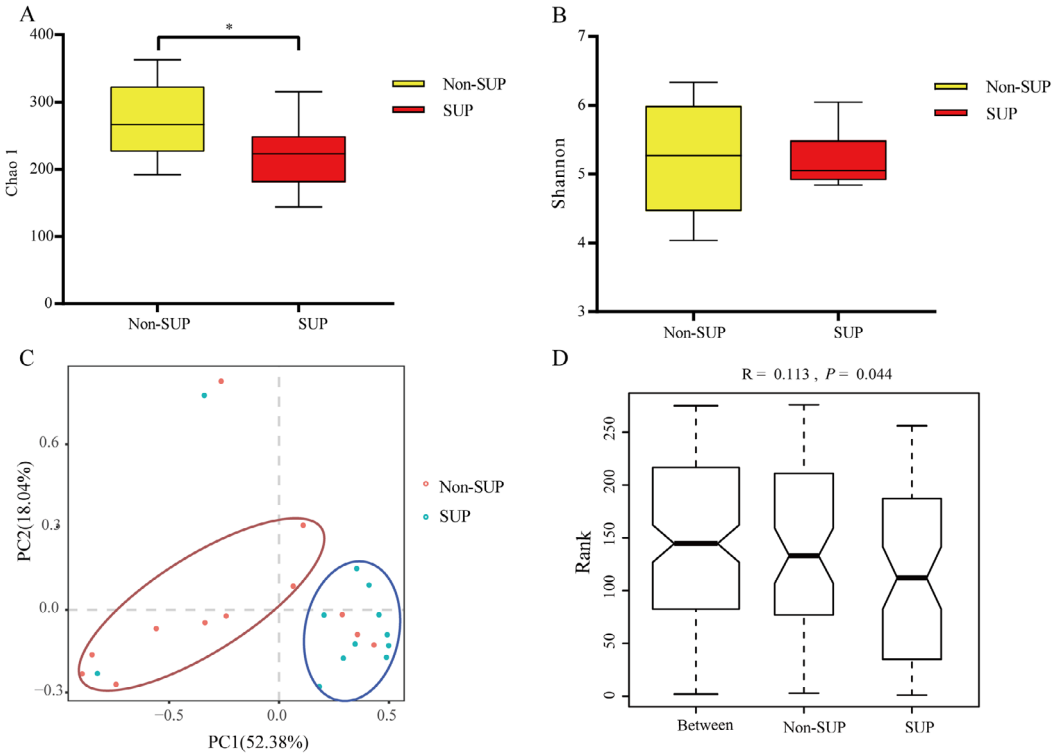

FIGURE 1 Comparisons of alpha diversity and beta diversity between peri-implant mucositis sites with or without suppuration. (**A**) Microbial richness between SUP group and Non-SUP group presented by Chao1, $^{*}P < 0.05$. (**B**) Microbial diversity between SUP group and Non-SUP group presented by Shannon. (**C**) Principle Component Analysis by Unifrac distance. (**D**) ANOSIM between SUP group and Non-SUP group. SUP, suppuration group; Non-SUP, Non-suppuration group

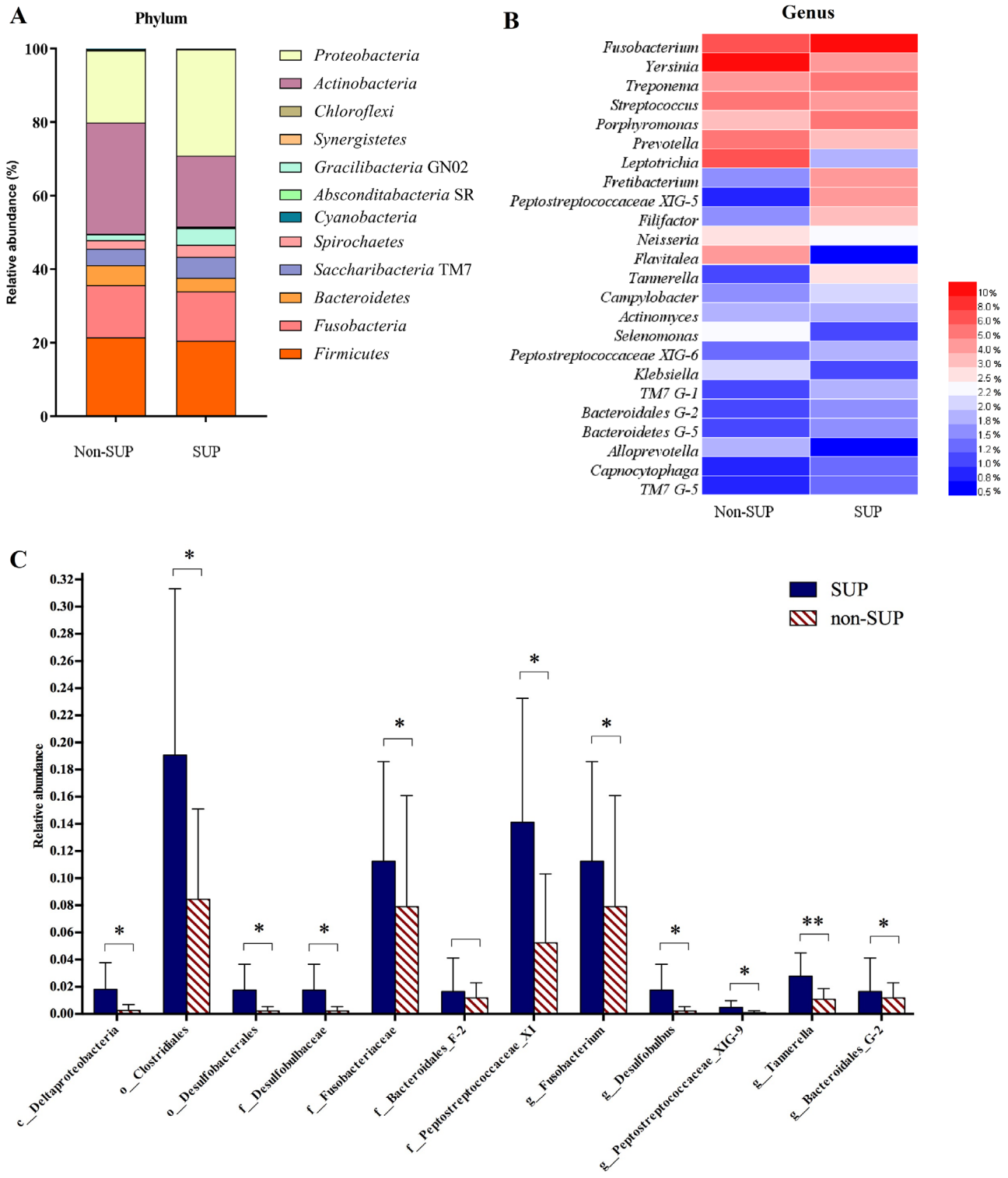

FIGURE 2 Composition and difference of sequences between peri-implant mucositis sites with or without suppuration. (**A**) Microbial composition at phylum level. (**B**) Core genera with relative abundance > 1%. (**C**) microbial differences between SUP group and Non-SUP group from phylum level to species level. $^{*}P < 0.05$, $^{**}P < 0.01$, tested by General linear mixed model

group and Non-SUP group after the correction of age, gender, location of implants, and multiple implants per patient. Some microbiota such as genera of *Fusobacterium*, *Desulfobulbus*, *Peptostreptococcaceae* XIG-9, *Tannerella*, *Bacteroidales* G-2, which were frequently detected at periodontitis and peri-implantitis sites, were significantly more abundant at SUP group compared with non-SUP group ($P < 0.05$) (Figure 2C).

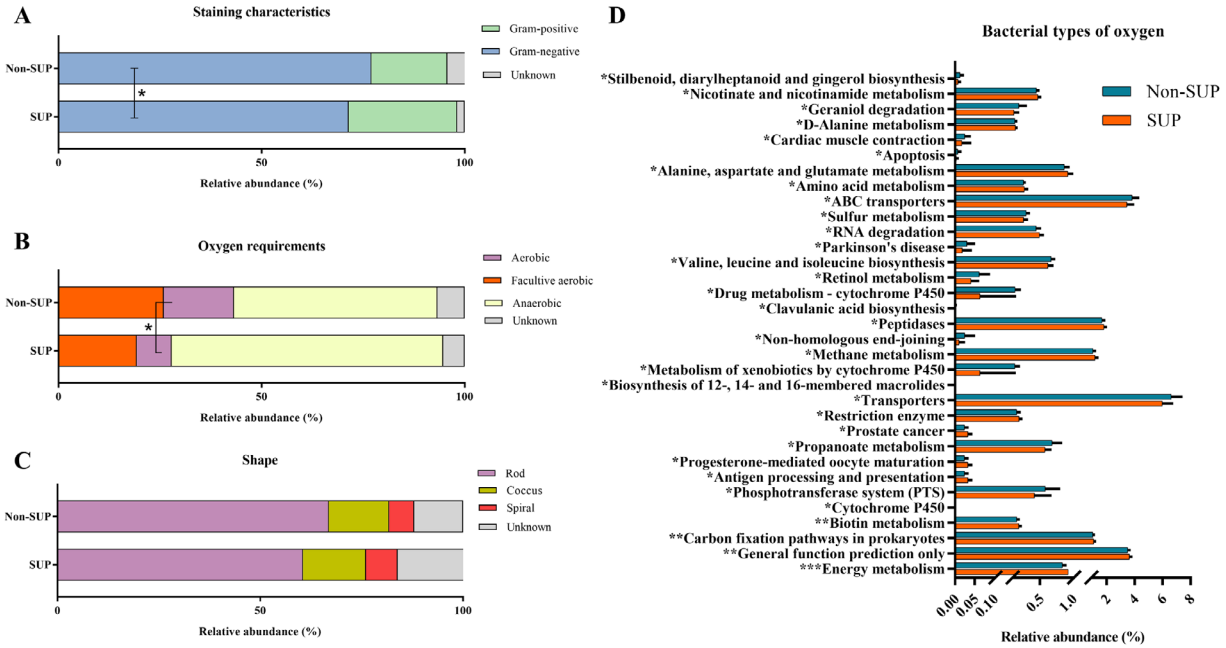

FIGURE 3 The comparisons of bacterial types and functional analysis. (**A**) Bacterial types by staining characteristics. (**B**) Bacterial types by morphotype. (**C**) Bacterial types by aerotolerance. (**D**) Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) based on Kyoto Encyclopedia of Genes and Genomes (KEGG).[32] SUP, suppuration group; Non-SUP, Non-suppuration group. * $P < 0.05$. ** $P < 0.01$

3.3 | The difference in bacterial types and metabolism between peri-implant sites with or without suppuration

The comparison of staining characteristics showed that Gram-positive bacteria were significantly less abundant ($P < 0.05$), whereas Gram-negative bacteria were more abundant ($P > 0.05$) in SUP group than Non-SUP group (Figure 3A). Difference in oxygen requirement was also found among groups, which appeared as significantly lower abundance of aerobic bacteria in SUP group ($P < 0.05$) (Figure 3B). Anaerobic bacteria and spiral-shaped bacteria were also more abundant in SUP group than Non-SUP group, although there were no significant differences (Figure 3B–3C).

Functional analysis showed that SUP group harbored more pathways related to prosperous life activity than Non-SUP group, such as energy metabolism and amino acid metabolism. These pathways might contribute to the colonization and reproduction of bacteria. Although Non-SUP group demonstrated more advanced metabolic pathways as well as apoptosis, which might cause less tissue destruction (Figure 3D).

3.4 | The potential interaction and symbiotic relationship among taxa

Co-occurrence networks of submucosal community was performed with core bacteria (relative abundance > 0.5%) to show the potential interaction and symbiotic relationship (Figure 4). Comparing Non-SUP group, the network of SUP group was slightly more complex and disordered. Besides, some pathogenic genera, such as *Porphyromonas*, *Treponema*, and *Tannerella*, showed more robust relationship among taxa in SUP group than Non-SUP group.

3.5 | The classification model of microbiota to distinguish the clinical conditions with or without suppuration

To define the characteristics of submucosal microbiome at peri-implant sites with or without suppuration, we constructed a classification model with Canonical Discriminate Function based on the Fisher method. It screened out 12 genera and the formulas were as follows:

Non-SUP group:

$$= 3446\,G1 + 48621\,G2 - 415261\,G3 + 981726\,G4 - 116036\,G5 - 106877\,G6 + 2161\,G7 - 1731\,G8 + 1858\,G9 + 10216\,G10 - 912588\,G11 + 3251561\,G12 - 404$$

SUP group:

$$= 147828\,G1 + 134620\,G2 - 1156706\,G3 + 2704652\,G4 - 325562\,G5 - 297696\,G6 + 5899\,G7 - 4999\,G8 + 5019\,G9 + 285863\,G10 - 2537674\,G11 + 9034524\,G12 - 3058$$

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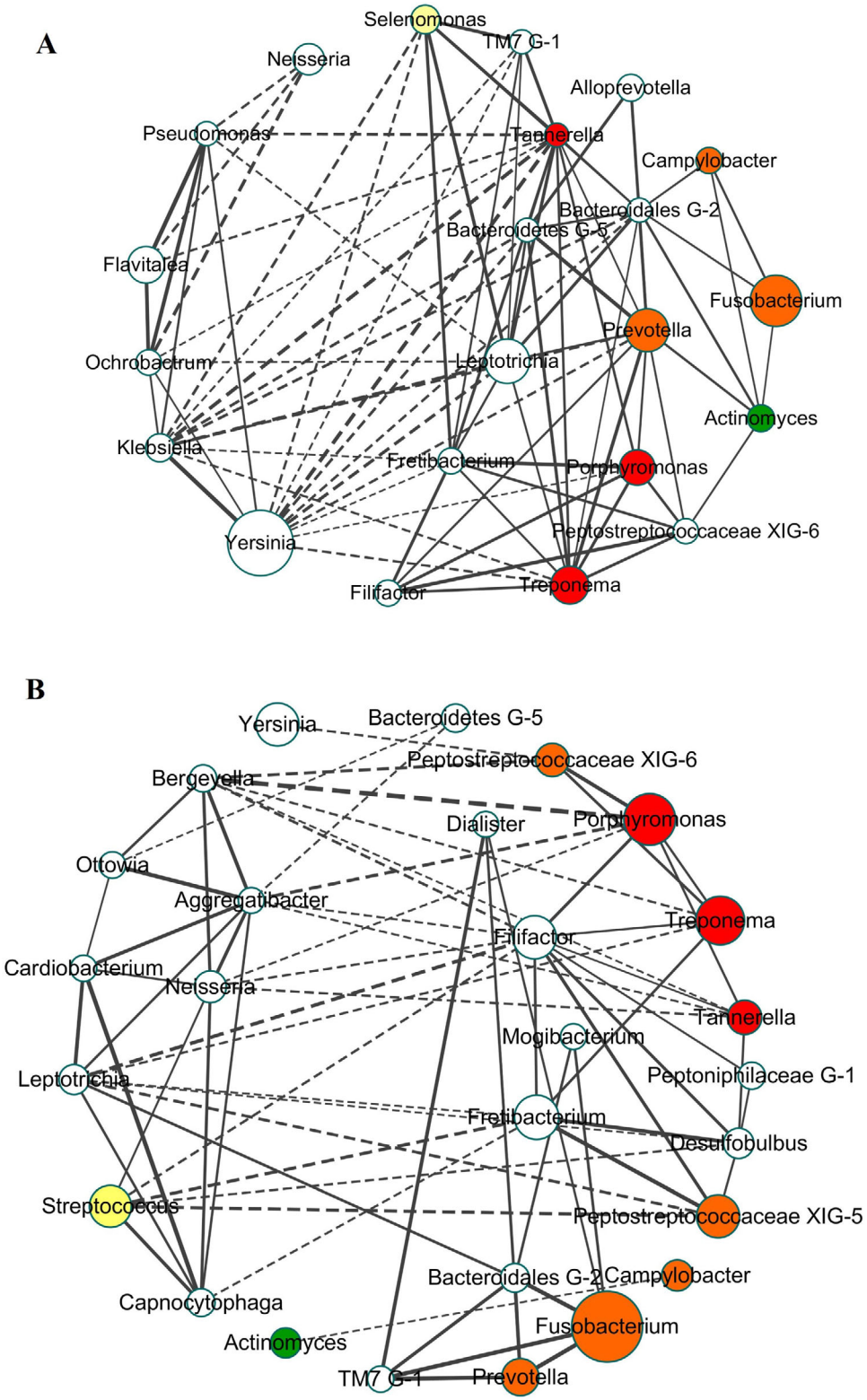

FIGURE 4 The co-occurrence network of core genera. The co-occurrence network of core genera (relative abundance > 1%) tested by Spearman correlation with $P < 0.05\%$. (**A**) Non-SUP group. (**B**) SUP group. The size of the nodes was determined by relative abundance. The thickness of the edges was determined by the correlation coefficient. The color of the nodes was determined by complexes to which they belonged to, as described by Socransky.[33] SUP, suppuration group; Non-SUP, Non-suppuration group

(G1, *Tannerella*. G2, *Aggregatibacter*. G3, *Arsenicicoccus*. G4, *Eggerthella*. G5, *Lactobacillus*. G6, *Mollicutes* G-1. G7, *Yersinia*. G8, *Bacteroidales* G-2. G9, *Bacteroidetes* G-5. G10, *Bulleidia*. G11, TM7 G-6. G12, *Caulobacter*).

For an unknown sample, the relative abundance of 12 genera (biomarkers) included should be calculated with the formulas and get a value. The sample belongs to the group of which got the larger value when tested by the formulas. With the generated discriminant value, submucosal microbiome was completely divided with an accuracy of 100% validated by receptor operative curve (ROC).

4 | DISCUSSION

This is the first study to investigate microbiome at peri-implant mucositis sites with and without clinical signs of suppuration using 16S rRNA gene sequence analysis. Sites with suppuration demonstrated a more disordered microbiome than sites without suppuration, with lower microbial richness, more pathogenic microbiota, and more disordered structure. The higher pathogenicity of microbiome at per-implant mucositis sites with suppuration might increase the risk of the transition from peri-implant mucositis to peri-implantitis, which could help us to understand the underlining mechanism of the strong association between suppuration and bone loss.[10,11]

Peri-implant mucositis sites with suppuration showed distinct microbiome from peri-implant mucositis sites without suppuration, characterized by more pathogenic bacteria such as genera of *Fusobacterium* and *Tannerella*, which are strongly associated with peri-implantitis confirmed by several studies.[20,35,36] *Fusobacterium* serves as bridge in biofilm, providing condition for colonization of subsequent pathogenic bacteria.[37] Research has indicated that the submucosal presence of *Fusobacterium* was associated with peri-implant disease status.[38] *Tannerella* was also identified as an important promoting factor in peri-implantitis development in several studies.[38,39] Besides, biofilms at peri-implantitis sites have been reported to be dominated by Gram-negative anaerobic rods with a proteolytic metabolism such as *Fusobacterium* and *Tannerella*.[40] In this study, the reduced percentage of Gram-positive bacteria and aerobic bacteria at peri-implant mucositis sites with suppuration indicated higher pathogenicity of submucosal microbiome.[41] In addition, peri-implant mucositis sites with suppuration showed a more disordered structure with excessive proliferation of some pathogenic bacteria, which might result in the dysbiosis of submucosal community.[42]

Although there were several studies comparing bacteria in sites with different conditions, studies providing global profile in sites with peri-implant mucositis were very limited. The dominating genera at peri-implant mucositis sites in current study were in agreement with previous studies, including *Streptococcus*, *Prevotella*, *Fusobacterium*, *Leptotrichia*, *Actinomyces*, and etc.[43,44] Besides, the proportions of coccus and rod bacteria analyzed in our study were about 60% and 20% respectively, which accords previous observation by culture.[45] However, most of the previous studies lack the description of suppuration. When comparing with periodontal sites with suppuration, peri-implant sites with suppuration showed more gram-negative, anaerobic, and rod-shape bacteria (55% versus 71%; 45% versus 67%; 42% versus 61%).[9] High prevalence of putative periodontal pathogens, including *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Bacteroides forsythus*, were found in periodontal sites with suppuration,[9,46] which was basically consistent with our finding at peri-implant sites with suppuration.

In the current study, *Peptostreptococcaceae* were observed with significantly higher relative abundance at suppurative peri-implant mucositis sites. Species of *Peptostreptococcus* are part of the human oral microbiome, which have been shown to be closely correlated with periodontal diseases and have been frequently observed at peri-implant diseases sites.[21,22,47,48] *Peptostreptococcus spp.* are symbiotic bacteria in humans that can cause abscesses and necrotizing soft tissue infections.[49] The role of *Peptostreptococcaceae* in the presence of suppuration at peri-implant disease sites is noteworthy and further study is needed to confirm our finding.

Every ecological type has the capability to maintain health or elicit disease. The current results screened out 12 biomarkers, such as the genus of *Tannerella*, *Aggregatibacter*, *Bacteroidales* G-2, *Bacteroidetes* G-5, to build a classification model. It could help to distinguish the microbiome of SUP group and Non-SUP with a high accuracy. It's reported that sites with suppuration might be an indication of active tissue breakdown.[9,10,11] Therefore, the classification model could help us to distinguish the two ecological types associated with active progression of inflammation. Indeed, the classifier would require validation in independent and larger population before application on a large scale. Further study could consider microbiota-targeted treatment to hinder the progress of peri-implant diseases.

It is established that clinical presence of suppuration is associated with increased susceptibility of bone loss by threefold.[10,11] The higher pathogenicity and disordered community associated with suppuration presented in this study could give an explanation to the increased risk of aggravating destruction. Therefore, it is possible that suppuration is a sign of active progression of inflammation, and peri-implant mucositis sites with suppuration are more prone to progress into peri-implantitis. Notably, one of the implants in the current study with the presence of suppuration progressed into peri-implantitis at 5 years after crown rehabilitation and was finally

lost 8 years after crown rehabilitation. The data in our study may have some implications for future therapeutic strategies of peri-implant diseases. Firstly, implants with clinical signs of suppuration may be a sign of inflammation progression. Secondly, the microbial composition of implants with clinical signs of suppuration should be considered when the antimicrobial therapy is performed. It is of great clinical significance to hinder the progression from peri-implant mucositis to peri-implantitis because the prognosis of peri-implantitis is uncertain, and if left untreated, it could ultimately lead to implant failure.

This is the first study to evaluate the submucosal microbiome of suppuration in peri-implant mucositis, which supports early-stage suppuration as an important indicator of the progress of peri-implant diseases because of higher microbial risk. Furthermore, this study recorded the long-term clinical outcome, which could confirm suppuration as a reminder for subsequent peri-implantitis, even implant failure. The potential limitation might be that more than one implants from one patient were recruited in this study. However, GLMM was applied to adjust the confounding factor, and made the results more reliable. Further studies are needed to clarify the prediction effect of suppuration for peri-implantitis and implant failure. Besides, it should be considered to evaluate the clinical and microbiological outcome of clinical intervention at peri-implant mucositis sites with suppuration.

5 | CONCLUSIONS

Significant differences were observed in the microbial composition between peri-implant mucositis sites with and without suppuration. Sites with suppuration showed a more disordered microbiome with lower microbial richness, significantly higher pathogenic taxa, more disorder structure than sites without suppuration. It supports that suppuration is an important clinical indicator for higher microbial risk and appropriate treatment should be performed against biofilm to prevent progression of peri-implant disease. Besides, classification model with microbiota has a high accuracy in distinguishing peri-implant mucositis sites with or without suppuration.

AUTHOR CONTRIBUTIONS

QW and HL analysed the data and wrote the manuscript. LZ designed the clinical trial and did clinical examination. BZ and XY performed clinical examination and laboratory assays. HM designed the clinical trial, performed clinical intervention and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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SUPPORTING INFORMATION

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

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