Journal of Immunology Research

Oral Microbiota and Immunology

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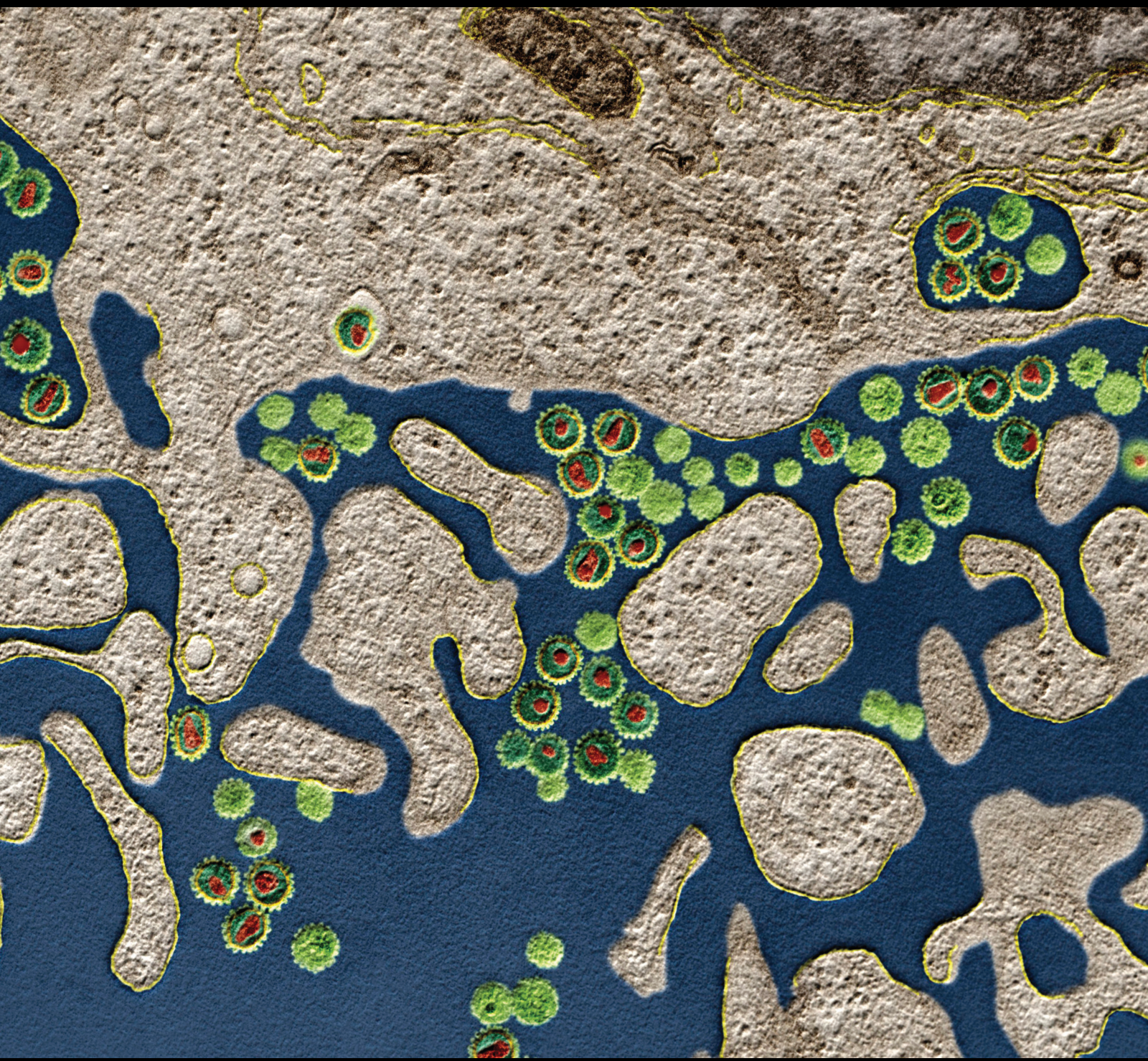

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Contents

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Research Article

Gender Variations in the Oral Microbiomes of Elderly Patients with Initial Periodontitis

Jie Zhao,[1] Ying-Hui Zhou,[2] Ya-Qiong Zhao,[1] Yao Feng,[1] Fei Yan,[3] Zheng-Rong Gao,[1] Qin Ye,[1] Yun Chen,[1] Qiong Liu,[1] Li Tan,[1] Shao-Hui Zhang,[1] Jing Hu,[1] Marie Aimee Dusenge,[1] Yun-Zhi Feng,[1] and Yue Guo[1]

[1]*Department of Stomatology, The Second Xiangya Hospital of Central South University, Changsha, 410011 Hunan, China*
[2]*National Clinical Research Center for Metabolic Diseases, Hunan Provincial Key Laboratory of Metabolic Bone Diseases, Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha, 410011 Hunan, China*
[3]*Hunan Key Laboratory of Oral Health Research & Hunan 3D Printing Engineering Research Center of Oral Care & Hunan Clinical Research Center of Oral Major Diseases and Oral Health & Xiangya Stomatological Hospital & Xiangya School of Stomatology, Central South University, Changsha 410008, China*

Correspondence should be addressed to Yun-Zhi Feng; fengyunzhi001@csu.edu.cn and Yue Guo; guoyue@csu.edu.cn

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Periodontitis is a globally prevalent disease that imposes a functional and aesthetic burden on patients. The oral microbiome influences human health. The aim of this study was at assessing gender variation in the subgingival bacterial microbiome of elderly patients with initial periodontitis and to determine the causes of this variation. Twelve males and twenty females (range 50–68 years old) with initial periodontitis provided subgingival plaque samples. 16S rRNA gene sequencing, QIIME-based data processing, and statistical analyses were carried out using several different analytical approaches to detect differences in the oral microbiome between the two groups. Males had higher Chao1 index, observed species, and phylogenetic diversity whole tree values than females. Analysis of β-diversity indicated that the samples were reasonably divided by the gender. The linear discriminant analysis effect size showed that the most representative biomarkers were the genus *Haemophilus* in males, whereas the dominant bacteria in females were *Campylobacter*. Kyoto Encyclopedia of Genes and Genomes analysis showed that predicting changes in the female oral microbiota may be related to the immune system and immune system diseases are the main factor in males. These data suggest that gender may be a differentiating factor in the microbial composition of subgingival plaques in elderly patients with initial periodontitis. These results could deepen our understanding of the role of gender in the oral microbiota present during initial periodontitis.

1. Introduction

The human oral cavity is composed of unique niches colonized by a variety of microorganisms, including bacteria, fungi, viruses, and archaea, which create a rich microbial community known as the oral microbiome [1]. These communities of microbes are highly dynamic and responsible for various functions that can both maintain and deplete oral health. Oral microbiomes often exist in a homeostatic equilibrium with the host, but under certain conditions, this equilibrium is disturbed. Oral microbiome dysbiosis can cause a wide range of systemic illnesses, including diabetes [2], cancer [3], Alzheimer disease [4], rheumatoid arthritis [5], and cardiovascular disease [6]. For example, a higher pancreatic cancer risk has been associated with the enrichment of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [7]. Furthermore, oral *P. gingivalis* infection in mice results in brain colonization of an Alzheimer's disease model and increased production of Aβ1-42, a component of amyloid plaques [8]. Oral microbiome

dysbiosis also directly affects oral health, potentially causing periodontitis and dental caries, two of the most prevalent and typical microbially induced disorders worldwide. Such periodontal disease will also affect systemic health, including the immune system [9] and digestive system [10].

Age correlates with the severity of periodontal disease and dental caries. Because of the decline in immune system function and low salivary flow rates brought on by the natural advancement of age, there is a clinical increase in the prevalence and severity of periodontitis and dental caries in older adults. Age is also an important factor in oral microbiome dysbiosis. Rodenburg et al. found that the prevalence of subjects colonized by *A. actinomycetemcomitans* appeared to be age related, as it decreased with increasing age [11]. In addition, the incidence and severity seem to have a gender predilection with the incidence and severity of periodontal disease appearing to be greater in males than in females [12, 13]. Lira-Junior et al. found that male gender presented higher counts of 16 bacteria in saliva than female gender, which may be related to the slightly worse periodontal condition in male than in female [13]. Furthermore, the NHANES III survey has demonstrated, in most age groups, a higher prevalence and greater extent of attachment loss, as well as a higher prevalence of periodontitis and deeper probing depths in males compared to females [14]. However, females have been shown to be more susceptible to caries [15]. Thus, gender can logically be assumed to be an important factor affecting the oral microbiome.

The immunological response produced by the oral microbiome is important and complex. Tissue homeostasis is maintained by innate immunity, which prevents destruction of the periodontal tissue. Severe periodontitis is characterized by neutropenia, agranulocytosis, neutrophil adhesion, deficiencies in chemotaxis, and diseases affecting lysosomal degranulation [16]. The cellular infiltrate in human gingivitis is primarily composed of T helper (Th) cells. The phenotypes of Th cells are directed by phagocytic antigen-presenting cells, including dendritic cells (DCs) and Langerhans cells [17]. The immune response in the gingiva, during an undefined transition, switches from neutrophil recruitment and activation to pathogenic bacteria to chronic infiltration of T and B cells and plasma cells [18] that induces vascular prolifera and the destruction of connective tissue. At the same time, sex steroids are related to the immune system. Youssef and Stashenko indicated that estrogen directly stimulates IL-1 production by macrophages, leading to heightened immune responses and the resistance of females to disseminating dentoalveolar infections [19]. In contrast, androgen has been reported to play a suppressive role in the immune response and to act as a potential promoter of tumor growth and protector from autoimmune diseases [20]. Androgen-deficient male C57BL/6 mice were shown to be significantly more susceptible to endotoxic shock, possibly due to the significantly higher expression of surface TLR4 on macrophages [21].

Therefore, the aim of the present study was to assess gender variation in the subgingival bacteria microbiome of elderly patients with initial periodontitis and determine the causes of this variation. Specifically, we examined whether bacterial taxa were differentially abundant, whether gender variation exists in the α- and β-diversity using high-throughput sequencing data, and whether the variation is related to the immune system.

2. Materials and Methods

2.1. Study Population and Sample Data. All participants were recruited from The Second Xiangya Hospital of Central South University in Changsha, China. The Ethics Committee of The Second Xiangya Hospital of Central South University provided ethics and regulatory approval. Verbal and written informed consent were obtained from all participants. The clinical trial registration number is ChiCTR2100046828.

The participants included in this study were 50 to 68 years old [22–24], with ≥15 teeth present and at least one natural tooth in all six sections. Exclusion criteria included smoking, infection, trauma or recent surgery, pregnancy, systemic disease (e.g., heart failure, autoimmune disease, musculoskeletal disorders, and malignancy), the use of antibiotics or immunomodulators in the previous 30 days, periodontal treatment in the previous 6 months, or oral disease (e.g., oral lichen planus, ulcer, oral leukoplakia, and pharyngitis). Questionnaires were used to obtain data from all patients who agreed to serve as subgingival plaque donors. We enrolled 32 patients with initial (stage I) periodontitis, including 12 males and 20 females. Before collecting samples, sites were air dried. The stage of periodontal disease was determined according to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions "Staging and grading of periodontitis: Framework and proposal of a new classification and case definition" [25]. The clinical attachment level (CAL) of the most severe site was recorded for each tooth; 1–2 mm was defined as stage I, 3–4 mm as stage II, and ≥5 mm as stage III. The number of missing teeth was also staged, with no missing teeth indicating stages I and II, ≤4 teeth missing stage III, and ≥5 teeth missing stage IV. Notably, the reasons for the missing teeth were not considered. The complexity of management was evaluated by reclassifying stage II patients as stage III if the maximum probing pocket depth (PPD) was ≥6 mm, and stage III patients as stage IV if <20 teeth or 10 opposing pairs remained. The subgingival plaques of the four first molars were scraped at the bottom of the periodontal pocket using a sterile Gracey scraper. The samples were transferred to PBS, immediately frozen, and maintained at −80°C.

2.2. DNA Extraction and Sequencing of the 16S rRNA V3-V4 Region. Based on the manufacturer's instructions, total genomic DNA was extracted using a DNA Extraction Kit (DNeasy PowerSoil Kit, QIAGEN). The extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the quality confirmed by agarose gel electrophoresis. The universal PCR primers 343 F (5′-TACGGRAGGCAGCAG-3′) and 798 R (5′-AGGGTATCTAATCCT-3′) were designed to amplify the V3-V4 variable regions of bacterial 16S rRNA genes. 16S rRNA gene sequencing was completed by

OEBiotech (Shanghai, China). PCR was performed using the following program: initial denaturation at 94°C for 5 min, 26 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 20 s, and a final extension at 72°C for 5 min. Amplicons were purified using AMPure XP beads (Agencourt, Beckman Coulter, USA) and quantified using the Qubit dsDNA Assay Kit (Life Technologies, USA) following the manufacturers' instructions. Purified amplicons were pooled for sequencing. The raw reads were generated by Illumina MiSeq (V1.9.1), and the sequences were processed and analyzed in QIIME (v1.9.1). Briefly, the effective sequences were clustered to the same OTUs with ≥97% identity using VSEARCH (v.2.4.2) [26]. Next, the annotation information of representative sequences of each OTU was analyzed using the RDP classifier Naive Bayesian method [27] and the SILVA database.

2.3. Bioinformatics and Statistical Analysis. The α-diversity indices (Chao, Shannon, Simpson, Good's coverage, and phylogenetic diversity (PD) index) were calculated at 97% identity by the Wilcoxon rank-sum test. Samples from different groups were compared by Student's *t*-test and the Kruskal-Wallis test. Beta diversity analysis was performed by principal coordinates analysis (PCoA) based on unweighted UniFrac distances at the OTU level. Principal component analysis (PCA) was also conducted. The analysis of nonmetric multidimensional scaling (NMDS) based on weighted UniFrac distances was conducted to compare different groups. The relative abundance of predominant bacteria was compared between different groups using the Wilcoxon rank-sum test. The core microbiome was defined at the species level using a Venn diagram. We performed linear discriminant analysis (LDA) of effect size (LEfSe) to define biomarkers in the four groups. The logarithmic LDA score threshold for distinguishing features was 2.0. The functional content from the16S rRNA gene sequences was predicted using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software [28] by linking taxonomic information to the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations of the reference genome. Differences were considered significant when $P < 0.05$. SPSS 25.0 software was used for statistical analyses.

3. Results

3.1. Characteristics of Selected Patients and OTU Basic Analysis. Patient age ($n = 32$) ranged from 50 to 68 years. The male patients had a mean age of 54 years (range 51–60 years) and female patients 58.6 years (range 50–68 years). There was no statistically significant difference in age between male and female ($P > 0.05$, Table 1). The Venn diagram of differences in the OTUs (Figure 1(a)) showed that males and females had 3477 common OTUs. However, 1642 OTUs were higher in females and 1165 in males.

3.2. Diversity Analyses. Alpha diversity reflects the abundance and diversity of microbial communities. The Chao1 index and observed species were used to calculate community richness, whereas the Shannon and Simpson indices were able to evaluate community diversity. Compared to females, males had a significantly higher number and/or diversity of taxa based on the Chao1 index ($P < 0.01$, Figure 1(b)) and observed species ($P < 0.01$, Figure 1(c)). No significant difference was apparent between the two groups in the Shannon (6.37 versus 6.56, Figure 1(d)) and Simpson (0.96 versus 0.97, Figure 1(e)) indices. Higher Good's coverage index values were associated with a higher probability of species being measured in the sample. Although Good's coverage was significantly different between the two groups ($P < 0.01$), both groups reached 0.99 for the depth of sequence representing the majority of bacterial species in the plaque samples. The PD whole tree index reflects the relationships of species within the community and was found to decrease in females compared to males (Figure 1(g)).

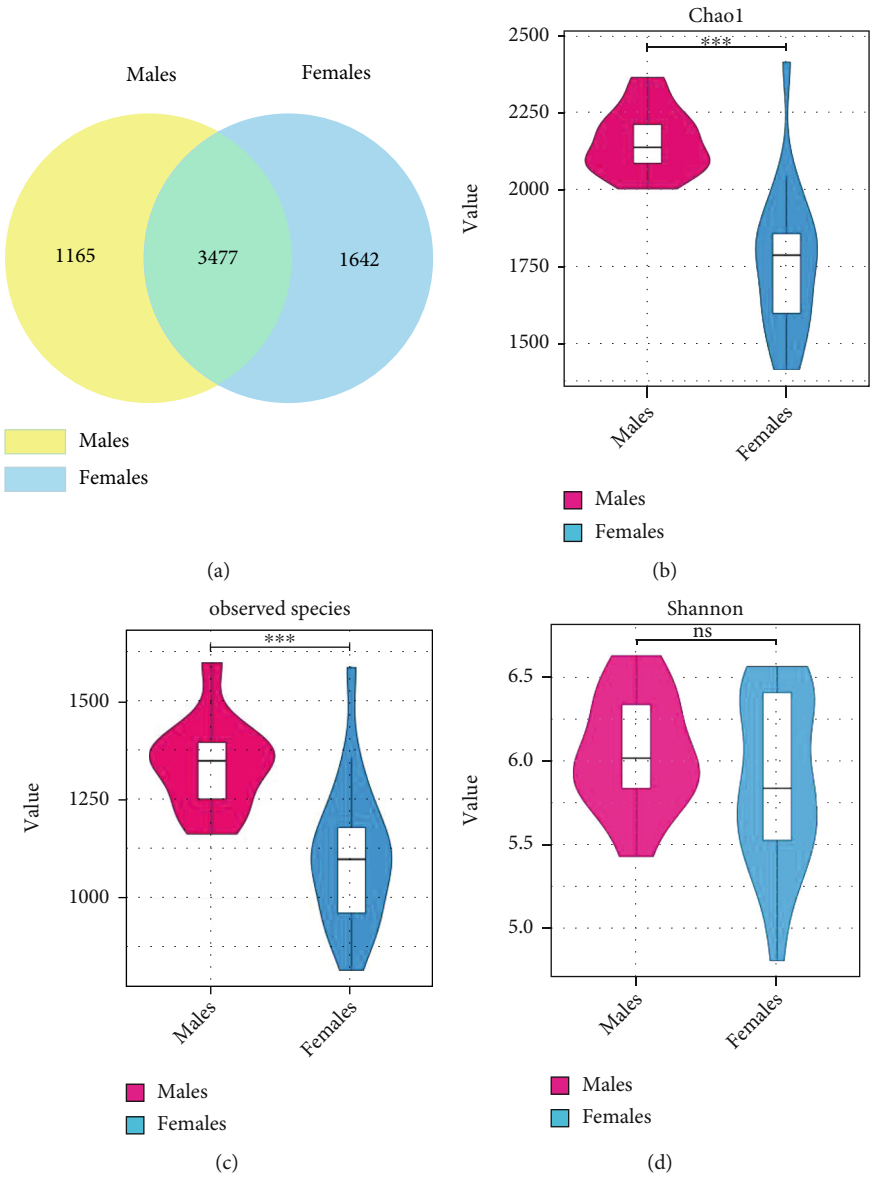
Table 1: Age information of the enrolled participants.

Group	Number	Age (year)
Group 1	12	54.00 ± 3.54
Group 2	20	58.55 ± 7.45
P		>0.05

Beta diversity refers to visualization of the differences in the diversity of microbiota in the two groups. The PCA (Figure 2(a)) and PCoA (Figure 2(b)) indicated the extent of the difference in the spatial distance, especially in PCoA analysis based on unweighted UniFrac distance. In NMDS analysis based on the binary Jaccard distance, we found a significant spatial distance and value of stress < 0.2, representing certain explanatory significance in the two-dimensional point graph (Figure 2(c)). The male and female groups were far apart (Figure 2), indicating that the oral microbiotas in elderly with initial periodontitis vary by gender.

3.3. Composition of the Oral Microbiota. The relative abundance of the top 15 bacteria was assessed at the phylum level (Figure 3(a)) and genus level (Figure 3(b)). Multivariate analysis estimated further differences between the two groups and identified bacteria that differed significantly between them. A boxplot shows the top 3 different species at the phylum (Figure 3(c)) and top 10 different species at the genus (Figure 3(d)) levels. Compared to females, the relative abundance of cyanobacteria at the phylum level increased in males, whereas the relative abundance of Epsilonbacteraeota and Fibrobacteres was decreased ($P < 0.05$; Figure 3(c)). At the genus level, females had higher relative abundance of *Campylobacter* than males ($P < 0.05$). However, the relative abundance of *Haemophilus*, *Bacteroides*, *Prevotellaceae_UCG-001*, *Clade_Ia*, *Acinetobacter*, *Ruminococcus_1*, *Prevotellaceae_NK3B31_group*, *Ruminococcaceae_UCG-005*, and *Parabacteroides* was lower in females than in males ($P < 0.05$; Figure 3(d)). Figure 4 shows the different species between the two groups in the form of a heat map.

We conducted LEfSe to identify the differential bacterial composition between the male and female patients and



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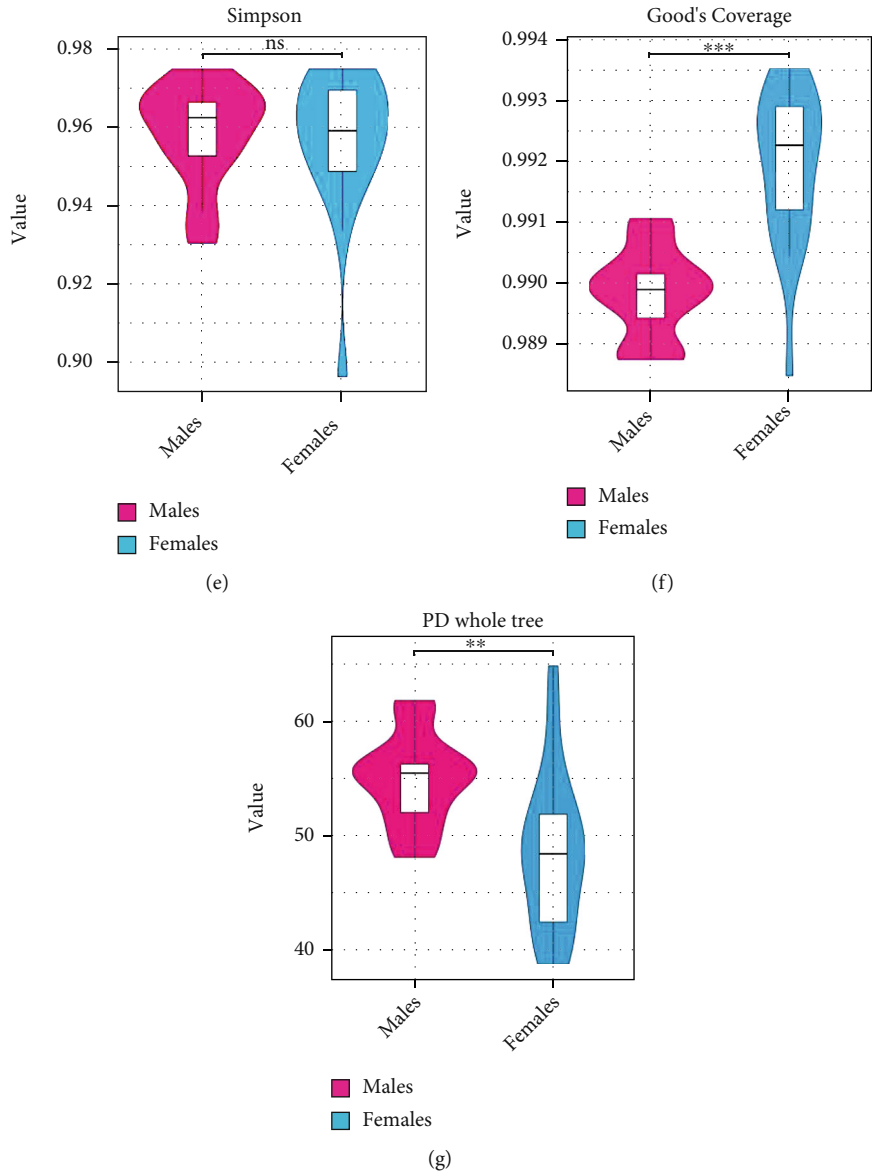


Figure 1: Venn diagram and α-diversity analysis of elderly males and females with stage 1 periodontitis. (a) Venn diagram based on operational taxonomic units (OTUs). (b–g) Violin plots comparing α-diversity indices (Chao1, observed species, Shannon index, Simpson index, Good's coverage, and PD whole tree) between males and females. $^{***}P < 0.001$; $^{**}P < 0.01$; ns: not significant.

screen for potential biomarkers. As stated above, significant differences in the microbiota were observed between males and females with initial periodontitis (Figure 5(a)). A cladogram showing the most discriminative bacterial clades identified by LEfSe is shown in Figure 5(b). The following species were more predominant in the supragingival plaques of men than women: the class Alphaproteobacteria, its order SAR11_clade or Rhodobacterales, its family Clade_I/Clade_II or Rhodobacteraceae, and its genus Clade_Ia/Ambiguous_tax, along with the family Muribaculaceae/Bacteroidaceae and the genus Prevotellaceae_UCG_001/Prevotellaceae_NK3B31_group. In males, genus *Haemophilus* had the largest LDA score. However, these results show the significantly higher level of the phylum Epsilonbacteraeota and the corresponding class Campylobacteria, order Campylobacterales, family Campylobacteraceae, and genus *Campylobacter*, in the subgingival microbiotas of females. The microbiotas of women were also enriched with genera mainly belonging to the Bacteroidetes phylum, particularly the F082 and Bacteroidales_BS11_gut_group family. In addition, the phylum Actinobacteria and its genus *Gardnerella*, along with the phylum Firmicutes and its genus Selenomonas_1, were high in females. The phylum Proteobacteria and its genus *Pelomonas* were also high in females. Detailed data and statistics are provided in Table 2.

3.4. Function Prediction. By linking the genomes to pathways via KEGG orthologue annotation, we determined differences in the estimated bacterial functional capabilities in the 12 males and 20 females and compared them using

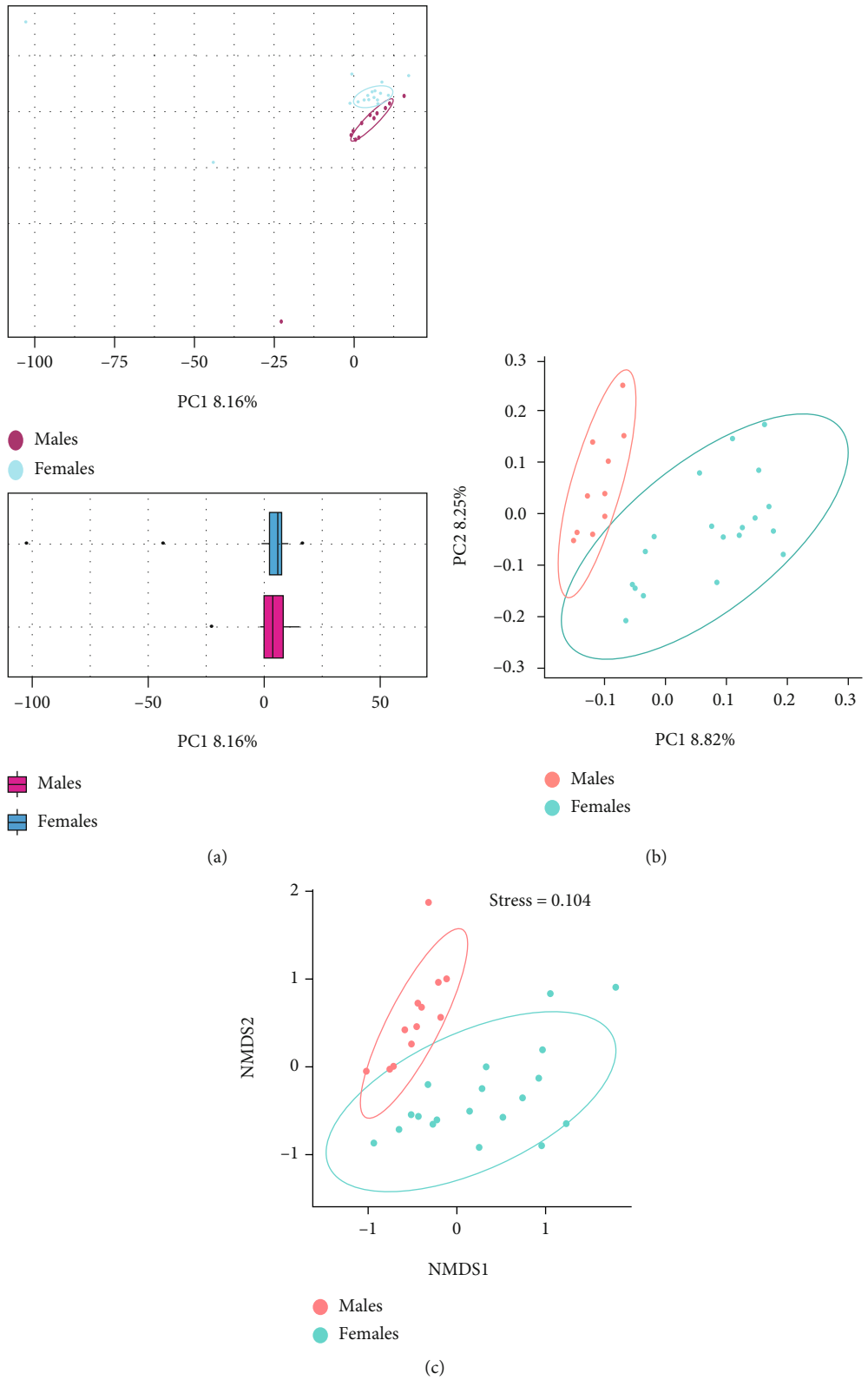


Figure 2: Similarity of microbial communities between elderly males and females with stage 1 periodontitis based on the unweighted UniFrac distance. (a) 2D diagram of principal component analysis (PCA). (b) 2D diagram of principal coordinate analysis. (c) 2D diagram of nonmetric multidimensional scale (NMDS) used to analyze the β-diversity of microbial communities between males and females.

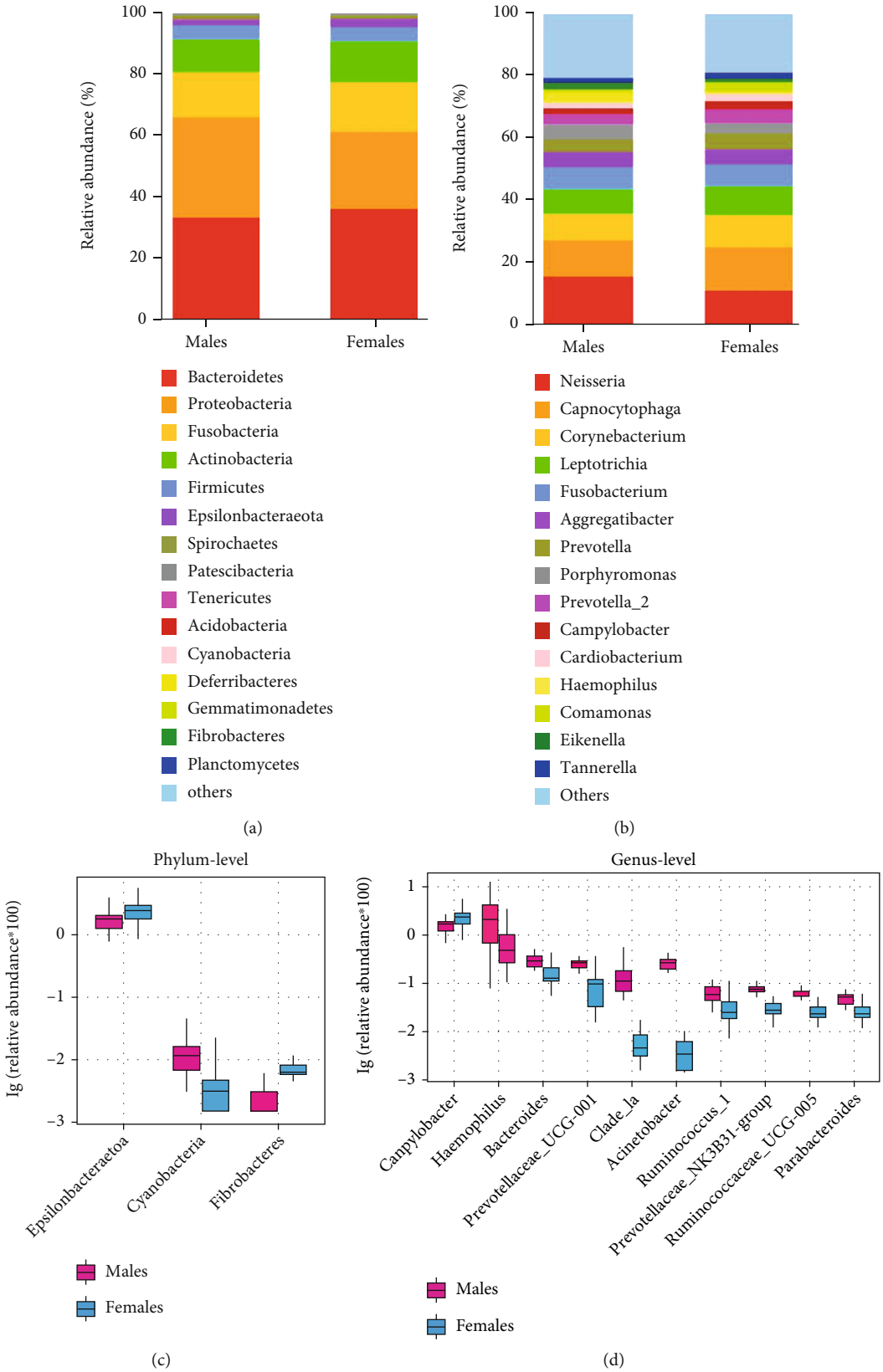


Figure 3: The oral microbiome composition of elderly males and females with stage 1 periodontitis. (a) Phylum level and (b) genus level composition. (c) Species difference analysis between males and females (group 2) by the Kruskal-Wallis test at the phylum and (d) genus levels.

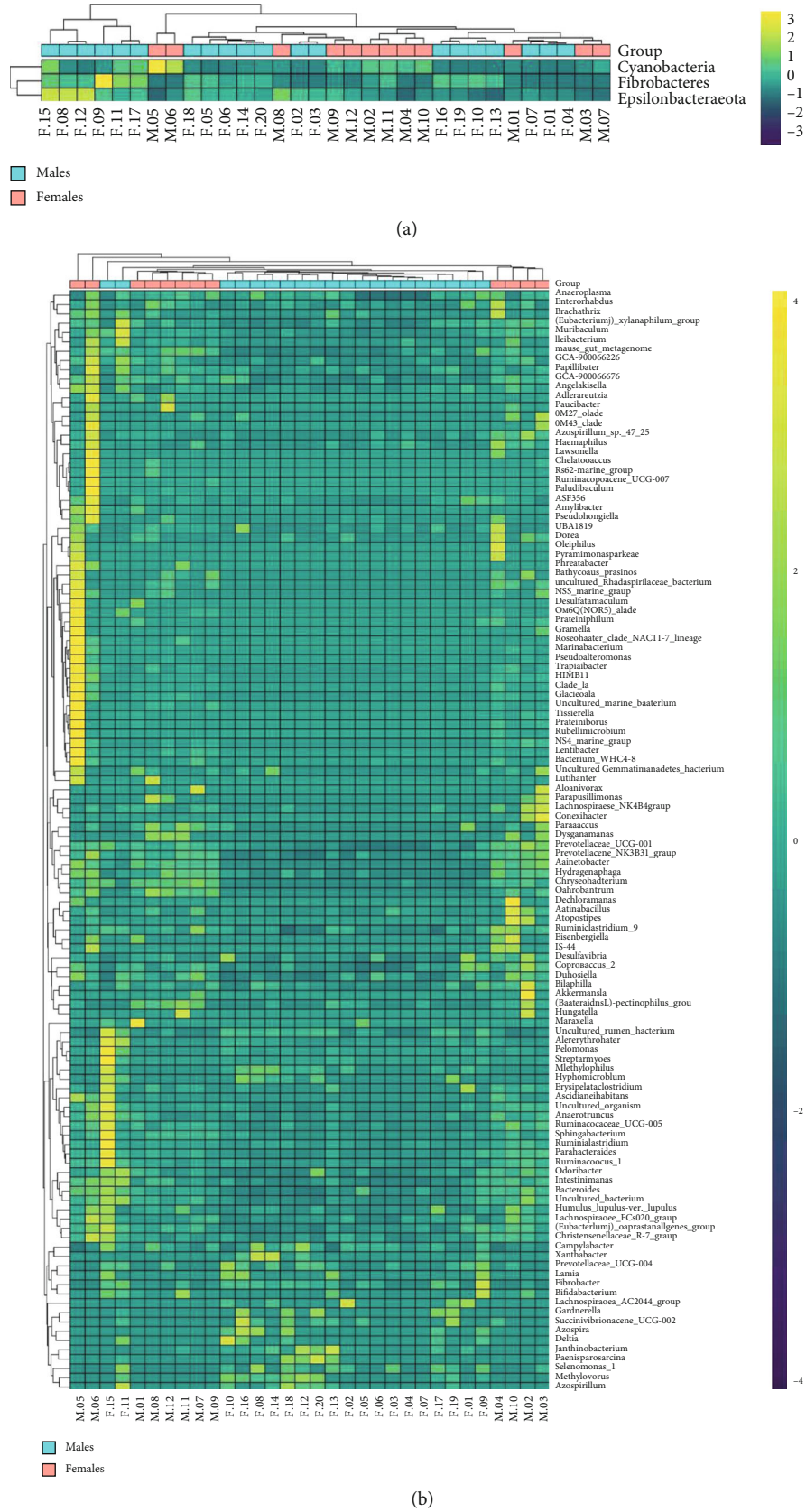
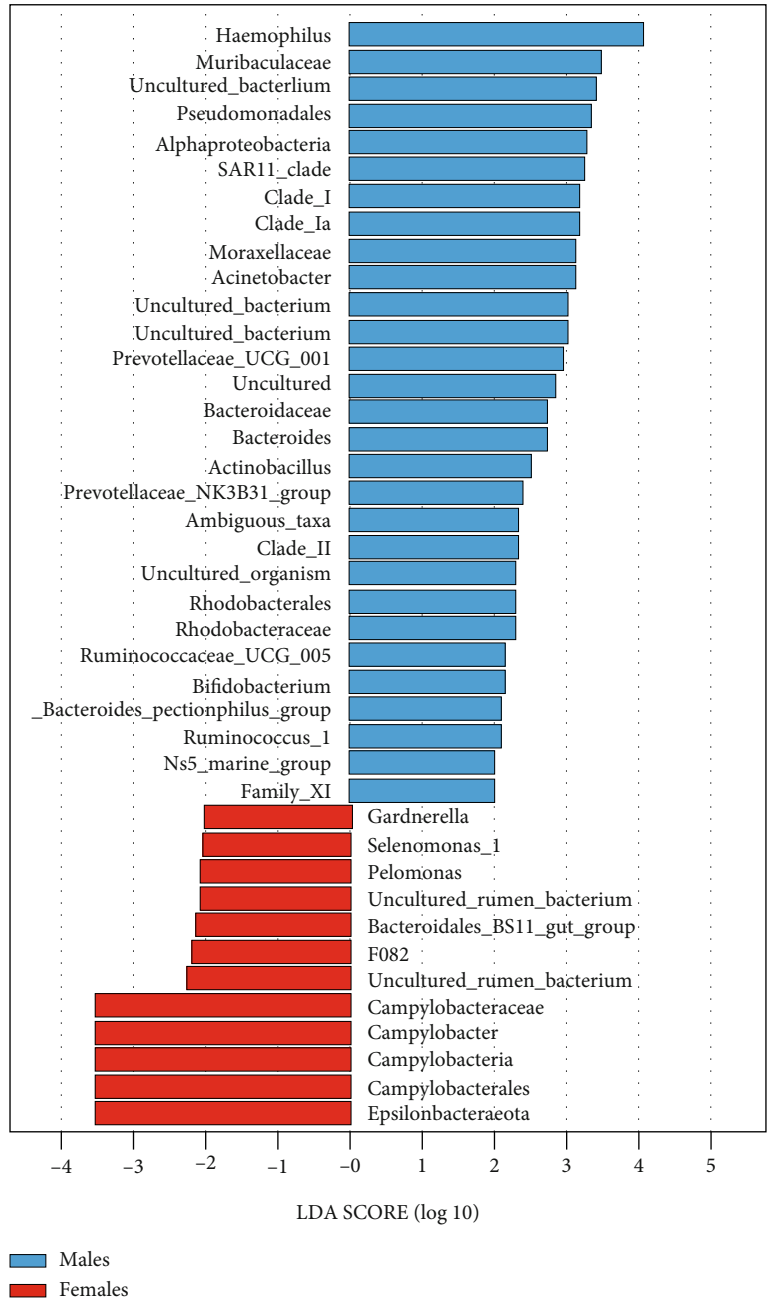


Figure 4: Heat map of differential oral microbiomes between elderly males and females with stage 1 periodontitis. (a) Phylum level. (b) Genus level. The sample information (group and number) and species annotation information are displayed on the horizontal axis and vertical axes, respectively. Colors indicate the Spearman rank correlation.



(a)

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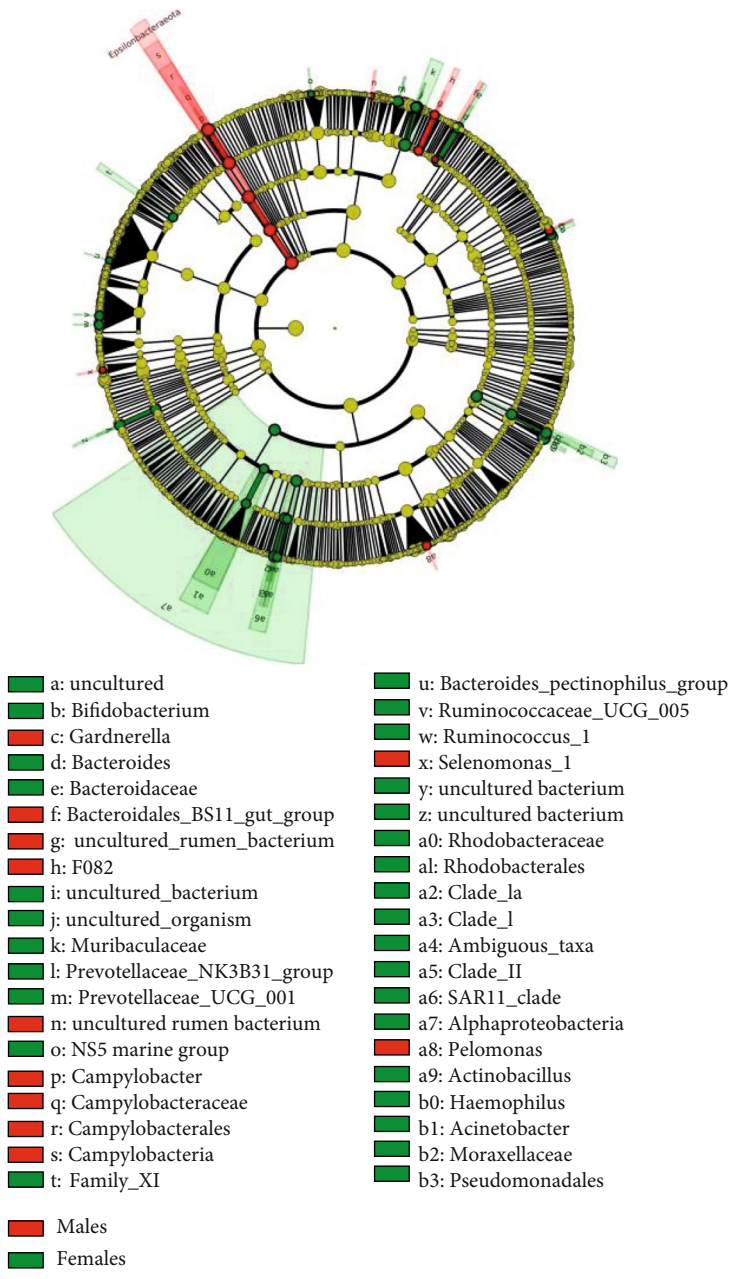


(b)

Figure 5: Different rich taxa between elderly males and females with stage 1 periodontitis analyzed by the linear discriminant analysis effect size. (a) Histogram of the LDA scores. (b) The phylogenetic tree in the form of a cladogram.

QIIME2 and PICRUSt2. At the phylum level, the relative proportions of functions associated with immune system diseases, neurodegenerative diseases, the circulatory system, and cardiovascular diseases appeared to increase in male patients. However, the functions associated with immune response, including the immune system, increased in females, whereas the immune-related functions decreased in males (Figure 6).

4. Discussion

In this study, we performed a detailed analysis of deep sequencing data and showed that the diversity and abundance of oral bacteria varies significantly between elderly male and female patients with initial periodontitis. The α-diversity, reflecting species richness, was higher in males than females. The results of the β-diversity analysis showed that the samples were reasonably divided into different groups by gender. According to the relative abundance of species composition, LEfSe analysis showed that biomarkers in males were *Haemophilus*, family Muribaculaceae, and Clade_I/Clade_II, among others, whereas the dominant bacteria in females were *Campylobacter*, family F082, Bacteroidales_BS11_gut_group, *Selenomonas_1*, and *Pelomonas*. KEGG analysis showed that predicting changes in the female

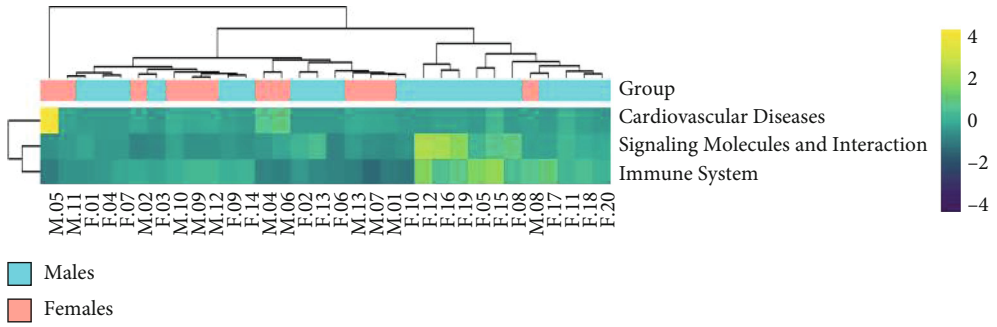
Table 2: Linear discriminant analysis (LDA) effect size (LEfSe) results of the distinct taxa between males and females.

Biomarker	Groups	LDA value
Bacteria.Proteobacteria.Gammaproteobacteria.Pasteurellales.Pasteurellaceae.Haemophilus	Males	4.05
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Muribaculaceae	Males	3.46
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Muribaculaceae.uncultured_bacterium	Males	3.40
Bacteria.Proteobacteria.Alphaproteobacteria	Males	3.28
Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales	Males	3.33
Bacteria.Proteobacteria.Alphaproteobacteria.SAR11_clade	Males	3.25
Bacteria.Proteobacteria.Alphaproteobacteria.SAR11_clade.Clade_I	Males	3.18
Bacteria.Proteobacteria.Alphaproteobacteria.SAR11_clade.Clade_I.Clade_Ia	Males	3.16
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidaceae.Bacteroides	Males	2.72
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidaceae	Males	2.72
Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Moraxellaceae	Males	3.12
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_UCG_001	Males	2.92
Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Moraxellaceae.Acinetobacter	Males	3.10
Bacteria.Patescibacteria.Gracilibacteria.Absconditabacteriales__SR1_.uncultured_bacterium	Males	3.02
Bacteria.Patescibacteria.Gracilibacteria.Absconditabacteriales__SR1_.uncultured_bacterium.uncultured_bacterium	Males	3.02
Bacteria.Actinobacteria.Actinobacteria.Actinomycetales.Actinomycetaceae.Uncultured	Males	2.84
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Muribaculaceae.uncultured_organism	Males	2.27
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Prevotellaceae.Prevotellaceae_NK3B31_group	Males	2.39
Bacteria.Proteobacteria.Gammaproteobacteria.Pasteurellales.Pasteurellaceae.Actinobacillus	Males	2.51
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcus_1	Males	2.07
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae_UCG_005	Males	2.14
Bacteria.Proteobacteria.Alphaproteobacteria.Rhodobacterales.Rhodobacteraceae	Males	2.27
Bacteria.Proteobacteria.Alphaproteobacteria.Rhodobacterales	Males	2.27
Bacteria.Proteobacteria.Alphaproteobacteria.SAR11_clade.Clade_II	Males	2.31
Bacteria.Proteobacteria.Alphaproteobacteria.SAR11_clade.Clade_II.Ambiguous_taxa	Males	2.32
Bacteria.Actinobacteria.Actinobacteria.Bifidobacteriales.Bifidobacteriaceae.Bifidobacterium	Males	2.13
Bacteria.Firmicutes.Clostridia.Clostridiales.Family_XI	Males	2.01
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae._Bacteroides__pectinophilus_group	Males	2.10
Bacteria.Bacteroidetes.Bacteroidia.Flavobacteriales.Flavobacteriaceae.NS5_marine_group	Males	2.03
Bacteria.Epsilonbacteraeota	Females	3.55
Bacteria.Epsilonbacteraeota.Campylobacteria.Campylobacterales	Females	3.55
Bacteria.Epsilonbacteraeota.Campylobacteria	Females	3.55
Bacteria.Epsilonbacteraeota.Campylobacteria.Campylobacterales.Campylobacteraceae.Campylobacter	Females	3.53
Bacteria.Epsilonbacteraeota.Campylobacteria.Campylobacterales.Campylobacteraceae	Females	3.53
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.F082	Females	2.18
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.F082.uncultured_rumen_bacterium	Females	2.08
Bacteria.Proteobacteria.Gammaproteobacteria.Betaproteobacteriales.Burkholderiaceae.Pelomonas	Females	2.06
Bacteria.Actinobacteria.Actinobacteria.Bifidobacteriales.Bifidobacteriaceae.Gardnerella	Females	2.00
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidales_BS11_gut_group	Females	2.13
Bacteria.Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Selenomonas_1	Females	2.00
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.uncultured.uncultured_rumen_bacterium	Females	2.25

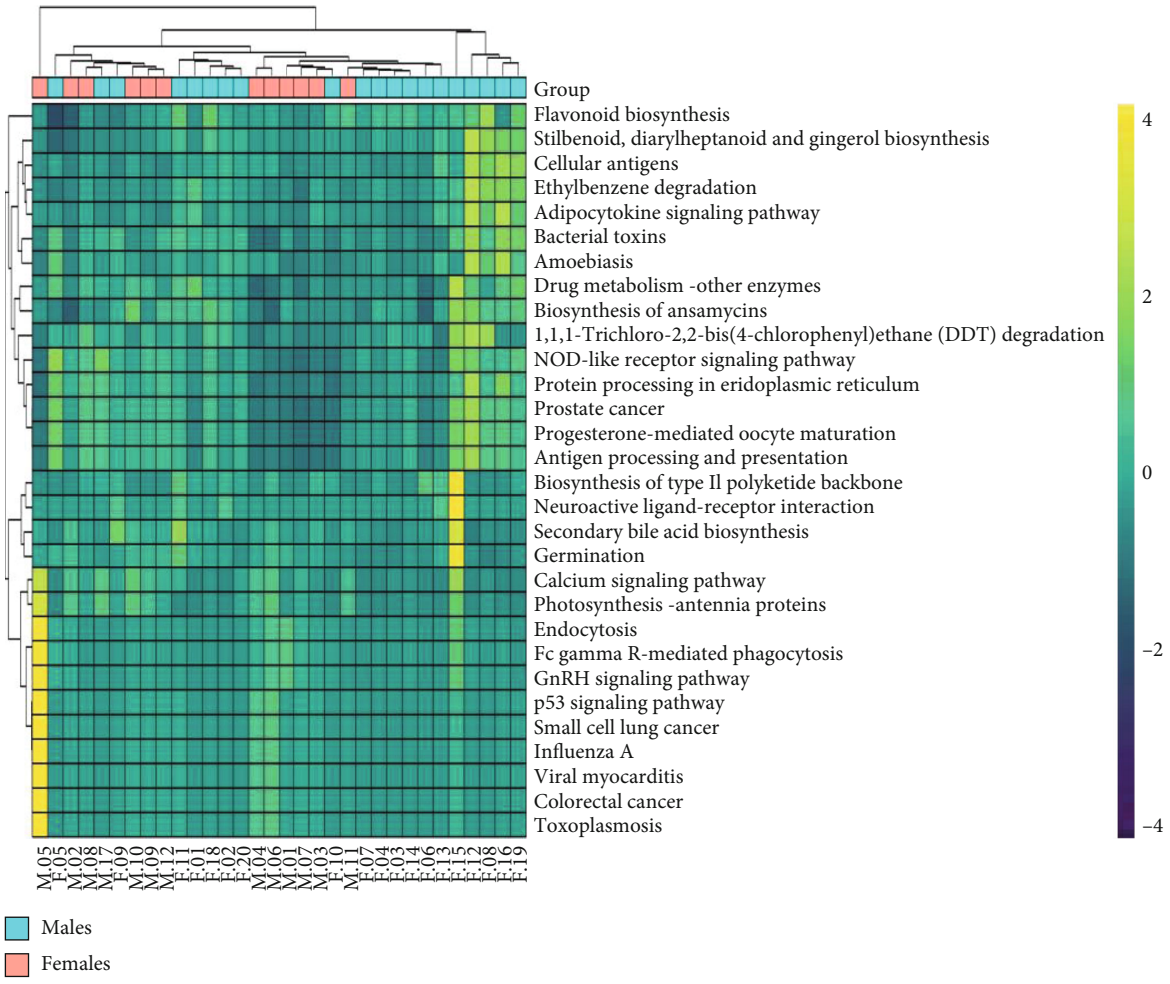
Only an LDA score of >2.0 is shown.

oral microbiota may be related to the immune system and immune system diseases are the main predictor of periodontitis in males.

Using the Kruskal-Wallis test for different species, the relative abundance of phyla Epsilonbacteraeota and Fibrobacteres and genus *Campylobacter* was higher in females than in males. The LEfSe analysis also identified *Campylobacter* as important. *Campylobacter* is Gram-negative microaerophilic bacteria that live as commensal organisms in the gastrointestinal tract. In addition to their own bacterial components, *Campylobacter* produces several different cytotoxins, including cytolethal distending toxin (CDT) and 1,3 galactosyltransferases involved in lipopolysaccharide (LPS) production, which play a role in colonization [29].



(a)



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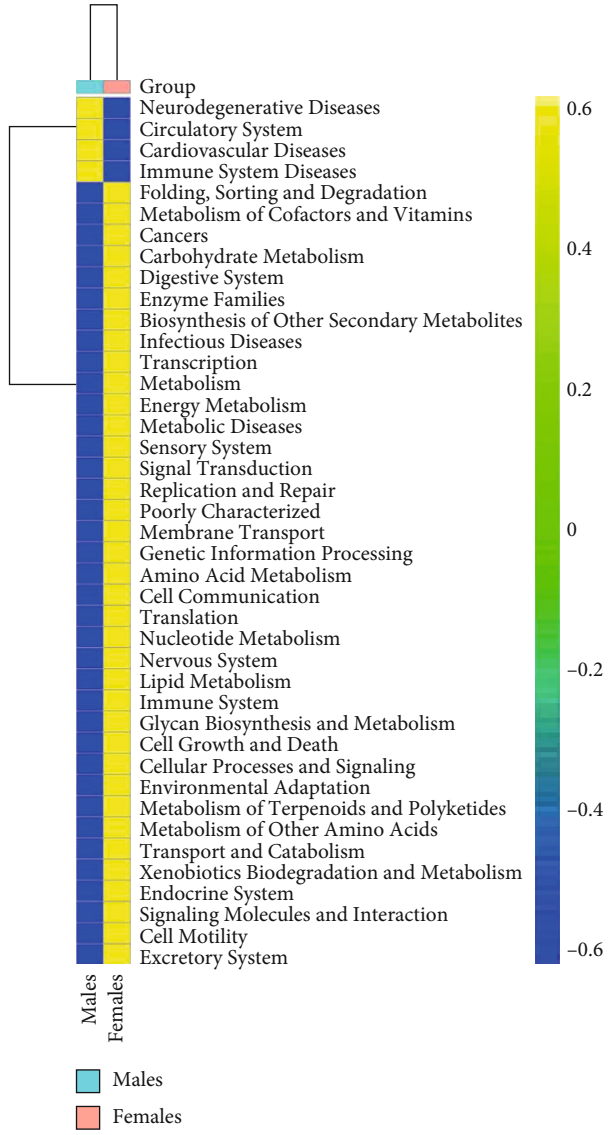

(c)

Figure 6: KEGG pathway enrichment analysis. (a) The different pathways in individual patients are shown in clustering heat maps at level 2 and (b) level 3 of KEGG. (c) At level 2 of KEGG, the different pathways between the two groups were clustered into a heat map.

Lundmark et al. found that *Campylobacter conisus* is more abundant in healthy individuals through 16S rRNA sequencing of saliva samples from patients with chronic periodontitis and healthy periodontal controls [30]. An observational cross-sectional study of 76 postmenopausal women found *Campylobacter rectus* in the oral microflora of these subjects by real-time PCR [31]. Our results also showed that Campylobacter was abundant in the postmenopausal women compared with elderly men. Thus, the increase in *Campylobacter* may play a potential positive role in periodontal health in postmenopausal women. Interestingly, a previous study investigated the composition of the tongue microflora in 16 intraoral halitosis (IOH) patients and 10 healthy subjects and found that *Campylobacter* was significantly abundant in the IOH group [32]. This difference may be due to age, hormone levels, noncomparable study populations, and the diverse mechanisms of oral diseases.

An important factor affecting the oral microbiome is age [33]. The outer membrane vesicles or gingipains of *P. gingivalis* and free soluble bacterial components of *A. actinomycetemcomitans* released into the circulation can induce a proatherogenic responses in endothelial cells [34], suggesting that the microbiota plays a role in cardiovascular disease in older adults. This may be associated with a general decrease in immune function and the development of chronic inflammation during aging. In men, age correlates with a more pronounced decrease in the total number of T and B cells and larger increase in senescent CD8+T effector memory cells compared to women [35, 36]. In contrast, menopause is associated with increased levels of IL-1β, IL-6, and TNF-α (proinflammatory) and reduced levels of IFN-γ (anti-inflammatory). In aged women, monocytes have a proinflammatory phenotype and NK cell robust cytotoxic activity [37]. Furthermore, periodontitis has been reported

to occur more often in postmenopausal women who do not receive hormone replacement compared to premenopausal women [31]. Our results showed that *Haemophilus* had the largest LDA score in males. *Haemophilus* is a Gram-negative bacteria that can only grow with fresh blood during artificial culture. It is deposited mainly in the throat and oral mucosa of humans and animals, which could cause primary suppurative infection and serious secondary infection [38]. The main virulence factors include capsule and lipooligosaccharide (LOS). The LOS of *Haemophilus ducreyi* induces immunosuppressive enzyme expression in DCs, largely through type I IFN- and TNF-α-dependent mechanisms, as well as the MAPK, NF-κB, and JAK-STAT pathways [39]. Other studies have shown that *Haemophilus* is related to sex differences. A 16S rRNA gene sequencing analysis of sputum samples from patients with asthma and normal controls found that *Haemophilus* spp. are associated with asthma in men but not in women [40]. On the other hand, in contrast to the present study, Raju et al. found a high relative abundance of *Haemophilus* in the saliva microbiota of females [41]. The LEfSe analysis identified additional biomarkers, including Rhodobacteraceae, Actinobacillus, Prevotellaceae, Muribaculaceae, and Bacteroidaceae. The immunomodulatory effects of LPS from *Rhodopseudomonas sphaeroides* are mainly the result of eliminating the inhibitory effects of T cells; this permitted the positive effects of amplifier T cells to be more fully expressed, resulting in an increased antibody response [42]. Furthermore, *Actinobacillus actinomycetemcomitans*, one of the major causative agents of chronic inflammatory periodontal disease, has been shown to cause a specific immune response by the host [43].

The dominant bacteria mentioned above may change the host immune response. We used KEGG to predict signaling pathways involved by the bacteria. Immune system diseases, neurodegenerative diseases, circulatory system diseases, and cardiovascular diseases were enriched in males, whereas immune system diseases, infectious diseases, metabolic diseases, and endocrine system diseases were increased in females. Jansen et al. have indicated that female-biased Gene Ontology categories are highly enriched for various immune system functions, including the TLR3 and TLR4 pathways, as well as genes linked to autoimmune diseases and genes regulated by estrogen and LPS [44]. As mentioned above, *Campylobacter* is a commensal organism in the gastrointestinal tract. Commensal microorganisms are greatly involved in maintaining homeostasis and health not only by blocking microbial activity but also by reinforcing the human immune system via specialized mechanisms [45], which further indicates a correlation between the oral microbiome and immune system. In KEGG pathway analysis, the endocrine system was enriched in females. The differences may be related to the change in female sexual hormones throughout life, one of the factors that plays an essential role in microbiota modulation. With increasing age, estrogen and androgen levels vary in both males and females with some commonality, as well as significant differences [46]. Though estrogen levels drastically decrease with menopause in women, androgens progressively decrease in both sexes starting at approximately 30 years of age [47–49]. Changes in periodontal status have also been found to be associated with variations in levels of sex hormones [50]. Finally, specific bacterial species, such as *P. gingivalis* and *Tannerella forsythensis*, have been shown to be important in postmenopausal women regarding the etiology of periodontitis [51].

5. Conclusion

Overall, our findings indicate that gender may be a differentiating factor in the microbial composition of the subgingival plaques of elderly patients with initial periodontitis. Future oral microbiome studies may yield better resolution if the context of sex-specific differences is considered.

Data Availability

Raw reads have been deposited at NCBI under the BioProject accession number PRJNA763744.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jie Zhao and Ying-Hui Zhou contributed equally to this manuscript.

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Research Article

The Microbiota Profile Analysis of Combined Periodontal-Endodontic Lesions Using 16S rRNA Next-Generation Sequencing

Ping Sun,[1] Zhiyong Guo,[2] Daiping Guo,[1] Jian Wang,[1] Tingting Wu,[1] Tingjun Li,[1] Jiannan Liu,[2] and Xinhua Liu[1]

[1]*The First People's Hospital of Jinzhong, Jinzhong City, 030600 Shanxi Province, China*
[2]*Department of Oromaxillofacial-Head & Neck Oncology, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine, National Clinical Research Center for Oral Diseases, Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology, Shanghai 200011, China*

Correspondence should be addressed to Jiannan Liu; laurence_ljn@163.com and Xinhua Liu; xinhua765@163.com

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Objective. The primary aim of this investigation was to analyze the microbiome in patients with combined periodontal-endodontic lesions. *Method*. Patients with loose and/or painful teeth referred for treatment from March 2020 to December 2020 in the First People's Hospital of Jinzhong were recruited. Samples were collected from teeth diagnosed as chronic periodontics (PE), ulcerative pulpitis (PU), and retrograde pulpitis (RE). Genomic DNA was extracted. The quantitative polymerase chain reaction, targeting the 16S ribosomal RNA (rRNA), was adopted for the quantification of bacteria. Then, the V3-V4 hypervariable regions of the 16S rRNA gene were amplified and subjected to next-generation sequencing. The statistical analysis was performed by R software (V3.5.1). *Results*. A total of 57 qualified samples were collected from 48 patients and analyzed (7 PE, 21 PU, and 19 RE). By linear discriminant analysis effect size, *Kingella* and *Barnesiella* were significantly increased in the periodontal pocket of retrograde pulpitis (RE-PE), compared with PE. The relative abundance of *Clostridiales Incertae Sedis XI*, *Fusobacteriaceae*, *Fusobacterium*, *Parvimonas*, *Micrococcaceae*, and *Rothia* was significantly increased in the pulp of retrograde pulpitis (RE-PU) than PU and RE-PE. *Prevotella*, *Leptotrichia*, *Porphyromonas*, *Streptococcus*, and *Fusobacterium* are consistently at a high abundance, across PU, RE-PE, and RE-PU. *Conclusion*. The current study highlighted the evidence that a specific microbial community is associated with the occurrence of retrograde pulpitis. The microenvironment of the root canal and pulp chamber will select microbiota. This study offered insights into the pathogenesis of retrograde pulpitis.

1. Introduction

Firstly described by Simring and Goldberg in 1964 [1], combined periodontal-endodontic lesions have been classified into three categories based on the primary site [2]: (a) endodontic lesions with secondary periodontic involvement (retrograde pulpitis), (b) periodontic lesions with secondary endodontic involvement (retrograde periodontics), and (c) "TRUE" combined lesions. The periodontium and endodontium are tightly bonded embryonically, anatomically, and functionally, making combined periodontal-endodontic lesions a dilemma to diagnose and treat thoroughly [3, 4]. It has been widely accepted that the anatomical interconnection of periodontium and endodontium with pathogenic microorganism transmission is accused of inducing the former two types of diseases as above [5]. Several researchers have found the common microbial composition between infected root canals and advanced periodontitis through the traditional bacterial culture method [6–9]. However, restricted to the conventional research technique, only cultivable microbes could be detected, and the panoramic difference between infected root and derived periodontitis is still not known.

Being different from caries-caused pulpitis [10, 11], the infections of retrograde pulpitis are thought to be derived from those microorganisms that exist deep in the periodontal pocket [4, 12]. However, not all teeth with chronic periodontitis will progress into retrograde pulpitis eventually,

inspiring us that apart from anatomical factors of root canals, discrepant microbiome profiles of periodontal pockets may also lead to the onset of pulpitis or not.

Several approaches have been applied in identifying pathogens. Culture and biochemical testing, with the advantage of low cost, is considered the golden standard of pathogen identification [13]. The main limitation of the culture and biochemical testing is that not all pathogen is cultivable. Next-generation sequencing (NGS), developed in 2005, can sequence billions of DNA fragments independently and simultaneously [14]. The characteristic of NGS is the ability to identify unculturable bacteria, shorter turnaround time, and more accurate results [15]. Thus, NGS has been widely adopted in the investigation of the association between microorganisms and oral diseases [16–19].

The principal aim of the present study is to compare the microbiome composition of chronic periodontitis (PE), ulcerative pulpitis (PU), and retrograde pulpitis (RE). The 16S rRNA gene sequencing technique was adopted to analyze the composition of the microbial community, aiming to elucidate the possible driving force for the development of periodontitis into retrograde pulpitis and to provide a theoretical basis for the early intervention of the disease.

2. Materials and Methods

2.1. Collection of Clinical Samples and Sampling Procedures. Patients with loose and/or painful teeth referred for treatment from March 2020 to December 2020 in the First People's Hospital of Jinzhong were screened and eligible patients were included. Teeth with probing depth (PD) ≥ 6 mm, attachment level (AL) ≥ 5 mm, looseness ≥ II°, and obvious alveolar bone resorption were diagnosed as PE. A tooth that had extensive caries lesions that led to pulp exposure without root canal treatment history and sensitivity to cold and or heat tests was diagnosed as PU. The diagnosis of RE included classical periodontics symptoms and the following criteria: (1) caries-free and intact crown; (2) spontaneous, cold, and/or heat-evoked localized or diffused pain; (3) no history of trauma; and (4) lack of periapical lesion radiographically with no sinus tracts. The summary of grouping abbreviations is presented in Table 1.

Exclusion criteria include (1) long-term medication history or antibiotics are taken within the past 3 months; (2) women during pregnancy, lactation, or menstruation; (3) have periodontal and endodontic treatment during the past 3 months; (4) history of orthodontic treatment; and (5) smoking history.

The study was approved by the Ethics Committee of the First People's Hospital of Jinzhong (2020-006-01). Written informed consent was obtained from each patient.

2.2. Microbial DNA Extraction and Sequencing. Genomic DNA was extracted from samples by using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA quality was evaluated by absorbance ratios of A260 to A280 using spectrophotometry (NanoDrop8000, Thermo Scientific). Only DNA samples with a ratio of A260 to A280 higher than 1.8 were recognized as qualified samples and used for subsequent analysis. The hypervariable V3-V4 region of the bacteria 16S ribosomal RNA genes was amplified using the primer pair 5′-CCTA CGGGRSGCAGCAG-3′ (forward primer) and 5′-GGAC TACVVGGGTATCTAATC-3′ (reverse primer), with the following PCR conditions: 95°C for 3 min, followed by 30 cycles at 98°C for 20 s, 58°C for 15 s, and 72°C for 20s and a final extension at 72°C for 5 min. PCR reactions were performed in 30 μL mixture containing 15 μL of 2× KAPA Library Amplification ReadyMix, 1 μL of each primer (10 μM), 50 ng of template DNA, and ddH_2O.

Table 1: The summary of grouping abbreviations.

Abbreviation	Grouping
PE	Chronic periodontics
PU	Ulcerative pulpitis
RE	Retrograde pulpitis
RE-PE	Periodontal pocket of retrograde pulpitis
RE-PU	Pulp of retrograde pulpitis

Sequencing was performed according to a previously described protocol [20, 21]. In brief, amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's protocols and quantified using Qubit®2.0 (Invitrogen, USA). All quantified amplicons were pooled to equalize sequencing concentrations using Illumina MiSeq/PE250 (Illumina, Inc., CA, USA). The paired-end reads of 425 bp were overlapped on their 3′ ends for concatenation into original longer tags by using PANDAseq (https://github.com/neufeld/pandaseq, V2.9). DNA extraction, library construction, and sequencing were performed at Realbio Genomics Institute (Shanghai, China). Operational taxonomic units (OTUs) were clustered with 97% similarity by using USEARCH (V7.0.1090). Each representative tag was assigned to taxa by RDP Classifier (http://rdp.cme.msu.edu/). QIIME (V1.9.1) and R (V3.5.1) were also used to analyze and profile differences of results.

2.3. Statistical Analysis. The statistical analysis of alpha diversity, beta diversity, and statistically significant differences analysis was performed by R (V3.5.1) and QIIME (V1.9.1). Only average relative abundance > 0.1% would be counted in the statistical analysis. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Subject Characteristics. A total of 48 patients fulfilling the criteria were recruited. Among them, 23 (47.9%) were males and 25 (52.1%) were females. The average age of included patients was 45.2 years old (range 12-76 years old). Most of the teeth were molar (71.7%). The characteristics of recruited patients are shown in Table 2.

3.2. 16S rRNA Gene Sequencing. We sequenced 16S rRNA gene amplicons from 57 qualified samples (PE 17, PU 21,

Table 2: Study population characteristics.

Characteristics	PU	PE	RE
	Gender		
Male	8	9	6
Female	15	5	5
Age (mean, range)	36.7 (12-66)	52.2 (38-76)	53.9 (45-62)
	Location		
Anterior tooth	4	2	—
Premolar	5	4	—
Molar	15	12	11
	Systematic conditions		
Hashimoto's disease	1	—	—
Hypertension	1	3	2
Diabetes	—	3	2

and RE 19). For RE, 10 samples were collected from the periodontal pocket (RE-PE) and 9 samples were collected from the pulp (RE-PU). All samples passed quality control. The number of total valid reads from 16S rRNA was 2.04×10^6, ranging from 29,733-38,987 reads (mean 35,763 reads). Using a 97% similarity level, a total of 1163 OTUs were detected. The detailed distribution of OTUs is shown in Figure S1.

3.3. The Differences of Microbial Community Structure among the PE, RE-PE, and RE-PU Groups. The alpha diversity analysis included community richness (Chao1 diversity index) and community evenness (Shannon diversity index). In general, the RE-PU yielded the lowest community richness, followed by RE-PE and PE ($P < 0.05$) (Figure 1(a)). For the community evenness, RE-PU was significantly lower than PE (Figure 1(b)). The beta-diversity was analyzed to depict divergence among PE, RE-PE, and RE-PU groups. The principal coordinates analysis (PCoA) diagram showed that three groups were relatively independent indicating significantly different microbial communities (Adonis analysis, $P = 0.001$) (Figure 1(c)). The distribution of the top 20 genera is depicted in Figure 1(d). Compared with RE-PE and PE, *Porphyromonas*, *Leptotrichia*, *Saccharibacteria*, and *Selenomonas* were significantly reduced in RE-PU, while *Streptococcus*, *Parvimonas*, *Rothia*, and *Murdochiella* were significantly rich in RE-PU. The linear discriminant analysis (LDA) effect size (LEfSe) analysis was adopted to find the species with an abundance that is significantly different among multiple groups. As shown in Figure 2, a total of 32 genera (represented by *Clostridiales Incertae Sedis XI*, *Actinobacteria*, and *Parvimonas*) were significantly abundant in RE-PU, 17 genera (represented by *Porphyromonadaceae*, *Porphyromonas*, *Treponema*, and *Spirochaetes*) were abundant in RE-PE, and 20 genera (represented by *Synergistetes*, *Synergistales*, *Synergistia*, and *Synergistaceae*) were abundant in PE.

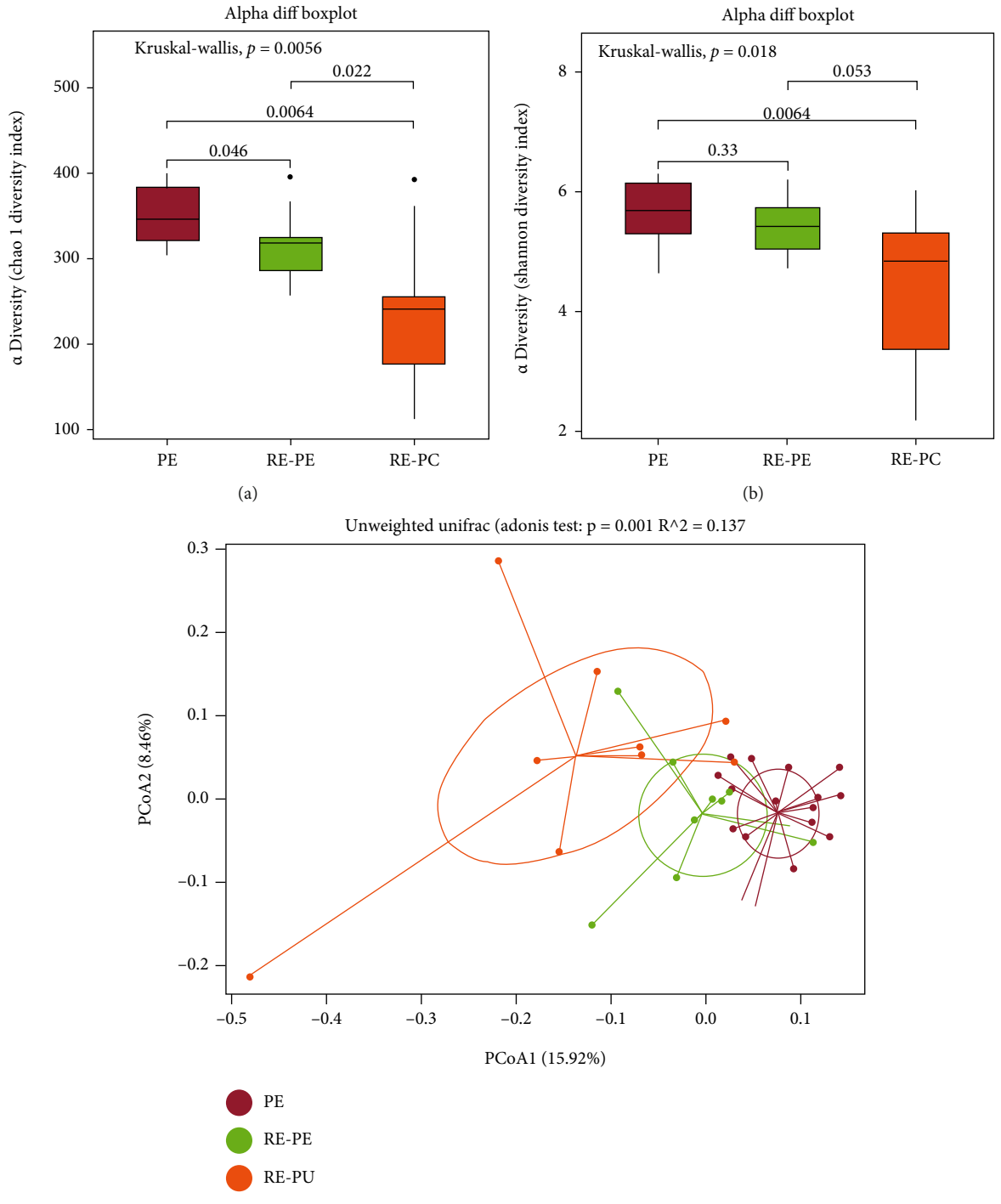
3.4. The Differences in Microbial Community Structure among the PU, RE-PU, and RE-PE Groups. Alpha diversity showed higher richness and evenness in RE-PE, while there are no statistically significant differences between PU and RE-PU (Figures 3(a) and 3(b)). PCoA diagram indicated three relatively independent groups (Figure 3(c)), testified by Adonis ($P = 0.001$), Anosim ($P = 0.003$), and MRPP ($P = 0.002$) analyses. Genera of the top 20 relative abundances were marked in Figure 3(d). In detail, the abundance of *Leptotrichia*, *Selenomonas*, and *Capnocytophaga* were significantly lower in RE-PU than PU and RE-PE. Meanwhile, the abundance of *Streptococcus*, *Parvimonas*, and *Murdochiella* was significantly higher in RE-PU, compared with PU and RE-PE. LEfSe analysis filtered genera with a significant difference in relative abundance are shown in Figure 4. In specific, RE-PE was rich in *Porphyromonadaceae*, *Bacteroidia*, *Bacteroidales*, *Porphyromonas*, and *Bacteroidetes*. For RE-PU, the relative abundance of *Clostridiales Incertae Sedis XI*, *Fusobacteriaceae*, *Fusobacterium*, *Parvimonas*, *Micrococcaceae*, and *Rothia* was significantly increased, while the most selectively enriched abundant genera for PU were *Actinobacteria*, *Actinomycetales*, *Lactobacillales*, and *Bacilli*.

3.5. The Function Prediction of Microbial Community. The function of a differently detected microbial community was predicted using KEGG (http://www.kegg.jp/). The top 30 genes enriched by a differently detected microbial community are presented in Figure 5 ($P < 0.05$). The function of a differently detected microbial community was close for PE and RE-PE, compared with RE-PU. In specific, RNA polymerase sigma, methyl-accepting chemotaxis protein, glutathione S-transferase, acetyl-CoA C-acetyltransferase, and acyl-CoA thioester hydrolase were rich in PE and RE-PE (Figure 5(a)). Gene families involved in fatty acid biosynthesis, glycolysis/gluconeogenesis, fatty acid degradation, glutathione metabolism, pyrimidine metabolism, fatty acid metabolism, iron complex outer-membrane receptor protein, RNA polymerase sigma-70 factor, ECF subfamily, and chromosome partitioning protein were significantly enriched in RE-PU and RE-PE, while those responsible for galactose metabolism, proteasome, starch, and sucrose metabolism were significantly associated with PE (Figure 5(b)).

4. Discussion

Combined periodontal-endodontic lesions, which can establish independently with or without communication between endodontic and periodontal components, are a kind of disease characterized by the interrelationship between the periodontal pocket and tooth pulp [22]. The prognosis of combined periodontal-endodontic lesions treated with traditional management is poor [23, 24]. Targeted therapy, aiming at the trigger event of combined periodontal-endodontic lesions, may have better performance and is eagerly needed. It is very important for clinicians to understand the pathogenesis process of combined periodontal-endodontic lesions.

The important role of microorganisms in oral diseases has been well recognized [20, 21, 25–28]. The specific mechanism of periodontal-endodontic lesions occurrence is still under debate; however, the important role of bacterial



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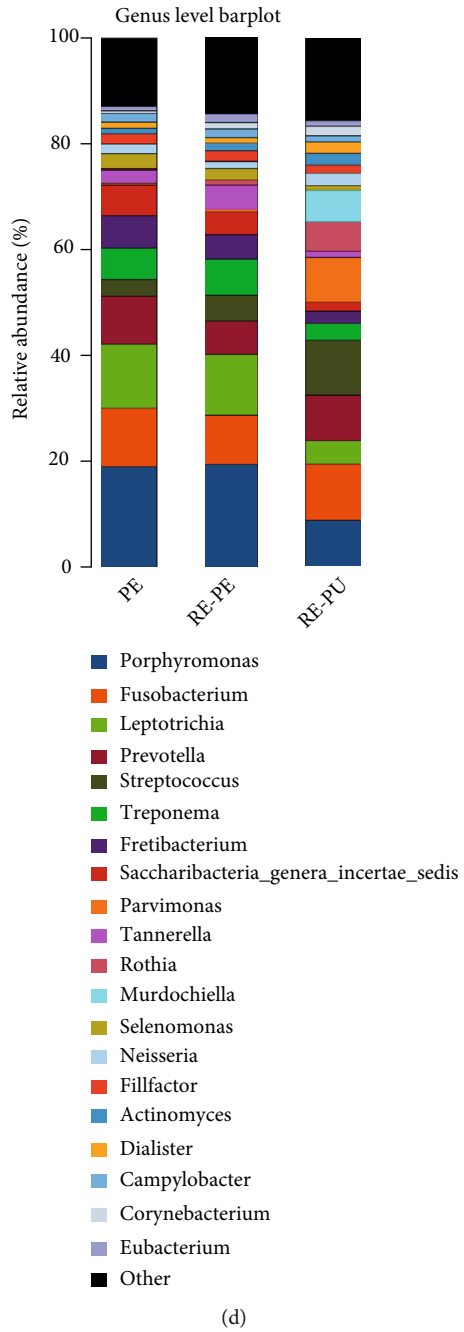
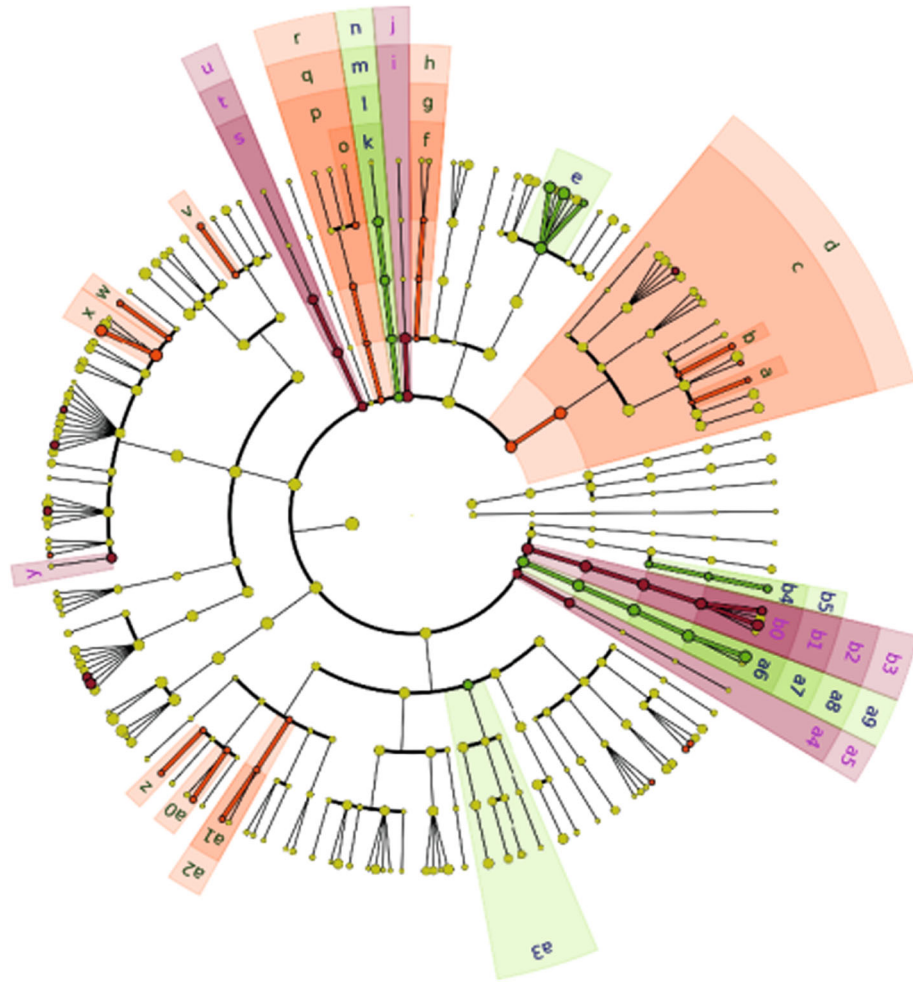


Figure 1: The analysis of microbial community structure of PE, RE-PE, and RE-PU groups. (a, b) The boxplot diagrams show alpha diversity index among PE, RE-PE, and RE-PU. (c) The plot of principal coordinates analysis (PCoA) shows intergroup distances by 2 principal coordinates. (d) The relative abundance of bacterial taxa at the genus level of PE, RE-PE, and RE-PU.

infection has been identified [6–8, 29, 30]. The transmission of microbial community and toxins between the periodontal pocket and the root canal was speculated to be the irritation [7]. Kipioti et al. found the similarity of flora between root canals and adjacent periodontal pockets of teeth with advanced periodontitis [6]. Kobayashi et al. also detected common microflora from root canals and periodontal pockets of caries-free teeth with advanced periodontitis [8]. Both of the studies above were conducted using the culture method only. Xia and Qi compared bacterial profiles using denaturing gradient gel electrophoresis (DGGE) between dental plaque and pulp of 13 teeth with combined periodontal-endodontic lesions [31]. However, the similarity of bacteria from dental plaque and pulp of the same tooth ranged from 13.1% to 62.5%. The conclusion might not be valid. Li et al. identified 43 genera/species from 20 patients' teeth with combined periodontal-endodontic lesions via DGGE [32]. The predominant genera were *Porphyromonas* sp. (13.9%), *Filifactor* sp. (12.5%), and *Parvimonas* sp. (11.1%). The most prevalent bacteria in the root canal and



(a)

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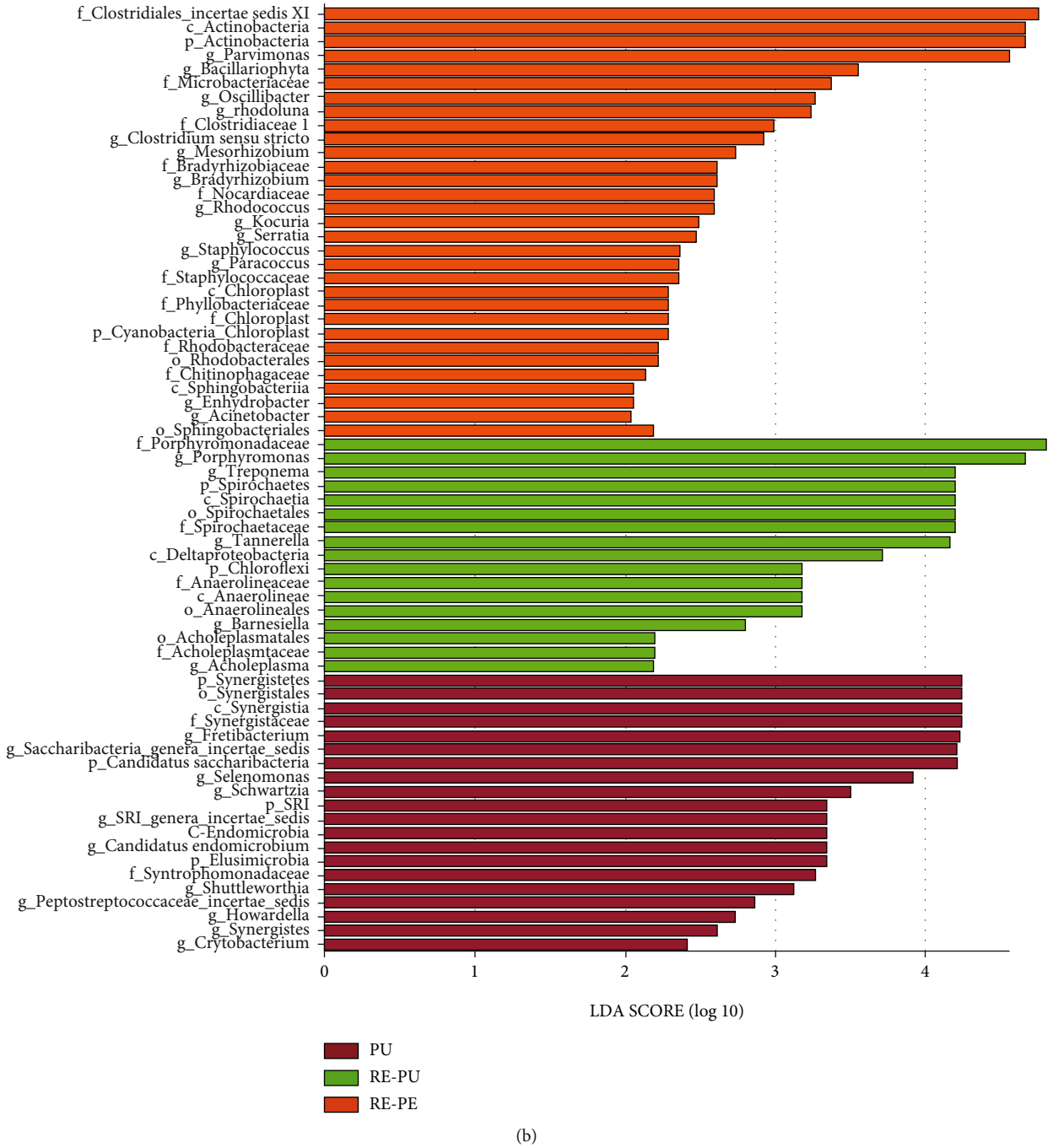
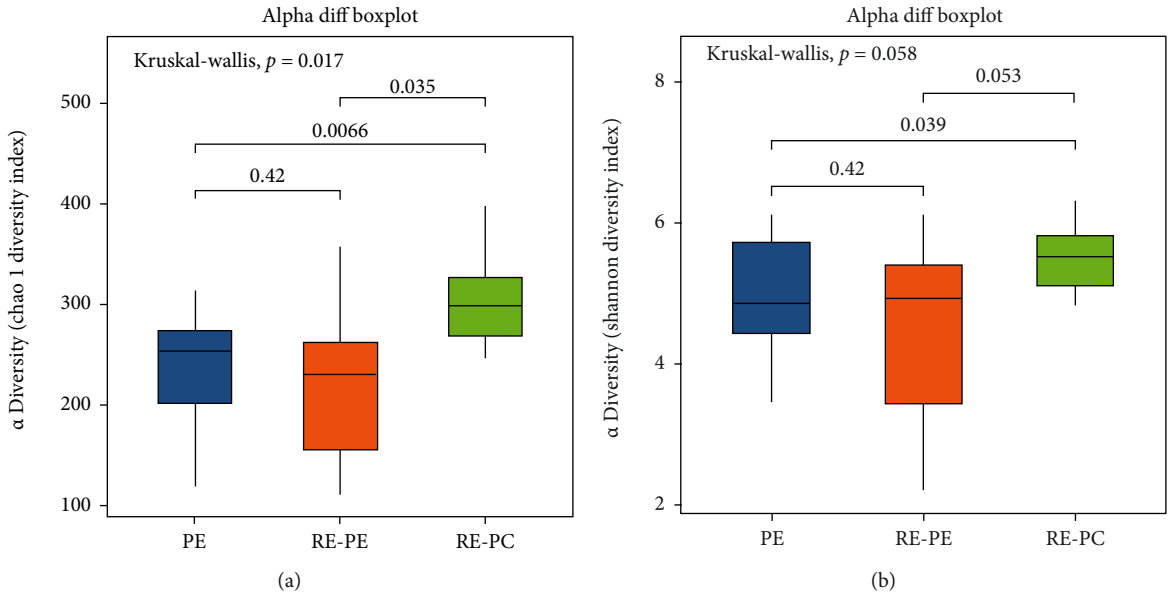


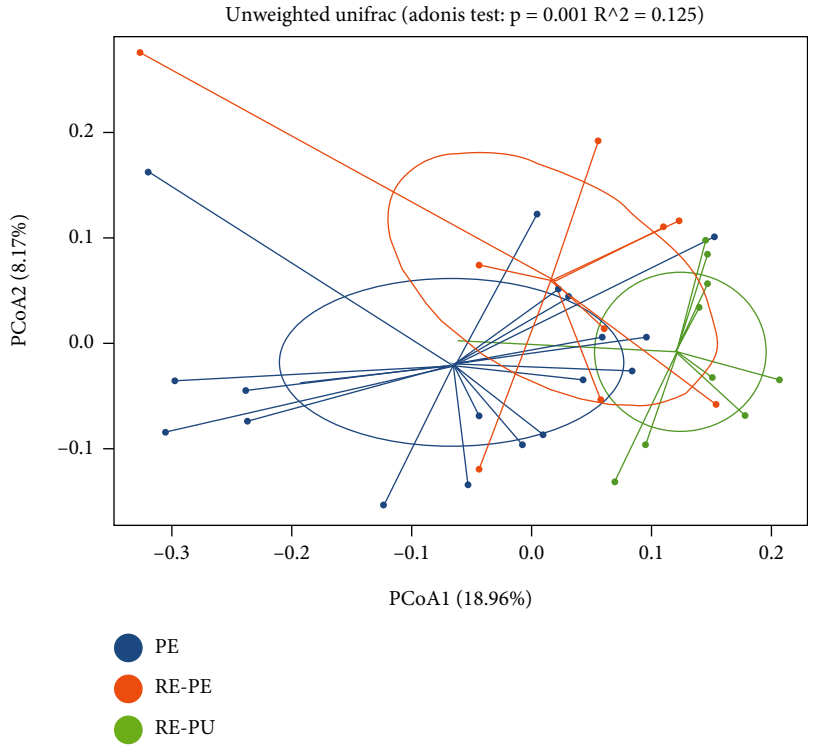
(b)

Figure 2: The linear discriminant analysis (LDA) effect size (LEfSe) profiles of PE, RE-PE, and RE-PU. (a) Cladograms indicating the phylogenetic distribution of bacterial lineages among 3 groups. The phylum, class, order, family, and genus levels are listed in order from inside to outside of the cladogram, and the labels for levels of order, family, and genus are abbreviated by a single letter. (b) LDA along with effect size measurements was applied to present the enriched bacterial genera of each group.

periodontal pocket were *Filifactor alocis*, *Parvimonas micra*, *Porphyromonas gingivalis*, and *Tannerella forsythia*. In general, previous studies revealed the possibility that the periodontal pocket was a source of root canal infection; however, due to the limited technique, the profile of differently distributed bacterial was not comprehensive. There was also a lack of follow-up research to elucidate the changing process after the root canal infection derived from the periodontal pocket.

It is interesting to note that not all teeth, which suffered from chronic periodontitis, will develop retrograde pulpitis and result in combined periodontal-endodontic lesions eventually. It is unclear which factor will decide the fate of the tooth. The present study showed the composition of





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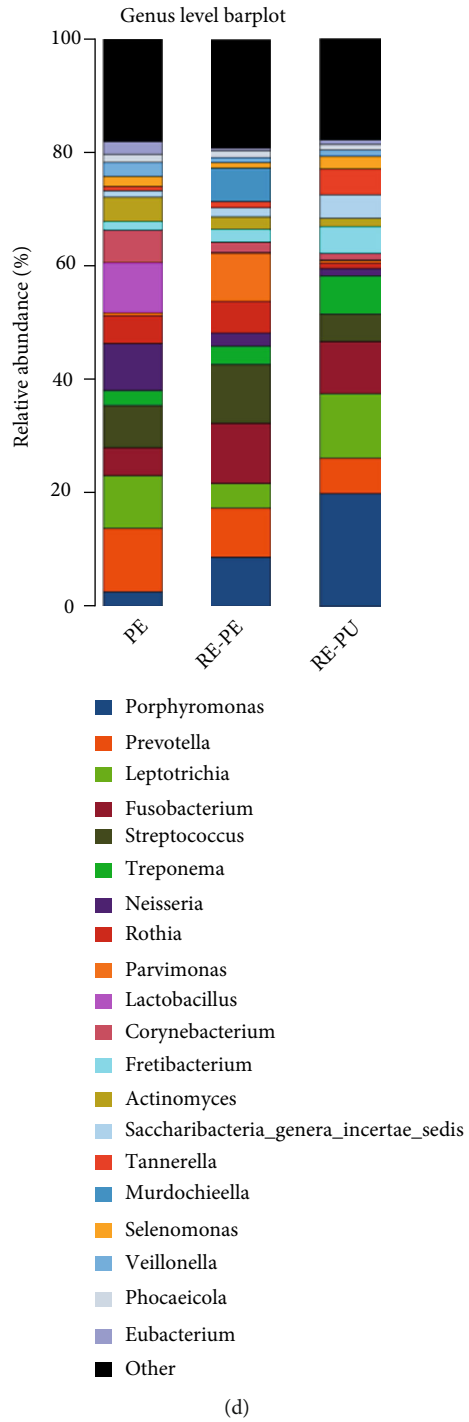
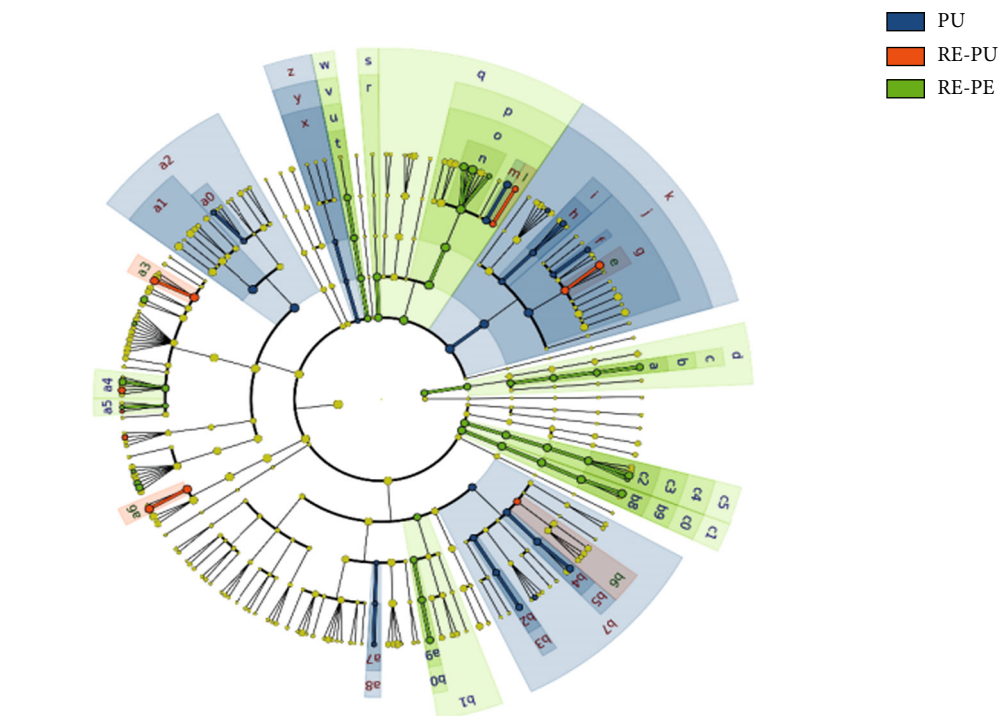


Figure 3: The analysis of microbial community structure of PU, RE-PU, and RE-PE groups. (a, b) The boxplot diagrams showed alpha diversity index among PU, RE-PU, and RE-PE. (c) The plot of principal coordinates analysis (PCoA) showed intergroup distances by 2 principal coordinates. (d) The relative abundance of top 20 bacterial taxa at the genus level for PU, RE-PE, and RE-PU.

the microbiota of RE-PE is differed from PE, indicating that different genera in periodontal pockets are the key to the occurrence of retrograde pulpitis. The fact that the microbiome of RE-PU was closer to RE-PE rather than PE further supported this viewpoint (Figure 1(c)). Highly rich genera in RE-PE may exert a pivotal role in the pulp invasion, leading to retrograde pulpitis. By LEfSe analysis, *Kingella* and *Barnesiella* are found to be highly rich in RE-PE than PE. *Kingella* is a genus of Gram-negative, aerobic, and facultatively anaerobic bacilli. *Kingella* is a normal flora in the oral cavity, being detected in both healthy and periodontitis subjects [33, 34]. To note, a study showed that *Kingella* was rich in biofilm collected from children with severe caries, indicating that *Kingella* might associate with invasion and

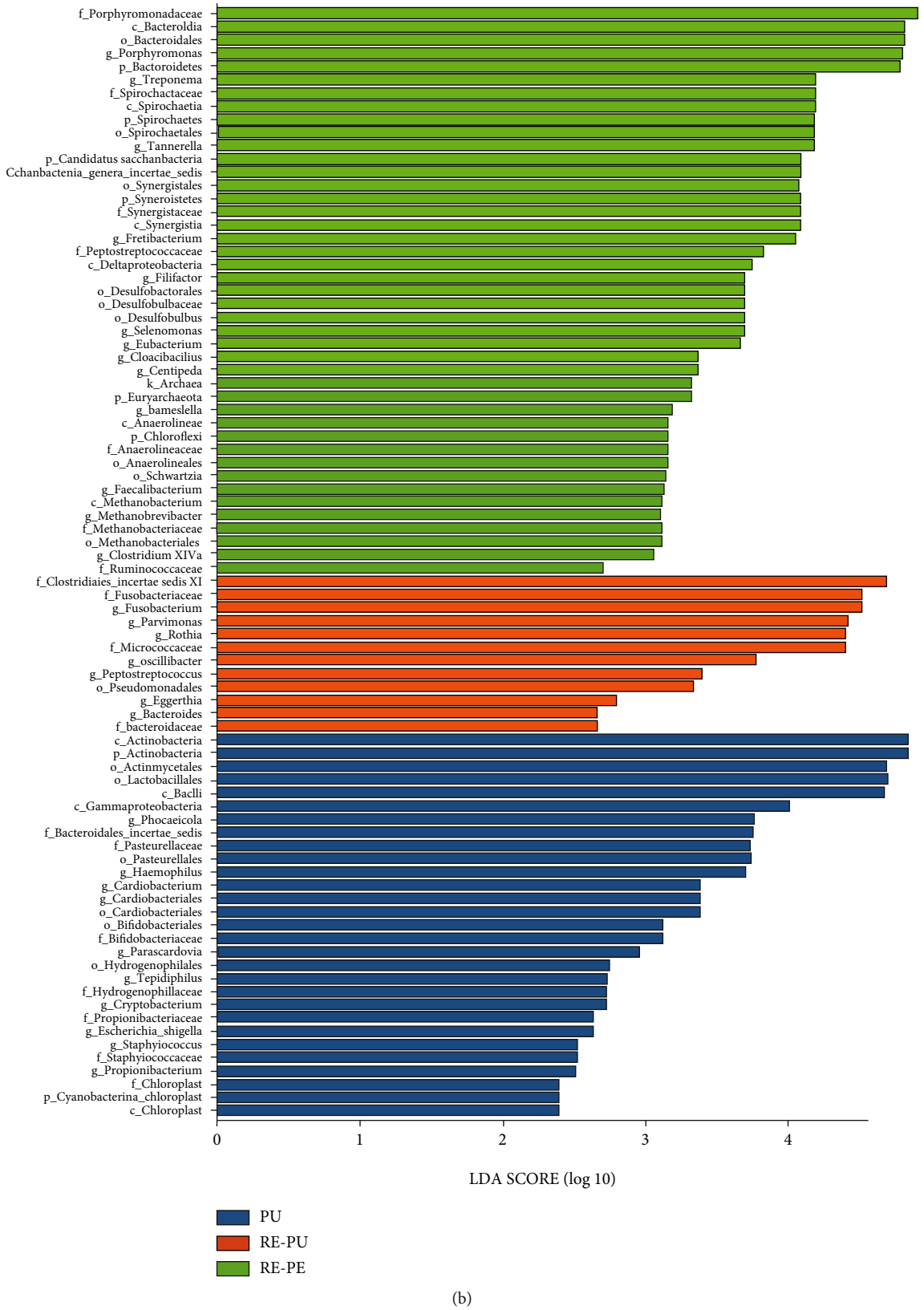


a:f_Methanobacteriaceae
b:o_Methanobacteriales
c:c_Methanobacteria
d:p_Euryarchaeota
e:f_Micrococcaceae
f:f_Propionibacteriaceae
g:o_Actinomycetales
h;f_Bifidobacteriaceae
i:f_Bifidobacteriales
j:c_Actinobacteria
K:p_Actinobacteria
l:f_Bacteroidaceae
m:f_Bacteroidales_incertae_sedis
n:f_Porphyromonadaccae
o:o_Bacteroidales
p:c_Bacteroidia
q:p_Bacteroidetes
r:g_Saccharibacteria_genera_incertae_sedis
s:p_Candidatus saccharibacteria
t:f_Anaerolineaceae
u:o_Anaerolineales
v:c_Anaerolineae
w:p_Chloroflexi
x:f_Chloroplast
y:c_Chloroplast
z:p_Cyanobacteria_chloroplast

a0:f_staphylococcaceae
a1:o_lactobacillales
a2:c_Bacilli
a3:f_Clostridiales_incertae sedis XI
a4:f_Peptostreptococcaceae
a5:f_Ruminococcaceae
a6:f_Fusobacteriaceae
a7:f_Hydrogenophilaceae
a8:o_Hydrogenophilales
a9:f_Deslfobulbaceae
b0:o_Desulfobacterales
b1:c_Deltaproteobacteria
b2:f_Cardiobacteriaceae
b3:o_cardiobacteriales
b4:f_Pasteurellaceae
b5:o_Pasteurellales
b6:o_Pseudomonadales
b7:c_Gammaproteobacteria
b8:f_Spirochaetaceae
b9:o_Spirochaetales
c0:c_Spirochaetia
c1:p_Spirochaetes
c2:f_Synergistaceae
c3:o_Synergistales
c4:c_Synergistia
c5:p_Synergistetes

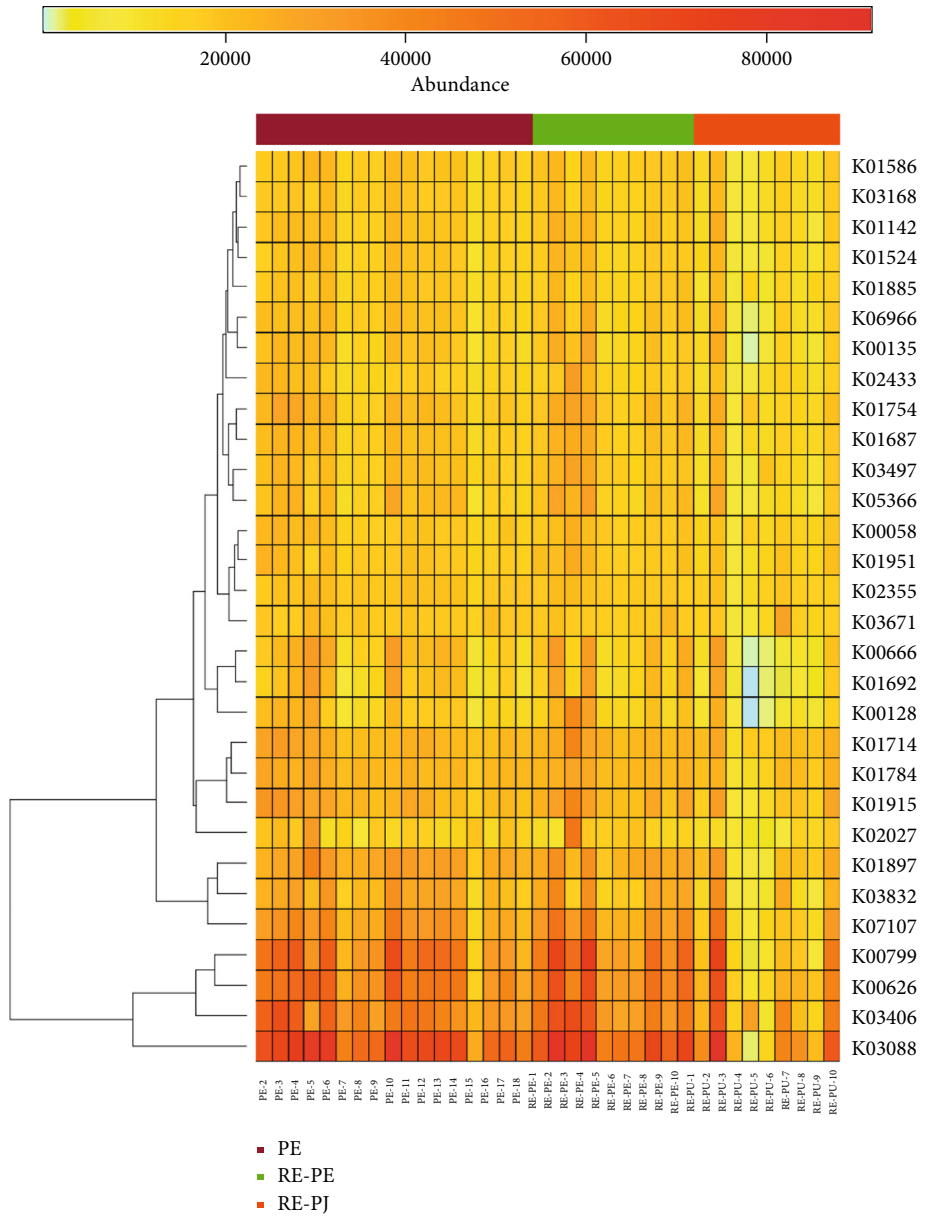
(a)

Figure 4: Continued.



(b)

Figure 4: The linear discriminant analysis (LDA) effect size (LEfSe) profiles of PU, RE-PU, and RE-PE. (a) Cladograms indicating the phylogenetic distribution of bacterial lineages among 3 groups. The phylum, class, order, family, and genus levels are listed in order from inside to outside of the cladogram, and the labels for levels of order, family, and genus are abbreviated by a single letter. (b) LDA along with effect size measurements was applied to present the enriched bacterial genera of each group.



(a)

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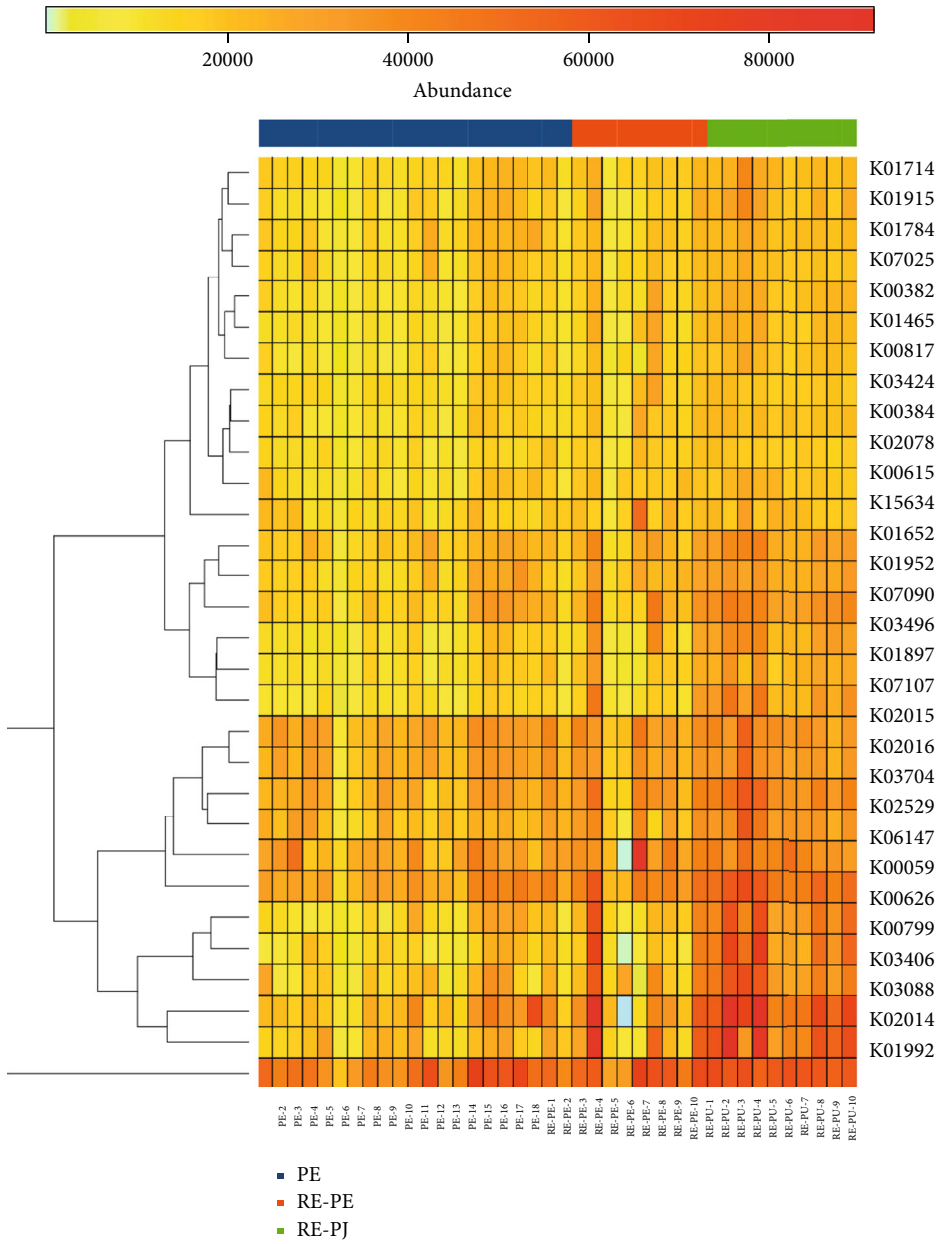

(b)

Figure 5: The heat map of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway categories based on differently detected genera of (a) PE, RE-PE, and RE-PU and (b) PU, RE-PE, and RE-PU.

erosion of enamel and dentin [35]. We speculate that predominate *Kingella* can degrade mineral substances and form the channel through which microbiota and secreted toxins can affect dental pulp. *Barnesiella* is reported to play an important role in several diseases as a major component of the gut microbiome [36–38]. A recent published study demonstrated that the abundance of *Barnesiella* would increase after *Porphyromonas gingivalis* infection, which was the etiological agent of periodontal disease [39]. It has been recognized that the gut microbiome is significantly associated with host immunity [40]. Thus, we speculated that *Barnesiella* may be able to affect the immune microenvironment of the periodontal pocket, damage the immune barrier, and accelerate the invasion of pathogenic microorganisms. The function of *Barnesiella* in periodontitis should be further investigated.

Previous studies showed that as the periodontal inflammation progresses, alteration of local flora might happen gradually [41–43]. To our knowledge, there is no study investigating the microbiota shift in retrograde pulpitis. The PCA analysis revealed that the composition of the microbiota of RE-PU is closer to PU rather than RE-PE. To note, all teeth diagnosed as combined periodontal-endodontic lesions are caries-free. Thus, the infection resource of retrograde pulpitis is only periodontal pockets. The significantly different microbiota composition between

RE-PE and RE-PU indicates that the microenvironment has selected the microorganism by local pressures. The results of the alpha diversity analysis also support the assumption. The score of RE-PU is significantly lower than the PE and RE-PE groups, indicating that some predominant microorganisms are existing in the infected dental pulp.

The composition of microorganisms is significantly different among PU, RE-PE, and RE-PU. In detail, *Clostridiales Incertae Sedis XI*, *Parvimonas*, *Clostridium*, *Peptostreptococcaceae*, and *Filifactor* are more frequently detected in RE-PU, compared with PU and RE-PE. *Clostridiales Incertae Sedis XI* was created in 2001, and family members of *Clostridiales Incertae Sedis XI* were pathogen of septic arthritis, necrotizing pneumonia, chronic rhinosinusitis, and bacterial vaginosis [44–50]. This is the first time to illustrate the significant role of *Clostridiales Incertae Sedis XI* in oral disease. The function of *Clostridiales Incertae Sedis XI* is merit to be further explored. Delima et al. found that smoke cessation would lead to a decrease in the prevalence of *Parvimonas* [51]. The abundance of *Parvimonas* was proved to be rich in smokers with periodontitis than nonsmokers [52, 53]. *Clostridium* is a genus of gram-positive obligate anaerobes, including several significant human pathogens. Vigil et al. isolated *Clostridium* from 22 refractory periapical cases via growth culture [54]. Medina-Palacios et al. identified that *Clostridium* was one of the most common genera in refractory apical periodontitis [55]. One possible mechanism that *Clostridium* contributed to destruction is degrading Type IV collagen, which is the major component of basement membranes, via proteinases [56]. Several researchers have identified the abnormal accumulation of *Peptostreptococcaceae* in both chronic periodontitis and aggressive periodontitis [57–61]. In this study, we firstly reported *Peptostreptococcaceae* is rich in RE-PU. This adds a growing body of evidence that the periodontal pocket is the resource of infection for retrograde pulpitis. The role of *Peptostreptococcaceae* in pathogenesis of retrograde pulpitis needs to be further investigated. *Filifactor* is a genus of Gram-positive and anaerobic bacterium, containing *Filifactor alocis*, which is a diagnostic indicator of periodontal disease [62]. The most important characteristic of *Filifactor alocis* is the ability to survive in the oxidative stress-rich environment, especially periodontal pocket. Compared with *P. gingivalis*, *Filifactor alocis* is more resistant to H_2O_2-induced oxidative stress [63]. Moreover, it is interesting to note that the survival of *P. gingivalis* is significantly increased when cocultured with *Filifactor alocis*, indicating that *Filifactor alocis* may have the ability to detoxify the microenvironment and thereby improve the survival of pathogenic microorganism. Further study revealed the core role of "superoxide reductase" of *Filifactor alocis* in resistance to superoxide radicals [64]. Profound synergistic interactions among *Filifactor alocis* and oral bacteria have been confirmed by in vitro studies, suggesting the central role of *Filifactor alocis* in the microbial community of periodontal pocket [62, 63, 65, 66] and may majorly contribute retrograde pulpitis development.

Overall, this study is the largest cohort so far to investigate the microbiota for combined periodontal-endodontic lesions. The present study suggests that specific microbiota of periodontal pocket may be associated with the occurrence of retrograde pulpitis. The shift of microbial communities from periodontitis to retrograde pulpitis is investigated and revealed that the microenvironment of the root canal and pulp chamber can select predominate microflora. Due to the poor prognosis of retrograde pulpitis, more profound research and a better understanding of microorganisms might give us a hint on targeted medication to prevent the occurrence of retrograde pulpitis in the future.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Ping Sun and Zhiyong Guo contributed data collection, analysis, and interpretation equally to this work and share first authorship. Daiping Guo, Jian Wang, Tingting Wu, and Tingjun Li collected and processed samples. Jiannan Liu and Xinhua Liu contributed conception, design, and data acquisition and drafted the manuscript equally to this work and are co-corresponding authors. Ping Sun and Zhiyong Guo contributed equally to this work.

Acknowledgments

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Supplementary Materials

Figure S1: the Venn diagram contouring the distribution of operational taxonomic units (OTUs) for (a) PE, RE-PE, and RE-PU and (b) PU, RE-PE, and RE-PU. *(Supplementary Materials)*

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Research Article

Sex Variations in the Oral Microbiomes of Youths with Severe Periodontitis

Ya-Qiong Zhao,[1] Ying-Hui Zhou,[2] Jie Zhao,[1] Yao Feng,[1] Zheng-Rong Gao,[1] Qin Ye,[1] Qiong Liu,[1] Yun Chen,[1] Shao-Hui Zhang,[1] Li Tan,[1] Marie Aimee Dusenge,[1] Jing Hu,[1] Yun-Zhi Feng,[1] Fei Yan ,[3] and Yue Guo [1]

[1]*Department of Stomatology, The Second Xiangya Hospital of Central South University, Changsha 410011, Hunan, China*
[2]*National Clinical Research Center for Metabolic Diseases, Hunan Provincial Key Laboratory of Metabolic Bone Diseases, and Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha 410011, Hunan, China*
[3]*Hunan Key Laboratory of Oral Health Research & Hunan 3D Printing Engineering Research Center of Oral Care & Hunan Clinical Research Center of Oral Major Diseases and Oral Health & Xiangya Stomatological Hospital & Xiangya School of Stomatology, Central South University, Changsha 410008, China*

Correspondence should be addressed to Fei Yan; yfann2004@csu.edu.cn and Yue Guo; guoyue@csu.edu.cn

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Objective. Periodontitis is an inflammatory disease of microbial etiology caused primarily by dysbiosis of the oral microbiota. Our aim was to compare variations in the composition of the oral microbiomes of youths with severe periodontitis according to gender. *Methods*. Subgingival plaque samples collected from 17 patients with severe periodontitis (11 males and 6 females) were split for 16S rRNA gene sequencing. The composition, α-diversity, and β-diversity of the patients' oral microbiomes were compared between the males and the females. Linear discriminant analysis effect size (LEfSe) was used to analyze the specific taxa enriched in the two groups. Functional profiles (KEGG pathways) were obtained using PICRUSt based on 16S rRNA gene sequencing data. *Results*. The Chao1 index and phylogenetic diversity whole tree were significantly higher in males than in females. The Simpson and Shannon indices were not significantly different between the two groups. β-Diversity suggested that the samples were reasonably divided into groups. The Kruskal-Wallis test based on the relative abundance of species, combined with the LEfSe analysis showed that the dominant bacteria in males were *Pseudomonas* and *Papillibacter*, whereas the dominant bacteria in women were Fusobacteriales and *Tannerella*. KEGG analysis predicted that the variation in the oral microbiome may be related to the immune system in women, whereas immune system diseases were the dominant pathway in men. *Conclusion*. We found sex-specific differences in the oral microbiome in a sample of youths with severe periodontitis. The differences may be related to changes in immune homeostasis and lead to a better understanding of periodontitis.

1. Introduction

Disturbances in the oral microbiota can cause an immune response by the host that affects the protection and support of the periodontium, resulting in the development of periodontal disease [1]. Periodontitis is a highly prevalent oral disease among adults, with a prevalence of up to 50% in developed countries [2]. The prevalence is even greater (~90%) in developing countries [3]. The global burden of periodontitis is increasing with life expectancy and due to a worldwide decrease in tooth loss. In the 4th National Oral Health Survey in mainland China, the frequency of adults with periodontitis was 52.8% and with severe periodontitis (stage III or IV) 10.6% [4]. Understanding the composition and structure of oral microbiomes could improve periodontitis prevention, making it important for public health.

Periodontitis is directly preceded by a dynamic, polymicrobial oral microbiome. As a bacterial community develops,

the ecological succession from a microbial community to a state of dysbiosis manifests as emergence of newly dominant community members rather than the appearance of novel species [5]. The specific groups and combinations of bacteria, including the "red complex," *Porphyromonas gingivalis (P. gingivalis)*, *Treponema denticola* (*T. denticola*), and *Tannerella forsythensis (T. forsythensis)*, have been strongly associated with the pathology of periodontitis [6]. These bacteria can alter host immune competence with increased production of virulence factors. For example, the *P. gingivalis* virulence factors have been shown to decrease the host response [7] by subverting innate immune signaling. Though the relationship with the host is normally homeostatic, by manipulating the crosstalk between complement and Toll-like receptors (TLRs) [8], a destructive change is triggered [9]. In addition, the virulence factors of *P. gingivalis* can specifically recognize epitope-specific CD4+ T cell phenotypes [10]. Furthermore, a cluster of species with a less stringent association with disease was defined as the "orange complex" and includes *Prevotella* spp., *Fusobacterium* spp., and *Parvimonas micra* (formerly *Peptostreptococcus micros*) [11]. They were also found to be associated with immunity. *Fusobacterium nucleatum* (*F. nucleatum*) could have the capacity to induce a downregulation of antimicrobial peptides, such as hβD-1 and LL-37, but this downregulation of the host defense may be another bacteria-mediated virulence mechanism [12].

Previous studies have suggested that both host genetic and immunological factors are important in oral microbiome dysbiosis, further demonstrating the complex nature of this condition. Race/ethnicity, psychosocial stress, socioeconomic status, gender, and other sociodemographic factors are also gaining more and more importance in the incidence and severity of periodontitis and alterations to oral microbiomes [13]. However, the influence of gender in the process remains controversial. No sex relationship was found for yeasts and staphylococci in a microbiological analysis of 3075 "refractory" periodontitis patients [14]. Similarly, a study that analyzed the relationship of gender and race with components of the subgingival microflora from individuals with different degrees of periodontal disease and periodontal health found no significant differences between males and females [15]. In contrast, Umeda et al. suggested an association between gender and carriage of a specific organism, demonstrating that *Prevotella intermedia/nigrescens* (*P. intermedia/P. nigrescens*) is more likely to be found in the saliva and subgingival and supragingival plaques of males than females [16].

In the current study, we identified sex-specific differences in the oral microbiomes of youths with severe periodontitis through 16S rRNA gene sequencing and predicted the variation of the oral microbiomes that may be related to immunity in order to gain further understanding of periodontitis.

2. Materials and Methods

2.1. Participants and Inclusion Criteria. We enrolled a total of 17 participants (11 males and 6 females) who visited the medical examination center at The Second Xiangya Hospital of Central South University. The inclusion criteria were patient age between 20 and 44 years; the presence of at least 15 existing natural teeth (excluding third molars); no removable partial denture, bridge, or implant; no antibiotic use within 1 month and no periodontal treatment within the last 6 months; no other bacterial infectious oral disease or systemic disease; and the patient was a nonsmoker. All of the participants received a comprehensive oral examination, which included a professional assessment by a specialized dentist based on the standards of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions "Staging and Grading of Periodontitis: Framework and Proposal of a New Classification and Case Definition" [17]. Before measurement, the sites were air-dried. "Initial" periodontitis was defined as at least one tooth with probing depth (PD) $\geq$ 3 mm and attachment loss (AL) $\geq$ 3 mm or PD $\geq$ 4 mm and AL $\geq$ 3 mm in $\leq$30% of teeth. "Moderate" periodontitis was defined as PD $\geq$ 5 mm and AL $\geq$ 4 mm in <30% of teeth or PD $\geq$ 4 mm and AL $\geq$ 3 mm in 30-60% of teeth. "Severe" periodontitis was PD $\geq$ 5 mm and AL $\geq$ 4 mm in $\geq$30% of teeth or PD $\geq$ 4 mm and AL $\geq$ 3 mm in $\geq$60% of teeth. Patients with severe (stage III) and advanced (stage IV) periodontitis were recruited [18]. The participants were divided into two groups based on gender. The study was approved by the Ethics Committee of The Second Xiangya Hospital of Central South University. All participants were informed of the research aims and provided verbal and written consent. The clinical trial registration number is ChiCTR2100046828.

Table 1: Age information of the enrolled participants.

Group	Number	Age
Group 1	11	35.73 ± 5.93
Group 2	6	34.17 ± 7.49
P		0.643

2.2. Subgingival Plaque Collection. Patients were sampled after the oral examination. Supragingival plaque was removed carefully before prior to sampling. The subgingival plaque was collected using a sterile Gracey curette. The curette was introduced into the bottom of the site, and the plaque content was removed in a single stroke into a 1.5 mL tube containing 1 mL of PBS. Samples were stored at -80°C.

2.3. DNA Extraction and 16S rRNA Gene Library Preparation and Sequencing. Genomic DNA was isolated from each sample using the DNeasy PowerSoil Kit (QIAGEN) following the manufacturer's instructions. We assessed the yield, purity, and integrity of the DNA using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific Inc., MA USA) and agarose gel electrophoresis as appropriate. 16S rRNA was completed by oebiotech (Shanghai, China). 16S rRNA gene amplification was performed in two steps. First, the V3-V4 hypervariable region was amplified by PCR using the genomic DNA and the following

Table 2: Estimated tags, tag quality, and species diversity of the samples.

Sample ID	Clean tags	Valid tags	Valid percent	OUT counts	Total OTUs	Observed species	Chao1	Good's coverage
M.01	71022	62912	88.58%	1432	5181	1402.3	2363.8	0.989402
M.02	74730	66072	88.41%	1350	5181	1292.7	2087.1	0.990492
M.03	71745	66208	92.28%	1752	5181	1687.9	2435.0	0.989183
M.04	72892	61201	83.96%	1286	5181	1276	2142.8	0.990585
M.05	72755	68461	94.10%	1473	5181	1405.5	2110.3	0.991535
M.06	73836	63441	85.92%	1469	5181	1435	2197.9	0.990143
M.07	70749	63789	90.16%	1452	5181	1416.3	2212.6	0.989977
M.08	72424	65118	89.91%	1380	5181	1330.8	2317.6	0.989657
M.09	71942	65394	90.90%	1510	5181	1457.7	2395.8	0.98916
M.10	70637	62832	88.95%	1544	5181	1512.3	2364.3	0.989155
M.11	70569	63563	90.07%	1462	5181	1425.6	2083.2	0.990467
SM ± SEM	72118 ± 1304	64454 ± 1929	89.39 ± 2.65%	1465 ± 115	5181 ± 0	1422.0 ± 107.6	2246.4 ± 126.3	0.9900 ± 0.0007
F.01	71274	66186	92.86%	1235	5181	1188.4	1831.3	0.99166
F.02	74255	68779	92.63%	1328	5181	1250.9	2082.6	0.990988
F.03	73743	68612	93.04%	1325	5181	1245.7	1934.6	0.99093
F.04	70808	64919	91.68%	1249	5181	1207.8	1909.5	0.991316
F.05	72305	67972	94.01%	1240	5181	1179.6	1817.0	0.991786
F.06	64237	60115	93.58%	1171	5181	1170.6	1672.0	0.99285
SM ± SEM	71104 ± 3307	66097 ± 3006	92.97 ± 0.74%	1258 ± 55	5181 ± 0	1207.2 ± 31.2	1874.5 ± 125.4	0.9916 ± 0.0006

SEM: standard error of the sample mean; SM: sample mean.

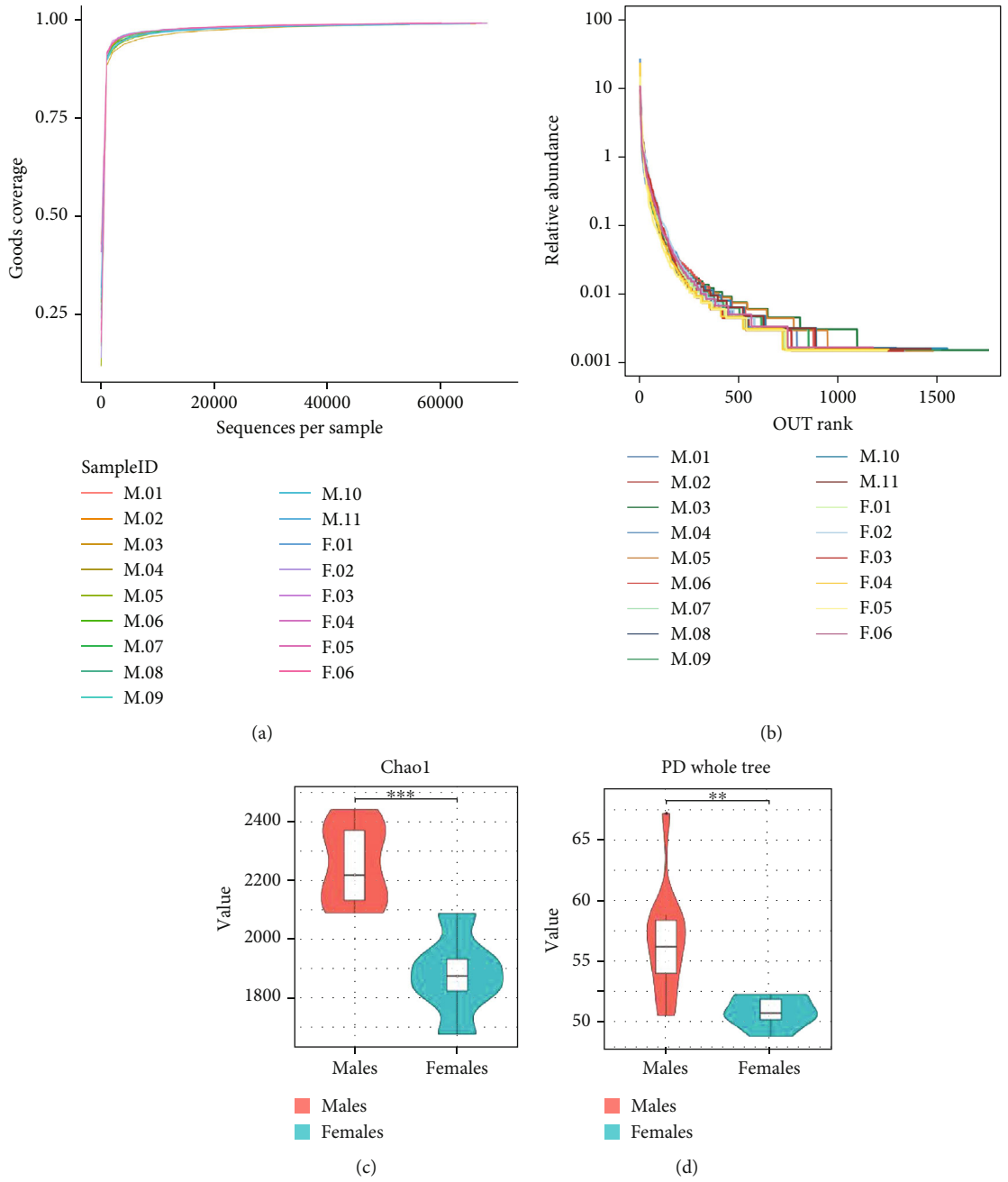


Figure 1: Continued.

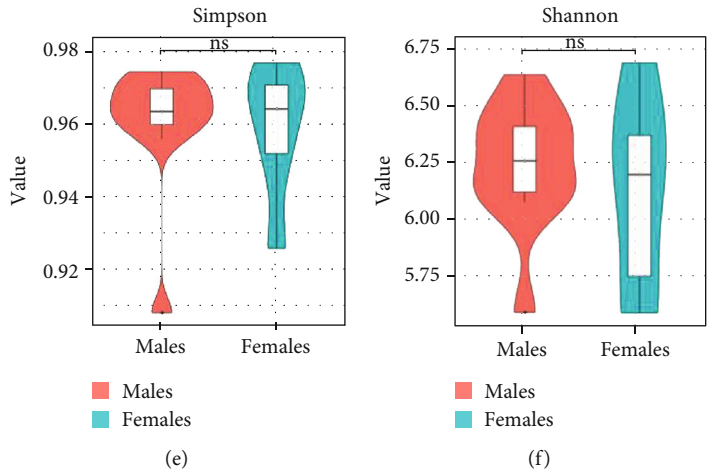


Figure 1: Sequence quality and α-diversity analysis of samples from young male and female patients with severe and advanced periodontitis. (a) Rarefaction curves based on Good's coverage for all samples from males and females. The horizontal axis shows the number of operational taxonomic units (OTUs), and appropriate number of sequences is shown on the vertical axis. (b) Rank-abundance curve representative of all samples from males and females. (c–f) Violin plots comparing α-diversity indices Chao1, PD whole tree, Simpson index, and Shannon index between the two groups. $^{***}P < 0.001$; $^{**}P < 0.01$; ns: not significant.

primers: forward, 343 F (5′-TACGGRAGGCAGCAG-3′); reverse, 798 R (5′-AGGGTATCTAATCCT-3′). Amplification was performed in a reaction mixture containing 15 μL of 2x Gflex PCR Buffer, 5 μM primer 343 F, 5 μM primer 798 R, 0.75U Tks Gflex DNA Polymerase (Takara), and 50 ng of template DNA in a total volume of 30 μL/sample. Reactions were run in a PCR thermocycler (BIO-RAD) according to the following cycling program: 5 min of denaturation at 94°C, followed by 26 cycles of 30 s at 94°C (denaturing), 30 s at 55°C (annealing), and 20 s at 72°C (elongation), with a final extension at 72°C for 5 min. The amplified products were checked by 1% agarose gel electrophoresis, purified using AMPure XP beads (Agencourt), and amplified in another round of PCR.

In the second step, sequencing primers and adaptors (1x KAPA HiFi Hotstart ReadyMix, 0.5 μM fusion forward and 0.5 μM fusion reverse primer, and 30 ng Meta-gDNA) were added to 2 μL of the diluted amplicons to a total volume of 50 μL. The PCR was run as described above except with 7 cycles. The Qubit quantification system (Life Technologies) was used for quantification of amplicons according to the manufacturer's instructions. In a single tube, the amplification products were pooled in equimolar amounts and the concentration was determined using the Qubit system. Amplicons were sequenced on the Illumina MiSeq System (Illumina Inc., CA, USA).

2.4. Bioinformatic and Statistical Analysis. Fastq files were demultiplexed using MiSeq Controller Software (Illumina Inc.). Vsearch (v. 2.4.2) [19] was used for operational taxonomic unit (OUT) clustering at or above 97%. The taxonomy of the OTUs was assigned, and sequences were aligned according to the RDP classifier Naive Bayesian method [20] and the Silva database. The OTUs were analyzed by phylogenetic methods in the Quantitative Insights into Microbial Ecology (QIIME) software (v.1.9.0). We calculated the α-diversity (observed species number, Shannon index, Simpson index, Chao index, PD whole tree index, and Good's coverage index) and β-diversity (binary Jaccard, unweighted UniFrac distances, and weighted UniFrac distances) based on the rarefied OTU counts. We used the principal component analysis (PCA) and principal coordinate analysis (PCoA) to plot the similarity or difference in the composition of the sample community. Sequences were used for microbial community metagenome prediction with PICRUSt [21] based on the GreenGenes database [22]. The differential taxa analyses were performed with the linear discriminant analysis effect size (LEfSe) using default parameters (the significance threshold of alpha parameter is set to 0.05, and the logarithmic LDA score cutoff value is set to 2.0). Functional inference was identified using Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways. The Wilcoxon test was used to determine differentially abundant KEGG pathways between the two groups.

3. Results

3.1. Clinical Characteristics of Participants. The mean ± standard deviation (SD) patient age was 35.73 ± 5.93 and 34.17 ± 7.49 for males and females, respectively. There was no statistically significant difference in age between males and females ($P > 0.05$, Table 1).

3.2. Sequence Information. We used Illumina MiSeq and QIIME to analyze the microbiome composition in subgingival plaques from patients with severe and advanced periodontitis. An average of 72,118 (min. 70,569, max. 74,730) clean tags was generated from males and 71,104 (min. 64,237, max. 74,255) clean tags from females. After removing chimeras, the number of analyzed tags decreased to an average of 64,454 (min. 61,201, max. 68,461) in males and 66,097 (min. 60,115, max. 68,779) in females. Using Vsearch (v.2.4.2) software to cluster individual sequences with 97% genetic similarity at the species level, we identified 5181

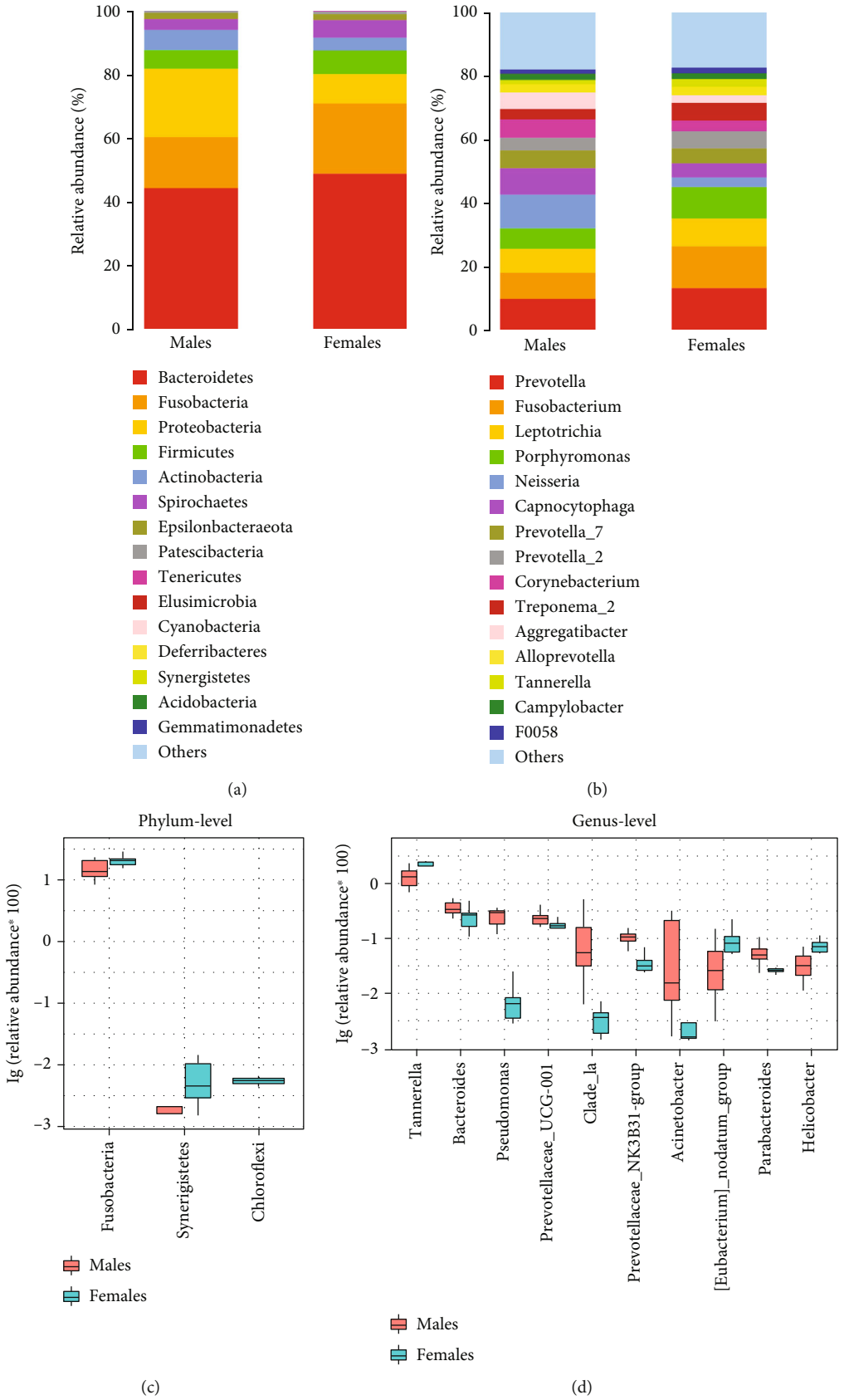


Figure 2: The oral microbiome composition of young male and female patients with severe and advanced periodontitis. (a) Phylum level composition. (b) Genus level composition. Species difference analysis between males and females by the Kruskal-Wallis test at the level of the (c) phylum and (d) genus.

OTUs among 17 samples, with a minimum of 1171 OTUs in a single sample and a maximum of 1752 OTUs (Table 2).

The quality of sequencing can generally be determined based on the index of the rarefaction curve, rank-abundance curve, or species accumulation curve. In our sample, the rarefaction curves based on Good's coverage tended to be close to saturated, indicating that the number of samples was ample, and there were no obvious differences in the rarefaction curves between the two groups (Figure 1(a)). Similar results were obtained with the rank-abundance curves, which indicate that the amount of sequencing data was abundant enough to reflect most of the microbial species information in the sample (Figure 1(b)).

3.3. Diversity Analysis. α-Diversity reflects the richness and diversity of oral microbial communities. Bacterial richness and evenness within each group (α-diversity) was estimated using the Chao1 index, PD whole tree measurements, and the Simpson and Shannon indices (Figures 1(c)–1(f)). The Chao1 index and PD whole tree were significantly higher in males than females (Wilcoxon rank-sum test, $P < 0.01$). The Simpson and Shannon indices were not significantly different between the two groups (Wilcoxon rank-sum test, $P > 0.05$). Thus, the community diversity was not different in patients with severe and advanced periodontitis according to gender.

Subsequently, for community structure, relative abundance was assessed at the top 15 phylum (Figure 2(a)) and genus (Figure 2(b)) level, respectively. Although the dominant florae were similar, the abundance of each species was vastly different. The main microbiota (>1%) at the phylum and genus level and their abundance are listed in Table 3. Statistical analyses of the major bacteria between the two groups were carried out, and boxplots representing the abundance of the top 3 different species at the phylum and top 10 different species at the genus levels ($P < 0.05$) are shown in Figures 2(c) and 2(d). Compared to males, the relative abundance of *Fusobacteria*, *Synergistetes*, and *Chloroflexi* at the phylum level increased in females ($P < 0.05$), in which the abundance of *Chloroflexi* in males is 0 (Figure 2(c)). At the genus level, the relative abundance of *Tannerella*, *Eubacterium_nodatum_group*, and *Helicobacter* was higher in females than in males ($P < 0.05$) while the relative abundance of *Bacteroides*, *Pseudomonas*, *Prevotellaceae_UCG-001*, *Clade_la*, *Prevotellaceae_NK3B31-group*, *Acinetobacter*, and *Parabacteroides* in females was lower than that in males ($P < 0.05$; Figure 2(d)). Figure 3 shows the Wilcoxon analysis of differences between males and females in the relative proportions at the phylum (Figure 3(a)) and genus (Figure 3(b)) levels in the form of a heat map.

β-Diversity reflects the differences of microbial diversity between two groups. Therefore, we evaluated the extent of the similarity between the microbial communities using PCA, PCoA, and nonmetric multidimensional scaling (NMDS) analysis based on unweighted UniFrac distances. Males and females with severe and advanced periodontitis could form relative clusters (Figures 4(a)–4(c)) and generally be separated into a two-dimensional spatial distribution, indicating that the two communities were different (Figures 4(b) and 4(c)). An ANOSIM test of the β-diversity demonstrated a significant difference (R value with the unweighted UniFrac distance matrix = 0.4515; $P = 0.001$).

Table 3: The main microbiota (>1%) at the phylum and genus level and their abundance.

Level	Microbiota	Males	Females
Phylum level	*Bacteroidetes*	44.23%	48.77%
	Fusobacteria	15.92%	22.01%
	Proteobacteria	21.57%	9.29%
	Firmicutes	5.83%	7.36%
	Actinobacteria	6.46%	4.07%
	Spirochaetes	3.26%	5.56%
	Epsilonbacteraeota	1.97%	1.87%
Genus level	*Prevotella*	9.89%	13.29%
	Fusobacterium	8.25%	13.16%
	Leptotrichia	7.6%	8.78%
	Porphyromonas	6.35%	9.87%
	Neisseria	10.63%	4.07%
	Capnocytophaga	8.31%	4.43%
	Prevotella_7	5.62%	4.72%
	Prevotella_2	3.99%	5.42%
	Corynebacteriumand	5.72%	3.35%
	Treponema_2	3.26%	5.56%
	Aggregatibacter	5.23%	2.34%
	Alloprevotella	2.6%	2.69%
	Tannerella	1.37%	2.43%
	Campylobacter	1.92%	1.79%
	F0058	1.32%	1.79%

3.4. LEfSe Analysis. The linear discriminant analysis effect size (LEfSe) was used to identify taxa characterizing the differences between the two groups. Based on the results of the species-abundance comparison between the two groups of oral microbiotas, the community differences between groups were analyzed at the phylum to genus level using LEfSe analysis (Figures 5(a) and 5(b)). Our results suggest that the phylum Fusobacteria, its class Fusobacteria, and its order Fusobacteriales, along with the family Tannerellaceae and its genus *Tannerella*, were abundant in the female patients. In addition, the order Pseudomonadales and its family Pseudomonadaceae and its genus *Pseudomonas*, along with the genus *Papillibacter*, were abundant in the male patients. *Tannerella* was the key component that could make the difference between groups by selecting the first 30 genera of relative abundance and carrying out the random forest feature selection procedure (Figure 5(c)).

3.5. Functional Metabolism Pathway Prediction. Among the KEGG pathways predicted for microbial function, we identified immune-related pathways that exhibit a significant difference in abundance between males and females at

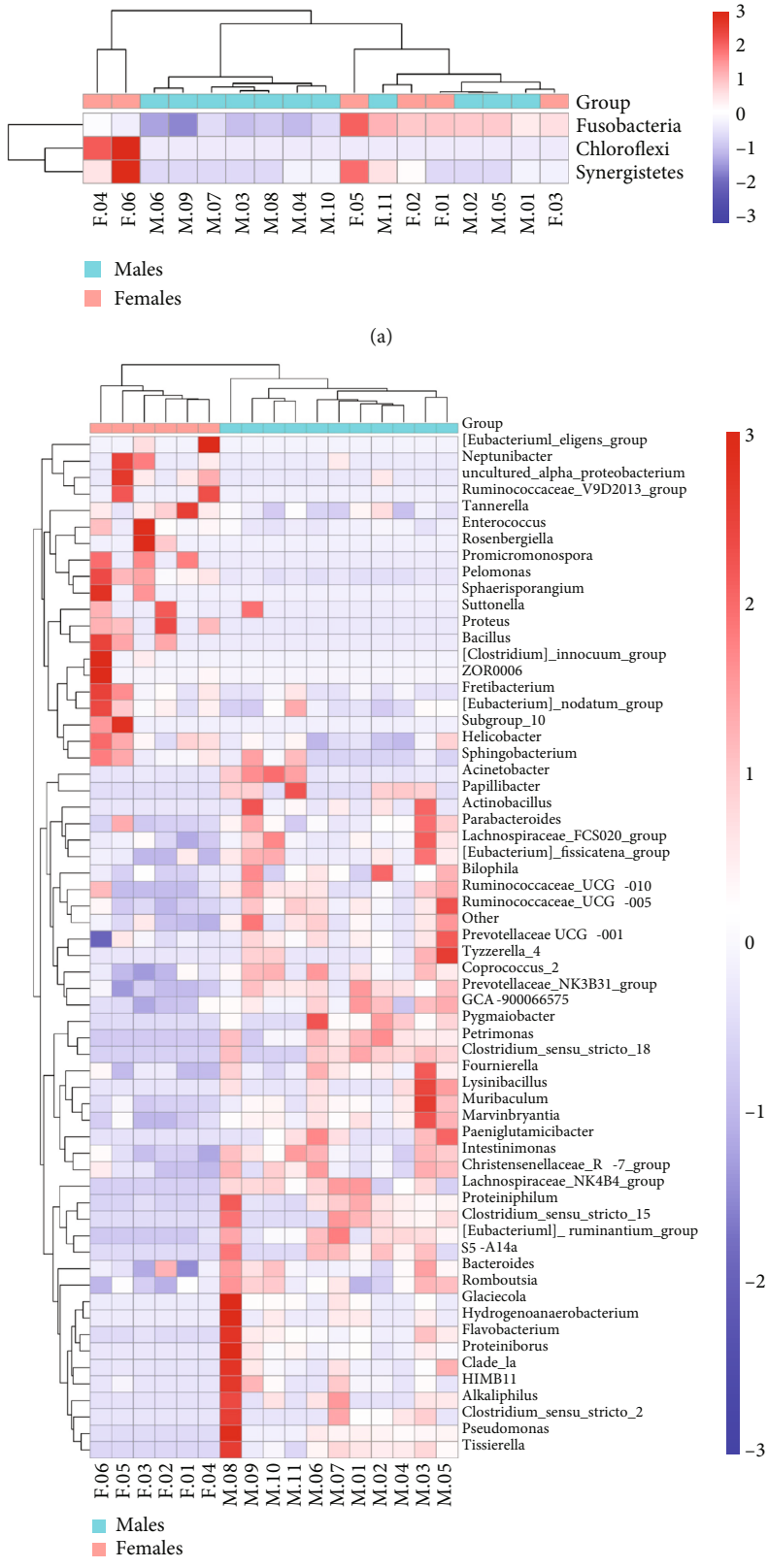


Figure 3: Heat map of the differential oral microbiomes between young male and female patients with severe and advanced periodontitis. (a) Phylum level. (b) Genus level. The horizontal axis is the sample information (group and number), and the vertical axis is the species annotation. Colors indicate the Spearman rank correlation.

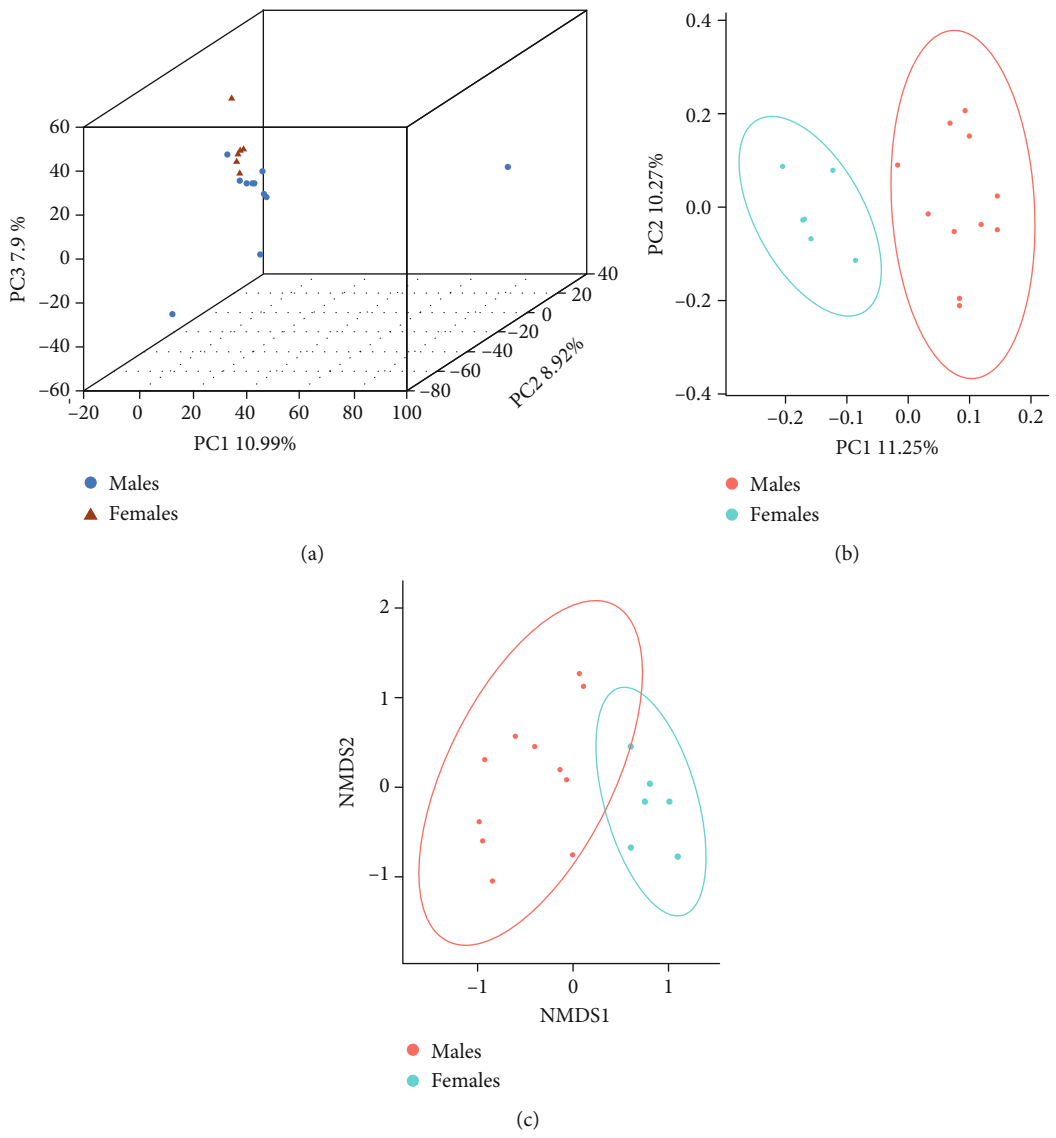


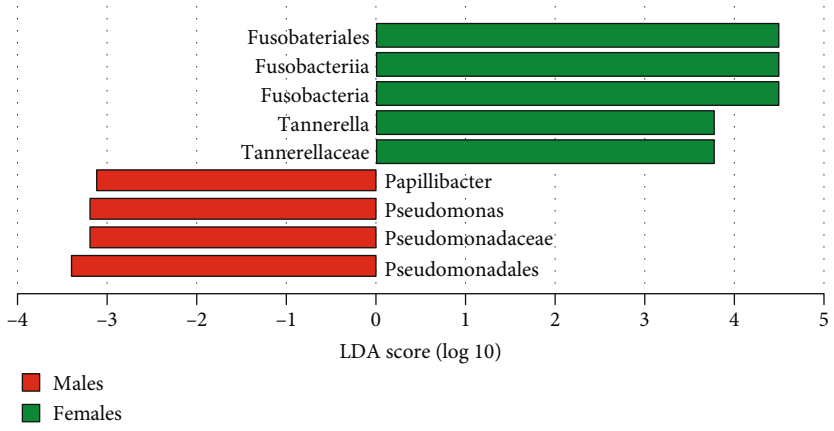
FIGURE 4: β-Diversity analysis of young male and female patients with severe and advanced periodontitis. (a) The similarity of the microbial communities between males and females was analyzed by principal component analysis, (b) principal coordinate analysis, and (c) nonmetric multidimensional scale (NMDS) based on the unweighted UniFrac distance.

different levels in Wilcoxon tests (Figure 6). The pathway analysis of the predicted KEGG pathways in each sample at the phylum level indicated that the microbiomes with altered abundance are mainly involved in pathways related to the immune system and cardiovascular disease (Figure 6(a)). The results at the class level also showed that the pathways were related to the immune system, including the NOD-like receptor signaling pathway and antigen processing and presentation (Figure 6(b)). The predicted KEGG pathways based on group showed that the immune system was also significantly enriched in females and immune system diseases concentrated in males (Figure 6(c)).

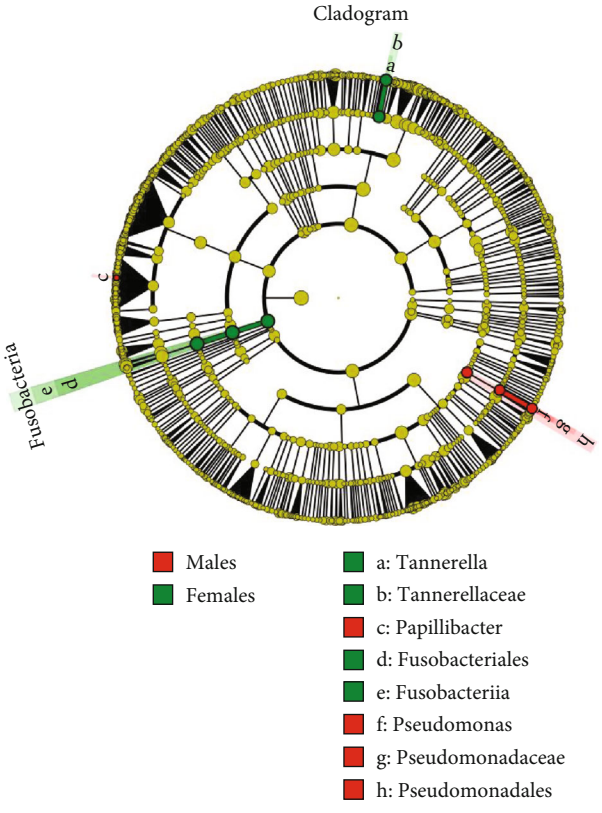
4. Discussion

In this study, we used 16S rRNA high-throughput sequencing to determine the differences in species abundance in the oral microbiomes of young men and women with severe periodontitis. Regarding α-diversity based on species richness, males had higher values than females. In addition, β-diversity suggested that the samples were divided into reasonable groups. The Kruskal-Wallis test based on the relative abundance of species composition, combined with LEfSe analysis showed that the dominant bacteria in males were *Pseudomonas* and *Papillibacter*, whereas the dominant bacteria in women were the order Fusobacteriales and the genus *Tannerella*. KEGG analysis predicted that the variation in the oral microbiome may be related to the immune system in women, whereas immune system diseases are the dominant pathway in men, offering further understanding of periodontitis.

Periodontitis is an inflammation that extends deep into tissue and leads to the loss of supporting connective tissue and alveolar bone [23]. Oral microbiomes are defined as



(a)



(b)

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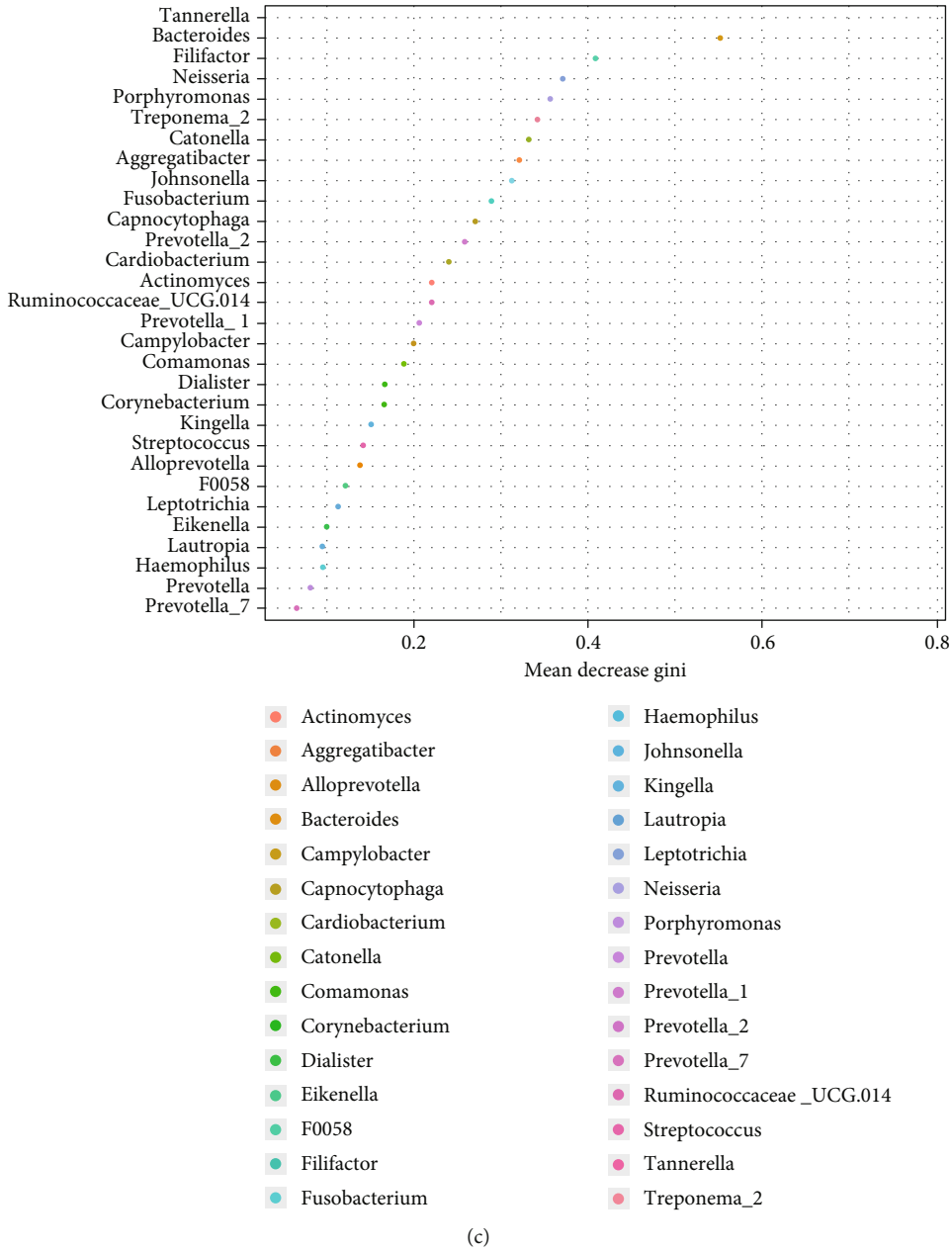
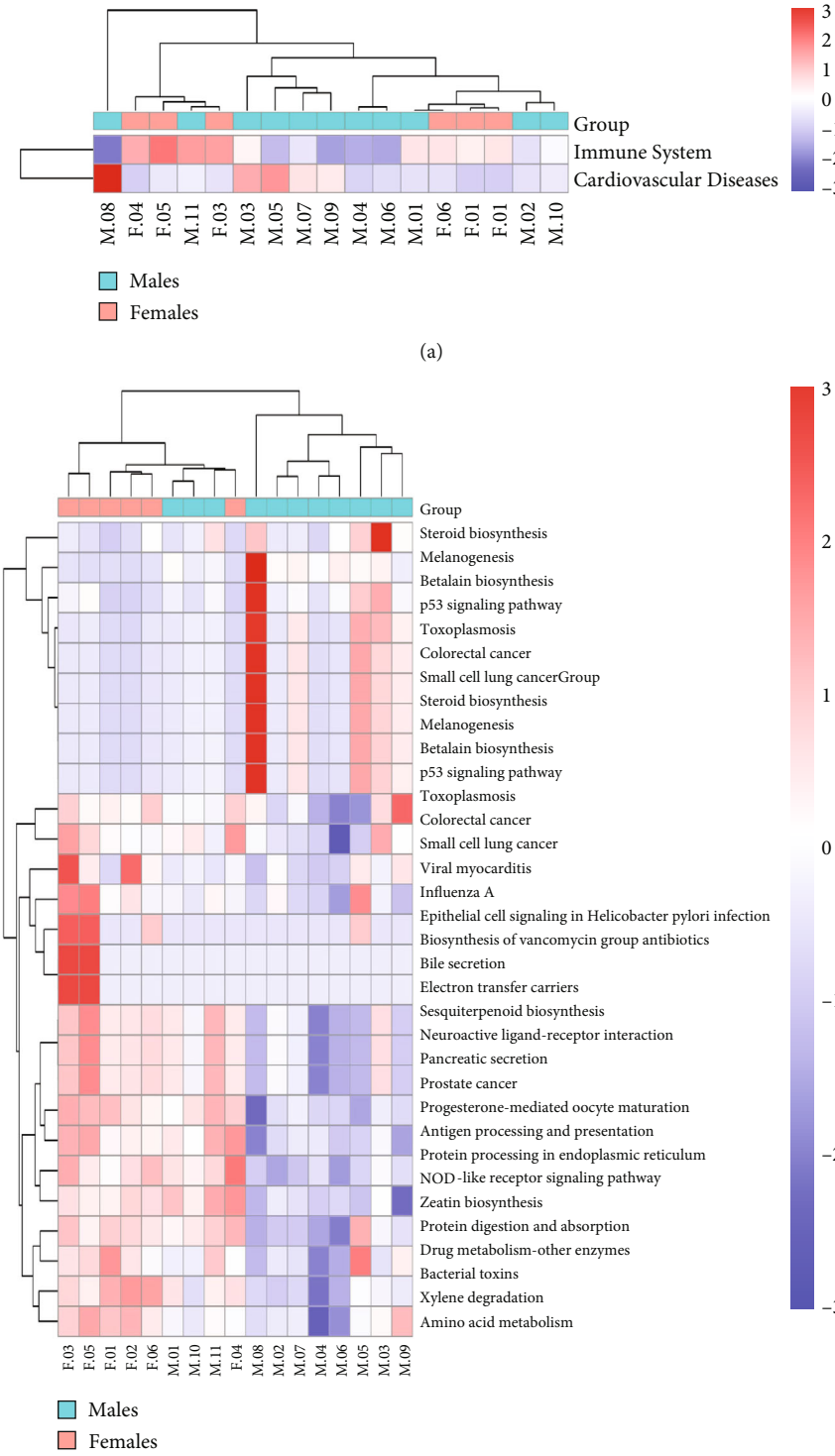


(c)

Figure 5: Linear discriminant analysis (LDA) effect size (LEfSe) analysis of the oral microbiomes in young male and female patients with severe and advanced periodontitis. (a) Histogram of the LDA scores of males and females. Female-enriched taxa are indicated by a positive LDA score (green), and taxa enriched in males have a negative score (red). (b) Taxonomic cladogram obtained from LEfSe analysis. Red indicates male, green indicates female, and yellow indicates nonsignificant between males and females. (c) Point map of species importance. The horizontal axis is the measure of importance, and the vertical axis is the name of the species sorted by importance.

the microorganisms found in the oral cavity, including bacteria, viruses, fungi, protozoa, and archaea [24]. The dysbiosis hypothesis states that the transition from periodontal health to disease occurs due to changes in species abundance among the bacteria in the periodontal pocket. This shift in the composition of the microbial community is sufficient to alter the host-microbe crosstalk, resulting in destructive inflammation and bone loss [7]. The healthy oral microbial community usually has low diversity and richness [25]. The species diversity of the general flora is lower in youth than in the elderly, which is often reflected in the periodontal condition of young people being healthier. In addition, young people are more affected by hormones, so there is clinical significance to discussing gender differences. We found that the Chao1 index and PD whole tree were significantly higher in males than females, representing the greater bacterial richness in men, and that they may have severe periodontal tissue destruction. An investigation of the gut microbiota in healthy Japanese subjects found that, although there were significant differences in the microbial structure



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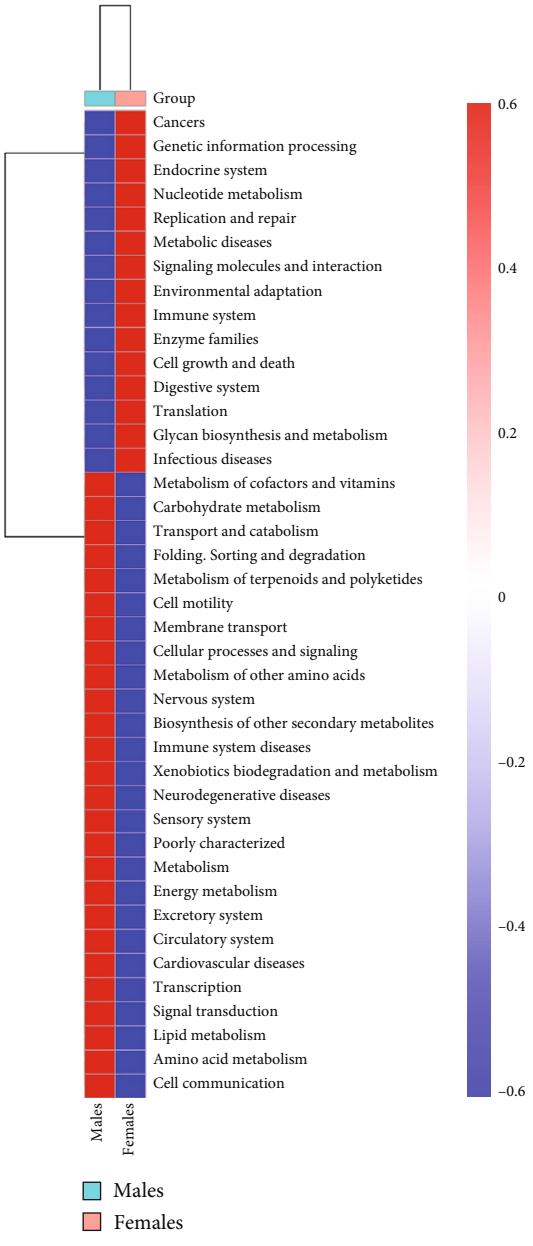

(c)

Figure 6: Pathway enrichment analysis based on KEGG. (a) Heat maps of differential pathways at the phylum level and the class level of KEGG in individual patients. (c) Heat map of differential pathways between males and females at the phylum level of KEGG.

between males and females aged 20–89 years, the α-diversity of the gut microbiota was not different between males and females or among age groups [26].

At the American Academy of Periodontology Workshop held in 1996, experts agreed there are 11 microorganisms that are closely related to periodontal disease, including gram-negative bacteria such as *P. gingivalis*, *T. forsythia*, *P. intermedia*/*P. nigrescens*, *F. nucleatum*, *Aggregatibacter actinomycetemcomitans* (*A.actinomycetemcomitans*), and *T. denticola* [27]. Among them, we found that *Fusobacteriales* are significantly higher in women than in men through the Kruskal-Wallis test at the level of phylum and LEfSe analysis. The most prevalent in females was *Fusobacteria*, anaerobic gram-negative bacilli that cause tissue necrosis, septicemia, intra-amniotic infections, premature labor, and disorders of the oral cavity, such as pulpal infections, alveolar bone abscesses, and periodontal disease. *F. nucleatum* induces production of inflammatory cytokines and cell proliferation and inhibits apoptosis, cellular invasion, and migration through host cell genomic alterations [12]. *F. nucleatum* reacts to the inflammatory response during periodontal disease and induces secretion of salivary antimicrobial peptides that have an impact on host cells and modulate the immune response [12]. Kostic et al. have indicated that Fusobacteria generates a proinflammatory microenvironment conducive to the progression of colorectal neoplasia

by recruiting tumor-infiltrating immune cells [28]. The phylum Proteobacteria was enriched in male patients. Guan et al. have found that high expression is also present in severe bronchiectasis [29].

Our results showed significant variation in the oral microbiomes of the youths of different genders. In our research, at the genus level, the increased *Porphyromonas*, *Prevotella*, and *Tannerella* in the oral microbiomes of females contributed to the periodontal destruction. *P. gingivalis* is the most extensively studied of all major periodontal pathogenic organisms. The virulence factor of *P. gingival* is lipopolysaccharides (LPS), fimbriae, and capsule [30]. *P. gingivalis* LPS and fimbriae stimulation led to the upregulation of TLR2 expression and proinflammatory cytokine production *in vivo* and *in vitro*, resulting in pathogenic inflammatory bone loss [31]. *P. intermedia* and *P. nigrescens* are often isolated from periodontal sites and were once considered to be two different genotypes of *P. intermedia* [32, 33]. *P. intermedia* LPS can participate in bone destruction by stimulating the differentiation and activity of osteoclasts and promoting the release of matrix metalloproteinases from osteoclasts and osteoblasts [34]. It is worth noting that we found that *Tannerella* is significantly increased in women through the LEfSe analysis. *Tannerella* is gram-negative, obligate anaerobic, nonmotile, pleomorphic bacilli [35] that can affect the host immune system through induction of proinflammatory cytokines (e.g., IL-1β and IL-6) via CD4+ T helper cells and TNF-α [36]. A clinical cross-sectional study found that the presence of *P. gingivalis* and high colonization by *T. denticola* and *P. intermedia* play an important role in severe periodontitis in a Thai population [37]. Besides, the severity of periodontitis in the youths was related to the high level of hormones. In a cohort study of 106 women (50–58 years old), hormone replacement therapy resulted in fewer positive samples for the periodontal pathogens *P. gingivalis*, *P. intermedia*, and *Tannerella forsythia* in the subgingival plaque [38].

Our results show that *Neisseria* and *Capnocytophaga* are increased in men with severe periodontitis, which is consistent with previous results. Minty et al. reported that *Capnocytophaga* were overrepresented in salivary samples from males compared to females [39]. *Capnocytophaga canimorsus* is a gram-negative bacillus with unique virulence factors that enable it to evade the human immune system; it is present in the oral cavities of 22% to 74% of healthy dogs [40]. Leptotrichia, Prevotella_7, and Prevotella_2 showed no significant difference between males and females. Furthermore, no difference was found between males and females regarding the Simpson and Shannon indices, indicating a large number of similar strains in males and females, which is consistent with the results reported by Chen et al. [41]. In contrast to our results, when Belstrøm et al. used microarrays to examine the oral microbiomes of 292 Danish individuals with low levels of dental caries and periodontitis, they found that diet, BMI, age, and sex did not significantly affect microbial abundance, though socioeconomic status affected the oral microbiome profiles [42]. Other possible factors could explain the differences observed in our study, including genetic variations, social factors, chronology of tooth eruption, and hormonal differences, which could affect the composition of the plaque microbiomes [14, 43].

In our study, KEGG analysis found that the immune system, endocrine system, metabolic disease, and infectious disease pathways were significantly changes in women. This may be related to the fact that the female dominant bacteria F. nucleatum had the ability to form a biofilm and coadhere, which can lead to systemic diseases such as urinary tract infection, bacteremia, pericarditis, and otitis media [44, 45]. But the specific mechanism needs to be further studied. This suggests that the imbalance in oral microbiota participates in the regulation of immunological and metabolic homeostasis according to gender differences. However, through the LEfSe analysis, the number of *Papillibacter* was significantly higher in males. Some studies have found that the high relative abundance of *Papillibacter ruminococcacea* is related to melanoma patients with a high response to PD-1 immunotherapy [46], indicating that *Papillibacter* is closely related to immune disease, which is consistent with the enrichment of KEGG analysis in males. Furthermore, *Papillibacter* can cause local inflammation, resulting in aggregation of α-synuclein and generation of Lewy bodies that can participate in the occurrence and development of Parkinson's disease in males [47]. This is possibly related to a phenomenon referred to as the mobile microbiome, which is thought to contribute to systemic disease onset and progression. The phenomenon comprises shifts in the oral microbiome; resulting alterations in the local host-immune response and spillage of proinflammatory mediators into the systemic circulation could influence systemic inflammation and immune system diseases [48]. Moreover, nearly all immune cells express the hormone receptors [49, 50], and many immune-related genes possess androgen receptor-responsive elements and estrogen receptor-responsive elements in their promoters, which may be another factor affecting sex differences in the immune responses [51, 52].

5. Conclusions

In summary, our study proved the hypothesis of a sex-specific association between the oral microbiomes and severe periodontitis in the youth through 16S rRNA gene sequencing. In addition, KEGG pathway analysis predicted that the variation in the oral microbiome may be related to immune homeostasis. Therefore, further investigation is needed to deepen our understanding of the mechanism.

Data Availability

Raw reads have been deposited at NCBI under the BioProject accession number PRJNA763727.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Ya-Qiong Zhao and Ying-Hui Zhou contributed equally to this work.

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