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Original article

Analysis of subgingival microbiome of periodontal disease and rheumatoid arthritis in Chinese: A case-control study

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ABSTRACT

Objective: Periodontitis (PD) and rheumatoid arthritis (RA) share similar pathogenesis. Evidences indicated that oral bacteria play an important role in the etiology of both diseases. For example, *Porphyromonas gingvialis* connect the two diseases through immune responses. We designed this study to compare bacterial diversity in RA, PD and healthy subjects and to investigate whether there are other oral bacteria play an potential role in linking the two diseases.
Methods: This study included 3 groups of Chinese participants who visited Sichuan Provincial People's Hospital during August 2018 and March 2019. Subgingival plaques were collected from RA group (n = 54), PD group (n = 45) and normal group (n = 44). Illumina MiSeq was used to compare the composition of subgingival microbiota and analyze correlations between oral bacteria and both diseases.
Results: Alpha diversity Analysis reflected similar microbiome profile in RA, PD and healthy groups. But we found *Treponema* was significantly more abundant in the PD and RA groups than healthy group at each taxonomic level from the phylum down to the genus level. *Porphyromonas, Prevotella,* and *Veilonella* were significantly more abundant in RA while *Streptococcus, Gemella, Planobacterium* were verified the opposite results.
Conclusions: We found no significant group differences referent to either microbial diversity or richness. But we picked out *Spirochaetes* which may link the two diseases. Upper/lower regulation of some microbia in RA may remind us a direction to explore the role they play in pathogenesis.

1. Introduction

Rheumatoid arthtitis (RA) is a chronic, immune-mediated inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints (Potempa et al., 2017). Similarly, periodontitis is a chronic inflammation of the tooth-supporting tissues, leading to the progressive destruction of periodontal ligament and alveolar bone, in which pathogenic biofilm cause a series of pathological events (de Pablo et al., 2009). *Porphyromonas gingvialis* is one of the most important pathogenic bacteria (Wegner et al., 2010).

RA and periodontitis have a similar pathophysiology, characterized by destructive inflammation (Abbasi, 2017). Furthermore, clinical and epidemiologic studies indicate that patients with RA have an increased prevalence of periodontitis and tooth loss. As one plausible but most convincing causal mechanism, the citrullination of proteins by *Porphyromonas gingvialis* and the subsequent generation of autoantigens that drive autoimmunity in RA represents a possible causative link between these two diseases (Wegner et al., 2010).

Multiple lines of investigation have suggested a link between oral microbes, periodontal diseases (PD) and RA (Potempa et al., 2017; de Pablo et al., 2009; Konig et al., 2016; Lundberg et al., 2010). However, most clinical studies implicating specific oral microorganisms as triggers for RA have relied only on serological

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methods. Detailed bacterial biological information help researchers learn more about the role of oral bacteria in RA, such as *Porphyromonas gingvialis*. Several studies have been done to compare the biodiversity in PD and RA but the data are limited and inconclusive.

In the present study, we aimed to determine subgingival microbial diversity of RA patients, PD patients and healthy controls and to directly correlate the subgingival microbiota with RA status utilizing high-throughput 16S rRNA sequencing. We focused our attention on the biodiversity in different groups and whether there is another specific oral microbiota appear in RA and the relationship with periodontitis.

2. Materials and methods

2.1. Patient and public involvement statement

This study was approved by the ethical review committee of Sichuan Provincial People's Hospital (ethics ID:2019210), and conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from the participants of all subjects before study. Study included 3 groups of participants who visited Sichuan Provincial People's Hospital during August 2018 and March 2019. The RA patients were diagnosed according to the 2010 ACR/EULAR classification criteria (Aletaha et al., 2010). And the PD patients were diagnosed according to the criteria proposed by Machtei et al. (1992). Healthy controls were picked randomly from the department of Stomatology of Sichuan Provincial People's Hospital. The study results were disseminated to participants by written informations.

2.2. Subject

54 random rheumatoid arthritis patients were picked for the RA group. 45 random periodontal disease patients consisted the PD group.44 random healthy participants were picked for control group. The age of the RA group varied from 27 to 69, and the average age were 51 ± 13.3. Whereas, the age of the PD group varied from 24 to 66, and the average age were 41.9 ± 18.69. To contrast, the age of healthy group varied from 23 to 50 with the average age of 38.3 ± 16.87 (Table 1).

All of the study participants were healthy and had no other systematic diseases, with no systematic antibiotics application in last three months, no medical history of hormone-based drugs or immunosuppressive drugs, and no local usage of antibiotics of oral cavity.

2.3. Sample collection and oral examination

For each of the subjects, microbial samples were collected approximately 2 h after lunch without tooth-brushing nor dental flossing according to the protocol described in Manual of Procedures for Human Microbiome Project (http://hmpdacc.org/tools_protocols/tools_protocols.php). We used a sterile Gracey curette to collected mixed supragingival plaque sample from the mesial surfaces of six index teeth:16;11;26;36;41;46 after isolating the sample sites with cotton rolls. The collected samples were released in 700 μl of TE buffer (10 mM Tris-Cl [pH 7.5] and 1 mM EDTA). The microbial samples were immediately transported on ice to the laboratory and stored at −80 °C until further DNA extraction and pyrosequencing analysis (Du et al., 2017).

2.4. DNA extraction and pyrosequencing

Total bacteria DNA was extracted from microbial samples and was stored at −80 °C before further analysis. DNA was amplified by using the 515f/806r primer set (515f: 59-GTG CCAGC MGCCGCGGTA A-39, 806r: 59-XXX XXXGGACTACHVGGGTWT CTA AT-39), which targets the V4 region of the bacterial 16S rDNA, with the reverse primer containing a 6-bp error-correcting barcode unique to each sample (Caporaso et al., 2012). PCR reaction was performed using Bio-Rad S-1000 (Bio-rad, American) with the following condition: 94 °C for 3 min(1 cycle), 94 °C for 45 s/50 °C for 30 s/72 °C for 60 s (30 cycles), and a last step of 72 °C for 10 min. Takara ExTaq™ HotStart Version (Takara, Japan) was used as PCR enzyme. The products were purified by using the Omega Gel Extraction Kit (Omega, American), quantified by using Qubit® dsDNA BR Assay Kit (Life Technologies, American) and divided to 500 ng for every sample. Pyrosequencing was conducted by using Illumina MiSeq.

Table 1
Characteristics of all subjects.

Group	RA	PD	Control
Age (years)	51 ± 3.3	41.9 ± 18.69	38.3 ± 16.87

2.5. Bioinformatics and statistical analysis.

Sample reads were assembled by using CASAVA v1.8.2 and FastQC. Chimeric sequences were removed according to standard principles. Operational Taxonomic Unit (OTUs) were picked using denovo OTU picking protocol. Taxonomy assignment of OTUs was performed by comparing sequences to the Greengenes database. Alpha diversity analysis included ACE, chao, Simpson and Shannon index. Beta diversity was calculated by weighted UniFrac distances.

2.6. Statistical analyses

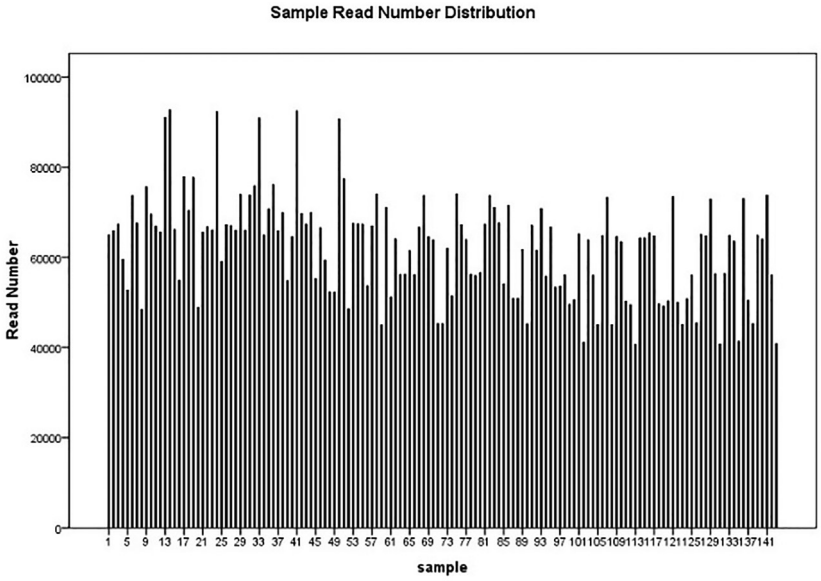
Differences were evaluated by independent *t* test, Mann–Whitney *U* test or chi-squared tests. We used SPSS V.22.0 software (SPSS, Chicago, Illinois, USA) to determine the statistical significance, two-tailed significance testing was used and significance was set as $p < 0.05$.

3. Results

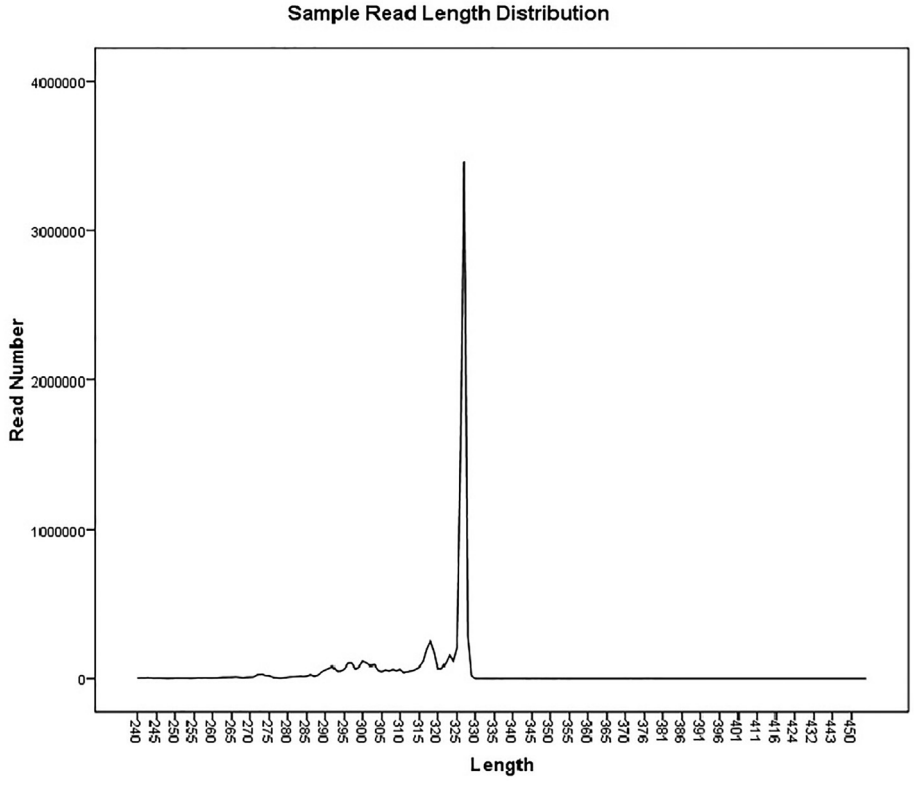
3.1. Overall results of pyrosequencing

In all the 143 subgingival plaque samples, over 8 million amplicons were sequenced. The average sequences of each sample were 63591. And the distribution of sample read length was from 240 bp to 468 bp (Fig. 1). Using a distance-based similarity of >97% for operational taxonomic unit (OTU) analysis, 14 phyla were identified. More than 98% OTUs is divided into 7 phyla or candidate divisions, that is *Firmicutes* (30.2%), *Proteobacteria* (29.3%), *Bacteroidetes* (23.8%), *Fusobacteria* (7.3%), *Actinobacteria* (5.6%), *Spirochaetes* (0.6%) and 1.4% OTUs were not recognized (Fig. 2). These main phyla is consistent with the result of B.J.F Keijser though the ranking is not exactly alike (Keijser et al., 2008).

We found 78 main genuses which compose 98% microbiota in all samples.Moreover, *Prevotella*, *Streptococcus*, *Neisseria*, *Escherichia/Shigella*, *Pasteurellaceae;Other*, *Leptotrichia*, *Veillonella*, *Rothia*, *Granulicatella*, *Porphyromonadaceae;Other*, *Fusobacterium*, *Lachnospiraceae;Other*, *Gemella, Capnocytophaga, Actinomyces* comprise the most (Fig. 3).



a. sample read number



b. read length distribution

Fig. 1. Sample read number and length distribution.

3.2. The oral microbiota is equally rich and diverse in RA, PD and control groups

To compare the oral microbial diversity of RA, PD and healthy groups, we used the Ace, chao, Simpson and Shannon indexes. A high index reflects a more diverse microbiota. Utilizing both calculations, no significant differences in microbial diversity were observed between RA groups and controls, PD groups and controls or RA groups and PD groups (Table 2) but Shannon index. The Shannon index in RA patients is higher than control group

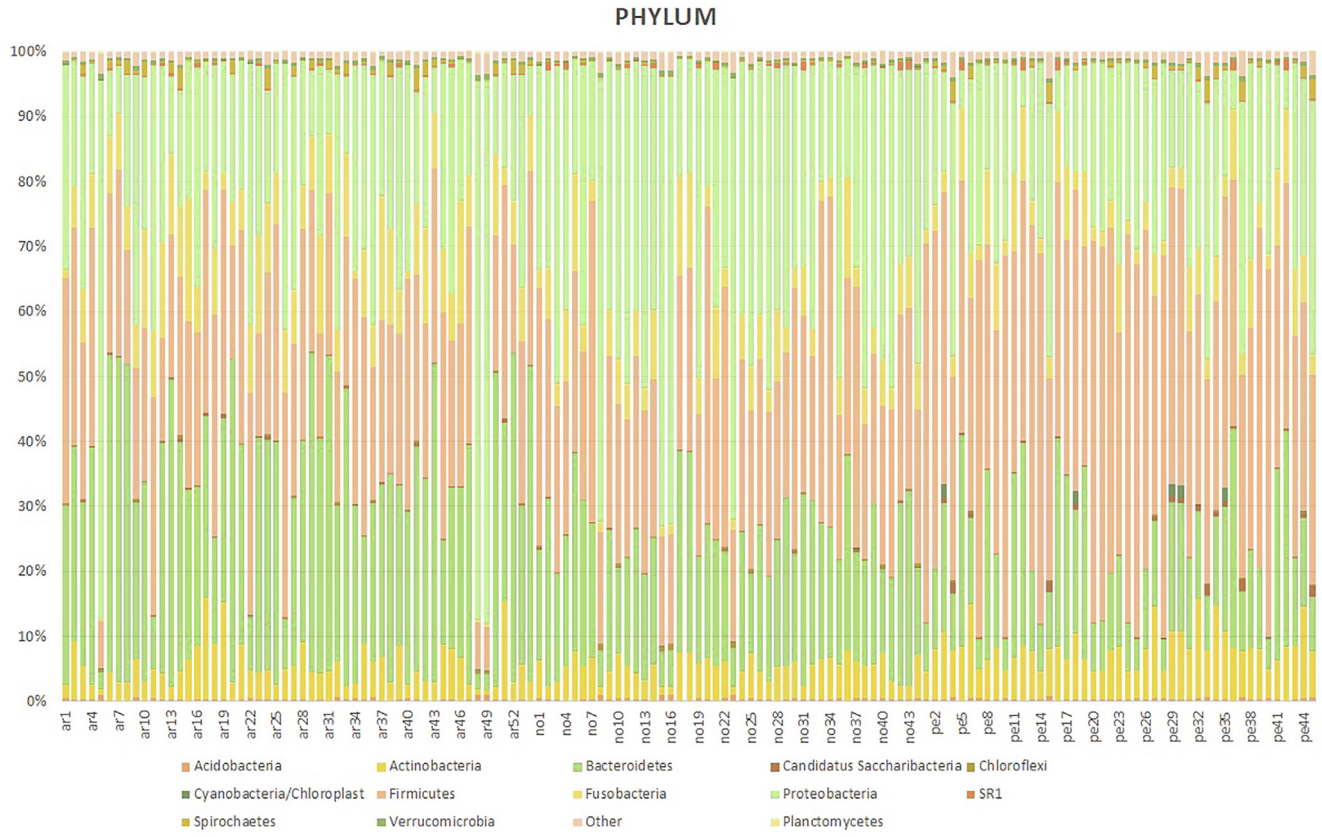


Fig. 2. Relative abundance of the main phyla identified in subgingival plaque.

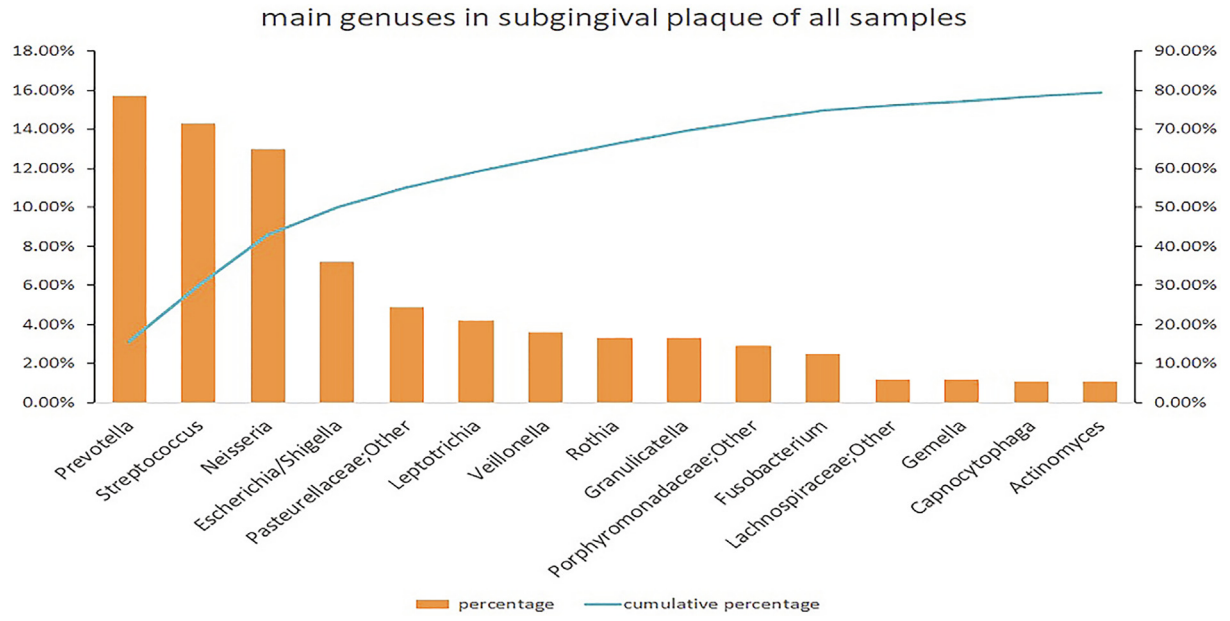


Fig. 3. Main genuses in subgingival plaque of all samples.

(P = 0.03), which is consistent with result from Bin Chen (Chen et al., 2018). Although the oral microbiota is equally rich in RA, PD and control groups, there is a significant difference about evenness between RA and healthy subjects.

To analyze if RA and PD microbiota was distinct from that of healthy controls, we used weighted Unifrac to the quantified similarity distances between samples and applied Principal Coordinate Analysis (PCoA) by clustering them along orthogonal axes of max-

Table 2
Diversity index of RA, PD and control groups.

	Ace	*Chao*	*Simpson*	*Shannon*
Control	8600.46 ± 1002.82	7904.81 ± 876.25	0.90 ± 0.04	5.58 ± 0.29
PD	8494.64 ± 695.60	7747.11 ± 718.30	0.90 ± 0.06	5.76 ± 0.80
P value	0.79	0.67	0.96	0.48
Control	8600.46 ± 1002.82	7904.81 ± 876.25	0.90 ± 0.04	5.58 ± 0.29
RA	9243.08 ± 1437.95	8487.25 ± 1228.95	0.92 ± 0.09	6.07 ± 0.66
P value	0.21	0.18	0.64	0.03
PD	8494.64 ± 695.60	7747.11 ± 718.30	0.90 ± 0.06	5.76 ± 0.80
RA	9243.08 ± 1437.95	8487.25 ± 1228.95	0.92 ± 0.09	6.07 ± 0.66
P value	0.16	0.11	0.663	0.30

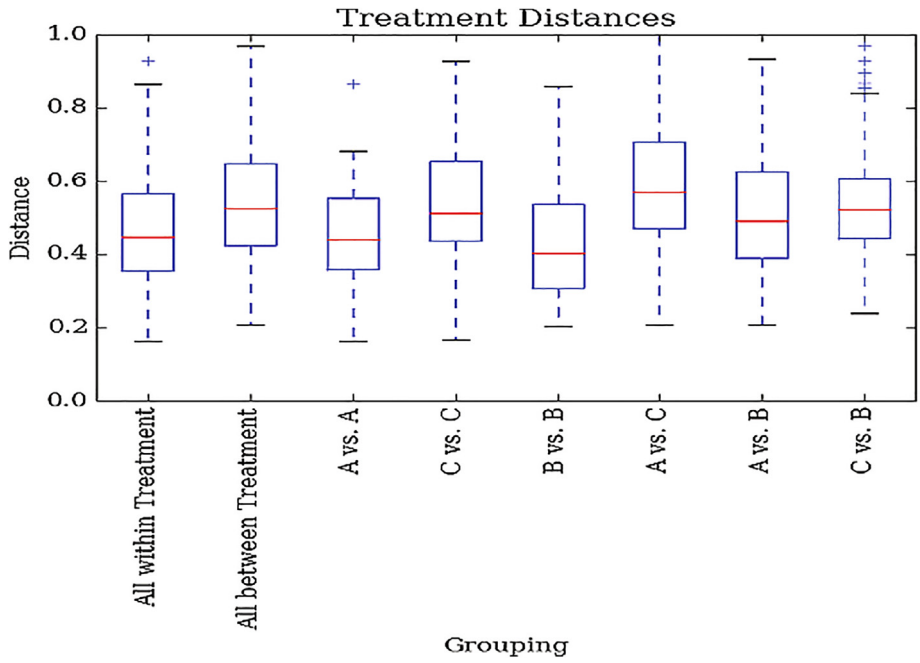


Fig. 4. UniFrac analysis Group A is RA group, Group B is control group, Group C is PD group. Unifrac distance metric was analysed. And there is no significant difference.

imal variance(Fig. 4a). Two principal coordinates (PC1 and PC2) explain most of the variation observed between the samples.

3.3. The comparision of oral microbiome in RA patients, PD patients and healthy people

Taking taxonomic lineages into account, although oral microbiota is equally rich and diverse in RA, PD and control groups, different dominant lineages were found in RA, PD and healthy people. At Phylum level, RA patients have a higher portion of *Bacteriodetes* while in PD patients *Firmicutes* comprise most (Fig. 5).

Furthermore, we found one lineage was more abundant in RA group than control at each taxonomic level from the phylum down to the genus level. The lineage was *Spirochaetes* at the phylum level (P = 0.008), *Spirochaetia* at the class level (P = 0.026), *Spirochaetales* at the order level (P = 0.008), *Spirochaetaceae* at the family level (P = 0.008, *Treponema* at the genus level (P = 0.006) (Fig. 5). In PD group, although it seems the *Spirochaetes* lineage is more abundant than control group at each taxonomic level, there is no statistical significance (P = 0.112).

4. The profile of periodontal pathogens in RA and PE groups compared with controls

Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia are called 'red complex' which is a prototype polybacterial pathogenic consortium in periodontitis (Holt and Ebersole, 2000; Jiao et al., 2014; Ng et al., 2016). Besides, coninfection of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Trponema denticola* cause more serious damage to periodontium (Konig et al., 2016; Chen et al., 2005). So we assessed proportion of these PD-related bacteria compared to RA and control group at genus level. First; *Porphyromonas* was present in 13 RA patients and 8 PD patients while the prevalence in healthy controls is 10/11. Further, in the samples which detected *Porphyromonas*, there is a significant difference between RA patients and controls on relative abundance of *Porphyromonas* (P = 0.001, Fig. 6). *Actinomyces* is also appeared in all samples, but there was no statistical significance between RA patients and controls or RA and PD patients (P = 0.680, P = 0.455, Fig. 6). *Treponema* were more abundant in RA (P = 0.008). *Tannerella* composed 0.2% of all the sample bacteria. Like *Treponema, Tannerella* was more abundant in RA (P = 0.010).

4.1. The profile of related genuses of periodontal pathogens in RA and PE groups compared with controls

In previous study, bacteriocin from *Prevotella nigrescens* is bactericidal against *Porphyromonas gingvialis* (Kaewsrichan et al., 2004). But compared to control group, we found *Prevotella* in RA patients is more abundant, which appeared in all samples (P = 0.007, Fig. 7). Then we analyzed the relative proportion of Streptococcus which provide a site for *Pg* binding to oral biofilm

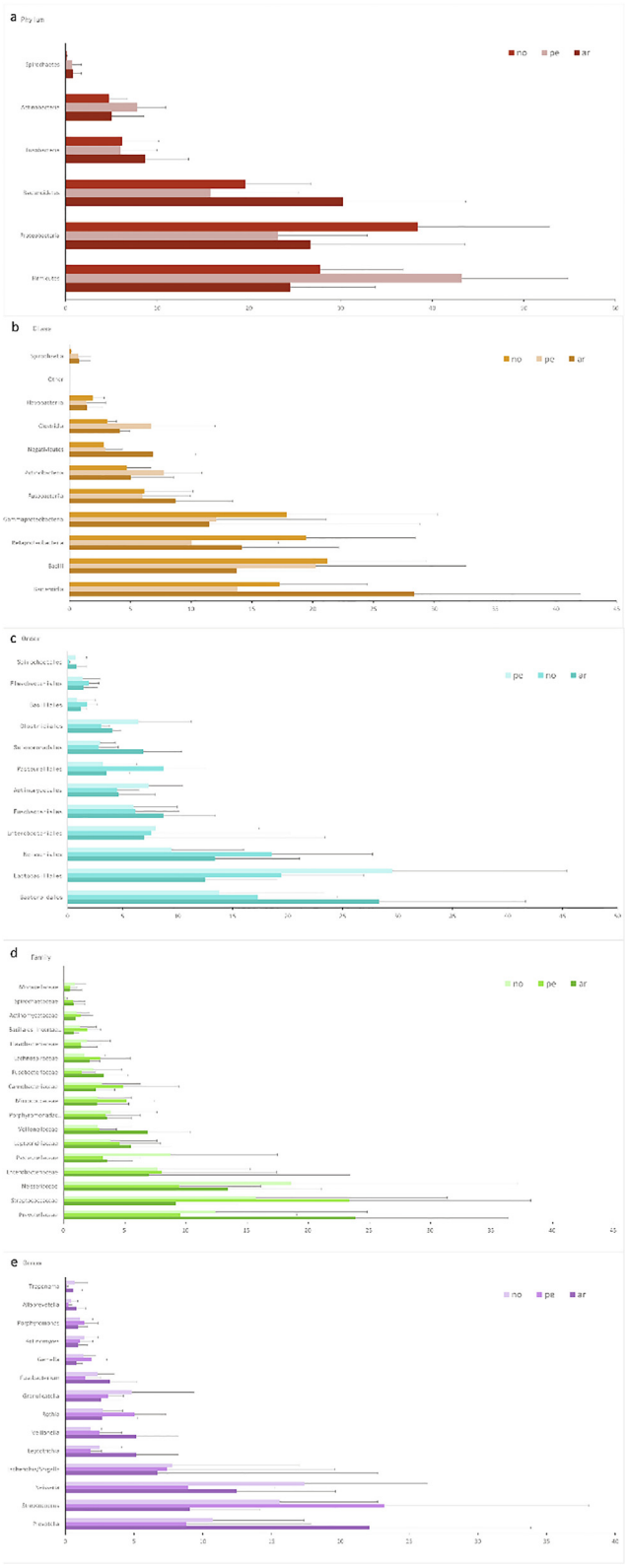


Fig. 5. Different relative abundance of bacterial taxonomy profiles of RA, PD and control group (a) phylum, (b) class, (c) order, (d) family, (e) genus. (*P < 0.05 in no and ar; #P < 0.05 in ar and pe; P < 0.05 in pe and no, RA(n = 54), PD(n = 45) and control group(n = 44)).

(Curtis et al., 2011). And there is a significant difference between RA patients and controls on relative abundance of *Streptococcus* (P = 0.008, Fig. 7).

5. Discussion

The human mouth has one of the most diverse microbiomes in the human body, which is only second to that of the colon in terms of species-richness (Human Microbiome Project Consortium, 2012). The number of microorganisms that colonize in the oral cavity may be more than 19,000 phylotypes (Keijser et al., 2008).Most of the microorganisms can't be cultured in laboratory. So culture-independent methods such as pyrosequencing are used to analyze the bacterial diversity in oral cavity (Aas et al., 2005; Bik et al., 2010; Dewhirst et al., 2010; Zaura et al., 2009). The microorganisms' inevitable interaction plays a key role in changing the balance between health and disease (Human Microbiome Project Consortium, 2012). So understanding the difference of microbiome structure in oral cavity between healthy and disease people is an important method to study the impact of oral microbiome (He et al., 2015).

In the last decade correlations were observed between periodontitis and some systemic disease, such as cardiovascular disease, RA, Alzheimer's disease, pulmonary disease, pre-term delivery of low birth weight infants and metabolic disease (Kumar, 2017; Dominy et al., 2019; Seoudi et al., 2015). Periodontal pathobionts may play a causal role in the initiating or exacerbating certain diseases either by direct invasion or by stimulating a florid immune-inflammatory response (Kumar, 2017; Dominy et al., 2019; Seoudi et al., 2015). *Porphyromonas gingvialis;* which is the only organism produce peptidyl deiminase, has been implicated in the etiology of RA for over two decades. Evidence shows that patients with both RA and periodontitis are more likely to be citrullinated proteins positive (Dissick et al., 2010; Perricone et al., 2019; Aliko et al., 2018). We designed this study to explore whether there is common and special characteristics of microbiomes in RA and PD patients compared to healthy people.

Up to now, we searched only several articles about characterizing oral microbial composition by 16S rRNA pyrosequencing in RA (Chen et al., 2018; Scher et al., 2012; Mikuls et al., 2018; Zhang et al., 2015; Mikuls et al., 2014). There is no exactly the same result. That may relate to different methods used (454 pyrosequencing, Illumina sequencing) as well as different samples (subgingival plaque, saliva) from different population.

In our study we found 7 main phylum in subgingival sample of Chinese healthy people, which is *Firmicutes* (27.7%), *Proteobacteria* (38.4%), *Bacteroidetes* (19.6%), *Fusobacteria* (6.2%), *Actinobacteria* (4.7%), *Spirochaetes* (0.1%). When compared with healthy adult Americans, a higher relative abundance of *Fusobacteria* (24%) and *Actinobacteria* (21.8%) and a lower relative abundance of *Firmicutes* (10.9%) and *Proteobacteria* (16.9%) were found in Americans' subgingival microbiota (Scher et al., 2012). This may relate to different life styles and host genotypes.

We explored links between PD and RA by profiling both the abundance and diversity of the subgingival microbiome in patients with established disease, using healthy people for comparison. We compared the oral microbiota in RA, PD patients and healthy subjects by Illumina MiSeq. We found that oral microbiota is equally rich and diverse in RA, PD and healthy people. This result was consistent to the report from Scher *et al.* but contrasted with results from Bin Chen *et al* (Chen et al., 2018; Scher et al., 2012; Mikuls et al., 2018). The difference may relate to different sample sites or different stage of the disease (Zhang et al., 2015).

In our research, some oral microbes related to PD were upper or lower regulated in RA compared to PD and healthy groups. Contrasted with study of Miluls *et al.*, *Porphyromonas* and *Prevotella* was more abundant in RA. And the result is consistent with reports from Bin Chen *et al* and Scher *et al* (Chen et al., 2018; Scher et al., 2012; Mikuls et al., 2018). Besides, although all of above bacteria

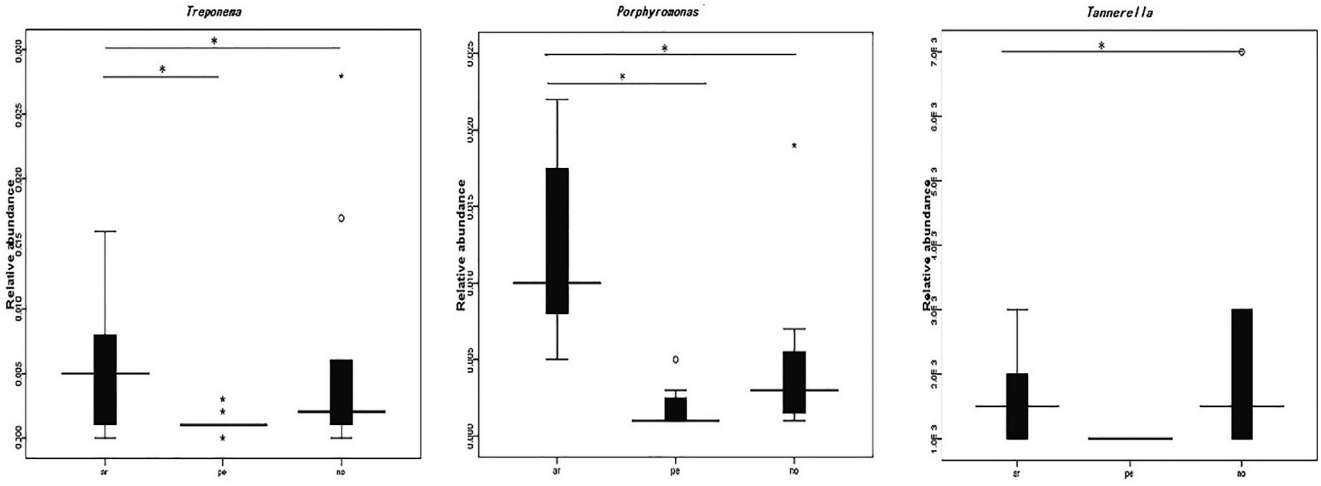


Fig. 6. The profile of periodontal pathogens in RA and PE groups compared with controls (*P < 0.05).

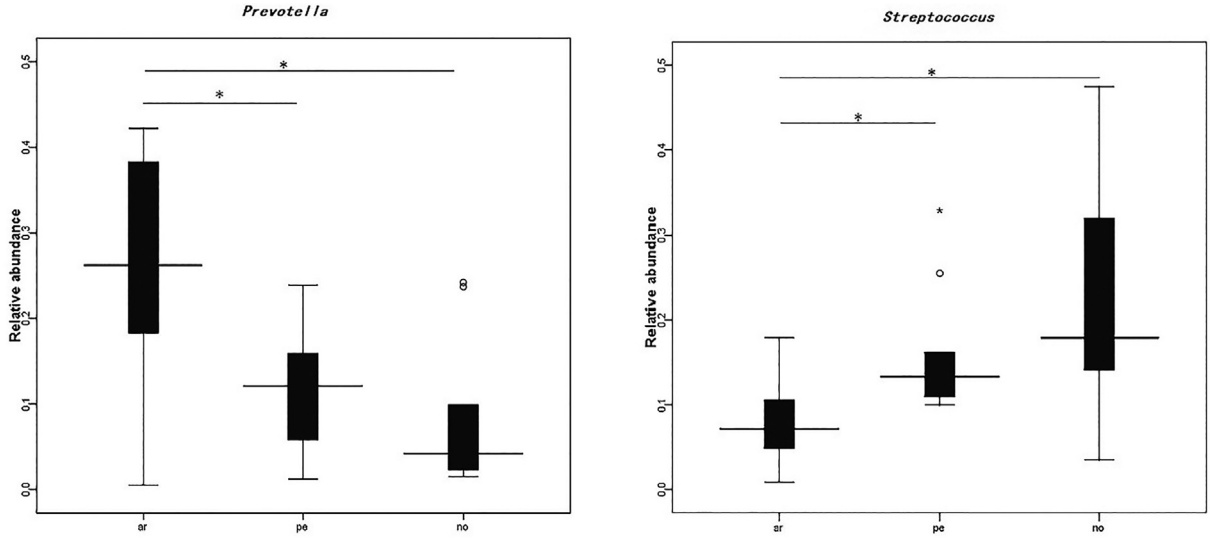


Fig. 7. The profile of competitive genuses of periodontal pathogens in RA and PE groups compared with controls (*P < 0.05).

are considered to be reasonable for PD, these bacteria in PD group suggested a surprised result which beyond our expected. In previous study, Bo Liu also found similar result, that is, neither Fusobacterium, nor *Porphyromonas* were found to be significantly more abundant in the PD samples, despite being previously implicated in this disease (Liu et al., 2012). This may be due to the high variance in abundance of the microbiome in our samples. Otherwise, different stage or severity of PD may also contribute to this result. Besides, *Streptococcus* were lower regulated in RA group compared with controls. But in research of Mikuls *et al.*, *Streptococcus* was found in greater abundance in patients with RA, which was contrasted with reports from Bin Chen *et al* and Scher *et al.* (Chen et al., 2018; Scher et al., 2012; Mikuls et al., 2018). Otherwise, PD group presented an independent result. Interestingly, we found one lineage was more abundant in the RA groups than control at each taxonomic level from the phylum down to the genus level, that is *Spirochaetes*, *Spirochaetia*, *Spirochaetales*, *Spirochaetaceae, Treponemes*. In PD group, although there is no statistical significance, it seems that *Treponemes* has a richness tendency. But in Bo Liu's study, *treponemes* in PD patients was significantly enriched (Liu et al., 2012). Evidence supports oral *treponemes* play an important role in the progression of periodontal diseases (Holt and Ebersole, 2000; Dashper et al., 2011). During the progression to periodontitis there is a large increase in the number, proportion of the total population, and diversity of species. It has been estimated to account for approximately 50% of the total bacteria present in a periodontal lesion (Moore and Moore, 2000; Listgarten, 1976). But up to now, there is no research or article focusing on the relationship between *treponeme* and RA. Further study is needed.

Reasonable evidence has shown that *Porphyromonas gingivalis* is directly linked to RA through citrullination and induction of antipeptidyl citrulline antibodies reacting to citullinated human self-proteins (Potempa et al., 2017; Perricone et al., 2019). But the driver pathogenic factor is still unknown. Oral cavity is a microenvironment where bacteria battle or cooperate with each other (Kuramitsu et al., 2007). Whether other oral bacteria have an effect on the common Etiology of RA and PD or not is still a big question to be answered. We compared subgingival organism profile of RA, PD and control group by Pyrosequencing analysis. Interestingly, we picked out *Treponeme* which may link the two diseases just like *Porphyromonas gingvialis* But further study is

needed to confirm. Besides, upper/lower regulation of some microbes in RA may remind us a direction to explore the role they play in pathogenesis of RA.

Contributors

Liu X and Tian K are co-first authors, they contributed equally to this work. Du Q obtained funding. Du Q, Tian K and Liu X designed the study. Li X, Tang N and Xiao L collected the data. Liu X, Ma X, Wang S and Luo C were involved in data cleaning, verification and analysis. Liu X drafted the manuscript. All authors have read and approved the final manuscript. All authors revised the manuscript for important content and approved the final version.

Patient consent

Obtained.

Data sharing

No additional data are available.

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Ethical approval

This study was approved by the ethical review committee of Sichuan Provincial People's Hospital (ethics ID:2019210), and conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from the participants of all subjects before study.

Declaration of Competing Interest

We have read and understood SJBS policy on declaration of interests and declare that we have no competing interests.

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