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ORIGINAL ARTICLE

# Quantitative analysis of microbiota in saliva, supragingival, and subgingival plaque of Chinese adults with chronic periodontitis

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**Abstract**
*Objective* The aim of this study was to determine the profiles of periodontopathogenic bacteria in a Chinese population using quantitative real-time polymerase chain reaction (qRT-PCR).
*Materials and methods* Twenty-four periodontally healthy Chinese subjects and 60 patients with chronic periodontitis (CP) were enrolled in this cross-sectional study. qRT-PCR was used to quantify *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Prevotella intermedia* as well as total bacterial counts from 252 samples collected from the saliva, supragingival plaque, and subgingival plaque of all 84 subjects.
*Results* The detection frequency of *A. actinomycetemcomitans* was less than 50%. *F. nucleatum* was detected in all subjects and CP patients had higher bacterial loads than healthy subjects. The median proportion of *F. nucleatum* was significantly higher in subgingival plaque than in supragingival plaque and saliva. *P. gingivalis* and *P. intermedia* had higher detection frequencies and bacterial loads in CP patients than in healthy subjects. The median proportion of *P. gingivalis* was significantly different among the three intraoral locations in the CP group and its proportion in subgingival plaque was 9.01%. Moreover, strong positive Spearman's correlations were found in *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* counts across the three intraoral locations.
*Conclusion* The presence and bacteria loads of these four bacteria in this Chinese population are similar to those from other populations.
*Clinical relevance* Examination of bacterial detection frequency and loads in Chinese adults may assist microbial studies of periodontal disease and will shed light on periodontal disease diagnosis and treatment using antibiotics in the Chinese population.

**Keywords** Oral microbiota · Chronic periodontitis · Dental plaque · Saliva · Real-time PCR · Quantitative detection

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## Introduction

Periodontal diseases are distributed worldwide in varied epidemiologic patterns [1, 2]. Chronic periodontitis (CP), the most common type, causes an inflammatory host response leading to bone loss and connective tissue destruction and may act as an important risk factor for systemic chronic diseases such as atherosclerotic cardiovascular disease [3, 4]. CP is polymicrobial, and bacteria play an important role in its initiation. Although approximately 700 bacterial species inhabit the oral cavity, only a small number seems to have the potential to cause periodontal tissue destruction [5]. Many studies at the epidemiologic and molecular levels have been conducted to evaluate the microbial species in the etiology of periodontal disease [6, 7]. *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are ranked high on the list of periodontal pathogens due to their high prevalence worldwide and well-studied pathogenic mechanism and virulence factors [8, 9].

Other bacteria such as *Fusobacterium nucleatum* and *Prevotella intermedia* have also been shown to be associated with periodontal diseases [10, 11].

Microbiological tests are used for disease diagnosis and mainly for periodontal therapy, especially when antimicrobial drugs are considered for treatment [12]. Understanding the composition of periodontal pathogens along with periodontal disease status in a particular population is very important for rapid diagnosis and effective treatment. However, most of the current knowledge regarding oral microbial composition has been established from studies conducted in the USA and Western Europe. Moreover, wide variations in the prevalence and proportion of periodontal pathogens have been extensively documented for different geographic regions and ethnic groups [13–16].

To our knowledge, very few studies have been done on the microbial profile of the Chinese population [17–20]. These studies mainly focused on bacterial profiles in subgingival plaque and used checkerboard DNA–DNA hybridization, regular PCR, or culture methods. In this study, the quantitative real-time polymerase chain reaction (qRT-PCR) based on 16S rRNA genes was applied to investigate the presence of bacteria and bacterial loads in saliva, supragingival plaque, and subgingival plaque of Chinese adults. qRT-PCR can quantitatively detect oral microorganisms with great accuracy, a rapid processing time, and a broad detection range, and it is an important tool to detect and quantify oral microorganisms in epidemiological and clinical studies [21, 22]. *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* were assessed in saliva, supragingival plaque, and subgingival plaque from periodontally healthy subjects and patients with CP, and the corresponding frequencies of detection, absolute counts of bacteria, and proportions of target bacteria were determined. We also compared the distributions of these species in saliva, supragingival plaque, and subgingival plaque and the bacterial correlations among these three intraoral locations.

The aim of the present study was to investigate the presence and bacterial loads of four target species in Chinese adults. To the best of our knowledge, this study is the first to report quantitative measures of periodontal pathogens in a Chinese population using the qRT-PCR technique. This provides a foundation for comparative studies in various Chinese groups, ultimately to help periodontal disease diagnosis and treatment in the Chinese population.

## Materials and methods

### Subjects and clinical assessment

Fifty-five male and 29 female subjects between the ages of 21 and 52 years who visited the Affiliated Hospital of Stomatology in Zhejiang University were enrolled between November 2009 and May 2010. All subjects had ≥20 teeth and were systemically healthy. Exclusion criteria were: use of antibiotics within the last 3 months, periodontal treatment in the past 6 months, pregnancy, and conditions such as heart disease and hypertension that may influence the progression of periodontal disease. The protocol of the present study was approved by the Ethics Committee of Zhejiang University and written informed consent was obtained from each participant.

Periodontal examination and sample collection were performed by one dentist. Probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) were recorded at six sites (buccal-mesial, mid-buccal, buccal-distal, lingual-mesial, mid-lingual, and lingual-distal) on each tooth using a standard CPI probe (Shanghai Medical Instruments, Shanghai, China). Subjects were divided into two groups based on periodontal parameters and radiographic diagnosis: periodontally healthy controls, 24 Chinese subjects who showed no clinical evidence of periodontal disease, and the CP group, 60 Chinese patients who exhibited bleeding on probing and attachment loss, with radiographic alveolar bone loss in four or more teeth, and PD ≥4 mm in at least four sites that were not on the same tooth. The demographic and clinical characteristics of the study population are summarized in Table 1.

### Sample collection

Sample collection was performed 1 week after full-mouth periodontal examination. Participants refrained from eating, drinking, and brushing at least 1 h prior to the saliva and plaque collection. Subjects were guided to spit into a paper cup. Five hundred microliters of saliva was transferred into a 1.5 ml microcentrifuge tube. Supragingival and subgingival

**Table 1** Demographic and clinical characteristics of the study population

| | Control ($n$=24) | CP ($n$=60) |
|---|---|---|
| Age (years) ± SD* | 26.9±6.0 | 35.3±9.1 |
| % male* | 41.7 | 75.0 |
| % smoker | 12.5 | 25.0 |
| % of sites with BOP ± SD* | 12.5±14.5 | 41.0±18.7 |
| Mean PD (mm) ± SD* | 1.6±0.4 | 2.7±0.7 |
| Mean CAL (mm) ± SD* | 0.1±0.1 | 2.4±1.8 |
| Mean PD of 4 sampling sites (mm) ± SD* | 2.4±0.4 | 3.9±1.3 |
| Mean CAL of 4 sampling sites (mm) ± SD* | 0.0±0.1 | 3.9±2.7 |

Mean of PD (CAL) of a subject was calculated by averaging the PD (CAL) of all measured sites, with six sites per tooth, in the full mouth

*$p$<0.05, significantly different between the CP and control groups

plaque sampling was performed in a standardized way on four teeth [13]. Each target tooth was isolated using cotton rolls and gentle air drying. Supragingival plaque samples from the mesial-buccal surface of the first molar (second molar if first molar was missing) in each quadrant were collected. Supragingival plaque samples were carefully scraped with sterile explorers from the gingival margin to prevent contamination with subgingival plaque. The pooled supragingival plaque from each subject was placed in a microcentrifuge tube containing 1.5 ml sterile reduced transfer fluid (RTF) buffer following Syed et al. [23]. Subgingival plaque was sampled from the site of the deepest pocket of the same tooth where the supragingival plaque was collected. A sterile paper point (ISO 30, Dentsply-De-Trey, GmbH, Germany) was gently inserted for 20 s and immediately removed to RTF buffer. Four paper points from each subject were pooled in the same microcentrifuge tube with RTF buffer. Collections were stored at −80°C until they were analyzed by qRT-PCR. A total of 252 samples (84 samples each of saliva, supragingival plaque, and subgingival plaque) were collected.

## DNA extraction

Total DNAs from cultivated bacterial strains and clinical samples were isolated using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with some modifications. Saliva samples were washed three times with 1 ml sterile water in microcentrifuge tubes; subgingival plaque samples were dispersed in RTF buffer by vortexing for 3 min. Bacteria from saliva, supragingival, and subgingival plaque samples were pelleted, resuspended in 180 μl QIAmp-ATL and 20 μl proteinase K, and incubated in a rotating rocker at 56°C for 2 h. Subsequently, 20 μl lysozyme buffer (50 mg/ml lysozyme, 20 mM Tris·HCl, pH 8.0, 2 mM EDTA, 1.2% Triton) was added to lyse gram-positive bacteria and incubated at 37°C for 2 h and then at 95°C for 15 min. QIAmp-AL buffer (200 μl) was added and incubated at 70°C for 10 min. Then, 220 μl 100% ethanol was added to the samples and mixed by pulse-vortexing for 15 s. DNA was concentrated by ethanol precipitation and applied to the QIAmp mini spin column. After washing with 500 μl QIAmp-AW1 and QIAmp-AW2, purified DNA was eluted in 200 μl AE buffer (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0) and stored at −20°C.

The concentrations of DNA from cultured *A. actinomycetemcomitans* ATCC 29523, *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, and *P. intermedia* ATCC 25611 were determined by a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, NC, USA). The number of bacterial cell copies was quantified and calculated based on the molecular mass formula according to a previously published method [24]. Serial tenfold dilutions from $10^7$ to $10^2$ cell copies of the quantified cultured bacteria were prepared.

## Quantitative real-time PCR assay

The tested bacteria were identified using a 16S rRNA-based qRT-PCR analysis. The sequences of primers used are listed in Table 2. Bacteria-specific primer pairs were used to identify *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* based on the species-specific region on the 16S rRNA gene according to the literature [25–28]. In addition, a universal primer pair based on the conserved region of the 16S rRNA gene was used to quantify the total amount of eubacterial species in the sample [29].

All samples were run in duplicate in 96-well plates in a LightCycler® 480 qRT-PCR System (Roche Diagnostics, Mannheim, Germany). The 10 μl reaction mixture contained 1 μl template DNA, 5 μl LightCycler® 480 SYBR Green I Master, 1 μl 2.5 μM primer pair, and 3.5 μl dd$H_2O$. The amplification cycling conditions were 95°C for 10 min; 40 cycles of 10 s at 95°C, 15 s at a bacterium-specific annealing temperature (temperatures listed in Table 2) and 40 s at 72°C. Melting analysis was

**Table 2** 16S rRNA bacterial primers and annealing temperatures ($T_m$) used in qRT-PCR and the expected product size

| Primer | Sequence (5′→3′) | $T_m$ (°C) | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *A. actinomycetemcomitans* | CTTACCTACTCTTGACATCCGAA<br>ATGCAGCACCTGTCTCAAAGC | 60 | 77 | [25] |
| *F. nucleatum* | CGCAGAAGGTGAAAGTCCTGTAT<br>TGGTCCTCACTGATTCACACAGA | 65 | 101 | [26] |
| *P. gingivalis* | AGGCAGCTTGCCATACTGCG<br>ACTGTTAGCAACTACCGATGT | 60 | 404 | [27] |
| *P. intermedia* | CGTGGACCAAAGATTCATCGGTGGA<br>CCGCTTTACTCCCCAACAAA | 64 | 259 | [28] |
| Universal | TCCTACGGGAGGCAGCAGT<br>GGACTACCAGGGTATCTAATCCTGTT | 58 | 466 | [29] |

performed for each run by 10 s at 95°C, 40°C for 10 s, and the annealing temperature was increased from 60°C to 95°C at a ramp rate of 0.11°C/s. The melting temperature profile was used to verify the specificity of the PCR products. Quantification analysis of target bacterial DNA isolated from saliva, supragingival plaque, and subgingival plaque samples was performed using the LightCycler® 480 software. Quantification was based on the standard curve for each target species, ranging from $10^2$ to $10^7$ cells. The minimal detection limit is ~$10^2$ cells of these four species. A standard curve was prepared in each plate for each target bacterium using the genomic DNAs from pure culture and the bacterium-specific primer pair. Serially diluted genomic DNA of *P. gingivalis* ATCC 33277 was used as a DNA template to generate the standard curve to quantify the total number of bacteria as determined using universal 16S rRNA primers that detect all bacterial species. Genomic DNA of *P. gingivalis* was used to make the standard curve of total bacteria because it is commonly present in dental biofilm and is reported to be a good standard in qRT-PCR analysis [30].

### Data analysis

The demographics and clinical characteristics of the CP and healthy control groups were compared using standard statistical tests. The *t* test was used to compare continuous attributes such as age and average CAL of the two groups; Fisher's exact test was used to compare proportions such as gender and smoker ratios. Age and gender ratios were significantly different between the two groups (Table 1); therefore, age and gender were adjusted in the regression analyses. Considering the potential effect of smoking on bacterial profiles according to the literature, we also adjusted for smoking history even though there was no statistically significant difference between these two groups.

The bacterial detection frequency was calculated by dividing the number of subjects positive for a specific bacterium by the total number of subjects in each group. For each bacterium and intraoral location, logistic regression was used to compare the detection frequencies between the CP and control groups, adjusting for age, gender, and smoking history.

The absolute bacterial counts of four target species and the total number of bacteria in samples were exported from LightCycler® 480 software. The absolute bacterial counts were $\log_{10}$ transformed. Prior to the $\log_{10}$ transformation, 1 was added to all counts to handle cases of zero counts. For each species and intraoral location, quantile regression was applied to compare the medians of the $\log_{10}$ bacterial counts between samples from different disease statuses, controlling for age, gender, and smoking history.

The proportion of each species at a particular oral location was calculated by dividing the number of target bacteria by the total number of bacteria in each clinical sample. For each species in each oral location, quantile regression was applied to compare the medians of the bacterial proportions between samples from the CP and control groups, adjusting for age, gender, and smoking history. The Kruskal–Wallis test was used to compare the medians of the species proportions among saliva, supragingival plaque, and subgingival plaque, and the Wilcoxon rank sum test with Bonferroni's correction was applied to determine differences between groups with respect to target bacteria at different intraoral locations. Spearman's rank correlation coefficient was used to summarize the strength of the relationship in absolute bacterial counts in 84 subjects among three intraoral locations.

All tests were two-sided with a significance level of 0.05. All statistical analyses were performed with SAS version 9.2.

## Results

### Comparison of species detection frequencies between CP and control groups

The target species in the CP and control groups from the saliva, supragingival plaque, or subgingival plaque samples are listed in Table 3. Detection frequencies in the samples from the CP group were equal to or higher than those from periodontally healthy controls. *A. actinomycetemcomitans* was detected in 48.3% of the CP group, which was not significantly different from the control group; *F. nucleatum* was detected in all samples from subjects with or without periodontal disease; *P. gingivalis* was detected in 96.7% of the CP group and 45.8% of the control group; and *P. intermedia* was detected in 100% of the CP group and 83.3% of the control group. The differences in detection frequencies between the CP and control groups were significant for *P. gingivalis* ($p<0.01$) and *P. intermedia* ($p<0.05$) among saliva, supragingival plaque, and subgingival plaque.

### Comparison of bacterial loads between the CP and control groups

To quantitatively assess the loads of target species in the CP and healthy groups, we compared the absolute bacterial counts of each species and their corresponding proportions in saliva, supragingival plaque, and subgingival plaque (Table 3).

The differences in median bacterial counts between the two groups were statistically significant for *F. nucleatum* ($p<0.05$ in saliva and supragingival plaque; $p<0.01$ in subgingival plaque), *P. gingivalis* ($p<0.01$ in all three locations), and *P. intermedia* ($p<0.05$ in saliva and supragingival plaque; $p<$

**Table 3** Frequencies of detection and bacterial loads in saliva, supragingival, and subgingival plaque from periodontally healthy and CP subjects

| | | Frequency of detection *N* (%) | Median proportion (%) | $\log_{10}$ bacterial load | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Median | Mean | Min | 25th percentile | 75th percentile | Max |
| *A. actinomycetemcomitans* | | | | | | | | | |
| Saliva | CP | 29 (48.3) | 0 | 0 | 2.07 | 0 | 0 | 3.61 | 6.81 |
| | Control | 8 (33.3) | 0 | 0 | 1.50 | 0 | 0 | 3.33 | 5.86 |
| Supragingival plaque | CP | 21 (35.0) | 0 | 0 | 1.25 | 0 | 0 | 2.51 | 6.00 |
| | Control | 8 (33.3) | 0 | 0 | 1.03 | 0 | 0 | 2.10 | 5.03 |
| Subgingival plaque | CP | 12 (20.0) | 0 | 0 | 0.94 | 0 | 0 | 0.00 | 7.25 |
| | Control | 3 (12.5) | 0 | 0 | 0.44 | 0 | 0 | 0.00 | 4.30 |
| *F. nucleatum* | | | | | | | | | |
| Saliva | CP | 60 (100.0) | 0.26 | 6.65* | 6.57 | 6.65 | 6.29 | 6.83 | 7.64 |
| | Control | 24 (100.0) | 0.12 | 6.25 | 6.11 | 6.25 | 5.86 | 6.55 | 6.77 |
| Supragingival plaque | CP | 60 (100.0) | 1.68* | 7.29* | 7.22 | 7.29 | 6.97 | 7.50 | 7.75 |
| | Control | 24 (100.0) | 1.27 | 6.90 | 6.82 | 6.90 | 6.63 | 7.32 | 7.74 |
| Subgingival plaque | CP | 60 (100.0) | 2.43 | 6.38** | 6.35 | 6.38 | 5.94 | 6.79 | 7.36 |
| | Control | 24 (100.0) | 2.34 | 5.20 | 5.27 | 5.20 | 4.94 | 6.02 | 6.31 |
| *P. gingivalis* | | | | | | | | | |
| Saliva | CP | 58 (96.7)** | 0.88** | 7.15** | 6.90 | 7.15 | 6.88 | 7.53 | 8.03 |
| | Control | 11 (45.8) | 0 | 0 | 2.87 | 0 | 0 | 6.18 | 7.10 |
| Supragingival plaque | CP | 58 (96.7)** | 0.35** | 6.50** | 6.22 | 6.50 | 5.69 | 7.08 | 8.46 |
| | Control | 11 (45.8) | 0 | 0 | 2.35 | 0 | 0 | 5.19 | 6.75 |
| Subgingival plaque | CP | 57 (95.0)** | 9.01** | 6.90** | 6.31 | 7 | 6.18 | 7.26 | 8.36 |
| | Control | 10 (41.7) | 0 | 0 | 1.96 | 0 | 0 | 4.32 | 6.77 |
| *P. intermedia* | | | | | | | | | |
| Saliva | CP | 60(100.0)* | 0.09** | 6.14* | 6.00 | 6.14 | 5.56 | 6.53 | 6.96 |
| | Control | 20 (83.3) | 0.01 | 4.44 | 3.93 | 4.44 | 3.29 | 5.38 | 6.96 |
| Supragingival plaque | CP | 60(100.0)* | 0.11** | 6.20* | 5.88 | 6.20 | 5.30 | 6.76 | 7.55 |
| | Control | 17 (70.8) | 0 | 3.97 | 3.14 | 3.97 | 0.00 | 5.21 | 6.17 |
| Subgingival plaque | CP | 59 (98.3)* | 0.12** | 5.09** | 4.74 | 5.09 | 3.97 | 5.58 | 6.65 |
| | Control | 15 (62.5) | 0 | 2.24 | 2.07 | 2.24 | 0.00 | 3.68 | 5.21 |
| Total bacteria | | | | | | | | | |
| Saliva | CP | 60(100.0) | – | 9.26 | 9.18 | 9.26 | 9.01 | 9.42 | 9.86 |
| | Control | 24 (100.0) | – | 9.13 | 9.09 | 9.13 | 8.83 | 9.38 | 9.93 |
| Supragingival plaque | CP | 60(100.0) | – | 9.05 | 9.05 | 9.05 | 8.88 | 9.32 | 9.59 |
| | Control | 24 (100.0) | – | 8.93 | 8.79 | 8.93 | 8.59 | 9.04 | 9.94 |
| Subgingival plaque | CP | 60(100.0) | – | 7.99** | 7.94 | 7.99 | 7.63 | 8.23 | 8.97 |
| | Control | 24 (100.0) | – | 6.82 | 6.93 | 6.82 | 6.55 | 7.36 | 8.26 |

Statistical differences in detection frequency between the CP and control groups were calculated from multivariate logistic regression adjusted for age, gender, and smoking history. Median, mean, min, 25th percentile, 75th percentile, and max are presented for the detected bacterial load (log10 transformed) of *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* and total bacteria in saliva, supragingival plaque, and subgingival plaque from periodontally healthy individuals and CP patients. Tests of equal median of bacterial load between CP and control groups were performed using quantile regression, adjusted for age, gender, and smoking history

*$p$ <0.05; **$p$ <0.01

0.01 in subgingival plaque) (Table 3). Higher total counts of bacteria were detected in subgingival plaque in the CP group than the control group ($p$<0.01). No significant differences were found for the total bacterial counts between the CP and control groups in saliva and supragingival plaque.

The differences in the median proportions between the CP and control groups were statistically significant for *F. nucleatum* ($p$<0.05 in supragingival plaque), *P. gingivalis* ($p$<0.01 in all three locations), and *P. intermedia* ($p$<0.01 in all three locations) (Table 3). The median proportions of *F.*

*nucleatum* in the CP group were higher than those in controls in all three locations, though the differences were not significant for saliva and subgingival plaque. The median proportions of *P. gingivalis* and *P. intermedia* were significantly higher in the CP group than that in the control group at all three locations. Overall, the species proportions revealed increased bacterial loads of *F. nucleatum*, *P. gingivalis*, and *P. intermedia* in the CP patients. The result shown in proportions was consistent with those in absolute bacterial counts.

### Comparison of bacterial proportions among saliva, supragingival plaque, and subgingival plaque

The proportion of each target species present was compared across the different sampling locations: saliva, supragingival plaque, and subgingival plaque. The median proportions of *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* in the CP group showed overall significant differences across the three locations ($p<0.05$; Table 4). Significant differences between these three locations were found for *A. actinomycetemcomitans* ($p=0.04$, saliva vs subgingival plaque), *F. nucleatum* ($p<0.001$ for all pairwise comparisons between the three locations), and *P. gingivalis* ($p=0.002$, saliva vs supragingival plaque; $p<0.001$, supragingival plaque vs saliva and subgingival plaque). In the healthy group, the median proportion of *F. nucleatum* was significantly higher in both plaque surfaces than saliva and significantly higher in subgingival than supragingival plaque ($p<0.001$, saliva vs subgingival and supragingival plaque; $p=0.003$, subgingival vs supragingival plaque).

### Correlations of bacterial loads among saliva, supragingival plaque, and subgingival plaque

Spearman correlation analysis was used to evaluate the correlations of the target bacteria among saliva, supragingival plaque, and subgingival plaque. There were significant positive correlations of bacterial counts of *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* across the three intraoral locations (Table 5). The correlation coefficients of bacterial counts of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* among saliva, supragingival plaque, and subgingival plaque ranged from 0.65 to 0.75, from 0.63 to 0.77, and from 0.58 to 0.72, respectively, which indicated strong correlations. However, the bacterial counts of *F. nucleatum* had weak correlations across the three locations ($r$, 0.25–0.33).

## Discussion

It is widely accepted that the development of periodontal disease is accompanied by a shift of bacterial profile [31, 32]. Many observational studies showed that the presence and amount of some periodontal pathogens such as *P. gingivalis* in periodontal disease patients are different from those in healthy subjects [33–35]. It was also noted that

**Table 4** Bacterial proportions (in percent) in saliva, supragingival, and subgingival plaque from periodontally healthy and CP subjects

| | Saliva median [25th, 75th percentile] | | Supragingival plaque median [25th, 75th percentile] | | Subgingival plaque median [25th, 75th percentile] | | $p$[a] | Saliva vs supragingival | Saliva vs subgingival | Supragingival vs subgingival |
|---|---|---|---|---|---|---|---|---|---|---|
| *A. actinomycetemcomitans* | | | | | | | | | | |
| CP | 0 | [0, <0.01] | 0 | [0, <0.01] | 0 | [0, 0] | 0.03 | | * | |
| Control | 0 | [0, <0.01] | 0 | [0, <0.01] | 0 | [0, 0] | 0.28 | | | |
| *F. nucleatum* | | | | | | | | | | |
| CP | 0.26 | [0.15, 0.39] | 1.68 | [1.21, 1.93] | 2.43 | [1.34, 5.27] | <0.01 | ** | ** | ** |
| Control | 0.12 | [0.06, 0.24] | 1.27 | [0.89, 1.85] | 2.34 | [1.57, 3.89] | <0.01 | ** | ** | ** |
| *P. gingivalis* | | | | | | | | | | |
| CP | 0.88 | [0.54, 1.63] | 0.35 | [0.05, 1.09] | 9.01 | [2.69, 15.20] | <0.01 | ** | ** | ** |
| Control | 0 | [0, 0.12] | 0 | [0, 0.02] | 0 | [0, 0.58] | 0.80 | | | |
| *P. intermedia* | | | | | | | | | | |
| CP | 0.09 | [0.03, 0.15] | 0.11 | [0.02, 0.40] | 0.12 | [0.01, 0.32] | 0.48 | | | |
| Control | <0.01 | [<0.01, 0.02] | <0.01 | [0, 0.02] | <0.01 | [0, 0.04] | 0.87 | | | |

*$p<0.05$; **$p<0.01$, pairwise comparisons with Bonferroni's correction was used to calculate the significance of differences between any two of three intraoral locations

[a] Overall $p$ values for testing equality of the bacterial proportions among saliva, supragingival plaque, and subgingival plaques for each bacterial species and each disease status group

**Table 5** Correlations of bacterial loads between three intraoral locations

| Correlation coefficient | Subgingival plaque and saliva | Subgingival plaque and supragingival plaque | Supragingival plaque and saliva |
|---|---|---|---|
| *A. actinomycetemcomitans* | 0.71** | 0.75** | 0.65** |
| *F. nucleatum* | 0.33** | 0.25* | 0.26* |
| *P. gingivalis* | 0.77** | 0.69** | 0.63** |
| *P. intermedia* | 0.58** | 0.72** | 0.63** |

*$p<0.05$; **$p<0.01$, $p$ values for testing zero correlation of bacterial loads between intraoral locations

the distribution of bacteria varies in different populations and geographic regions. Understanding the bacterial profile for a particular population sheds light on the etiology of periodontal diseases and antibacterial treatment for people in that population. Therefore, this study assessed the presence and bacterial loads of *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* in Chinese adults.

*A. actinomycetemcomitans* is closely associated with aggressive periodontitis and commonly present in CP [36]. Previous studies showed that the presences of *A. actinomycetemcomitans* ranges from 20.0% to >50% depending on the sampling method and study population [14, 37, 38]. Our study conducted in eastern China found that it was up to 48.3% and there was no significant difference between CP and healthy controls. This is consistent with a checkerboard hybridization study performed on northern rural Chinese adults by Papapanou et al. (1997), who found that among 18 bacterial species examined *A. actinomycetemcomitans* was the least prevalent (83.1% at the subject level and 37.7% at the site level) and no significant difference was found with regard to disease status [20]. On the other hand, Meng et al. (2009), using 16S rRNA PCR analysis, found that *A. actinomycetemcomitans* is more frequent in Chinese periodontitis patients (33.62%) than in healthy controls (0.9%) in southwestern China [18]. This discrepancy may be explained by the difference in detection technique as well as the geographic difference in the study population. Taking into account the low presence and small proportion of total bacterial counts in our study, the role of *A. actinomycetemcomitans* needs to be further examined.

*F. nucleatum* has been shown to have a close association with periodontal disease and it is also well known as a bridge organism between early and late colonizers of the tooth surface [39]. High detection levels have been reported in adults worldwide using cell culture. Herrera et al. (2007) found that the detection frequency of *Fusobacterium* spp. in CP patients from Chile, Columbia, and Spain were 63.9%, 82.9%, and 100%, respectively [13]. van Winkelhoff et al. (2002) reported a high prevalence of *F. nucleatum* in the Netherlands, and it was significantly higher in periodontitis patients than in non-periodontitis subjects (95.7% vs 85.5%) [40]. In our study, a high presence of *F. nucleatum* was confirmed in the CP group; however, the periodontally healthy group also had 100% detection frequencies, suggesting that *F. nucleatum* is commonly present in the Chinese population regardless of disease status. Moreover, we found that the absolute counts of *F. nucleatum* increased in CP patients as compared to healthy subjects, as well as the proportion in each intraoral location, although the differences in proportions in saliva and subgingival plaque did not reach significance. The discrepancy of nonsignificant differences in proportions as opposed to significant absolute bacterial counts of *F. nucleatum* in saliva and subgingival plaque may be explained by the variation of the total number of bacteria in each sample. The presence and bacterial load of *F. nucleatum* is in agreement with a previous report by Boutaga et al. (2006) using the qRT-PCR technique [41]. These authors found a high prevalence of *Fusobacterium* spp. (>95%) in both periodontitis patients and non-periodontitis subjects, and significantly higher bacterial loads in periodontitis patients than that in controls. The distribution of *F. nucleatum* in this study supports the idea that not only does the presence of specific pathogenic bacteria influence the development of periodontitis, but also the numbers of these bacteria may be important to evaluate disease status [10].

*P. gingivalis* is strongly associated with periodontitis and widely accepted as an important periodontopathogen [9]. Studies have demonstrated that it is present in low numbers in health but more frequently detected with higher counts in periodontitis. Ximenez-Fyvie et al. (2006) examined 40 CP and 20 periodontally healthy Mexican subjects and reported a significantly higher detection frequency of *P. gingivalis*, higher mean levels, and higher proportions in periodontitis subjects, using checkerboard DNA-DNA hybridization [42]. Nonnenmacher et al. (2005) used the qRT-PCR technique and reported a statistically higher detection frequency and higher bacterial load of *P. gingivalis* in 83 periodontally diseased patients as compared to 43 periodontally healthy subjects in Germany [43]. In accord with previous reports in other populations, our study found a statistically higher detection frequency and bacterial load of *P. gingivalis* in CP groups than in controls, confirming the association of *P. gingivalis* with CP. Moreover, our result is in accord with previous reports showing a high presence of *P. gingivalis* in Chinese adults [19, 20].

*P. intermedia* has been implicated in the development of periodontal disease [44]. A high prevalence is associated with CP and its detection frequency varies from 50% to ~100% [34, 40, 45, 46]. The presence of *P. intermedia* in our study is consistent with previous studies; it was more frequently detected in the CP group than in the control group, indicating that its presence is significantly related to periodontal status. Moreover, we found significantly higher levels of *P. intermedia* in terms of bacterial counts and proportions in CP patients than in periodontally healthy subjects. Our results support previous reports that the bacterial load of *P. intermedia* is significantly associated with CP [40, 41].

It is noteworthy that all four species studied were found among periodontally healthy subjects as well as CP patients. The control group showed high prevalence of *F. nucleatum* (100%), *P. gingivalis* (45.8%), and *P. intermedia* (83.3%), and this has been documented in other populations. Wara-aswapati et al. (2009) found that the prevalence of *P. gingivalis* was as high as 45% in healthy Thai adults [15]. Braga et al. (2010) reported that the detection frequencies of *F. nucleatum*, *P. gingivalis*, *a*nd *P. intermedia* were 100%, 46.6%, and 80%, respectively, in female Brazilian adults without periodontal disease [47]. Moreover, our findings showed that the bacterial loads were lower in periodontally healthy subjects than in CP patients, which suggests that these species are opportunistic pathogens. Armitage (2010) stated that periodontally healthy subjects carry putative periodontal pathogens as part of the normal oral microbiota because these bacteria are detected at low numbers and are often found in periodontally healthy individuals in the absence of periodontal disease [8]. The results of our study are in accord with this idea. The presence of these bacteria at low levels may have a beneficial effect on the host for developing innate and adaptive immunity.

Periodontal tissue breakdown is associated with the dynamic intraoral translocation of periodontopathogens. Many periodontopathogenic bacteria can colonize multiple ecological sites, but these bacteria are not uniformly distributed. Mager et al. (2003), using checkerboard hybridization, found that the microbial profile varies distinctly among different intraoral surfaces with 34 of 40 bacterial species differing significantly [48]. In our study, the target species were present in almost all subjects at high detection frequencies, but the compositions of the microbiota at the different intraoral locations were different. In comparison to supragingival plaque and saliva, the proportions of *F. nucleatum* and *P. gingivalis* were significantly higher in subgingival plaque in CP patients, with 2.43% and 9.01% of the total microbiota, respectively. These two species are strictly anaerobic and they often colonize an anaerobic environment in host tissues such as subgingival plaque to establish themselves in the oral cavity. A study has also described how these two species are present in high proportions in the subgingival plaque of CP subjects from Brazil, Chile, Sweden, and USA [6].

Bacterial species in tooth and saliva samples are known to be strongly associated in individuals, and periodontopathic bacteria are more frequently detected in saliva than in subgingival plaque [49–51]. Saliva is more often colonized by bacteria, and anaerobic bacteria which are established around the teeth may also thrive in saliva. Our study found that the presence of the four target species in saliva was higher than or as high as in plaque samples, which is in agreement with the findings of Sakamoto et al. (2001) where higher detection frequencies for periodontopathic bacteria were found in saliva than in subgingival plaque [27], indicating that saliva serves as a reservoir and deserves therapeutic attention and preventive intervention. Moreover, a consistent pattern emerged from our study—for each species that had a significantly different detection frequency between CP and healthy subjects in saliva, a significant difference was also found in plaque. The same pattern was found for the median proportions and absolute bacterial loads. Furthermore, our results quantitatively showed that the bacterial loads of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* were highly correlated among the three locations. Taken together, we found that bacterial presence and loads in saliva were correlated with those in dental plaque.

## Conclusion

In summary, our study found that *F. nucleatum*, *P. gingivalis*, and *P. intermedia* occurred frequently in both periodontally healthy and CP subjects, but bacterial loads were higher in the CP subjects. Significant correlations of the bacterial loads of studied species between different intraoral locations were found. These results help us to understand the bacterial profiles in the oral cavity and further help in the development of microbiological testing in periodontal disease diagnosis and treatment. The differences of bacterial proportions in saliva and supragingival and subgingival plaque and the association of these three species of bacteria with periodontal status need to be taken into consideration in periodontal disease prevention or treatment.

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**Conflicts of interest** The authors declare that they have no conflict of interest.

## References


1. Corbet EF, Zee KY, Lo EC (2002) Periodontal diseases in Asia and Oceania. Periodontol 2000 29:122–152
2. Demmer RT, Papapanou PN (2010) Epidemiologic patterns of chronic and aggressive periodontitis. Periodontol 2000 53:28–44
3. Chen H, Zheng P, Zhu H, Zhu J, Zhao L, El Mokhtari NE, Eberhard J, Lins M, Jepsen S (2010) Platelet-activating factor levels of serum and gingival crevicular fluid in nonsmoking patients with periodontitis and/or coronary heart disease. Clin Oral Investig 14:629–636
4. Zheng P, Chen H, Shi S, Jepsen S, Eberhard J (2006) Periodontal parameters and platelet-activating factor levels in serum and gingival crevicular fluid in a Chinese population. J Clin Periodontol 33:797–802
5. Paster BJ, Olsen I, Aas JA, Dewhirst FE (2006) The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000 42:80–87
6. Haffajee AD, Bogren A, Hasturk H, Feres M, Lopez NJ, Socransky SS (2004) Subgingival microbiota of chronic periodontitis subjects from different geographic locations. J Clin Periodontol 31:996–1002
7. Socransky SS, Haffajee AD (2005) Periodontal microbial ecology. Periodontol 2000 38:135–187
8. Armitage GC (2010) Comparison of the microbiological features of chronic and aggressive periodontitis. Periodontol 2000(53):70–88
9. Slots J, Ting M (1999) *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. Periodontol 2000 20:82–121
10. Teles RP, Haffajee AD, Socransky SS (2006) Microbiological goals of periodontal therapy. Periodontol 2000 42:180–218
11. Riep B, Edesi-Neuss L, Claessen F, Skarabis H, Ehmke B, Flemmig TF, Bernimoulin JP, Gobel UB, Moter A (2009) Are putative periodontal pathogens reliable diagnostic markers? J Clin Microbiol 47:1705–1711
12. Shaddox LM, Walker C (2009) Microbial testing in periodontics: value, limitations and future directions. Periodontol 2000 50:25–38
13. Herrera D, Contreras A, Gamonal J, Oteo A, Jaramillo A, Silva N, Sanz M, Botero JE, Leon R (2008) Subgingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. J Clin Periodontol 35:106–113
14. Psoter WJ, Ge Y, Russell SL, Chen Z, Katz RV, Jean-Charles G, Li Y (2010) PCR detection of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* in dental plaque samples from Haitian adolescents. Clin Oral Investig 15:461–469
15. Wara-aswapati N, Pitiphat W, Chanchaimongkon L, Taweechaisupapong S, Boch JA, Ishikawa I (2009) Red bacterial complex is associated with the severity of chronic periodontitis in a Thai population. Oral Dis 15:354–359
16. Yang HW, Huang YF, Chou MY (2004) Occurrence of *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontally diseased and healthy subjects. J Periodontol 75:1077–1083
17. Dahlen GG, Luan WM, Baelum V, Fejerskov O, Chen X (1995) Periodontopathogens in elderly Chinese with different periodontal disease experience. J Clin Periodontol 22:188–200
18. Meng S, Zhao L, Yang H, Wu Y, Ouyang Y (2009) Prevalence of *Actinobacillus actinomycetemcomitans* in Chinese chronic periodontitis patients and periodontally healthy adults. Quintessence Int 40:53–60
19. Mombelli A, Gmur R, Frey J, Meyer J, Zee KY, Tam JO, Lo EC, Di Rienzo J, Lang NP, Corbet EF (1998) *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in young Chinese adults. Oral Microbiol Immunol 13:231–237
20. Papapanou PN, Baelum V, Luan WM, Madianos PN, Chen X, Fejerskov O, Dahlen G (1997) Subgingival microbiota in adult Chinese: prevalence and relation to periodontal disease progression. J Periodontol 68:651–666
21. Jervoe-Storm PM, AlAhdab H, Koltzscher M, Fimmers R, Jepsen S (2010) Quantification of periodontal pathogens by paper point sampling from the coronal and apical aspect of periodontal lesions by real-time PCR. Clin Oral Investig 14:533–541
22. Sakamoto M, Umeda M, Benno Y (2005) Molecular analysis of human oral microbiota. J Periodontal Res 40:277–285
23. Syed SA, Loesche WJ (1972) Survival of human dental plaque flora in various transport media. Appl Microbiol 24:638–644
24. Novak MJ, Novak KF, Hodges JS, Kirakodu S, Govindaswami M, Diangelis A, Buchanan W, Papapanou PN, Michalowicz BS (2008) Periodontal bacterial profiles in pregnant women: response to treatment and associations with birth outcomes in the obstetrics and periodontal therapy (OPT) study. J Periodontol 79:1870–1879
25. Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S (2003) Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. FEMS Immunol Med Microbiol 39:81–86
26. Suzuki N, Yoshida A, Saito T, Kawada M, Nakano Y (2004) Quantitative microbiological study of subgingival plaque by real-time PCR shows correlation between levels of *Tannerella forsythensis* and *Fusobacterium spp*. J Clin Microbiol 42:2255–2257
27. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y (2001) Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. Microbiol Immunol 45:39–44
28. Baumgartner JC, Watkins BJ, Bae KS, Xia T (1999) Association of black-pigmented bacteria with endodontic infections. J Endod 25:413–415
29. Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266
30. Kuboniwa M, Amano A, Kimura KR, Sekine S, Kato S, Yamamoto Y, Okahashi N, Iida T, Shizukuishi S (2004) Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with TaqMan probes. Oral Microbiol Immunol 19:168–176
31. Ledder RG, Gilbert P, Huws SA, Aarons L, Ashley MP, Hull PS, McBain AJ (2007) Molecular analysis of the subgingival microbiota in health and disease. Appl Environ Microbiol 73:516–523
32. Parahitiyawa NB, Scully C, Leung WK, Yam WC, Jin LJ, Samaranayake LP (2010) Exploring the oral bacterial flora: current status and future directions. Oral Dis 16:136–145
33. Lafaurie GI, Contreras A, Baron A, Botero J, Mayorga-Fayad I, Jaramillo A, Giraldo A, Gonzalez F, Mantilla S, Botero A, Archila LH, Diaz A, Chacon T, Castillo DM, Betancourt M, Aya MD, Arce R (2007) Demographic, clinical, and microbial aspects of chronic and aggressive periodontitis in Colombia: a multicenter study. J Periodontol 78:629–639
34. Nonnenmacher C, Dalpke A, Mutters R, Heeg K (2004) Quantitative detection of periodontopathogens by real-time PCR. J Microbiol Methods 59:117–125
35. Thiha K, Takeuchi Y, Umeda M, Huang Y, Ohnishi M, Ishikawa I (2007) Identification of periodontopathic bacteria in gingival tissue of Japanese periodontitis patients. Oral Microbiol Immunol 22:201–207
36. Fine DH, Kaplan JB, Kachlany SC, Schreiner HC (2006) How we got attached to *Actinobacillus actinomycetemcomitans*: a model for infectious diseases. Periodontol 2000 42:114–157
37. Koenoenen E, Paju S, Pussinen PJ, Hyvonen M, Di Tella P, Suominen-Taipale L, Knuuttila M (2007) Population-based study of salivary carriage of periodontal pathogens in adults. J Clin Microbiol 45:2446–2451

38. Hamlet SM, Cullinan MP, Westerman B, Lindeman M, Bird PS, Palmer J, Seymour GJ (2001) Distribution of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. J Clin Periodontol 28:1163–1171
39. Bolstad AI, Jensen HB, Bakken V (1996) Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. Clin Microbiol Rev 9:55–71
40. van Winkelhoff AJ, Loos BG, van der Reijden WA, van der Velden U (2002) *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. J Clin Periodontol 29:1023–1028
41. Boutaga K, Winkelhoff AJv, Vandenbroucke-Grauls CMJE, Savelkoul PHM (2006) The additional value of real-time PCR in the quantitative detection of periodontal pathogens. J Clin Periodontol 33:427–433
42. Ximenez-Fyvie LA, Almaguer-Flores A, Jacobo-Soto V, Lara-Cordoba M, Sanchez-Vargas LO, Alcantara-Maruri E (2006) Description of the subgingival microbiota of periodontally untreated Mexican subjects: chronic periodontitis and periodontal health. J Periodontol 77:460–471
43. Nonnenmacher C, Dalpke A, Rochon J, Flores-de-Jacoby L, Mutters R, Heeg K (2005) Real-time polymerase chain reaction for detection and quantification of bacteria in periodontal patients. J Periodontol 76:1542–1549
44. Lovegrove JM (2004) Dental plaque revisited: bacteria associated with periodontal disease. J N Z Soc Periodontol 87:7–21
45. Ximenez-Fyvie LA, Haffajee AD, Socransky SS (2000) Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol 27:648–657
46. Ali RW, Johannessen AC, Dahlen G, Socransky SS, Skaug N (1997) Comparison of the subgingival microbiota of periodontally healthy and diseased adults in northern Cameroon. J Clin Periodontol 24:830–835
47. Braga RR, Carvalho MA, Bruna-Romero O, Teixeira RE, Costa JE, Mendes EN, Farias LM, Magalhaes PP (2010) Quantification of five putative periodontal pathogens in female patients with and without chronic periodontitis by real-time polymerase chain reaction. Anaerobe 16:234–239
48. Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS (2003) Distribution of selected bacterial species on intraoral surfaces. J Clin Periodontol 30:644–654
49. Boutaga K, Savelkoul PHM, Winkel EG, van Winkelhoff AJ (2007) Comparison of subgingival bacterial sampling with oral lavage for detection and quantification of periodontal pathogens by real-time polymerase chain reaction. J Periodontol 78:79–86
50. Umeda M, Contreras A, Chen C, Bakker I, Slots J (1998) The utility of whole saliva to detect the oral presence of periodontopathic bacteria. J Periodontol 69:828–833
51. Matto J, Saarela M, von Troil-Linden B, Alaluusua S, Jousimies-Somer H, Asikainen S (1996) Similarity of salivary and subgingival *Prevotella intermedia* and *Prevotella nigrescens* isolates by arbitrarily primed polymerase chain reaction. Oral Microbiol Immunol 11:395–401