Smoking May Lead to Marginal Bone Loss Around Non-Submerged Implants During Bone Healing by Altering Salivary Microbiome: A Prospective Study

Xiaobo Duan,* Tingxi Wu,† Xin Xu,* Demeng Chen,† Anchun Mo,* Yiling Lei,† Lei Cheng,* Yi Man,* Xuedong Zhou,* Yongyue Wang,* and Quan Yuan*

**Background:** This prospective and controlled study elucidates the impact of smoking on the salivary microbiome and its further influence on marginal bone loss (MBL) around an implant during a 3-month bone-healing period.

**Methods:** Saliva samples were collected preoperatively from 20 periodontally healthy patients with single-tooth replacement in the posterior mandible (smokers [n = 10] and non-smokers [n = 10]). Sequencing of 16S recombinant RNA gene amplicons was used to characterize the salivary microbiome. Each patient received implant surgery after oral clinical assessment, and MBL around the implant was measured during a 3-month healing period.

**Results:** In total, 871,389 sequences were compared against the Human Oral Microbiome Database for bacterial identification. Microbial signatures of smokers exhibited lower diversity and richness, with a significant decrease in uncultured species. The phyla Gracilibacteria and Saccharibacteria showed a significant decrease in smokers. The genera Streptococcus, Lachnoanaerobaculum, Stomatobaculum, and Eubacterium were significantly increased in smokers, whereas Selenomonas, Selenomonas [G-3], and Catonella were significantly decreased. Specifically, Porphyromonas gingivalis was significantly more abundant in smokers, which was positively related to the severity of MBL during bone healing.

**Conclusions:** Smoking shapes the salivary microbiome in states of clinical health, and further may influence MBL during bone healing by creating high at-risk-for-harm communities. Understanding of the distinctly divergent oral microbiome in smokers and non-smokers is a base for personalized therapeutics for this high-risk cohort and also a base for further study on the pathologic mechanisms. *J Periodontol* 2017;88:1297-1308.

**KEY WORDS**
Alveolar bone loss; dental implants; microbiota; smoking.

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According to the World Health Organization Report on the Global Tobacco Epidemic,1 nearly 360 million people in China are current smokers. Nicotine is the most important constituent among >4,000 potentially toxic chemical substances in tobacco products. Chronic exposure to various chemical compounds is responsible for numerous diseases, according to WHO data.2 Tobacco smoking is also an accepted potential risk factor for oral health. Smokers present a high-at-risk cohort for periodontitis3 and have higher rates of tooth loss,4 suggesting that the increasing number of this cohort are expected to require replacement of missing teeth.

Since Cherche`ve developed the first root-form implants in 1966,5 they have become a consolidated treatment for replacing missing teeth. Implants are inserted into the jawbone to support a dental prosthesis and remain stable due to the maintenance of bony support around the implants.6 The role of smoking as a risk indicator for prognosis of implants has been strongly substantiated.7-10 Studies have identified greater marginal bone loss (MBL) over time in smokers compared with non-smokers.11,12 Moreover, tobacco smoking has been shown to be a risk factor for bone healing around implants;13 however, the mechanism has not been fully
elucidated. In addition, it has been reported that presence of bacteria is one of the important aspects for determining peri-implant bone loss and long-term stability of an implant. Smoking has been demonstrated to negatively affect subgingival microbial communities, and recent evidence also indicates that smoking shapes the peri-implant microbiome, even in states of clinical health, by supporting a pathogen-rich community.

The oral cavity is exposed to the external environment, and saliva is the first biologic medium encountered during inhalation of cigarette smoke. According to the literature, smokers have been demonstrated to have poorer oral hygiene compared with non-smokers. The concentration of the oligoelement composition in saliva is affected by smoking, and these elements may unlock various metabolic processes that lead to functional or infectious diseases. Salivary antioxidant levels may also be changed by smoking, which can generate deleterious effects on oral health. In addition, smoking has been suggested to favor early acquisition and colonization of pathogens in oral biofilms, and a positive correlation between commensal bacteria and proinflammatory cytokine level has been demonstrated in smokers. Tobacco smoke can change the salivary environment in various ways, however, there are limited studies on its influences on constituents of the salivary microbiome.

The large community of microbes residing in the oral cavity constitutes a dynamic and symbiotic ecosystem that has a major role in maintaining oral and systemic health. Disruption of the ecology of oral bacteria might cause oral infectious and systemic diseases. Previous studies have taken unstimulated saliva samples as representative of the entire oral ecosystem. Studies have already demonstrated correlations among the salivary microbiome and periodontal diseases, oral lichen planus, and even oral cancers. The salivary microbiome can trigger an inflammatory and immune response, resulting in an immuno-osteolytic reaction. This accounts for ongoing bone resorption and is often a final determination of the fate of implants. Elucidating effects of smoking on this ecosystem is critical to understanding the impact of smoking on bone healing around implants and also on whole oral health.

The present study was designed to test the hypothesis that the habit of smoking can lead to significant changes in the structure and composition of the salivary microbiome and further affect MBL around implants during bone healing.

MATERIALS AND METHODS

Study Population

The study protocol was approved by the Institution Review Board of the West China Hospital of Stomatology, Sichuan University, Chengdu, China before initiation (authorization numbers WCHSIRB-ST-2016 to 072). Participants provided written informed consent via a signed statement before participation. Patient recruitment was from March 2015 to the end of June 2016. Twenty systemically healthy participants (11 males and nine females, aged 29 to 60 years; mean age: 44.1 ± 10.76 years) with single-tooth replacement in posterior mandibles where bone density was evaluated as normal (Types II and III) were recruited from those seeking care at the Implant Center of West China Hospital of Stomatology, Chengdu, China.

Patients were excluded if they: 1) were pregnant or lactating; 2) were postmenopausal women or patients with osteoporosis; 3) had chronic and/or aggressive periodontitis, >30% of sites with suppuration and/or bleeding on probing (BOP), or any site with probing depth (PD) and clinical attachment level ≥4 mm; 4) had antibiotic therapy or oral prophylactic procedures within the preceding 3 months; 5) had a need for antibiotic coverage before dental treatment; 6) had <20 teeth present; 7) had a need for immediate implant or early implant placement; and 8) had a need for osseous grafting or other augmentation procedures. Tobacco exposure was assessed by questionnaire at baseline recruitment. Patients were asked to fill out information on smoking status (current, past, never) and frequency exposure was assessed by questionnaire at baseline recruitment. Patients were asked to fill out information on smoking status (current, past, never) and frequency of smoking (number of cigarettes per day, week, or month). Patients were categorized into two groups: smokers, who smoked >10 cigarettes a day for at least 5 years (n = 10), and non-smokers, who had never smoked (n = 10).

Clinical Data Collection

All visits were carried out in the same setting, and measurements were examined by a trained and calibrated examiner (YL) who was masked to the study groups. Prior to initiation of the study, an examiner repeatability exercise was undertaken to confirm adequate intraexaminer reliability using 10 non-study patients. The $\kappa$ score for intraexaminer reliability was 0.93. Number of remaining teeth, full-mouth plaque index (Pl), full-mouth gingival index (GI), and PD (mm) were measured preoperatively. PI and GI were measured at four sites per tooth (mesio-buccal, buccal, disto-buccal, and lingual). PD was measured at six sites per tooth (mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual) using a standard periodontal probe. Likewise, peri-implant PD and BOP were measured by one masked investigator (YL). The $\kappa$ score for intraexaminer reliability was 0.94. All measurements were performed once a week over two consecutive weeks to monitor intraexaminer agreement. Medical records were used to collect the following information: 1) age and sex; 2) smoking status; 3) current systemic disorders; and 4) medical histories.
Surgical Procedure
All surgical procedures were performed by one trained clinician (QY). The single tissue-level implant with a rough surface§ was placed with the rough surface of the implant up to the crestal bone, and the smooth neck part of 1.8 mm projecting above the crestal bone. Healing abutments were connected to the implants using hand torque. Postoperative antibiotics (amoxicillin 500 mg) and analgesics (ibuprofen 600 mg) were prescribed, and verbal oral hygiene instructions were given to the patients.

Marginal Bone Level Measurements
Patients were examined with the same cone beam computed tomography device¶ immediately after implant surgery (baseline) and 3 months post-surgery. Analyses of tomographic exams to assess MBL were carried out by two independent investigators (Yuchen Guo and Xiaofei Zheng, Department of Oral Implantology, West China Hospital of Stomatology, Sichuan, China) who were masked and unrelated to the study. The two examiners were found to have an agreement coefficient (κ statistic) of 0.92. The reference line for bone-level evaluation was defined as the implant platform (the horizontal interface between the implant and abutment). Vertical distances from the reference line to the most coronal level of bone-to-implant contact at both mesial and distal sites were measured at two time points. MBL was measured by subtracting the obtained data from the reference line for bone-level evaluation was defined as the implant platform (the horizontal interface between the implant and abutment). The mean average of mesial and distal measurements of each implant during the 3-month bone healing was calculated (see supplementary Fig. 1 in online Journal of Periodontology).

Collection of Clinical Samples and Preparation
Saliva was collected before surgery according to the techniques as described: 5 mL spontaneous, whole unstimulated saliva was collected in a sterile DNA-free conical tube from each patient between 8:00 am and 9:00 am. Patients were instructed to refrain from drinking and eating for at least 2 hours before sampling and oral hygiene (i.e., brushing or flossing of teeth) for 12 hours before sampling. All samples were stored at –80°C before further processing.

16S ribosomal RNA (rRNA) Gene Amplification and Sequencing
Total DNA was extracted from saliva using a DNA purifying kit,§ with minor modifications by adding an extra lysozyme (3 mg/mL water bath for 1.5 hours) treatment step for lysing the bacterial cell. Positive controls were included by adding a known bacterial culture and negative controls for the assessment of sample contamination by foreign DNA. The V4 regions of the bacterial 16S rRNA gene were amplified using polymerase chain reaction (PCR) (95°C for 3 minutes, followed by 27 cycles at 95°C for 30 seconds, 72°C for 45 seconds, and a final extension at 72°C for 10 minutes). The primers 515F 5’-GTGCA-GCMGCCGCGG-3’ and 907R 5’-CCGTAATTCMTTTRAGTTT-3’ were used. PCR reactions were performed in triplicate in a 20-μL mixture containing 4 μL of 5X DNA polymerase buffer, # 2 μL of 2.5 mM deoxyribonucleotide triphosphate, 0.8 μL of each primer (5 μM), 0.4 μL of DNA polymerase,** and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels, purified using a DNA gel extraction kit†† according to instructions provided by the manufacturer, and quantified using a single-tube fluorometer.‡‡ Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on a sequencing platform§§ according to standard protocols. The raw reads were deposited into the National Center for Biotechnology Information Sequence Read Archive database.28

Sequence Analyses
Raw FASTQ files were demultiplexed and quality-filtered using a bioinformatic pipeline.‖ Operational units (OTUs) were clustered with 97% similarity cutoff using an OTU clustering method, and chimeric sequences were identified and removed using chimera checking. The taxonomy of each 16S rRNA gene sequence was analyzed by the Ribosomal Database Project classifier against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70%. The α-Diversity indexes were estimated from the number of observed OTUs, Chao1, and the Shannon diversity index, which reveals both species richness and evenness. Non-metric multidimensional scaling (NMDS) ordination was used to determine the degree of dissimilarity between pairs of bacterial communities using the Bray–Curtis distance method. Changes in the core microbiota and species associated with diseases were evaluated. Relative abundances of each bacterial taxon were calculated and typically presented as mean ± standard error (SE). Species name and HOT (Oral Taxon ID) are based on the Human Oral Microbiome database. Mann–Whitney U test¶¶ was used to compare differences between the two groups (smokers and non-smokers). Correlation between salivary microbiome and severity of MBL during bone healing was explored by calculating the Pearson correlation coefficient.## Significance level was established as P = 0.05.
RESULTS

Clinical Characteristics and Overall Sequence Statistics of Patients

A total of 10 non-smokers and 10 smokers were recruited in this study; Table 1 shows the clinical and demographic characteristics of patients. Smokers demonstrated significantly greater MBL during the bone-healing period compared with non-smokers (P = 0.003). There were no significant differences between smokers and non-smokers in other clinical characteristics or demographics (P > 0.05). Clinical characteristics of the implants after the 3-month bone-healing period are shown in supplementary Table 1 in online Journal of Periodontology.

To investigate changes in structure and composition of salivary microbial communities, 20 samples were sequenced. In total, 871,389 raw 16S rRNA sequences with an average length of 395 base pairs were obtained. After preprocessing, 29 known phyla and 337 genera were identified. A total of 634 OTUs were detected at 3% dissimilarity using a high-performance clustering, alignment, and search algorithm program with 202 – 46 second-OTUs in each individual. Of these, 107 were singletons (detected in only one sample) and were excluded from further statistical analysis.

Comparison of the Bacterial Community Structures Between Non-Smokers and Smokers

Differences in the phylogenetic structures between non-smokers and smokers were visualized by performing an NMDS ordination, based on the Bray–Curtis distance (Fig. 1). Each data point represents one sample, and spatial distance between points in the plot is interpreted as the relative difference in the composition of substrate marking. Hence, points that are closer are more similar than points that are more distant. NMDS for these taxa indicated a tendency of separation between non-smoking and smoking patients. Furthermore, analysis of similarities testing on the Unifrac distances showed significant difference between the two groups (P = 0.04).

Next, an estimation was made of the community diversity for all samples to compare the complexity between non-smokers and smokers. Comparisons of bacterial richness and diversity between smokers and non-smokers using observed OTUs and Chao and Shannon indices are shown in Figures 2A through 2C. Smokers had a lower diversity and shared smaller numbers of species than did non-smokers (P > 0.05). Uncultivated phylotypes accounted for an average (mean ± SE) of 18.40% ± 2.27% and 10.52% ± 1.05% of the salivary microbiome in non-smokers and smokers, respectively. Significantly more uncultured phylotypes were observed in non-smokers (P = 0.02) (Fig. 2D). Furthermore, a comparison was made of the characteristics of constituent species of non-smokers and smokers based on their oxygen requirements and Gram-staining statuses; no significant differences between the two groups were found (Figs. 2E and 1F).

Comparison of the Bacterial Community Composition Between Non-Smokers and Smokers

The salivary microbial community composition of smokers and non-smokers was analyzed at different taxonomic levels. The microbial community composition of the two groups at the phylum level is shown in Figure 3A. The top 10 phyla are Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria,

Table 1.
Demographics and Clinical Parameters of all Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group</th>
<th>P Value</th>
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<tbody>
<tr>
<td></td>
<td>Non-smokers (n = 10)</td>
<td>Smokers (n = 10)</td>
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<tr>
<td>Males/Females</td>
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<td>6/4</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>44.40 ± 10.57</td>
<td>43.80 ± 11.52</td>
</tr>
<tr>
<td>Cigarettes smoked per day</td>
<td>—</td>
<td>11 ± 2.11</td>
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<tr>
<td>Number of teeth*</td>
<td>27.4 ± 1.71</td>
<td>27.4 ± 2.07</td>
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<tr>
<td>PI*</td>
<td>1.40 ± 1.17</td>
<td>1.60 ± 0.84</td>
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<tr>
<td>GI*</td>
<td>0.80 ± 0.56</td>
<td>0.84 ± 0.47</td>
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<tr>
<td>PD (mm)*</td>
<td>1.94 ± 0.45</td>
<td>1.99 ± 0.41</td>
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<tr>
<td>MBL (mm)*</td>
<td>0.53 ± 0.65</td>
<td>1.19 ± 0.49</td>
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</table>

— = vacancy.

Data are presented as mean ± SD.

* Significance of differences between groups was assessed using Mann–Whitney U test. Significance level was established as P = 0.05.
Synergistetes, Saccharibacteria, Spirochaetes, Gracilicilibacteria, and Tenericutes. The phyla Gracilicilibacteria \((P=0.004)\), Saccharibacteria \((P=0.001)\), and the total relative abundance of the other phyla \((P=0.01)\) were found at significantly higher levels in non-smokers. Comparing between the two groups, the top five phyla (99.84%) were found at similar levels. Proteobacteria \((P=0.05)\) was also more abundant in non-smokers, whereas Synergistetes \((P=0.58)\) showed a slight increase in smokers.

The microbial shift was investigated in more detail at the genus level. The top 30 genera are shown in Figures 3B through 3D. Compared with non-smokers, the relative abundance of genus *Streptococcus* \((P=0.02)\), *Lachnoanaerobaculum* \((P=0.03)\), *Stomatobaculum* \((P=0.04)\), and *Eubacterium nodatum* group \((P=0.02)\) were significantly increased in smokers. In contrast, *Selenomonas* [G-3] \((P=0.03)\), *Catonella* \((P=0.01)\), and *Selenomonas* \((P=0.02)\) showed a significant overabundance in non-smokers.

**Changes in Core Microbiota Between Non-Smokers and Smokers**

An attempt was made to determine the core microbiota of saliva in non-smokers and smokers based on a previous study.\(^{16}\) It was found that some species had significantly different abundance levels between non-smokers and smokers. The core salivary microbiome (species shared by at least 75% of individuals) in the two groups is shown in supplementary Table 2 in online *Journal of Periodontology*. Only the core microbiome with average relative abundance >1% or species with statistical differences between smokers and non-smokers are demonstrated in Figure 4. Both smokers and non-smokers exhibited a core microbiome comprising 23% of the 527 species; 78 species were common to both smokers and non-smokers, 23 species were unique to smokers, and 23 species were unique to non-smokers. Although many species were prevalent in both groups, *Streptococcus parasanguinis* \((P=0.01)\), *Streptococcus salivarius* \((P=0.01)\), *Eubacterium infirmum* \((P=0.02)\), and *Megasphaera micronuciformi* \((P=0.04)\) were significantly more abundant in smokers. *Catonella morbi* \((P=0.01)\), *Oribacterium asaccharolyticum* \((P=0.04)\), and *Solobacterium moorei* \((P=0.02)\) were significantly more abundant in non-smokers. No unique species with a mean relative abundance >1% was found in non-smokers or smokers. *Alloprevotella rava* \((P=0.04)\), *Shuttleworthia satelles* \((P=0.002)\), *Treponema sp. [HOT-258] (P=0.01)*, *Prevotella sp. [HOT-304] (P=0.04)*, and unclassified *Tannerella sp. (P=0.02)* were unique species with statistical overabundance in smokers. However, *Selenomonas sp. [G-3] (P=0.03)*, *SR1 bacterium sp. [HOT-875] (P=0.002)*, *Acinetobacter bohemicus* \((P=0.04)\), *Acinetobacter sp. [HOT-408] (P=0.02)*, *Saccharibacteria [G-3] sp. [HOT-351] (P=0.004)*, *Pseudomonas orientalis* \((P=0.04)\), *Vibrio gigantis* \((P=0.02)\), and some unclassified phylotypes were only detected in non-smoking samples, showing significantly higher proportions compared with smoking samples.

**Species Associated With Diseases**

Considering that smoking is a significant risk factor for periodontal health\(^3\) and dental implant therapy,\(^{11}\) species that are the important periodontal pathogens belonging to the “red complex” and “orange complex”\(^{33}\) were evaluated (Table 2). It was found that *Porphyromonas gingivalis* \((Pg) (P=0.04)\) was significantly more abundant in smokers. Although *Tannerella forsythia* \((Tf) (P=0.48)\) and *Treponema denticola* \((Td) (P=0.19)\) were numerically more abundant in smokers, there was no statistical difference. Smokers also appeared to have higher levels of species belonging to the orange complex than non-smokers.

**Species Associated With MBL During Bone Healing**

It was hypothesized that some bacteria might be associated with the severity of MBL during bone healing and, therefore, an investigation was made of the relationship between microbial characteristics and bacterial taxa (29 core species with statistical difference between groups) with the severity of MBL during bone healing by calculating Pearson ranked correlations (see supplementary Table 3 in online
Comparison of bacterial diversity, richness, and observed OTUs between smokers and non-smokers.

Comparison of uncultured species between smokers and non-smokers.

Comparison of the characteristics of constituent species of non-smokers and smokers based on their oxygen requirements and Gram-staining statuses. Significance of differences between groups was assessed using Mann–Whitney U test. Significance of differences within patients in the same group was assessed using Wilcoxon test. *P < 0.05; †P < 0.01.
Figure 3.
A) Relative abundance of the top 10 bacterial phyla. Relative abundance of top 30 bacterial genera in two groups. B) Comparison among top 10 abundant genera. C) Comparison among top 11 to 20 abundant genera. D) Comparison among top 21 to 30 abundant genera. Significance of differences between groups was assessed using Mann–Whitney U test. *P<0.05; †P<0.01; ‡P<0.001: significant difference between smokers and non-smokers.
The species, including \textit{Pg}, uncultured \textit{Stomatobaculum} sp., and \textit{Eubacterium infirmum}, were positively correlated with the severity of MBL, whereas uncultured \textit{Selenomonas} sp. and \textit{Saccharibacteria \text{[G-3]} sp. [HOT\_351]} \text{\textsuperscript{1}} were negatively correlated with MBL. Uncultured species also showed a positive correlation with MBL.

**DISCUSSION**

Oral health plays an important role in overall health and is strongly affected by the complex oral microbial ecosystem. Some species promote healthy conditions, whereas others contribute to disease.\textsuperscript{34} The oral microbiome is one of the first ecosystems to come into contact with tobacco smoke and can be affected by smoking.\textsuperscript{19} Various studies have shown that smoking affects composition of the subgingival microbial flora\textsuperscript{20,35} and alters bacteria acquisition and colonization in oral biofilms in favor of periodontopathogens.\textsuperscript{20} Increased levels of salivary periodontal pathogens have been proposed as risk indicators for future peri-implant disease.\textsuperscript{36} Moreover, evidence demonstrated that levels of proinflammatory cytokines in gingival crevicular fluid samples and peri-implant sulcus fluid samples were observed to be higher in smokers.\textsuperscript{20,37} However, limited studies have focused on the influence of smoking on the composition of the salivary microbiome, which is more responsible for maintaining the integrity of the oral cavity.\textsuperscript{38} The impact of smoking on shifting the balance from health to disease cannot be understood without a comprehensive view of the oral microbiome.

Furthermore, peri-implant bone healing is a complex phenomenon. Factors hypothesized to be associated
with MBL during bone healing include plaque control,39 smoking,40 surgical trauma,41 and implant design characteristics (e.g., platform-switching).42 The detrimental role of smoking on bone healing has been demonstrated to be due to less collagen production,43 reduced peripheral blood flow and nutrient delivery,44 and compromised function of polymorphonuclear leukocytes and macrophages.45 Bacteria in saliva are the primary etiologic agents of oral diseases.46 Increases in salivary periodontal pathogen and host inflammatory biomarker burden are associated with periodontal attachment loss.47 Microbiology and immunopathology are two important aspects for peri-implant diseases.48 The goal of the present study, therefore, was to investigate whether a smoking habit can lead to significant changes in structure and composition of the salivary microbiome and whether species that are more or less frequent in smokers can be correlated with MBL during bone healing.

NMDS analysis graphically displayed different bacterial profiles between smokers and non-smokers (Fig. 1). The analysis revealed a tendency of separation between non-smoking and smoking patients. Dissimilarity tests revealed significant difference between the two groups. The salivary microbiome of smokers exhibited a significant decrease in uncultured species and also a lower diversity and lower richness than that of non-smokers (Figs. 2A through 2D). Tsigarida et al.,16 using 454-pyrosequencing, found that microbial signatures of smokers exhibited lower diversity compared with non-smokers. These results indicate that tobacco smoking caused an alteration in the density of oral bacteria in healthy status, and cigarette smoking is composed of over 4,000 toxins that may cause an ecologic catastrophe for the commensals.

At the phylum level (Fig. 3A), relative abundances of phyla Gracilibacteria, Saccharibacteria, and 19 other phyla were significantly decreased in patients who smoked. In contrast, it has been reported that the phylum Saccharibacteria may have an association with periodontal diseases.49 Synergistetes and Spirochaetae were increased in smokers, although not statistically significantly so. Both Spirochaetae and Synergistetes are Gram-negative anaerobic taxa. Early research has shown that the subgingival biofilm around failing implants yields similar compositions, characterized by a high proportion of Gram-negative anaerobic rods.50 More significant differences have been observed when comparisons are made at the genus level (Figs. 3B through 3D). The smoking community was statistically enriched for genera previously regarded as positively associated with periodontal and/or peri-implant inflammation,51 including those belonging to Streptococcus, Stomatobaculum, and Eubacteria. However, both Catonella and Selenomonas were statistically more abundant in non-smokers, which is in accordance with findings of a previous study16 showing that Catonella and Selenomonas were elevated in the peri-implant biofilm samples of non-smokers.

The core salivary microbiome of both non-smokers and smokers comprised 124 species (see supplementary Table 2 in online Journal of Periodontology). However, although smokers and non-smokers shared 78 species, they differed by 46. The data indicates that there exists a core microbiome composed of species that are most suited to healthy non-smoking individuals, whereas a smoking habit might modify this environment. Streptococcus salivarius and Streptococcus

<table>
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<th>Species</th>
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<th>Smokers (n = 10)</th>
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<tr>
<td>Red complex</td>
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<tr>
<td>Porphyromonas gingivalis</td>
<td>0.15 ± 0.10</td>
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<td>Tannerella forsythia</td>
<td>0.09 ± 0.03</td>
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<tr>
<td>Treponema denticola</td>
<td>0.02 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Orange complex</td>
<td></td>
<td></td>
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<tr>
<td>Campylobacter showae</td>
<td>0.13 ± 0.06</td>
<td>0.01 ± 0.00</td>
<td>0.28</td>
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<td>Fusobacterium nucleatum</td>
<td>0.51 ± 0.13</td>
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<td>Prevotella intermedia</td>
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<td>Prevotella melaninogenica</td>
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<td>Prevotella nigrescens</td>
<td>0.03 ± 0.02</td>
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</table>

Data are presented as mean ± SD. Significance of differences between groups was assessed using Mann–Whitney U test. Significance level was established as P = 0.05.
Smoking Leads to Marginal Bone Loss by Altering Microbiome

parasanguinis, which play an important role in biofilm formation and promote aggregation with late tooth colonizers to form dental plaque, were present in healthy non-smoking individuals, and increased in smokers (Fig. 4). Various studies have demonstrated an increased risk of caries in smokers. Fifty core species that were unique in smokers showed significant differences compared with non-smokers (Fig. 4). Perhaps the more interesting species are those that are only present in smokers, since they seem more likely to account for pathogenesis. Further studies will be needed to test these possibilities. Notably, it was found that Pg, a proposed keystone organism in periodontitis, was significantly abundant in the smoking group (Table 2). Proportions of Tf, Td, Fusobacterium periodonticum, and Prevotella intermedia, belonging to the red complex or the orange complex, were slightly elevated in smokers but without a significant difference. This may be a result of the small sample size, which should be increased in any future study. Members of the red and orange complex were already shown to be the most important microbiota for progression of periodontitis and also for peri-implantitis. The high abundance of these pathogens in healthy smokers suggests smoking creates a pathogen-rich oral environment and warrants further investigation.

Considering that the overall composition of the salivary microbiome has been shown to be quite stable after a single antibiotic treatment, antibiotics have been prescribed postoperatively to reduce early dental implant failure. Moreover, previous studies have shown that without any changes in lifestyle and general and oral heath, the salivary microbiome tends to be relatively stable, implying that it has the potential capability for disease detection and disease prediction. However, the potential benefit of antibiotic regimens during routine dental implant insertion is a controversial subject, and antibiotic resistance should be more carefully considered.

Smokers demonstrated significantly more MBL 3 months postoperatively (Table 1). Meanwhile, Pearson correlation coefficients elucidated that the salivary microbiome was correlated with the severity of MBL during bone healing (see supplementary Table 3 in online Journal of Periodontology). These data indicate that a smoking habit might influence MBL during bone healing by shifting the structure and composition of the salivary microbiome.

CONCLUSIONS
A comprehensive, preliminary description of the oral salivary microbiota in smokers has been provided. Also, the current findings suggest that tobacco smoking has a role in creating an at-risk-for-harm microbiome and may further interfere with bone healing around an implant. To shed more light onto clinical mechanisms and disease progression, further studies with prospective design, rigorous control of confounding factors, and long-term follow-ups (i.e. 1 year after implant surgery) with an expanded sample size are needed in the future.

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