# High-Throughput Sequencing Analysis of Microbial Profiles in the Dry Socket

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**Purpose:** The aim of this study was to explore and describe the microbial profiles of dry socket (DS) and identify the key microbial population as a possible disease-related factor.

**Materials and Methods:** Bacterial samples were collected from patients who underwent surgical mandibular third molar extraction and were divided in 3 groups: the disease (D) group composed of patients who were diagnosed with DS; the treated (T) group composed of patients from the D group who received treatment; and the control (C) group composed of patients who did not have adverse reactions after tooth extraction. Bacterial DNA was extracted and the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene were amplified and subjected to sequencing. Sequence data were analyzed using alpha and beta diversity indices.

**Results:** In total, 772,169 high-quality sequences were detected from 31 samples. Using a 97% similarity level, 531 operational taxonomic units were detected. In addition, 10 phyla, 23 classes, 38 orders, 63 families, and 116 genera were found. Composition of the microbial community in the D group differed considerably from that of the T and C groups. Furthermore, a specific microbial pattern, which included *Parvimonas*, *Peptostreptococcus*, *Prevotella*, *Fusobacterium*, *Slackia*, *Oribacterium*, and *Solobacterium* species, appeared abundantly in the D group compared with the T and C groups. Moreover, *Parvimonas*, *Peptostreptococcus*, *Prevotella*, and *Fusobacterium* species had important roles in discriminating the D group from the other 2 groups.

**Conclusion:** These results suggest differences in the microbial community composition among DSs, normal-healing sockets, and post-treated sockets. These results provide better insight into the development of DS and enhance the understanding of DS. Nonetheless, further studies are necessary to investigate and confirm how these differential bacteria contribute to the development of the disease.
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Dry socket (DS) is a common postoperative complication after surgical extraction of the mandibular third molar; the reported incidence is 25 to 30%.[1] Moreover, the extraction of teeth with a pericoronitis history substantially increases the incidence of DS.[1,2] Typical clinical manifestations include postoperative pain in and around the extraction site, which increases in severity at any time from 1 to 3 days after extraction, and is accompanied by a partly or totally disintegrated blood clot within the alveolar socket.[3] Widely accepted treatments for DS involve the removal of food debris, necrotic tissue, and bacteria from the alveolar socket and blockage of external stimuli to achieve good analgesia.

The etiology of DS is unclear and has many influencing factors. Fibrinolysis of blood clots has been considered the main cause of DS[4]; however, the reason behind over-reacting fibrinolytic activity remains controversial. Birn[5-7] attributed the increased fibrinolytic activity in DS to excessive bone trauma. Mamoun[8] suggested that excessive trauma led to osteoblast necrosis, which disturbed metabolic integration of the blood clot. Some bacteria also could be involved in the pathogenesis through activation of fibrinolytic activity. *Bacteroides oralis* and *Treponema denticola* derived from the oral cavity exhibited the ability to activate fibrinolytic activity in vitro.[9] Furthermore, Nitzan[2] argued that *T denticola* was the main pathogenic bacteria, whose function was in line with the manifestation of DS. *Fusobacterium*, *Peptococcus*, and *Streptococcus* species isolated from the mandibular third molar extraction socket and subgingival plaque of teeth with advanced periodontitis could degrade fibrin or fibrinogen.[10] Moreover, local infections such as pericoronitis, advanced periodontal disease, and poor oral hygiene could markedly increase the incidence of DS.[11,12] In summary, all the evidence suggests that bacteria could be involved in the pathogenesis of DS.

Numerous studies have emphasized the relation between specific bacteria and disease. However, more recent studies have suggested that there is no specific pathogen, but rather a shifting of microbial community structures that lead to the occurrence of diseases.[13-15] To date, most studies that have focused on DS have concentrated on pathogens, and none of the existing studies have examined the detailed microbiome composition in DS using next-generation sequencing. In the present study, 16S rRNA gene sequencing was used to compare microbial communities in alveolar DSs, normal-healing sockets, and post-treatment sockets treated with iodoform gauze as intra-alveolar dressing to elucidate the changes of microbial profiles in DS and to identify the key microbial population as the possible disease-related factor.

## Materials and Methods

### PATIENTS

Patients with DS and gender- and age-matched healthy controls were recruited for this study. The disease (D) group included patients who were diagnosed with DS after surgical extraction of third molars; the treated (T) group included patients from the D group who received appropriate treatment; and the control (C) group included patients who did not have any adverse reactions after tooth extraction. Patients 18 to 50 years of age in good health were included. For the D group, the criteria were defined according to Blum[3]: postoperative pain in and around the extraction site, which increased in severity at any time 1 to 3 days after extraction and was accompanied by a partly or totally disintegrated blood clot within the alveolar socket with or without halitosis. For the T group, patients from the D group underwent inferior alveolar nerve block anesthesia and debriding with 3% hydrogen peroxide and physiologic saline solution, after which iodoform gauze was placed into the alveolar socket. Effective analgesia was achieved, and patients were required to visit the clinic 7 to 10 days after treatment; no halitosis, pus, spontaneous, or probing pain were observed in the socket. For the C group, patients were required to visit the clinic 7 to 10 days after surgical extraction of mandibular third molars. In addition, no adverse reaction was observed after surgery.

Excluded were patients with severe systemic disease, such as bone metabolic diseases or diabetes; those who used an antibiotic within 1 week before and after DS; those who refused to accept the treatment; and those with pain caused by local infection, bisphosphonate-related osteonecrosis, osteomyelitis, or other diseases.

The ethics committee of the Peking University School and Hospital of Stomatology (Beijing, China) approved this study (PKUSSIRB-201840164). Informed consent was obtained from all participants. The present research was conducted in full accordance with the World Medical Association's Declaration of Helsinki.

### SAMPLING

Patients in the D group received inferior alveolar nerve block anesthesia. Then, a cotton ball soaked with 0.1% benzalkonium bromide was used to disinfect the mucosa around the extraction socket and a dry sterile cotton ball was applied to block saliva. A sterile cotton swab (number 4383; Epicentre, Madison, WI) was inserted into the alveolar socket to thoroughly wipe the socket wall. Next, the samples were placed in frozen tubes and transferred to the Laboratory of Oral and Maxillofacial Surgery at the Peking University School and Hospital of Stomatology.

Patients in the T group underwent the same disinfection method as those in the D group, after which the iodoform gauze was removed and the identical procedures for sampling and storing were used. For the C group, specimens were obtained using these same methods.

### MICROBIAL DNA EXTRACTION AND SEQUENCING

Microbial genomic DNA was isolated using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). DNA quality was evaluated by absorbance ratios of A260 to A280 using spectrophotometry (NanoDrop 8000; Thermo Scientific, Inc, Wilmington, DE). Only DNA samples with a ratio of A260 to A280 higher than 1.8 were used for further analysis. Extracts were stored at −20°C until further use. The V3 and V4 hypervariable regions of the 16S rRNA gene were subjected to high-throughput sequencing (Beijing Auwigene Tech, Ltd, Beijing, China) using the Illumina Miseq PE300 sequencing platform (Illumina, Inc, San Diego, CA). The raw sequencing data were analyzed using the pipeline tools of MOTHUR and QIIME. After trimming and filtering, high-quality sequences were aligned to the Ribosomal Database Project[16] and clustered into operational taxonomical units (OTUs) using QIIME at a 97% similarity level.

### SEQUENCE ANALYSIS

Sequence data were further analyzed with the following statistical methods. *1)* Alpha diversity indices, including the Shannon index, Chao1, and observed species, were analyzed using QIIME 1.8 (http://qiime.org/scripts/alpha_rarefaction.html). Student *t* test was used to identify statistically relevant differences. *2)* Beta diversity indices, including principal coordinate analysis (PCoA) and principal component analysis (PCA), were used to compare the phylogenetic structures of the D, T, and C groups. In addition, ANOSIM was used to examine community differences among different groups. *3)* Statistically relevant differences in relative abundances of taxa in the D, T, and C samples were analyzed by Metastats analysis.[17] Statistically relevant differential bacteria were selected to plot the Heatmap figure by Heml 1.0.3.7.[18] Differential pairwise features of the D and C and D and T groups were plotted using the LEfSe online tool (http://huttenhower.sph.harvard.edu/galaxy/); the threshold logarithmic linear discriminant analysis score for discriminative features was 4.0. *4)* Samples of the D and T pairing were subjected to Wilcoxon signed rank test. All statistical analyses were performed with MOTHUR 1.8.0 (https://www.mothur.org/wiki/Mothur_v.1.8.0), R 3.5.0 (R Foundation, Vienna, Austria), and SPSS 19.0 (IBM Corp, Armonk, NY).

## Results

In total, 16S rRNA gene amplicons were sequenced from 31 qualified samples, including 11 samples from the D group, 7 samples from the T group, and 13 samples from the C group. Patient information is presented in Table 1. After processing, 772,169 high-quality sequences were generated (mean per sample, 22,932 ± 346; range per sample, 14,060 to 40,830). Using a 97% similarity level, 531 OTUs were detected (mean per specimen, 185 ± 40 OTUs; range per specimen, 109 to 245 OTUs). More OTUs were found in the T group (443 OTUs) than in the C group (418 OTUs) and D group (367 OTUs).

The alpha diversity analysis included OTU richness (Chao1 and observed species) and evenness (Shannon index; Fig 1A-C). A significantly higher Shannon index was observed in the C group compared with the T group ($P < .05$), showing a more stable microbial composition in the C group. In addition, the low level of microbial evenness implied that predominant bacteria with high relative abundance were present in the T group. Lower alpha diversity was found in the D group compared with the C group; however, the observed difference was not statistically relevant.

**Table 1. PATIENTS' BASIC INFORMATION**

| Number | Gender | Age | Impact Classification | Tooth |
|---|---|---|---|---|
| D1 | Female | 32 | Vertical | 38 |
| D2 | Female | 24 | Mesioangular | 38 |
| D3 | Female | 45 | Mesioangular | 48 |
| D4 | Male | 27 | Mesioangular | 38 |
| D5 | Female | 29 | Vertical | 48 |
| D6 | Male | 30 | Horizontal | 38 |
| D7 | Female | 31 | Vertical | 48 |
| D8 | Female | 27 | Vertical | 38 |
| D9 | Male | 24 | Horizontal | 48 |
| D10 | Male | 26 | Horizontal | 48 |
| D11 | Female | 28 | Distoangular | 38 |
| C1 | Male | 26 | Mesioangular | 48 |
| C2 | Female | 27 | Mesioangular | 38 |
| C3 | Female | 23 | Vertical | 48 |
| C4 | Male | 29 | Vertical | 38 |
| C5 | Male | 31 | Distoangular | 48 |
| C6 | Female | 37 | Mesioangular | 38 |
| C7 | Female | 21 | Distoangular | 48 |
| C8 | Female | 24 | Mesioangular | 48 |
| C9 | Male | 24 | Horizontal | 48 |
| C10 | Male | 29 | Horizontal | 48 |
| C11 | Female | 30 | Distoangular | 48 |
| C12 | Female | 22 | Horizontal | 38 |
| C13 | Female | 24 | Mesioangular | 38 |

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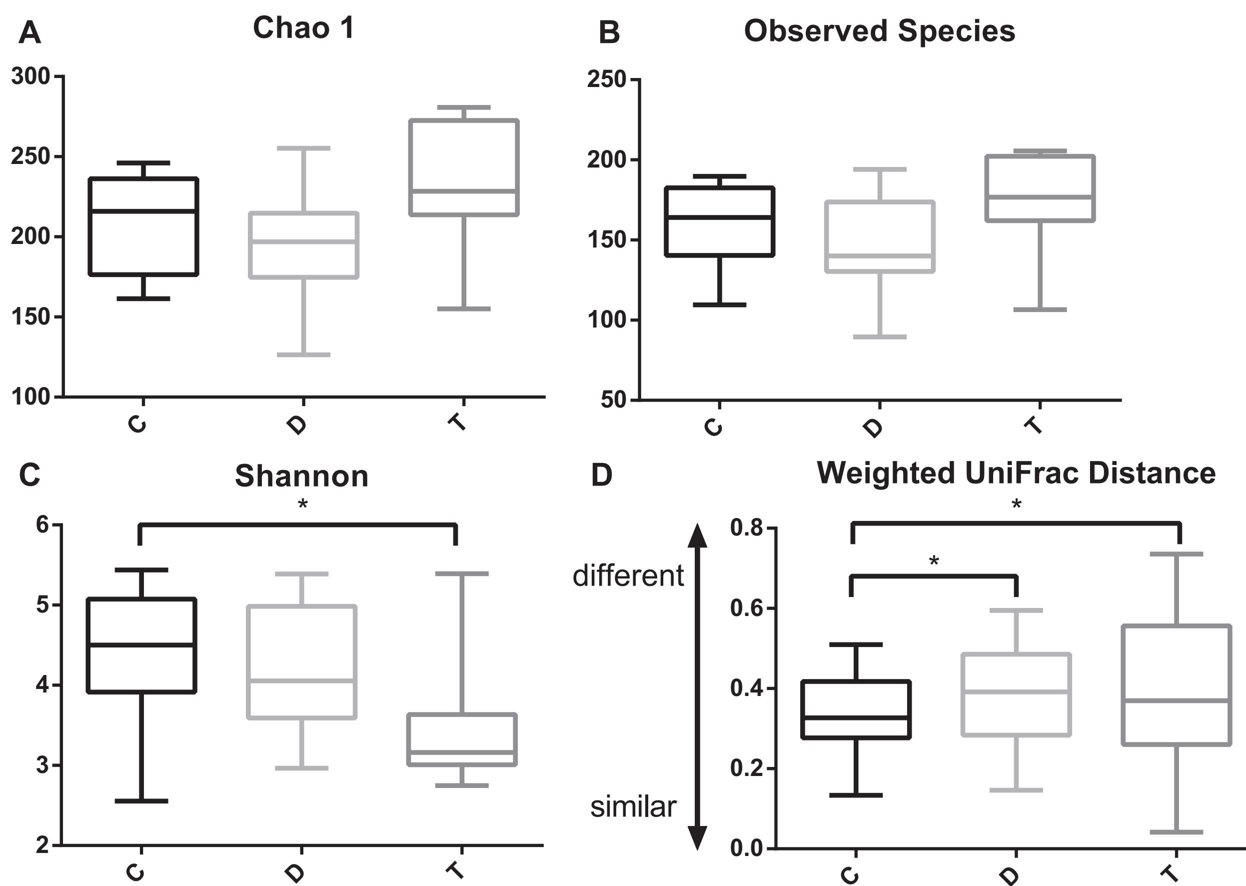


**FIGURE 1.** Alpha diversity and weighted UniFrac distance of microbiomes in the C, D, and T groups. Richness was estimated with *A,* Chao1 and *B,* observed species estimators. *C,* Evenness was analyzed based on the Shannon index. *D,* Microbial community variation within groups was analyzed using weighted UniFrac distance. * $P < .05$. C, control; D, disease; T, treated.

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Beta diversity was analyzed to examine divergence among the 3 groups. Overall microbial community compositions based on weighted UniFrac distance measurements were compared among the 3 groups (Fig 1D). Similarity of the microbial composition in the C group was greater than in the other 2 groups, with a significant difference ($P < .05$ for all comparisons), whereas the T group showed the greatest variation in composition. Moreover, PCoA showed that the T group could be easily discriminated from the other 2 groups, whereas the D and C groups were relatively similar (Fig 2A). In addition, the ANOSIM result indicated significant differences between the D and C, D and T, and T and C groups ($P < .05$ for all comparisons).

PCA was performed to screen out vital components from the microbial composition to discriminate different states based on relative abundance. To exclude irrelevant microbes with high relative abundance, Metastats analysis was applied to filter substantially different bacteria at the genus level between the D and C groups. The PCA chart showed that 2 groups were obviously different, showing a significant difference between groups D and C ($P < .01$; Fig 2B). PC1, which was the most discriminating dimension of the 2 sampling sites, was correlated with *Parvimonas* and *Peptostreptococcus* species, showing a positive relation with relative abundance. PC2 separated the 2 sampling sites by *Treponema* and *Peptostreptococcaceae_XIG-7* species. According to the identical analytical approaches, the D and T groups were significantly different ($P < .01$), mainly according to PC1 by *Fusobacterium*, *Parvimonas*, *Peptostreptococcus,* and *Prevotella* species, with a positive relation to relative abundance, whereas PC2 discriminated the 2 sampling sites by *Actinomyces* and *Streptococcus* species (Fig 2C).

The microbial community composition was characterized by the relative abundance of microbial taxa. Ten phyla, 23 classes, 38 orders, 63 families, and 116 genera were detected. At the phylum level (Fig 3A), more than 90% of sequences belonged to *Bacteroidetes* (2 to 67% of total sequences), *Firmicutes* (11 to 64%), *Fusobacteria* (0.2 to 35%), *Proteobacteria* (0.8 to 56%), *Actinobacteria* (0.1 to 68%), and

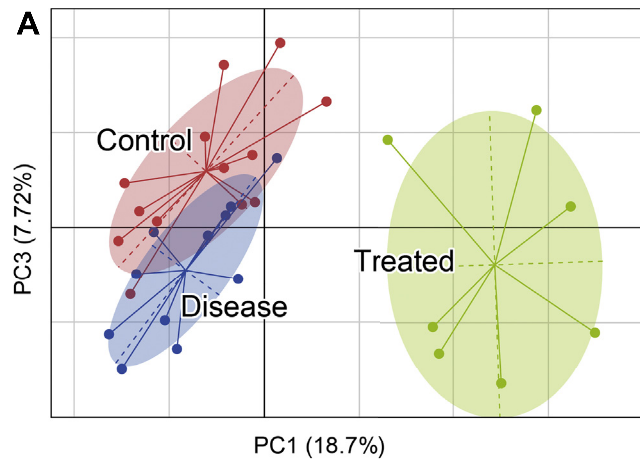


**FIGURE 2.** *A*, Principal coordinate analysis was performed based on weighted UniFrac distance values. The C, D, and T groups showed statistical differences ($P < .01$). **(Fig 2 continued on next page.)**

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*Spirochaetes* (0 to 12%) species. Except *Spirochaetes* species, all other phyla were detected in each sample. The remaining microbes belonged to the *Gracilibacteria_GN02*, *Saccharibacteria_TM7*, *SR1,* and *Synergistes* species. In addition, the average relative abundance of *Fusobacteria* species was higher in the D group than in the other 2 groups (Fig 3B).

A total of 116 genera were detected, and the top 48 are shown in Figure 3C. A significantly increased abundance of *Parvimonas*, *Peptostreptococcus*, *Prevotella*, *Fusobacterium*, *Slackia*, *Johnsonella*, *Oribacterium*, *Atopobium*, *Dialister*, *Filifactor,* and *Solobacterium* species was observed in the D group (average relative abundance, >0.1%) compared with the C and T groups ($P < .05$). Of these, the relative abundance of *Parvimonas*, *Peptostreptococcus*, and *Slackia* species differed substantially between the D and C groups. In addition, the relative abundance of *Actinomyces* and *Streptococcus* species in the T group was markedly higher compared with the D and C groups. *Aggregatibacter*, *Peptostreptococcaceae_XIG-7*, *Treponema*, and *Veillonella* species were considerably more abundant in the C group compared with the D group. A Heatmap chart was plotted to illustrate the relevant changes of relative abundance among these 3 groups (Fig 3D). Moreover, important characteristics for discriminating the D group from the other 2 groups, mainly by *Parvimonas*, *Peptostreptococcus*, *Fusobacterium*, and *Prevotella* species, were calculated by the LEfSe online tool.

The authors hypothesized that microbial communities that shared similar increasing and decreasing trends could be clustered to form a specific pattern. In the present study, *disease pattern* was used to describe those genera whose relative abundance increased in the disease state but decreased significantly in the healing and post-treated states (to improve accuracy, the $P$ value was set at .01), which included *Parvimonas*, *Peptostreptococcus*, *Prevotella*, *Fusobacterium*, *Slackia*, *Oribacterium*, and *Solobacterium* species. To verify this classification and exclude other confounding factors, a Wilcoxon signed rank test was performed on the 7 samples paired with the D and T groups. The changing mode of all these bacteria was perfectly consistent with the disease pattern.

## Discussion

According to the obtained results, the microbial composition in DS differed considerably from the normal-healing and post-treatment samples. The relative low alpha diversity observed in the D group implied that opportunistic bacteria might overgrow with dysbiosis. Samples from the C group exhibited the greatest evenness, species homogeneity, and sample-to-sample similarity, which could be due to their similar postoperative reactions and the fact they were extracted at similar time points. This suggested that the microbial community of the extraction socket might undergo a similar evolution and build a more stable ecologic microenvironment during the normal healing process. After treatment, the species homogeneity and sample similarity were considerably less compared with the disease and healing states, indicating that iodoform gauze selectively harbored iodoform-unsusceptible micro-organisms, which in turn led to uneven species distribution.

*Fusobacterium*, *Parvimonas*, *Peptostreptococcus*, and *Prevotella* species showed relatively high

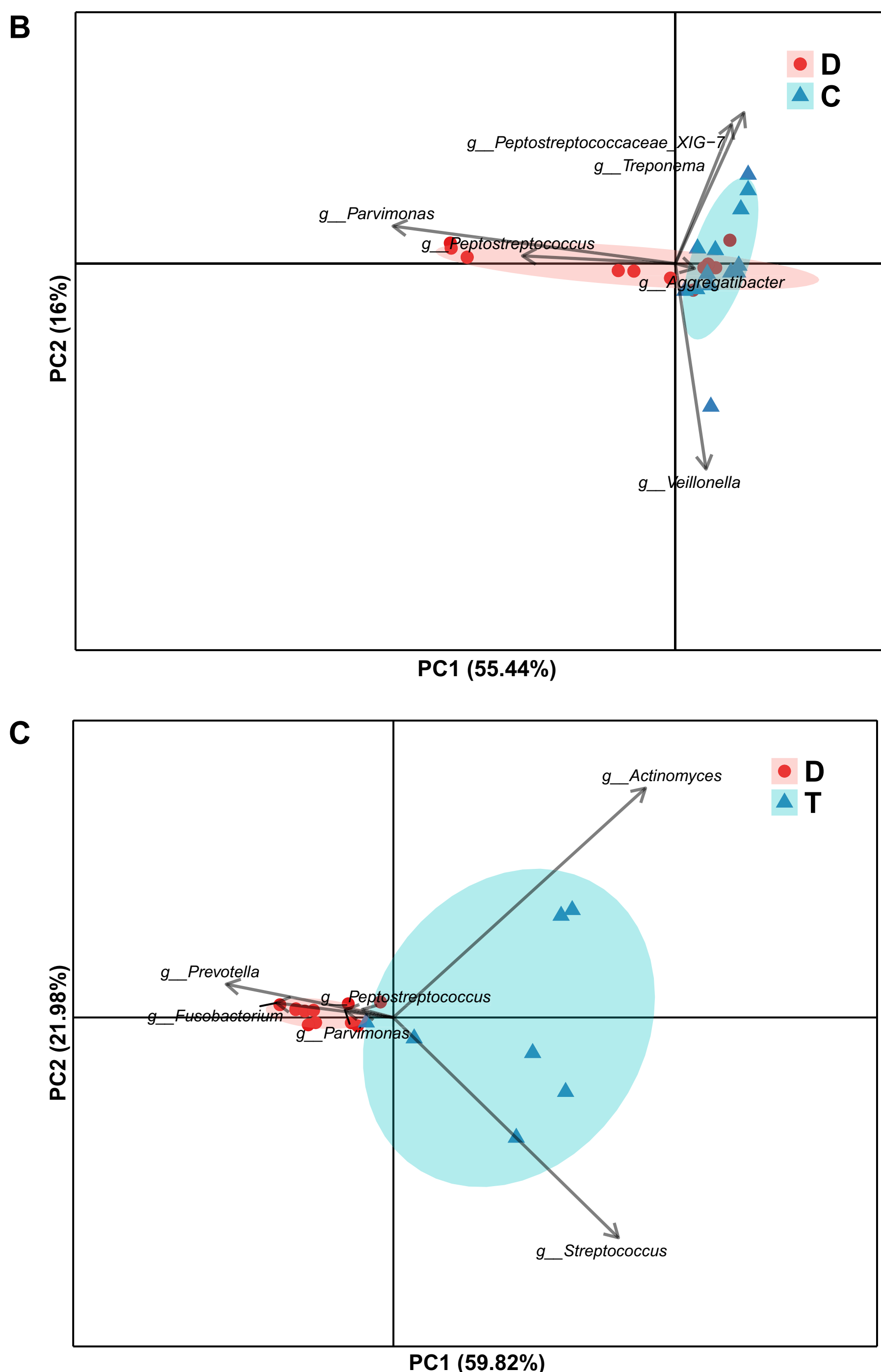


**FIGURE 2 (cont'd).** *B*, Principal component analysis was performed based on relative abundance. Pearson correlations were performed to highlight which genera were responsible for the divergence among samples. Correlation was considered significant when the coefficient of correlation was higher than 0.6 and the *P* value was less than .01. Genera with relative abundance differed markedly between the D and C groups. *C*, Genera with relative abundance differed markedly between the D and T groups. C, control; D, disease; PC, principal component; T, treated.

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abundance in the disease state but decreased in the healing and post-treated states, which suggested that these genera were likely associated with the onset or persistence of DS. Of these, *Fusobacterium* and *Prevotella* species showed the highest relative abundance in the D group at 16 and 24%, respectively, compared with 11 and 19% in the C group. The synergic relation between them promoted the formation of plaque biofilm, which is a prerequisite for further attachment of other disease-related bacteria.[19-21] Especially

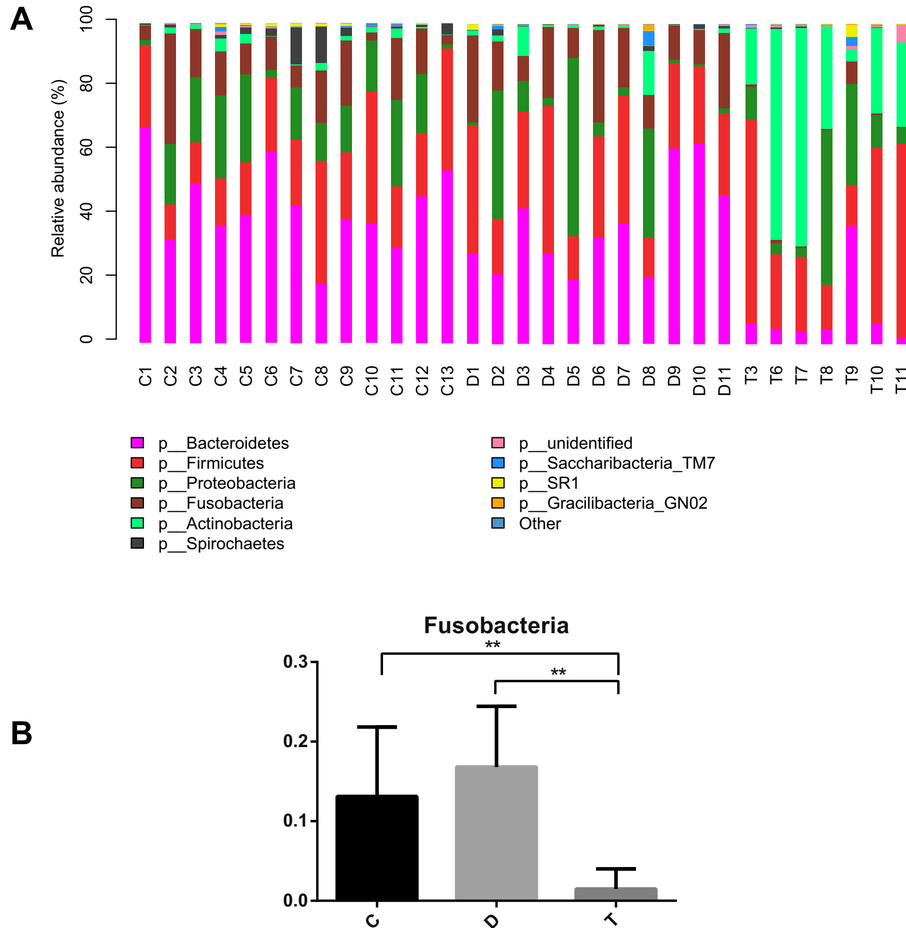


**FIGURE 3.** Relative abundance of *A*, bacterial phyla and *C*, genera at various sampling sites. *B*, Relative abundance of *Fusobacteria* species. **(Fig 3 continued on next page.)**

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*Fusobacterium* species have been reported to facilitate activation of fibrinolytic activity.[10] Mean relative abundance of *Peptostreptococcus* species was 4% in the D group, which was almost 4-fold higher compared with the C group. *Peptostreptococcus* and *Prevotella* species have been cultured from blood samples of extraction sockets, suggesting that these bacteria might colonize the socket wall and blood clot at an early stage.[22] Furthermore, because of their synergistic effect, *Peptostreptococcus* and *Prevotella* species have been identified as major pathogens in pulpitis with high abundance.[23] Moreover, *Fusobacterium*, *Prevotella*, and *Peptostreptococcus* species have been detected in pseudo-pockets of mandibular third molars,[24] which is consistent with the conclusion that DS is prone to occur in third molars with pericoronitis.[2,11] In the present study, *Parvimonas* species had a mean relative abundance of 7% in the D group compared with 1% in the C group and has been widely found in pulpitis, periapical periodontitis, and periodontitis.[25-27] It also can adhere to *Fusobacterium* species, promoting the release of proinflammatory cytokines and chemokines by macrophages[27] and has been positively correlated with *Prevotella* growth.[28] Van Dalen et al[29] found that *Parvimonas* species cocultured with *Prevotella* species mutually promote reproduction and pathogenicity. Grenier and Bouclin[30] found that *Parvimonas* species could activate plasmin and proteolytic capacity, which promotes the spread of bacteria to adjacent periodontal tissues and blood vessels.

Treatment of DS could be divided into non-dressing and dressing interventions. The application of antibacterial dressing to the extraction socket is widely recommended.[31] The present study showed that iodoform dressing substantially influenced the structure of the microbial community and predominant bacteria. The relative abundance of *Actinomyces* and *Streptococcus* species in the T group was considerably higher than that in the D and C groups. *Streptococcus*

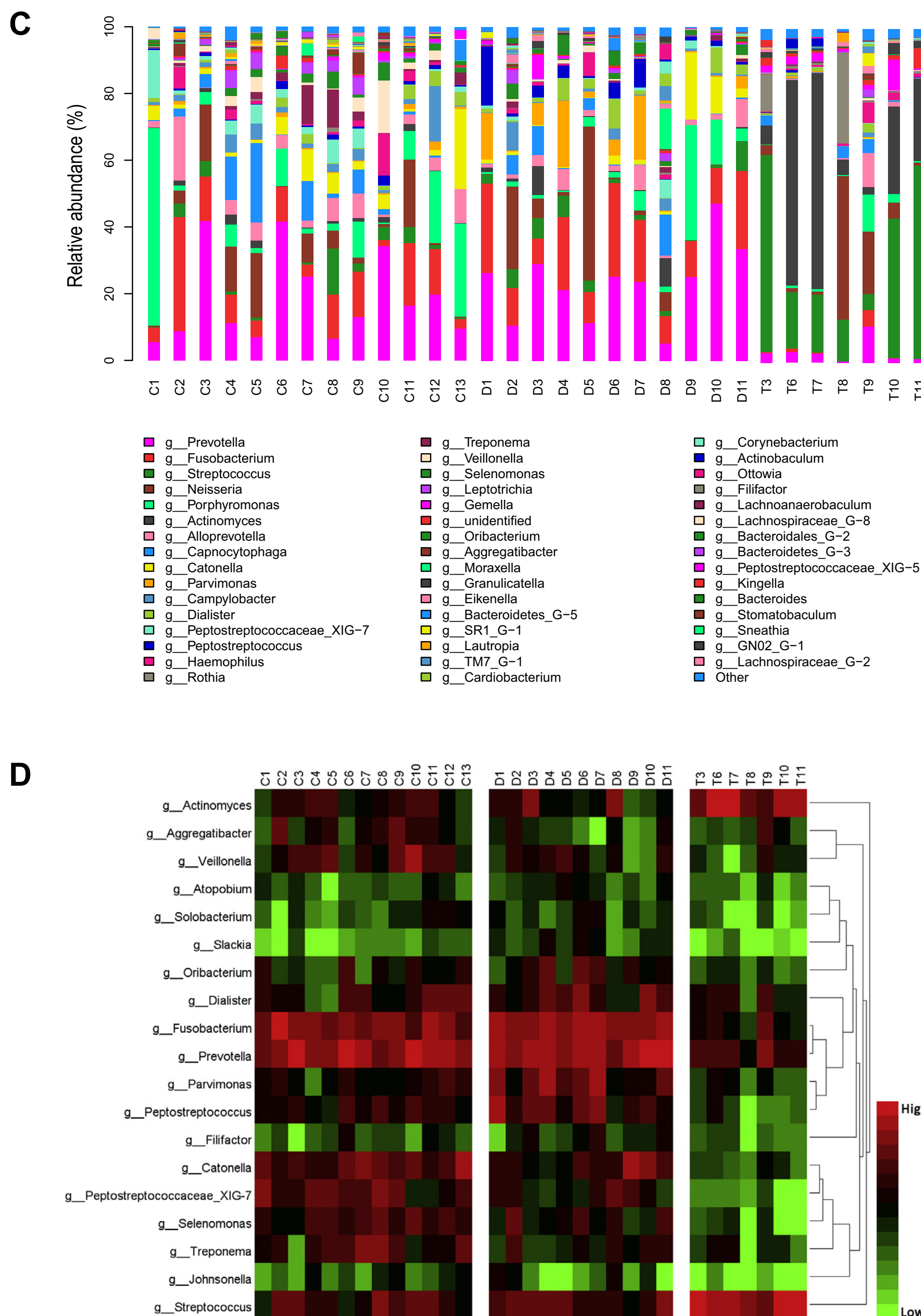


**FIGURE 3 (cont'd).** $^{**}P < .01$. *D*, Heatmap of genus level. Colors reflect the abundance of genera from high (*red*) to low (*green*). C, control; D, disease; T, treated.

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species has been proved to be capable of inhibiting the growth of *Fusobacterium* and *Prevotella* species in periodontal disease.[20] Furthermore, the antagonistic relation between *Actinomyces* and *Fusobacterium* has been reported.[32,33] The decreasing relative abundance of *Parvimonas* and *Peptostreptococcus* species could be attributed to the lack of *Fusobacterium* and *Prevotella* species.

Briefly, *Parvimonas*, *Peptostreptococcus*, *Fusobacterium*, and *Prevotella* species are considered the key population that could have crucial roles in the pathogenesis or maintenance of DS for their ability to form stable plaques, promote mutual growth, and activate fibrinolytic activity that increases pathogenicity, which to some extent is consist with the hypothesis of Birn.[5-7] *Actinomyces* and *Streptococcus* species suppress disease-associated bacteria in a nondominant state. Currently, more and more studies are prone to argue that disease occurrence is associated with a consortium of microbial species rather

than with 1 specific bacterium. In the present study, the proportion of bacteria that followed the disease pattern changed considerably, which could be the key link in the occurrence or maintenance of DS. The authors suggest that when the socket is appropriately treated, the disease pattern cannot maintain the specific proportion and pathogenicity without the support of the key population bacteria. Nevertheless, further studies are necessary to investigate and confirm how these bacteria contribute to DS.

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