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Supragingival Plaque Microbial Community Analysis of Children with Halitosis S

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As one of the most complex human-associated microbial habitats, the oral cavity harbors hundreds of bacteria. Halitosis is a prevalent oral condition that is typically caused by bacteria. The aim of this study was to analyze the microbial communities and predict functional profiles in supragingival plaque from healthy individuals and those with halitosis. Ten preschool children were enrolled in this study; five with halitosis and five without. Supragingival plaque was isolated from each participant and 16S rRNA gene pyrosequencing was used to identify the microbes present. Samples were primarily composed of Actinobacteria, Bacteroidetes, Proteobacteria, Firmicutes, Fusobacteria, and Candidate phylum TM7. The α and β diversity indices did not differ between healthy and halitosis subjects. Fifteen operational taxonomic units (OTUs) were identified with significantly different relative abundances between healthy and halitosis plaques, and included the phylotypes of *Prevotella* sp., *Leptotrichia* sp., *Actinomyces* sp., *Porphyromonas* sp., *Selenomonas* sp., *Selenomonas noxia*, and *Capnocytophaga ochracea*. We suggest that these OTUs are candidate halitosis-associated pathogens. Functional profiles were predicted using PICRUSt, and nine level-3 KEGG Orthology groups were significantly different. Hub modules of co-occurrence networks implied that microbes in halitosis dental plaque were more highly conserved than microbes of healthy individuals' plaque. Collectively, our data provide a background for the oral microbiota associated with halitosis from supragingival plaque, and help explain the etiology of halitosis.

Keywords: Halitosis, 16S rRNA gene pyrosequencing, PICRUSt, microbiome

Introduction

The oral cavity is a habitat to more than 600 bacterial species [9] and the oral microbial community plays a vital role in oral homeostasis. Many oral diseases, including dental caries and periodontitis, result from the disturbance of such communities [20]. As one of the primary complaints during dental visits, halitosis is associated with microbial activities [4]. Halitosis, which is also called oral malodor, is foul-smelling breath, exhaled from the oral cavity [17]. Affecting about one third of the worldwide population, halitosis is a great concern for the general public, causing personal and social discomforts [14]. Approximately 90% of halitosis cases are associated with oral conditions; others are caused by systemic diseases, including gastrointestinal disorders, hepatic diseases, and diabetes [4].

Volatile sulfur compounds (VSCs) are the major by-products of bacterial metabolic degradation, and are responsible for the foul smell associated with halitosis. The main components of VSCs include hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and dimethyl sulfide ($(CH_3)_2S$) [14]. Previous studies identified VSC-producing bacteria, of which *Porphyromonas*, *Prevotella*, *Actinobacillus*, and *Fusobacterium* species are the most common [18, 23].

16S rRNA gene pyrosequencing provides an overview of microbial communities as a whole, overcoming the limitations

of culture-based methods [15]. A recent computational approach called PICRUSt (Pylogenetic Investigation of Communities by Reconstruction of Unobserved States) allowed the prediction of functional composition using marker genes such as the 16S rRNA gene [19]. In addition, the majority of halitosis studies have focused on the tongue coating or saliva [11, 16, 24, 29, 30, 35, 38], and little is known about the effects of dental plaque. Preschool children rarely have periodontal diseases, which are confounding factors for halitosis; therefore, such children are good candidates for studying halitosis [24]. The objectives of this study were (i) to compare the microbial diversity and composition in healthy and halitosis samples, (ii) to identify candidate microbes associated with halitosis, and (iii) to predict functional profiles of healthy and halitosis microbiota.

Materials and Methods

Enrollment and Sample Collection

This project was approved by the Ethics Committee at Peking University School and Hospital of Stomatology (PKUSSIRB-2012062). The guardians of all participants in this study provided informed written consent. None of the participants caught cold or took antibiotics in 3 months prior to the study. They did not have systemic diseases, including gastrointestinal disorders, hepatic diseases, and diabetes. Halitosis was assessed via OralChroma (CHM-1, ABILIT Corporation, Japan). The reference values for each compound were 1.50 ng 10 ml^{-1}, 0.50 ng 10 ml^{-1}, and 0.20 ng 10 ml^{-1}, respectively [26]. A participant was considered as a halitosis subject if the concentrations of one or more of the three gases were above the reference values. Ten children, five with halitosis and the other five without halitosis, were recruited in this study. Supragingival plaque was collected from the enamel surface of each tooth and placed in a 1.5 ml Eppendorf tube containing 1 ml of TE (50 mM Tris-HCl, 1 mM EDTA; pH 7.6). All samples were immediately frozen at −20°C and stored at −80°C until use.

DNA Isolation and 16S rRNA Gene Sequencing

DNA was isolated using the TIANamp Bacteria DNA Kit (Tiangen Biotech, China), according to the manufacturer's instructions. DNA purity was determined using the NanoDrop 8000 Spectrophotometer (Thermo, USA). Amplicon libraries for the hypervariable V1−V3 regions of the 16S rRNA gene were generated using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-TTACCGCGGCTGCTGGCAC-3′). PCR was performed according to the GS FLX Amplicon DNA library preparation manual (Roche, Germany). The PCR cycling conditions were initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 45 sec, and extension at 72°C for 1 min; and a final extension at 72°C for 2 min. PCR amplicons were sequenced using the 454 GS FLX Titanium system (454 Life Sciences, USA) at the BGI Institute (China).

Data Processing and Statistical Analysis

The raw data for 10 samples were analyzed using the QIIME pipeline [10]. Sequences were de-multiplexed based on a unique barcode assigned to each sample. When the average quality score over a 50 bp sliding window dropped below 30, sequences were trimmed. A maximum of one barcode mismatch and two primer mismatches were allowed. Sequences >200 bp in length were retained after trimming [37]. Trimmed reads were clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff under the de novo OTU selection strategy. Taxonomies were assigned by the RDP classifier (ver. 1.27), with a confidence threshold of 0.8.

The α and β diversity indices were calculated using QIIME. The functional profiles of microbial communities were predicted via PICRUSt according to the pipeline at http://picrust.github.io/picrust/. We used the Nearest Sequenced Taxon Index (NSTI) to quantify the prediction accuracy [19]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) group descriptions were used as the basis for functional predictions [22]. The Wilcoxon rank-sum test was used to compare the α diversity, the relative abundances of OTUs. ANOSIM (analysis of similarity), Adonis (non-parametric multivariate analysis of variance), and MRPP (multi-response permutation procedure) were used to compare β diversity indices [37]. The linear discriminant analysis effect size (LEfSe) method was used to compare the abundances of functional profiles [28]. Pearson's correlation coefficients (PCC) were calculated between the OTUs in healthy (PH) and halitosis plaque (PD) samples. The significance of the PCC values was calculated using permutation tests. All statistical tests were performed using R software (ver. 3.2.0) with a p-value < 0.05 considered significant. Co-occurrence networks were generated and analyzed by Cytoscape software (ver. 3.2.1).

Sequences from this study were submitted to the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under Accession No. SRX831098.

Results

Basic Information of Sequencing Data and Microbial Diversity

Study participants' basic information is shown in Table 1. Pyrosequencing was used to analyze each participant. Participant gender, age, decayed-missing-filled tooth, gingival index, and debris index-simplified were not significantly different between PH (healthy) and PD (halitosis) samples. After processing by QIIME, a total of 85,291 clean reads were generated, ranging from 5,298 to 12,356 per sample (Table S1). Rarefaction curves revealed that sequencing coverage was adequate at the chosen depth (Fig. 1). In total,

Table 1. Basic characteristics of studied subjects.

ID	Group	Gender (M/F)	Age (year)	H_2S (ng 10 ml^{-1})	CH_3SH (ng 10 ml^{-1})	$(CH_3)_2S$ (ng 10 ml^{-1})	DMFT	GI	DI-S
P01	PH	F	4	0	0	0	0	1	9
P02	PH	M	4	0	0	0	3	1	12
P03	PH	M	4	0	0	0	2	1	10
P04	PH	F	4	0.02	0	0	0	1	8
P05	PH	M	4	0.05	0	0	0	1	16
P06	PD	F	4	1.58	0.49	1.54	0	1	12
P07	PD	M	4	5.97	0.56	0.31	4	1	6
P08	PD	F	4	6.03	0.11	0	0	1	6
P09	PD	F	5	4.34	0	0	1	1	10
P10	PD	M	4	0.52	0	1.05	8	1	10

PH: healthy plaque samples; PD: halitosis plaque samples; DMFT: decayed-missing-filled tooth; GI: ginginval index; DI-S: debris index-simplified.

14 phyla, 22 classes, 30 orders, 52 families, and 79 genera were assigned. Actinobacteria, Bacteroidetes, Proteobacteria, Firmicutes, Fusobacteria, and Candidate phylum TM7 were the most abundant phyla, accounting for 99.16% of all bacteria (Fig. 2). The α diversity indices, including the observed OTUs, Chao1, Simpson Evenness, and Simpson, were analyzed; however, no significant differences were observed between the PH and PD samples (Table S1). Similarly, no differences were observed between β diversity indices, as determined by weighted and unweighted UniFrac distances (Table S2).

Differences in Bacterial Communities between PH and PD

To investigate whether specific bacterial species were associated with halitosis, the relative abundances of OTUs between PH and PD samples were compared. A total of 15 OTUs had significantly different relative abundances, with 14 being increased and 1 being decreased in PD samples (Fig. 3A). The 15 OTUs included the phylotypes of *Prevotella*, *Leptotrichia*, *Actinomyces*, *Porphyromonas*, *Selenomonas*, *Selenomonas noxia*, and *Capnocytophaga ochracea*. To further visualize the variation between PH and PD samples, a heatmap was generated with the relative abundance of the 15 OTUs (Fig. 3B). With the exception of sample P09, all samples clustered well by Manhattan distance.

We next used PICRUSt to explore the functional profiles of the 30 samples. A total of 642 closed-reference OTUs were picked, which were then normalized by 16S rRNA copy number. The NSTI of our samples was 0.10 ± 0.05 (mean ± SD). In comparison, between the PH and PD groups, nine level-3 KO groups were significantly different based on the LEfSe method (Fig. 4). Five KOs were enriched

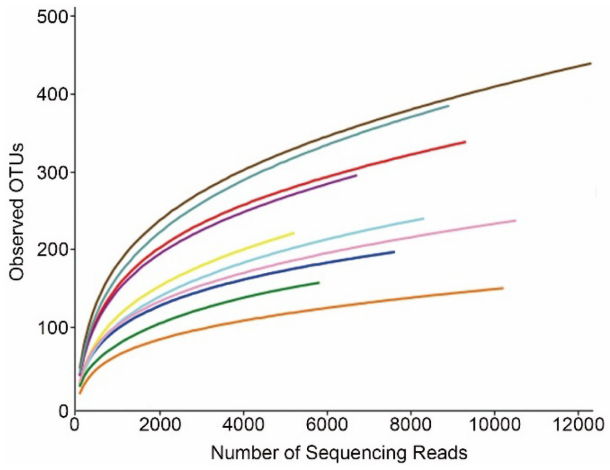

Fig. 1. Rarefaction curves for each sample.
A total of 5,200 sequences were randomly subsampled from each data set with 1,000 permutations.

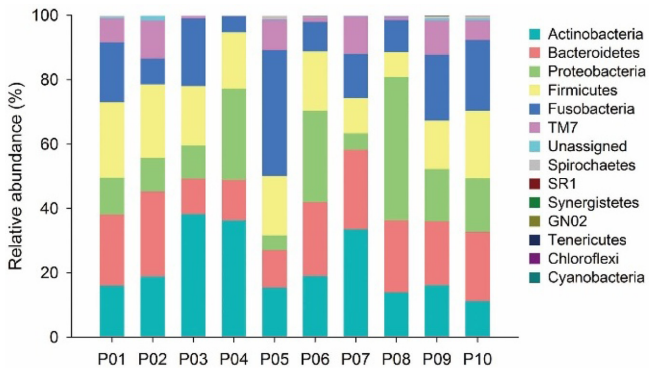

Fig. 2. Microbial composition at the phylum level.
The bar represents the relative abundance of a given phylum. The X-axis represents sample ID.

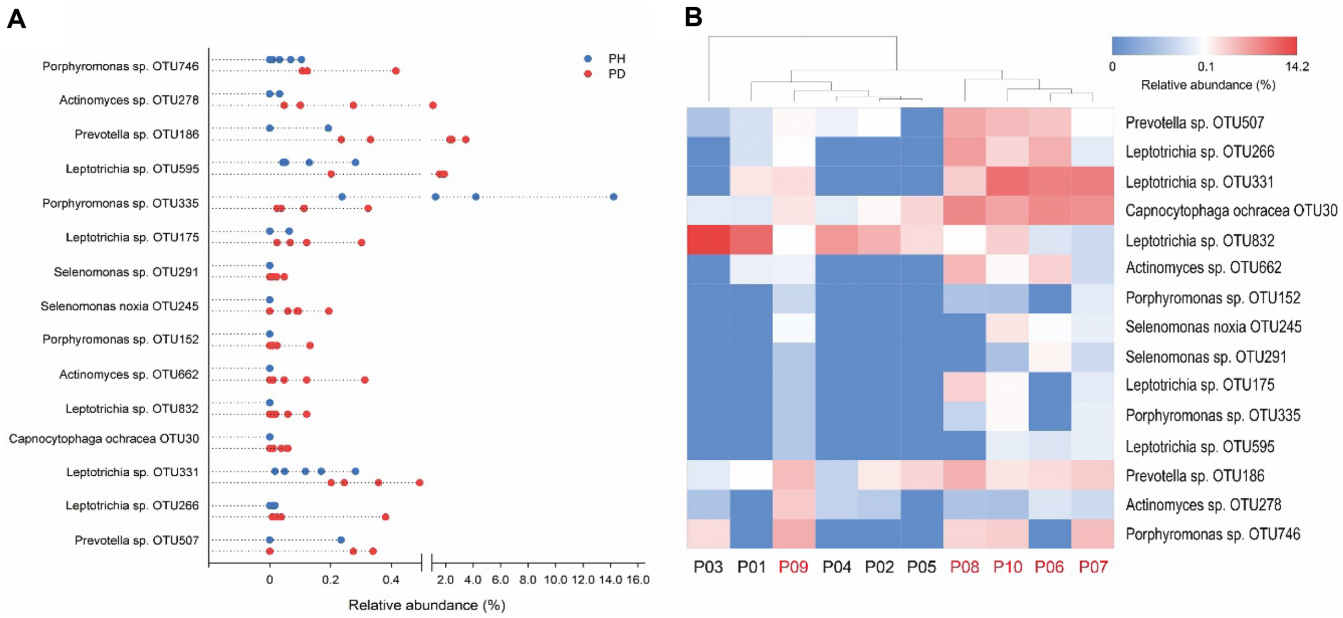

Fig. 3. Operational taxonomic units (OTUs) with different relative abundances between healthy (PH) and halitosis plaque (PD) samples.

(**A**) Each dot represents one sample. (**B**) The heatmap was constructed based on the OTUs in panel A using the Manhattan distance. P01–P05 are from the PH group, and P06–P10 are from the PD group.

in PH, whereas four KOs were enriched in PD. However, no differential level-1 or level-2 KOs were identified.

Co-occurrence Network Analysis

By calculating the Pearson's correlation coefficients between all OTUs in the PH and PD groups, two co-occurrence networks were constructed. To understand network interactions, OTUs (nodes) with the most numerous linkages (edges) were retained to make up the hub modules (Fig. 5). The PH hub module consisted of 45 OTUs with 250 correlation pairs (147 positive and 103 negative), whereas the PD hub module consisted of 24 OTUs with 177 correlation pairs (82 positive and 95 negative).

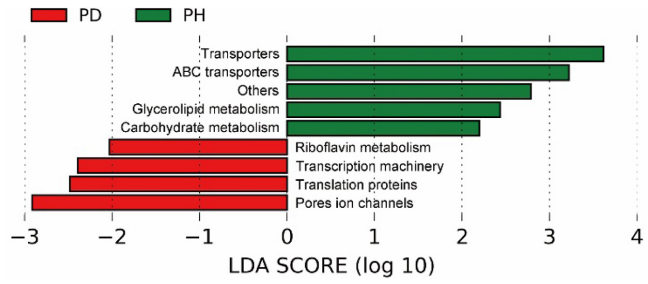

Fig. 4. Functional profiles with different relative abundances between healthy (PH) and halitosis plaque (PD) samples based on LEfSe results.

Bars represent linear discriminant analysis (LDA) scores.

Discussion

The oral cavity harbors hundreds of species, approximately 35% of which cannot be cultivated [6]. High-throughput sequencing of the 16S rRNA gene provides more comprehensive information than traditional culture-dependent approaches and has become an efficient way for identifying bacteria in the oral microbiome [2, 3, 36, 39]. In the present study, α and β diversity indices indicated that the microbial structure in healthy and halitosis plaques were similar (Tables S1 and S2).

The characteristics of differential OTUs are shown in Fig. 3 and Table 2. *Prevotella* spp. are associated with periodontal diseases and are the predominant H_2S-producing bacteria [5, 27, 31]. The relative abundance of *Prevotella* was also positively correlated with H_2S concentration in halitosis subjects [38]; however, Takeshita *et al.* [29] reported that the proportion of *Prevotella* was correlated with the level of CH_3SH, but not H_2S. *Porphyromonas* and *Selenomonas* can produce CH_3SH, which contributes to halitosis [23, 27, 29]. *Leptotrichia* is a constituent of the oral flora, normally found in human dental plaque [1], and is an opportunistic pathogen.

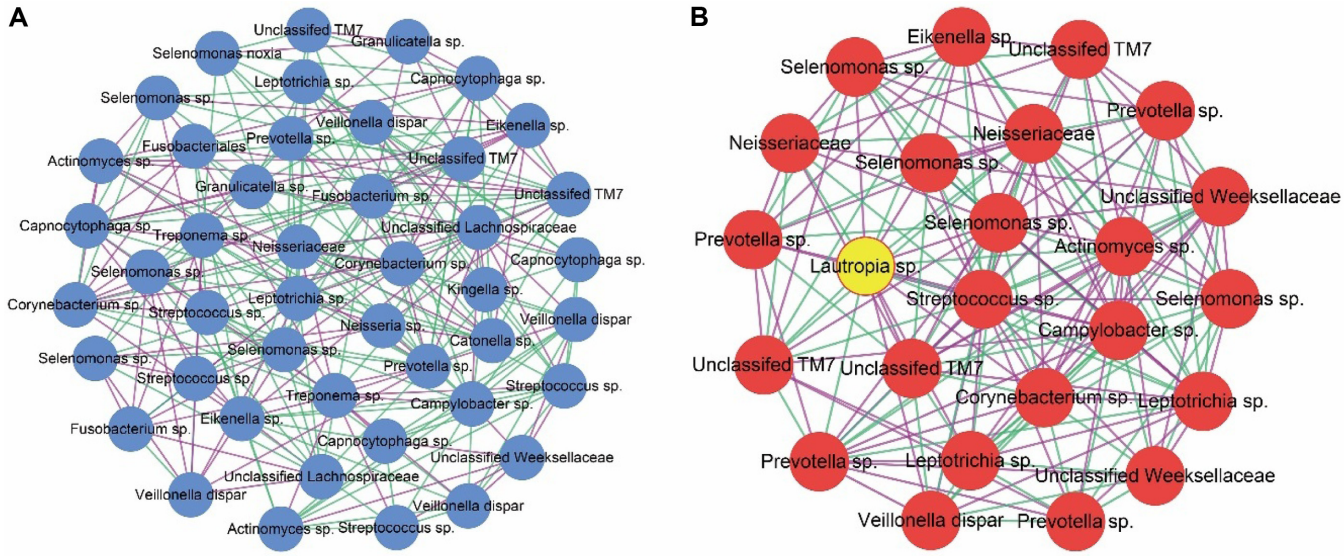

Fig. 5. Hub modules of healthy (PH) and halitosis plaque (PD) samples.
Each node represents one OTU (blue, PH nodes; red, PD nodes). Each linkage represents a correlation between two OTUs (green, positive correlation; pink, negative correlation). The OTUs in PD were also all present in the PH OTUs, except for one *Lautropia* sp. (yellow node, panel B).

Recent studies showed that *Leptotrichia* is associated with halitosis, particularly *L. wadei* [17, 29, 34, 38]. However, *L. wadei* cannot produce VSCs, and thus, the meaning of its association with halitosis remains unknown [13]. Most of the differential OTUs detected in this study could produce VSCs, and therefore, we recognized them as halitosis-associated species. Subsequently, we used PICRUSt to predict functional profiles. Although the NSTI of this study (0.10 ± 0.05) is comparable to those of other mammals (0.14 ± 0.06) [19], a large proportion of the OTUs were not matched to the database, and thus their functions were not imputed [40]. The differential pathways in Fig. 4 were not discussed in this study, and together with halitosis-associated species, need more validation experiments.

Dental plaque is a biofilm formed by the accumulation of bacteria in a timely manner together with the human salivary glycoproteins and polysaccharides secreted by microbes [21]. Meanwhile, microbes interact with each other, maintaining the ecosystem of dental plaque. From the PH hub module of the co-occurrence network, 45 OTUs interacted with 250 correlations, whereas the PD module had 24 OTUs and 177 correlations. OTUs in the PH module covered all of the OTUs in the PD module, except for *Lautropia* sp. (Fig. 5). These data implied that species in the PD module were dominant species and tended to form a more conserved ecosystem when halitosis occurred.

In summary, this study characterized the bacterial communities and predicted functional profiles in supragingival plaque from healthy preschool children and those with halitosis. However, owing to the limitations of the present study, investigations with larger sample sizes remain necessary.

Table 2. Characteristics of candidate halitosis-associated bacteria.

Bacterial phylotypes	Characteristics	References
Prevotella sp.	Produce VSCs, halitosis, periodontitis	[5, 27, 31]
Leptotrichia sp.	Halitosis, human infection	[12, 17]
Actinomyces sp.	Actinomycoses, abscess	[7]
Porphyromonas sp.	Produce VSCs, halitosis, periodontitis, and rheumatoid arthritis	[23, 25, 27]
Selenomonas sp.	Produce VSCs	[23]
Selenomonas noxia	Produce VSCs, periodontal pathogen	[32, 33]
Capnocytophaga ochracea	Periodontitis, sepsis	[8]

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