Microbial Basis of Oral Malodor Development in Humans

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ABSTRACT

To better understand the microbial basis of oral malodor development in humans, we used a cross-sectional and longitudinal study design and the pyrosequencing approach to track and compare the tongue microbiota associated with oral malodor in 29 Chinese adults who underwent a consecutive three-day evaluation for the amount of H_2S excreted orally. Three levels of the oral malodor state (healthy, oral malodor, and severe oral malodor) were defined based on the H_2S level. Community structure of the tongue plaques was more sensitive to changes of malodor state than to interpersonal variations or differences in sampling times. Within each individual, the structure of microbiota was relatively stable, while their variations were correlated with the change in the H_2S level. Severe oral malodor microbiota were the most conserved in community structure, whereas the healthy ones were relatively varied. Oral-malodor-associated bacteria were identified. The relative abundance of *Leptotrichia* and *Prevotella* was positively correlated with oral malodor severity, whereas *Hemophilus* and *Gemella* exhibited a negative relationship with oral malodor severity. Our study provides one of the first landscapes of oral microbiota changes associated with oral malodor development and reveals microbes potentially useful to the evaluation and control of oral malodor.

KEY WORDS: metagenome, tongue, high-throughput nucleotide sequencing, Prevotella, Leptotrichia, hydrogen sulfide.

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INTRODUCTION

Oral malodor is foul-smelling breath from the oral cavity in humans. It is a frequent health complaint that affects an estimated 30% to 50% of the general population (Liu *et al*., 2006). It can lead to embarrassment and compromise interpersonal social communication (Lee *et al.*, 2007). The ailment can be caused by oral diseases and systemic diseases (Scully and Greenman, 2012). However, most malodor originates from the hosts' tongue plaque alone and without any disease, defined as physiologic oral malodor (Yaegaki and Coil, 2000). The production of malodorants could be attributed to the tongue plaque that resides on the relatively large surface area of the tongue, with its papillary structure. Considerable amounts of bacteria inside the tongue plaque can cause amino acid and peptide by-products, as well as food debris, to putrefy, thus producing malodorants (Yaegaki and Sanada, 1992). Therefore, the tongue plaque microbiota may potentially serve as a proxy to help us understand and control physiologic oral malodor.

Unpleasant oral odor results from volatile sulfur compounds (VSC), which collectively include hydrogen sulfide, methyl mercaptan, other thiols, and dimethyl sulfide. Several studies have attempted to pinpoint VSC-producing bacteria through culture-based methods (Tyrrell *et al.*, 2003), in which oral bacteria such as *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythensis, Fusobacterium nucleatum*, and *Treponema denticola* were regarded as oral malodor pathogens, due to their potent production of VSCs *in vitro* (Persson *et al.*, 1990). However, the culture-dependency of such approaches has hindered the accurate identification of VSC-producing bacteria, due to the difficulties associated with cultivation and the inadequacy of microbial identification (Peterson *et al.*, 2011). Molecular approaches including 16S-clone-sequencing and T-RFLP have also been applied to the tongue microbiota; however, the clone-based sequencing depth of 12~29 reads *per* sample and the limited resolution of the T-RFLP technique have hindered the accurate profiling of the tongue microbiota during VSC production (Takeshita *et al*., 2010).

Pyrosequencing of 16S rRNA, which is independent of microbial cultivation, has allowed us and others to test the links of oral microbiota to oral diseases such as caries (Yang *et al*., 2012), periodontitis (Griffen *et al.*, 2012), and even systemic disease (Farrell *et al*., 2012). In a cross-sectional study, salivary microbiota from patients with extremely severe oral malodor were profiled for their organismal structure (Takeshita *et al.*, 2012). However,

bacterial coating on the dorsum of the tongue, not salivary bacteria, was generally recognized as the major VSC source (De Boever and Loesche, 1995). Thus, the microbial basis of oral malodor in the human population remains largely unknown. Furthermore, the intensity of oral malodor varies for the same host individuals at different times, whereas the absence of longitudinal studies to date has precluded investigations of how microbiota correlate with changes of oral malodor state, interpersonal variations, or differences in sampling times.

To probe the microbial basis of oral malodor development in humans, we used a combined cross-sectional and longitudinal study design to track and compare the tongue (dorsum) microbiota associated with oral malodor in 29 Chinese adults who underwent a consecutive three-day evaluation for the amount of H_2S excreted orally. Three levels of the oral malodor state (healthy, oral malodor, and severe oral malodor) were defined based on the H_2S value. The diversity and dynamics of bacterial communities residing in the dorsum of the tongue were analyzed by pyrosequencing of bacterial 16S rRNA genes.

MATERIALS & METHODS

A comprehensive description is provided in the online Appendix. All samples were collected at the Hai Tai He Chang Clinical Research Center in Beijing with approval from the Procter & Gamble Beijing Technical Center (China) Institutional Review Board and in accordance with the World Medical Association Declaration of Helsinki (1996 amendment). ICH Guidelines for Good Clinical Practice (GCPs) were followed, and voluntary informed consent was provided with the approval of the Research Ethics Board of P&G. This clinical investigation complied with STROBE guidelines for observational human studies. In total, 29 individuals with or without self-reported oral malodor were involved. Oral malodor was evaluated according to H_2S values measured *via* Portable Gas Analyzer Model 4170-1999b (Interscan, Chatsworth, CA, USA) on 3 consecutive days. After the H_2S measurement, tongue plaque was collected, and total DNA was extracted. PCR amplicon libraries of the small subunit ribosomal (16S) RNA gene V1-V3 hyper-variable region (*Escherichia coli* positions 5-534) were pyrosequenced according to our published protocols (Huang *et al*., 2011; Yang *et al.*, 2012).

Sequences were analyzed with MOTHUR (Schloss *et al*., 2009) for preprocessing, identification of operational taxonomic units (OTU), taxonomic assignment, and community-structure comparisons. Furthermore, we performed Principal Coordinates Analysis (PCoA) and Procruste analysis (Muegge *et al.*, 2011) to test the differences of tongue microbiota structure among the time points or among the multiple oral malodor levels. Unifrac distance and variation coefficient of the log2-transformed H_2S values were calculated to evaluate the degree of variation and then correlated with each other. Relative abundances of features in the taxonomic levels were correlated with H_2S value to calculate the Spearman correlation coefficient (r). Levels of confidence were denoted as: NS, not significant; $*.01 < p < .05$; $**p < .01$; $***p < .001$. All sequences were deposited at Sequence Read Archive under Accession ID SRA079871.

RESULTS

The 29 human participants sampled consisted of four men and 25 women ranging in age from 19 to 47 yrs. The level of H_2S was measured by gas analyzer once *per* day for 3 consecutive days. The mean value of H_2S was 156 ppb, ranging from 23 to 910 ppb (Appendix Table 1). The CV (coefficient of variance) of the log2-transformed H_2S value among individuals was from 0.01 to 0.15, with a mean of 0.07 (Appendix Table 1). No significant difference was found between the H_2S values for gender and age. According to the H_2S values, the oral malodor states of the participants were classified into 3 levels: Level 1 for healthy (below 100), Level 2 for oral malodor (above 100 and below 200), and Level 3 for severe oral malodor (above 200) (Appendix Table 2).

Tongue samples from each individual were collected after H_2S measurement and analyzed for microbial community structure. In total, 87 tongue plaque samples were collected for barcoded 16S rDNA amplicon sequencing, yielding a total of 492,776 processed reads (*i.e.*, reads after quality assessment and control measures (Schloss *et al.*, 2011)). The number of processed reads *per* sample ranged from 2,400 to 13,978, with an average 5,664 reads *per* sample (Appendix Table 2).

Richness and Biodiversity of Tongue Microbiota

Clustering the unique sequences into OTUs at a 3% genetic distance resulted in 482~1,211 different "species-level" taxa *per* microbiota (Appendix Table 2). The average level of Good's coverage (Hill *et al.* 2003) was over 94.1% in all samples, indicating that about 6 new phylotypes are expected for every 100 additional reads. Thus, the 16S rDNA sequences identified in the current study were able to represent the majority of bacterial members in these tongue samples.

For each of the plaque microbial communities analyzed, the microbial richness estimated by the Chao I and ACE indices (Hill *et al.* 2003) and the biodiversity assessed by Shannon index (Hill *et al.* 2003) did not show significant differences among the 3 oral malodor levels or among the different time points ($p > .05$). Rarefaction curve analysis of detected OTUs also exhibited similar richness among different groups (Appendix Figs. 1A, 1B).

Temporal Stability of Tongue Microbiota

To test whether the tongue microbiota were relatively stable, we applied multivariate analysis to compare the overall structure of microbiota from each individual based on weighted Unifrac and Braycurtis distance matrices. In weighted Unifrac-based PCoA analysis, no significant difference was found on tongue microbiota sampled at different time points (Fig. 1A). This result was also supported by PCoA based on Braycurtis distance (Appendix Fig. 2A). At all the time points sampled, the degree of the temporal variation of the tongue bacterial composition was evaluated *via* Procrustes analysis. Our results demonstrated the significant similarity of microbiotic structures within individuals among 3 time points: Day 1 to Day 2 ($p < .001$, observed

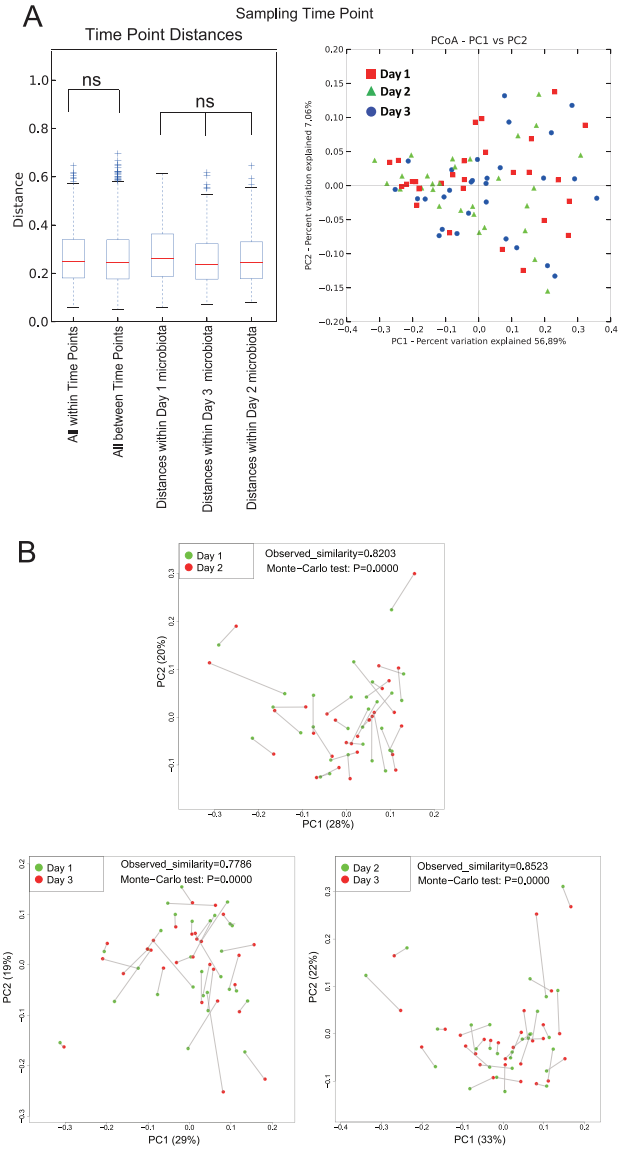

Figure 1. Microbial community analysis of tongue microbiota sampled continuously over 3 days. **(A)** Community structures were interrogated by principal coordinate analysis (PCoA) based on the FastUnifrac distance matrix. No significant difference was found in tongue microbiota from 3 time points. NS, not significant; *.01 < p < .05; **p < .01; ***p < .001. **(B)** Procrustes analysis of tongue microbiota from the different time points based on the distance matrix of species-level taxonomy results. Each point represented a tongue microbiota and was colored according to the time points sampled, with samples of each individual connected by a line. The fit of each Procrustes transformation over the first 4 dimensions was reported as the p value by 10,000 Monte Carlo label permutations.

similarity: .820), Day 2 to Day 3 (p < .05, observed similarity: .78), and Day 1 to Day 3 (p < .01, observed similarity: .85) (Fig. 1B). Thus, the tongue microbiota within a given individual was relatively stable.

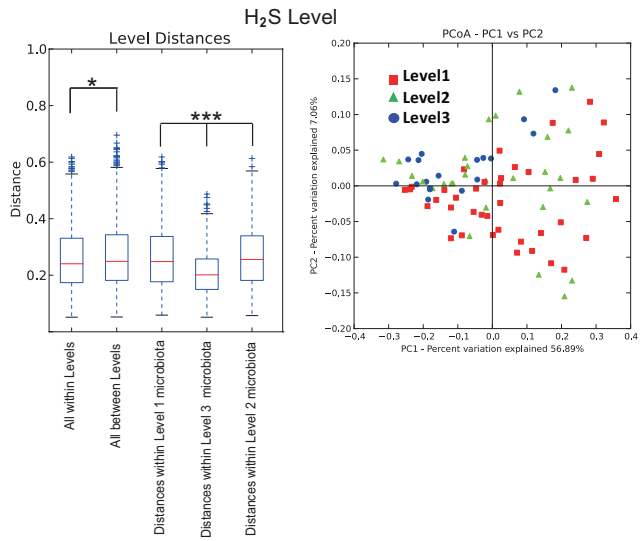

Figure 2. Microbial community analysis of tongue microbiota grouped by oral malodor severity. Community structures were interrogated by principal coordinate analysis (PCoA) based on the FastUnifrac distance matrix. Segregation of tongue microbiota from those 3 levels of oral malodor was observed. Severe oral malodor microbiomes were the most conserved, whereas the healthy ones were relatively varied. NS, not significant; *.01 < p < .05; **p < .01; ***p < .001.

Tongue Microbiota Associated with H_2S Levels

Tongue microbiota were grouped based on the level of H_2S. The weighted Unifrac-based distance analysis revealed significant differences among the 3 levels of oral malodor (Fig. 2). Severe oral malodor microbiota were the most conserved, whereas the healthy ones were relatively varied (Fig. 2). This result was also supported by the Braycurtis-based distance metrics (Appendix Fig. 2B). Thus, the effect of the oral malodor state is more prominent than that of inter personal variation in shaping the tongue microbiota. In addition, within-individual variation of tongue plaque community structure based on Unifrac distance (means of Unifrac distance within individuals) was significantly correlated with the variation of H_2S value (CV of log2-transformed H_2S value within individuals) (p < .05, Rho = 0.417), suggesting a significant bacterial contribution or link to the oral malodor state. Thus, community structure of tongue plaques was more sensitive to the changes of halitosis state than to inter personal variations or differences in sampling times.

Taxonomy/OTU-based Characterization of Tongue Microbiota

Bacterial taxa on the various taxonomic levels and OTU with 97% identity level were identified and quantified through taxonomic assignment against reference databases using MOTHUR, which revealed their relative abundance in each of the tongue microbiota (Appendix Figs. 3, 4). Over 300 (341) bacterial species were found inhabiting the tongue plaque. All sequences were found distributed in 12 bacterial phyla that included 6

predominant phyla (accounting for > 98% of the bacteria diversity) that were commonly encountered in the oral cavity: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and TM7 (Griffen *et al*., 2011) (Appendix Fig. 3). At the genus level, 101 genera were identified in the tongue microbiota. The top 20 taxa at genus and species levels were shown based on the different sampling days, and, statistically, no taxa were found differentially distributed among the 3 time points (Figs. 3A, 3B). The most frequently detected genera (the 5 most abundant genera that each represented at least 5% in average relative abundance) were *Prevotella* (~31.7%), *Streptococcus* (~23.4%), *Neisseria* (~11.5%), *Actinomyces* (6.7%), and *Rothia* (~5.6%), which together comprise approximately 80% of tongue microbiota.

Microbial Taxa Correlated with H_2S Value

To test the presence of oral-malodor-associated microbial taxa, relative abundance of phylotype/OTU in the tongue microbiota was correlated with the corresponding H_2S value. At the genus level, 4 bacterial genera (each with average relative abundance >1% at at least one time-point) were significantly correlated ($p < .05$, Rho > 0.3; False Discovery Rate [FDR] $q < 0.2$). Among them, *Prevotella* ($r = 0.31$) and *Leptotrichia* ($r = 0.32$) showed positive correlation, while *Hemophilus* ($r = -0.56$) and *Gemella* ($r = -0.31$) showed negative correlation (Fig. 4A). At the species level (each with average relative abundance > 0.5% at at least one time-point, $p < .05$, Rho > 0.4; FDR $q < 0.2$), 4 taxa, including *Prevotella tannerae* ($r = 0.47$), *Hemophilus_parainfluenzae* ($r = -0.57$), *Leptotrichia FP036* ($r = 0.44$), and *Leptotrichia wadei* ($r = 0.43$), were identified (Fig. 4B). At the 97% identity OTU level (each with average relative abundance > 0.2% at least one time point, $p < .05$, Rho > 0.5; FDR q < 0.2), 4 OUTs, including *Streptococcus unclassified* ($r = -0.5$), *Actinomyces odontolyticus_lingnae* ($r = 0.51$), *Streptococcus unclassified* ($r = -0.62$), *Prevotella tannerae* ($r = 0.52$), *Streptococcus unclassified* (r = -0.52), and *Hemophilus unclassified* ($r = -0.5$), were identified (Fig. 4C).

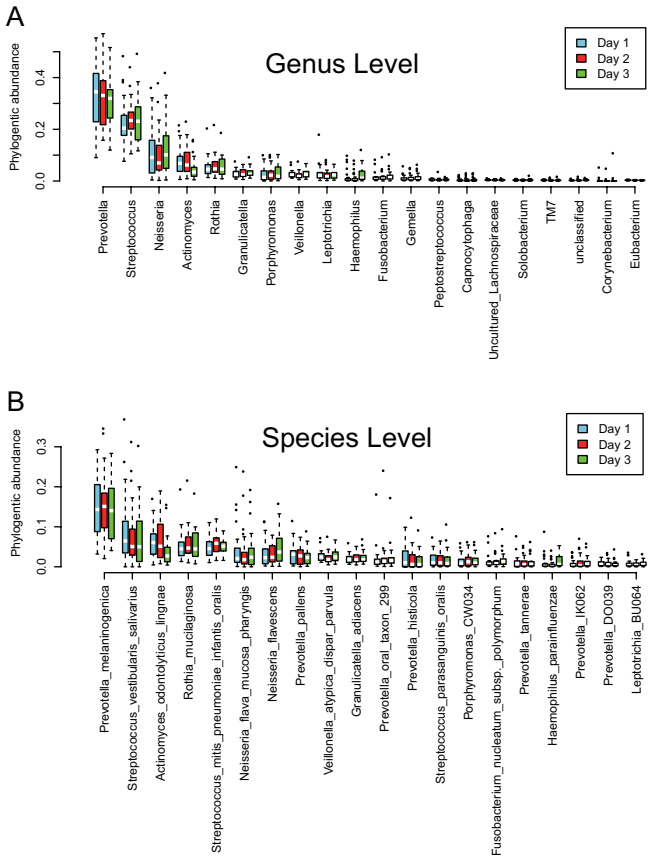

Figure 3. Comparisons of tongue microbiota between and among different sampling points. **(A)** Comparisons performed at genus level (top 20 predominant genera shown). **(B)** Comparisons performed at the species level (top 20 predominant species shown). Means of the relative abundance for each taxon at each taxonomical level were compared, and no taxa were found differentially distributed among the different time points.

DISCUSSION

This study used a combined cross-sectional and longitudinal study design to compare the tongue microbiota associated with oral malodor. Microbial community structure-based analyses suggested that severe oral malodor microbiota were the most conserved. Thus, healthy samples were more like a native forest that can be inhabited by diverse microbial members, whereas plaques from severe oral malodor were more like a fermenter that selects for only those tolerating the extreme environment. Temporal analysis showed that tongue microbiota within individuals were relatively stable among the different sampling times. Moreover, within host individuals, the variation in microbiotic structure was correlated with the change in the H_2S value. Therefore, tongue plaque may potentially serve as a proxy of the oral malodor states in human populations.

Our study revealed 4 bacterial taxa that were associated with the H_2S: the positively linked *Prevotella* and *Leptotrichia* and the negatively related *Hemophilus* and *Gemella*. Both links and distinctions were identified between these bacterial taxa and those oral-malodor-associated bacteria from other studies. Previous studies evaluating oral bacterial production of H_2S or VSCs reported that *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Treponema denticola, Solobacterium moorei*, and *Veillonella alcalescens* were correlated with oral malodor (Stassinakis *et al*., 2002; Kazor *et al.*, 2003; Tyrrell *et al.*, 2003; Tanaka *et al*., 2004; Washio *et al*., 2005; Haraszthy *et al.*, 2007; Takeshita *et al*., 2010). However, the findings were mostly based on tracking a few specific bacteria (Tanaka *et al*., 2004) or were dependent on bacterial culture (Tyrrell *et al*., 2003; Haraszthy *et al.*, 2007). In addition, those studies that adopted non-culture-based methods used Sanger sequencing of a small number of 16S rRNA gene clones (Kazor *et al*., 2003; Washio *et al*., 2005; Haraszthy *et al.*, 2007) or T-RFLP (Takeshita *et al.*, 2010), which provided limited resolution of the complex oral microbiota. Such differences in methodology could have contributed to the distinctions in the

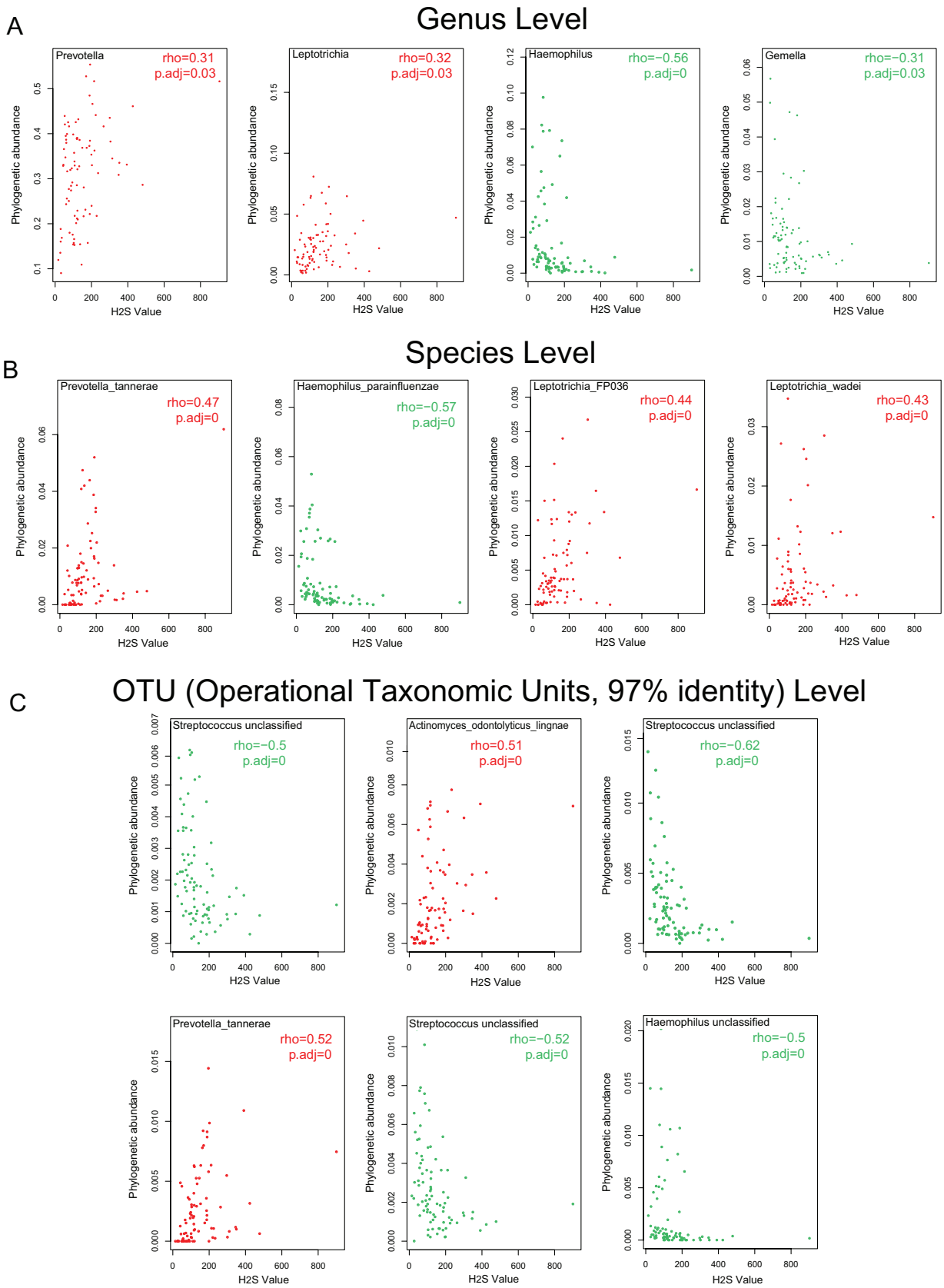

Figure 4. Bacterial taxa that were correlated with the H_2S value. **(A)** Genus-level taxa. **(B)** Species-level taxa. **(C)** OTUs.

identified oral-malodor-associated bacteria among the various studies.

Employing barcoded-pyrosequencing of salivary microbiota in oral malodor, Takeshita *et al*. identified a series of H_2S-producing bacteria such as *Neisseria, Fusobacterium, Porphyromonas*, and SR1 (Takeshita *et al.*, 2012), which were distinct from those found in our study. Potential reasons underlying the inconsistency can be numerous, *e.g.*, differences in the populations surveyed and distinctions in the oral niches sampled. In addition, the inclusion criteria for the individuals with oral malodor were different. Samples analyzed in Takeshita's study were associated with extreme oral malodor (*i.e.*, $H_2S > 1{,}000$ ppb as an inclusion criterion). Such patients represented only 10% of the general population (Takeshita *et al.*, 2012); however, in our study the highest individual H_2S level was 910 ppb, which allowed us to address a larger portion of the general population.

Prevotella are well-acknowledged periodontal pathogens and contribute to individual malodor (Ademovski *et al.*, 2013). *Prevotella intermedia* have been reported to generate significant amounts of CH_3SH and H_2S derived from L-methionine and L-cysteine, respectively (Persson *et al*., 1990). *Prevotella intermedia* and *Prevotella nigrescens* were also associated with oral hydrogen sulfide (Tanaka *et al.*, 2004). *Leptotrichia* spp. are part of the normal oral and intestinal human flora. Despite the absence of evidence for production of H_2S by *Leptotrichia in vitro*, one recent study suggested higher abundance of *Leptotrichia* in persons with malodor than in healthy control individuals (Takeshita *et al*., 2010). In our study, both *Prevotella* and *Leptotrichia* were found to be strongly associated with oral malodor.

Interestingly, periodontal-disease-associated bacteria such as *Porphyromonas gingivalis* or *Fusobacterium nucleatum* were found producing VSCs (Loesche and Kazor, 2002), but they were not correlated with H_2S concentration in our study. One potential reason for these observations is that these bacteria might contribute to the production of other, non-H_2S components within VSC.

Both *Hemophilus* and *Gemella* are negatively associated with H_2S concentration in this study *Hemophilus* are aerobic or facultatively anaerobic opportunistic pathogens, and, in our former study, they were found to be associated with health (unpublished observations). *Gemella* species are small Gram-positive cocci and are primarily regarded as normal human flora found in the mucous membranes of humans (Hung *et al*., 2012). This, to our knowledge, is the first report of a negative relationship of *Hemophilus* and *Gemella* with oral malodor *in vivo*, although no *in vitro* experiments have ever proved their capability to restrain H_2S production. The process of H_2S production can be very complex and involves many directly or indirectly contributing bacterial taxa. Some may directly participate in the production of H_2S, while others may be indirectly involved *via* providing protein substrates or accelerating H_2S production by competitive interactions.

Our study tracked temporal changes of oral-malodor-associated tongue microbiota and revealed microbes that can potentially be used to evaluate or perturb the state and development of oral malodor. Clinical efficacy of such positively or negatively correlated microbial agents with oral malodor should be determined by *in vitro* culture to clarify individual contributions of the oral-malodor-associated taxa. Furthermore, single-cell technologies that compare metabolic profiles of live bacterial cells in the biofilm should help to define the *in situ* roles of both individual members and the community in the pathogenesis of oral malodor (Li *et al.*, 2012).

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