

Tongue coating microbiome characterization of Chinese preschool children with halitosis

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Abstract

Background: Halitosis may affect individuals of any age. However, epidemiological surveys and etiological research into halitosis among children are limited. To assess the bacterial characteristics and environmental factors associated with intra-oral halitosis among Chinese preschool children.

Methods: An epidemiological study was conducted among 273 preschool children (aged 3 to 4 years) in Shanghai, China, followed by collection of 16 samples from the tongue coating of caries-free healthy and halitosis participants according to their organoleptic score. The characterization of associated microbial communities was performed using 16S rRNA gene sequencing on the MiSeq.

Results: Halitosis was observed in 13.1% of the preschool children. Logistic regression analysis showed that less frequent intake of sweet snacks and mothers' poor oral condition were associated with children's halitosis positively. Further analysis detected the presence of 15 phyla, 24 classes, 35 orders, 53 families, 93 genera, and 226 operational taxonomic units (OTUs) in the microbial communities. In terms of microbial diversity, the tongue coating of control and halitosis groups showed no significant difference ($P > 0.05$). Both groups shared a mass of common OTUs. The dominant bacterial genera observed in both groups included *Prevotella_7* (15.17% vs. 10.72%), *Veillonella* (13.52% vs. 10.86%), *Streptococcus* (13.95% vs. 18.68%), *Neisseria* (9.44% vs. 21.14%), *Actinomyces* (7.37% vs. 5.88%), *Haemophilus* (6.69% vs. 5.69%), and *Leptotrichia* (5.05% vs. 1.95%). At the genus level, the proportion of *Gemella* was statistically larger in the control group ($P = 0.018$).

Conclusions: Overall, halitosis is associated with multi-microbial mutual interactions. The oral flora microorganisms may exert potentiating or inhibiting effects on each other.

Background

Halitosis is an unpleasant condition resulting in a malodorous mouth, oropharynx, or nose [1]. It is highly prevalent and affects the quality of life of all age groups leading to social embarrassment and psychological restrictions. Halitosis mainly originates in the oral cavity [2, 3]. The production of volatile sulfur compounds (VSCs) [such as methyl mercaptan, hydrogen sulfide (H_2S), dimethyl sulfide] is associated with intra-oral halitosis. Moreover, short-chain fatty acids including propanoic acid, butyric acid, cadaverine, skatole, and indole are also involved [4, 5]. Sulfur-containing amino acids (cysteine, methionine, tryptophan, arginine, and lysine) are biodegraded by both the Gram-negative and Gram-positive anaerobic bacteria thereby producing VSCs and an unpleasant smell [6–8].

Due to the complex interaction of oral microbial flora, halitosis is mainly associated with mixed microbial infections rather than a single species infection [7, 8]. Indeed, a wide range of oral microorganisms including *Prevotella intermedia*, *Porphyromonas gingivalis*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Tannerella forsythensis* are associated with intra-oral halitosis [7, 8]. Identification of the underlying oral bacterial species is integral within halitosis-related investigations. The development of next-generation sequencing (NGS) [9] technology enabled researchers to evaluate microbial communities

including uncultured microorganisms associated with halitosis, leading to a deeper understanding of the micro-ecological changes related with halitosis in the pediatric population. The scientific evidence suggests that oral microbiomes differ in the oral environment of patients depending on whether halitosis is present. [10]. For instance, Ren et al. [11] reported that *Prevotella shahii* was frequently detected and was relatively abundant in saliva samples from children with halitosis.

Halitosis may affect individuals of any age. Indeed, parents frequently visit dentists complaining about the bad breath or odor coming from the oral cavities of their children. However, most halitosis studies [12–14] to date have investigated adults, and data from epidemiological surveys and etiological research into halitosis among children is limited [15–19]. Studies investigating the prevalence of halitosis in children have mainly focused on participants aged between 5 and 12 years or adolescents. Little is known of the prevalence, risk factors, and the microbiome associated with halitosis in preschool children. Since preschool children rarely have periodontal diseases, the microbial basis of halitosis in the oral environment remains largely unknown. Therefore, preschool children are considered good candidates for studying intra-oral halitosis and are likely to provide useful scientific data [11].

As no published articles had reported epidemiological data and microbial characteristics on halitosis among Chinese preschool children, the aim of this research was to assess the epidemiological features and environmental factors associated with intra-oral halitosis among Chinese preschool children. To investigate the bacterial variations associated with pediatric intra-oral halitosis in Chinese preschool children, tongue-coating samples were collected from selected children with and without halitosis.

Methods

Subjects

An epidemiological study was conducted on the oral health of pre-school children (aged 3 to 4 years) in Shanghai, China. Three kindergartens in Shanghai were randomly sampled: one from a central district and two from a suburban region. All children in the junior classes of these kindergartens were invited to participate in this study. Subsequently, 273 children underwent comprehensive oral examinations and were subjects of the study. The present study was reviewed and approval was obtained from the Ethical Committee of Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China (Ref No. 2015135). Prior to the examinations, all of the participants' guardians signed an informed consent form. All procedures were conducted in accordance with the Declaration of Helsinki.

Questionnaire

To gather data related to halitosis, all of the participants' guardians were requested to complete a questionnaire one day before their children's clinical oral examinations. Information including sociodemographic characteristics (gender, age, height, weight, parents' education level, and parents' income); disorders or diseases during gestation (e.g., premature birth); physical conditions (self-reported

oral malodor, systemic diseases, and discomfort); dietary habits (including frequency of fruit, sweets, and meat consumption); and oral hygiene habits (tooth brushing frequency, oral examination frequency, and parental assistance during tooth brushing) of the participants were collected using the questionnaires. Parents were also asked to disclose the medical history and any discomfort suffered by their children.

Methods, clinical oral examination, and halitosis assessment

Clinical examinations were performed on each child during the morning in the kindergartens. Organoleptic assessment criteria were used for diagnosing halitosis as previously described [20]. Briefly, children were asked to keep their mouth shut for 3 minutes and then to expel air from their mouth slowly using a paper-tube placed 10 cm from the observer's nose. Organoleptic assessment scores (OS) were determined by a qualified and registered dentist using the "0–5 Rosenberg scale" as shown below:

- No odor;
- Hardly noticeable malodor;
- Mild malodor;
- Moderate level of malodor;
- Strong malodor;
- Severe malodor.

Subjects scoring 2 or greater ($OS \geq 2$) were considered to have halitosis [20].

A qualified and registered dentist performed the clinical oral examination and evaluated every subject for various oral health indicators including tooth decay (dental caries), dmft (decayed, missed, filled teeth) and dmfs (decayed, missed, filled surfaces) indices, and plaque index (PLI). Evaluation of the tongue coating was estimated based on its area and thickness [3]. The area of tongue coating was graded from 0–3 corresponding to no coating (0), covering $<1/3$ of the tongue surface (1), covering $>1/3$ but $<2/3$ of tongue surface (2), and plaque covering $>2/3$ of tongue surface (3). Tongue-coating thickness was graded as 0 (none), 1 (thin, tongue papillae visible); or 2 (thick, tongue papillae invisible) [3].

Ten percent of the participants were reexamined to evaluate intra-examiner reliability. The Cohen's kappa values (κ) for all parameters (caries, indices, plaque accumulation, tongue-coating evaluation, and OS assessment) were >0.8 . The participants were divided into two groups (halitosis and control) according to the preset selection criteria.

The halitosis group participants ($n = 8$) fulfilled the following inclusion criteria: (1) $OS \geq 2$; (2) dmft = 0, dmfs = 0, and mean PLI <1 subjects; and (3) no history of systemic disorders. The selection criteria for control group ($n = 8$) were (1) OS = 0; (2) dmft = 0, dmfs = 0, and mean PLI <1 subjects; and (3) without systemic disorders. Participants who reported using antibiotics during the previous 6 months were excluded from the study ($n = 2$).

Sample collection

Tongue coating samples were collected from 16 participants by scrubbing from the dorsal to ventral surfaces of the tongue using a specialized tongue cleaning device (KC specimen collection flocked swab, Cleanmo, Shenzhen, China). The collected samples were then transferred to an empty 5-ml sterile Eppendorf tube. All samples were instantly placed in a bubble chamber containing dry ice and then delivered to a freezer at -80°C within 4 hours for storage until use in experiments.

PCR and MiSeq sequencing

The microbial DNA of tongue scrapped coating was extracted by soil DNA Kit (EZNA[®]; Omega Bio-tek, Norcross, GA, USA). A UV-vis spectrophotometer NanoDrop 2000 (Thermo Scientific, Wilmington, MA, USA) was used to evaluate purity and final concentration of DNA while the quality of DNA was detected using agarose gel (1%) electrophoresis. Primers 338F(5-ACTCCTACGGGAGGCAGCAG-3), 806R(5-GGACTACHVGGGTWTCTAAT-3), and a thermocycler PCR system (GeneAmp 9700, ABI, USA) were used to amplify bacterial 16S-rRNA gene (V3-V4 hypervariable segments). The whole PCR process was carried out as follows: denaturation (3 min; 95°C , 27×30 s cycles at 95°C), annealing (30s; 55°C), elongation (45s; 72°C), followed by final extension (10 mins; 72°C) [21]. All experiments were conducted in triplicate with 20 μL aliquots consisting of 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of DNA template. The present study used 2% agarose gels to extract PCR products and AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified by QuantiFluor[™]-ST (Promega, Fitchburg, WI, USA) for further purification. Once purified, an Illumina MiSeq platform (Illumina, San Diego, CA, USA) was used to pool the amplicons in the equimolar paired-end sequenced (2×300) following guidelines provided by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). All data in this study has been submitted to SRA database (SRA accession: PRJNA548494).

Sequencing data handling

The sequencing data was handled using Trimmomatic and FLASH software as previously described [22, 23]. The OTUs (similarity cutoff rate of 97%) were clustered using UPARSE v7.1 software (<http://drive5.com/uparse/>) [24]. The UCHIME was for identification and removal of chimeric sequences.

Each 16S-rRNA gene sequence was classified and measured using the RDP Classifier algorithm (<http://rdp.cme.msu.edu/>) against the Silva 16S-rRNA database (threshold of 70% confidence level). Representative sequences were divided at different classification levels.

Tongue coating and bacterial richness diversity were evaluated in the sample using the alpha (α) index. The analysis of similarities (ANOSIM) was performed to compare the differences in tongue community composition among the children based on the weighted UniFrac distance. The community ecological

package was used to obtain principal coordination analysis (PCoA) data. R-forge was applied to evaluate the beta diversity on the basis of weighted Unifrac and Braycurtis distance matrices (PCoA figure was generated by Vegan 2.0 package). The Circos software was used to construct the Circos graph. The linear discriminant analysis effect size (LEfSe) (<http://huttenhower.sph.harvard.edu/galaxy/>) at multiple levels was also applied to compare bacterial composition between the two groups. Wilcoxon rank-sum test was used to compare relative tongue coating microbiota abundance between two groups. Co-occurrence networks were generated using NetworkX (version 2.3).

Statistical analysis

All data were processed using SPSS v20 software (IBM, NY, USA). The significance level was set at the 5% level and all the test were two-sided. Descriptive statistics were used for children's sociodemographic characteristics and clinical measurements. Student's *t*-test was applied to analyze the significance of difference in continuous variables between the halitosis and control group children. Additionally, the Chi-square test was utilized for investigating differences both groups according to clinical and lifestyle factors. Logistic regression was conducted to estimate factors associated with children's halitosis. The differences in microbial diversity index were analyzed using the student's *t*-test. The Mann-Whitney U test was applied to assess variances of the bacterial compositions in tongue coatings.

Results

General and clinical characteristics

The present study analyzed the questionnaire information and clinical data of 273 (143 male and 130 female) kindergarten children aged 3–4 years. There was no significant difference in clinical and physical characteristics such as caries and body mass indices between children with and without halitosis ($P > 0.05$) (Table 1). In the organoleptic assessment, the majority of children ($n = 203$) scored 0 while 34 had a barely noticeable odor (organoleptic assessment score, OS = 1). Thirty-six children produced an organoleptic score greater than 2 ($OS \geq 2$) and thus were considered to have halitosis. Moreover, all the children with halitosis showed no significant differences in dental caries compared to the control group children (Table 1).

Halitosis and associated lifestyle factors

The distributions of the main demographic and socioeconomic characteristics of participants showed no significant differences in the educational level or the monthly income of parents with regard to the prevalence of halitosis (Table 1). In terms of any association between halitosis and lifestyle factors, less sweet snack consumption was associated with a higher prevalence of halitosis ($P = 0.047$) (Table 2). Furthermore, the study participants whose parents could always smell oral malodor in their children were observed to have a significantly higher percentage of halitosis ($P = 0.040$). Among child halitosis cases,

the logistic regression analysis revealed a strong association between less frequent intake of sweet snacks and mothers' poor oral condition (Table 3).

Tongue coating samples and sequence characteristics

Children (n = 8; 5 boys and 3 girls) who fulfilled the inclusion criteria for the halitosis group were selected. The control group also included 8 participants (3 boys and 5 girls) who fulfilled the required criteria. Both groups showed insignificant differences ($P > 0.05$) on the basis of their sociodemographic background and oral conditions (Table S1).

The analysis of participants' tongue coatings showed a total of 594,710 reads after screening and a mean of 37,169 reads per sample following. Sequence OTU clustering and notation performed at a 3% divergence level detected 15 phyla, 24 classes, 35 orders, 53 families, 93 genera, and 226 OTUs. The OTU distributions were showed by the Venn diagram (Fig.S1A), and the majority of OTUs (187) were preserved and shared in both groups.

Microbial richness and diversity of tongue coating

The rarefaction curves of all samples indicated an adequate sequencing depth in the present study (Fig. S1B). The alpha (α) diversity (observed at OTU level) of five different indices were calculated (Table S2). There were no statistical differences ($P > 0.05$) in the diversity of tongue microbial coatings between the halitosis and control groups, although the halitosis group showed lower indices by the Chao index and higher indices by the Shannon index (Fig. 1A and Fig. 1B).

The comparison of overall microbial composition and structure between halitosis and control group was conducted using principal coordination analysis (PCoA). At the genus level, there was no significant separation in the bacterial community composition between halitosis and control group samples (the analysis of similarities, ANOSIM: $R = 0.106$, $P = 0.115$) (Fig. 1C).

Characterization of tongue coating microbiota and microorganisms associated with halitosis in preschool children

At the genus level, genera with an average relative abundance greater than 1% were considered. The data showed that the dominant genera among samples of both groups were consistent (Fig. 2). A heat-map of the top 50 relatively abundant key bacteria genera of each sample was constructed on the basis of hierarchical clustering solution approach (Bray–Curtis distance metric & complete clustering). The data showed the Z-transformed relative percentage for every genus (Fig. 3). Of 93 genera obtained from all samples, the most frequently detected genera included *Prevotella_7* (15.17% vs. 10.72%), *Veillonella* (13.52% vs. 10.86%), *Streptococcus* (13.95% vs. 18.68%), *Neisseria* (9.44% vs. 21.14%), *Actinomyces*

(7.37% vs. 5.88%), *Haemophilus* (6.69% vs. 5.69%), *Leptotrichia* (5.05% vs. 1.95%), and *Granulicatella* (3.47% vs. 3.62%), which together comprised approximately 75% of children's tongue coating microbiota.

The microbiota abundance of both groups was compared using the linear discriminant analysis effect size (LEfSe) analysis (Fig. 4A) and the linear discriminant analysis (LDA) scores (Fig. 4B). The distribution of *Enterococcus* was significantly higher in halitosis samples ($P = 0.004$), whereas the distribution of *Gemella* and *Kingella* showed a higher abundance in the control samples ($P = 0.016$, $P = 0.001$, respectively).

We applied the Wilcoxon rank-sum test for further comparison between both groups' tongue coating microbiota for relative abundance (%) of the top 25 genus. At the genus level, the proportion of *Gemella* was statistically larger in the control group ($P = 0.018$). *Prevotella_7* and *Veillonella* showed higher relative percentages in the tongue coating samples of halitosis group compared to the control group, however, the differences were insignificant ($P > 0.05$) (Fig. 4C). Comparisons of bacterial average relative abundances between the halitosis and control groups at the genus level are presented in Supplementary Table S3.

Microbial correlation networks of tongue coating microbiota

As *Gemella* was the only dominant genus that showed statistical difference in the comparison of relative abundance between two groups (Fig.4), to explore the potential role of *Gemella* in microbial communities associated with halitosis, the co-occurrence network of 25 most abundant bacteria at the genus level were established (Fig.5). The *Gemella* genus node was more central and more complex in halitosis group. *Gemella* had 4 positive (*Oribacterium*, *Haemophilus*, *Porphyromonas* and *Fusobacterium*) and 5 negative (*Selenomonas*, *Campylobacter*, *Veillonella*, *Megasphaera* and *Atopotium*) correlations in the halitosis group (Fig. 5A). For the control group, *Gemella* showed a negative correlation with *Lachnoanaerobaculum* (Fig. 5B).

Discussion

The present study investigated the epidemiological characteristics, environmental factors and bacterial variations associated with intra-oral halitosis among Chinese preschool children. A structured questionnaire was used to gather data on sociodemographic characteristics, lifestyle, medical history, and oral hygiene. The tongue coating samples were analyzed for microbial communities in both healthy (control group) and halitosis participants.

The prevalence of halitosis was observed clinically in 13.1% of 273 preschool children. The percentage of halitosis cases in the present study was significantly lower compared to other studies [14, 25] reporting the prevalence of halitosis in adults. The previous studies reported that the prevalence of halitosis increases with age [16, 17, 26]. The positive correlation between children's halitosis and age can be caused by the eruption of permanent teeth providing a greater surface area for bacteria accumulation.

Generally, there are two types of established methods used for the diagnosis of halitosis: organoleptic assessment (OA) and by sulfide monitoring equipment. OA is considered the gold standard treatment [27], and is time-efficient for in-school investigations. Therefore, we used OA methods in the present study to assess halitosis. For the halitosis that originates from the oral cavity among adults, the main contributory factors are poor oral hygiene, periodontal conditions (such as periodontitis, gingivitis), tongue coating, and dry mouth [2, 28]. Since there was a low incidence of periodontal diseases and dry mouth in preschool children, the present study investigated fresh insight into factors that play a pivotal role in causing halitosis in children.

Our results showed that the children consuming sweet foods more frequently had a lower prevalence of halitosis. These findings are in line with the result of our previous study [25]. In the oral environment, the degradation of peptides resulting in an acidic environment (low pH) due to a trypsin-like enzyme contributes to halitosis. The frequent consumption of sweet snacks (such as carbohydrates including glucose and sucrose) by children may inhibit the enzymatic activities of various enzymes hence reducing the production of halitosis-associated metabolites [8, 29]. However, whether sweet foods cause halitosis in children needs to be investigated further. Our study also found that halitosis in children was related to the oral health status of their mothers. Indeed, parents' oral hygiene behaviors impact the oral health of their children [30, 31]. Appropriate oral health education and oral hygiene instructions from parents can play a major part in maintaining a healthy oral cavity and preventing halitosis in children.

The qualitative nature of bacterial tongue coating is a well-known indicator of halitosis [15]. For instance, the deep fissures in the tongue coating provides a low oxygen environment for halitosis-associated anaerobic bacteria thus potentiating their pathogenic colonization. In this study we compared features of bacterial tongue coatings obtained from halitosis and healthy children (control) groups. In the present study, both α and β diversity indices based on the weighted UniFrac distance metric indicated that the overall microbial structure was similar between healthy and halitosis children. Seerangaiyan et al [32] showed similar findings in that the qualitative bacterial composition of the tongue microbiome in the halitosis patients was largely the same as that of the control group with only a few exceptions of certain bacterial species and genera. In contrast, Ren et al [11] reported that the tongue coating of children aged 4–5 years with halitosis contained a significantly higher quantity of bacteria than those without halitosis. A follow-up study may be required to explore halitosis in children of different ages to detect any differences.

In the present study, the dominant genera of both groups were *Prevotella_7*, *Veillonella*, *Streptococcus*, *Neisseria*, *Actinomyces*, *Haemophilus*, and *Leptotrichia*. In addition, these bacteria were present in all of the tongue coatings and could adapt to the tongue coating environment of children. *Neisseria* accounted for more than a fifth (21.14%) of the bacterial community in the healthy group, and *Prevotella_7* (15.17%) was the most abundant genus in the halitosis group. *Prevotella* has been considered a pathogenic bacterium of halitosis and found to be positively correlated with VSC gases parameters [33–35]. As the major H₂S-producing bacteria, *Prevotella* showed a higher abundance in halitosis children in the present study; however, the difference was not significant and may have been due

to the subjects' ages and severity of their halitosis. As a constituent of the oral microbiota, *Leptotrichia* is an opportunistic pathogen normally present in the mouth. The present study detected relatively a higher abundance of *Leptotrichia* in the halitosis tongue coating samples. Several studies [11],[10],[36] reported an increased quantity of *Leptotrichia* in participants with halitosis. *Streptococcus* is the predominant species on the tongue dorsum. Previously, *Streptococcus* was reported to be associated with a lower percentage of halitosis and was negatively correlated to the H₂S value [12], which is in line with results of the present study. Kazoret et al [12] suspected *Streptococcus* as a common organism on the healthy tongue, and Takeshita et al [37] found the microbiota of the halitosis subjects was characterized by lower proportions of *Streptococcus*. In addition, Sterer and Rosenberg [6] suggested that *Streptococcus salivarius* contributed to mucin degradation and deglycosylate salivary glycoproteins, thus increasing the likelihood of halitosis. However, the role of these bacterial species in halitosis and their pathological mechanisms remain uncertain.

In terms of the genus, the proportion of *Gemella* (found in normal human flora) [38] was statistically higher in the control group. Yang et al [10] found a significantly negative relationship between *Gemella* and the H₂S value. However there is still insufficient evidence showing its effects in reducing H₂S value *in vitro*. Bacterial interactions within a microbial community can be revealed by co-occurrence analysis. The network of *Gemella* in the halitosis group displayed more intense clusters formation than in the control group, which suggested *Gemella* may be engaged in more complex interspecies competition and/or cooperation in the halitosis group and microbial interactions may be important in children halitosis pathogenesis. Interestingly, *Gemella* had a negative relationship with *Veillonella* and positively correlated with *Haemophilus* in the halitosis group network. *Haemophilus* and *Gemella* were reported that they can decrease the H₂S levels [10]. *Veillonella* was found to have a higher quantity in the halitosis group and was predominant in producing H₂S that may account for the halitosis [39]. These results indicated that various microorganisms interact to produce malodor in the oral cavity.

This is the first study focusing on halitosis characteristics among preschool children aged 3–4 years. There are a number of limitations; firstly, the oral cavity environment and bacterial community is dynamic and changes with age [40, 41] and lifestyle factors may affect the finding. Secondly, periodontal diseases are less prevalent in preschool children and correspondingly the periodontitis pathogens are reduced in their oral flora compared to the oral flora in adults. Compared to PCR, the present study applied 16S rRNA gene pyrosequencing that provided a comprehensive understanding of the oral flora, however further studies involving a larger sample size from various age groups are required to provide stronger statistical power and conclusions.

Conclusions

The present study provides new insights into halitosis in children. Halitosis in children is associated with multi-microbial mutual interactions, while the oral microflora may exert either potentiating or inhibiting effects on each species. Further follow-up and long term studies are required to fully understand the complex interaction of microbiota and various factors associated with halitosis in children.

Abbreviations

OTU: operational taxonomic units

VSCs: volatile sulfur compounds

OS: organoleptic assessment score

Dmft: decayed, missing, and filled teeth

Dmfs: decayed, missing, and filled surfaces

PLI: plaque index

ANOSIM: analysis of similarities

PCoA: principal coordination analysis

LEfSe: linear discriminant analysis effect size

LDA: linear discriminant analysis

Declarations

Ethics approval and consent to participate

The present study was reviewed and approval was obtained from the Ethical Committee of Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China (Ref No. 2015135). Prior to the examinations, written informed consent was obtained from all of the participants' parents. All procedures were conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and material

The datasets generated and analysed during the current study are available in the SRA database (SRA accession: PRJNA548494) <https://www.ncbi.nlm.nih.gov>.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

X.C and X. P.F designed the study. C. Z. and Y.Z completed the data analysis and statistics. X.C and Y.Z completed the sample collection. Y.Z completed the DNA extraction and amplification. All of the authors have read and approved the final manuscript.

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Tables

Table 1. Physical clinical characteristics and demographic and social characteristics of the participants in the two groups

	Halitosis (mean ± SD)	Control (mean ± SD)	<i>P</i>
Height (cm)	106.73±5.38	106.79±4.61	0.944 ^a
Weight (kg)	18.13±2.78	18.90±4.67	0.367 ^a
BMI (kg/m ²)	15.76±1.93	16.47±3.81	0.301 ^a
Plaque index	0.45±0.24	0.45±0.25	0.992 ^a
Dmft	4.97±5.77	3.05±3.90	0.060 ^a
Dmfs	10.00±14.73	5.19±8.36	0.063 ^a
Gender (N, % with OS≥2)			0.860 ^b
Male	140	13.6	
Female	133	12.8	
Father's monthly income, RMB* (N, % with OS≥2)			0.720 ^b
≤3000	7	0.0	
3001-6000	67	10.4	
6001-12 000	86	15.1	
12 001-24 000	47	14.9	
>24 000	45	11.1	
Mother's educational level* (N, % with OS≥2)			0.461 ^b
Middle School or below	15	13.3	
High School	30	20.0	
Vocational School	84	9.5	
College	120	15.0	
Graduate School	19	5.3	

^a Obtained by Student's *t*-test

^b Obtained by Chi-squared test

*Some data are missing for these variables.

SD: standard deviation

BMI: body mass index; Dmft: decayed, missing, and filled tooth; Dmfs: decayed, missing, and filled surface; N: Number of children examined; OS: Organoleptic assessment score

Table 2. Relationships between halitosis and health behaviors

	N	% with OS \geq 2	<i>p</i> ^a
Fruit intake*			0.456
\geq 1 \times /day	194	12.4	
1-6 \times /week	69	13.0	
Seldom/never	7	28.6	
Marmalade and honey intake*			0.346
\geq 1 \times /day	3	0.0	
1-6 \times /week	23	4.3	
Seldom/never	239	13.8	
Dessert intake*			0.451
\geq 1 \times /day	59	8.5	
1-6 \times /week	165	13.9	
Seldom/never	43	16.3	
Candy intake*			0.136
\geq 1 \times /day	29	6.9	
1-6 \times /week	132	10.6	
Seldom/never	105	18.1	
Beverage intake*			0.205
\geq 1 \times /day	6	16.7	
1-6 \times /week	72	6.9	
Seldom/never	192	15.1	
Any sweet snack intake*			0.047
\geq 1 \times /day	93	8.6	
1-6 \times /week	102	11.8	
Seldom/never	64	21.9	
Meat intake*			0.033
\geq 1 \times /day	0	-	
1-6 \times /week	28	0.0	
Seldom/never	240	14.6	
Snack intake before sleeping*			0.750
\geq 1 \times /day	91	14.4	
1-6 \times /week	57	14.0	
Seldom/never	118	11.0	
Regular oral examination within 6 months*			0.397
Yes	64	9.4	
No	205	14.6	

Seldom/never	185	39.5
Toothbrushing frequency*		0.479
≥2×/day	130	10.8
1×/day	99	16.2
Never or seldom	41	12.2
Assistance in toothbrushing		0.898
Yes	74	12.2
No	196	13.3
Parents perceived oral malodor*		0.040
Always	16	31.3
Sometimes	157	10.2
Never or seldom	95	13.7
Mother's oral condition*		0.062
Good	159	10.7
General	95	14.7
Poor	16	31.3

^a Obtained by Chi-squared test. * These variables have some missing data.

OS: Organoleptic assessment score.

Table 3. Multiple logistic regression model used for halitosis (0: OS<2, 1: OS ≥2)[#]

	Odds ratio	95% CI	<i>P</i>
Intake frequency of sweet food			0.037
≥1×/day ^a			
1-6×/week	1.62	0.61-4.27	
Seldom/never	3.41	1.30-9.03	
Mother's oral condition			0.043
Good ^a			
General	1.35	0.60-3.03	
Poor	4.88	1.41-16.85	

[#] Multiple logistic regression.

^a Reference group.

Figures

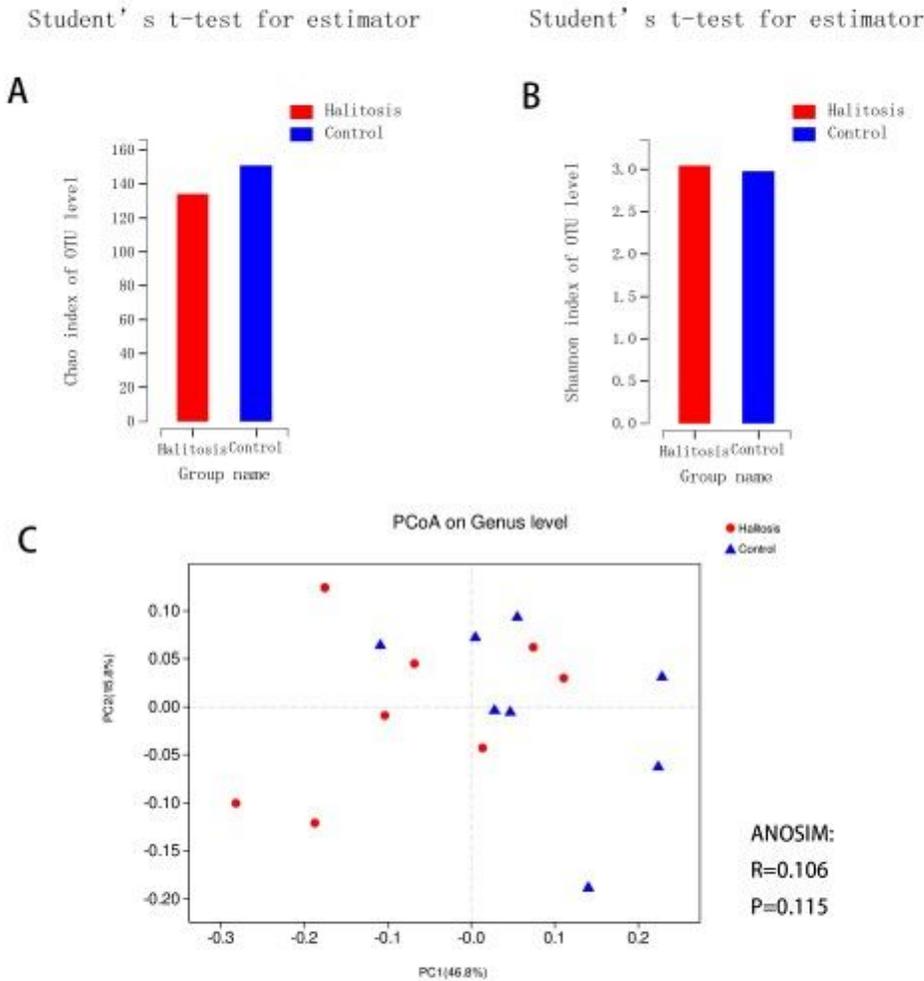


Figure 1

Microbial richness and diversity in the halitosis and healthy (control) groups. (A) Comparison of a diversity by the Chao index in healthy and halitosis tongue coating samples of children. (B) Comparison of a diversity by the Shannon index in healthy and halitosis tongue coating samples of children. (C)

Weighted UniFrac principal coordination analysis (PCoA) of children's tongue coating bacterial community composition on genus level.

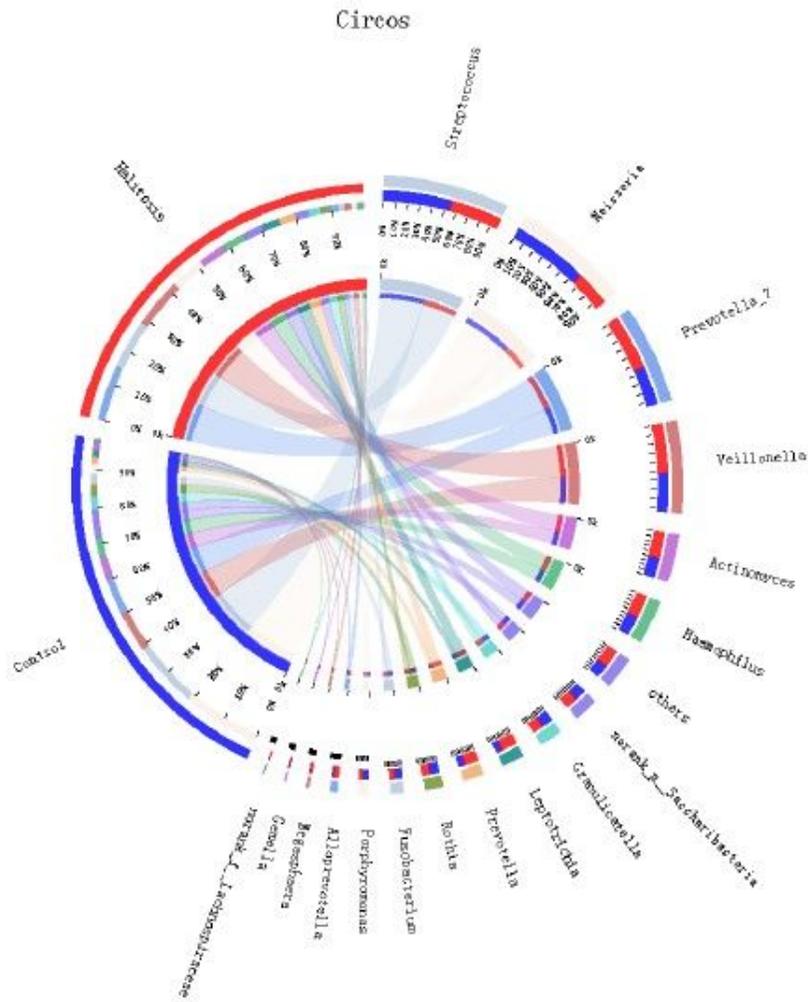


Figure 2

Distribution of bacterial community for the halitosis and control groups at genus level. The data were visualized by Circos. The length of the bars of each genus indicates the relative abundance.

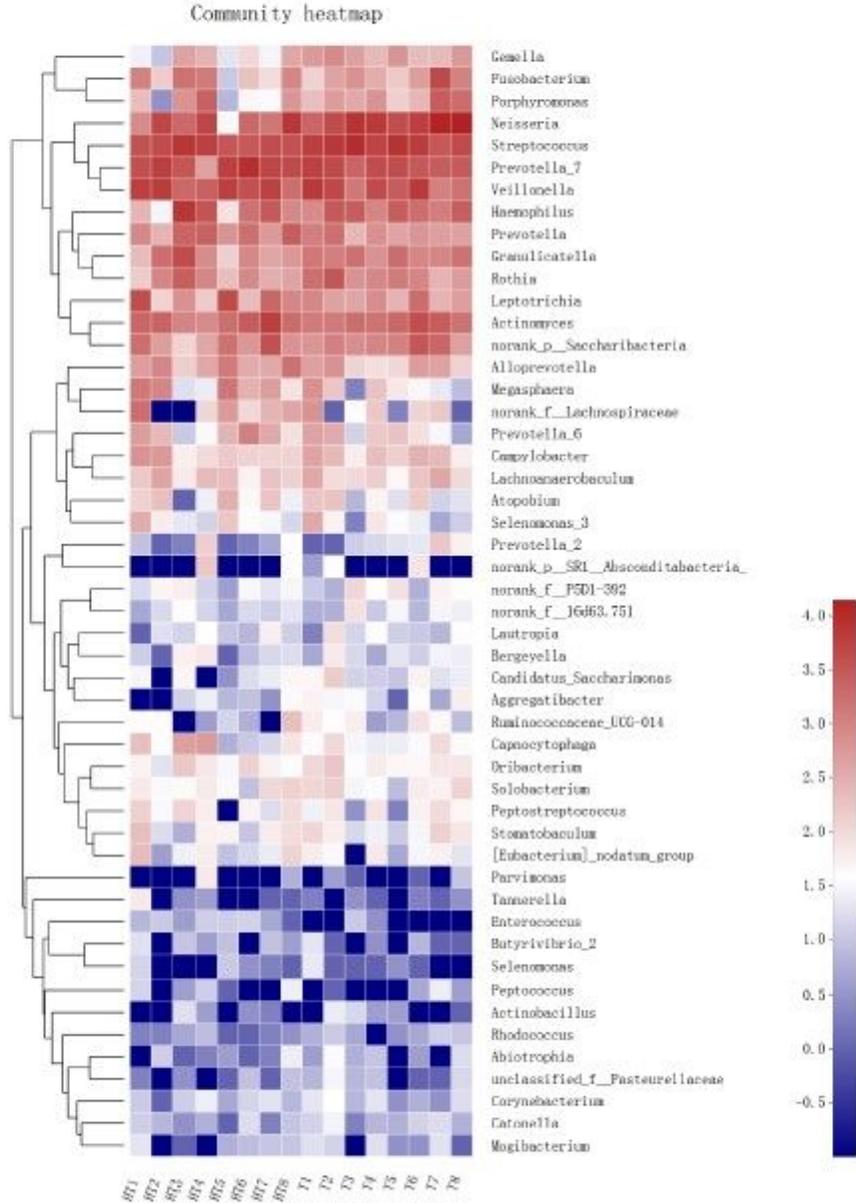


Figure 3

Composition of tongue coating bacterial communities across samples on genus level. Heat map analysis of the top 50 key genera among all tongue coating samples. The relative abundance of the genus is indicated by colour of the spots. HT: halitosis group tongue coating sample. T: control group tongue coating sample.

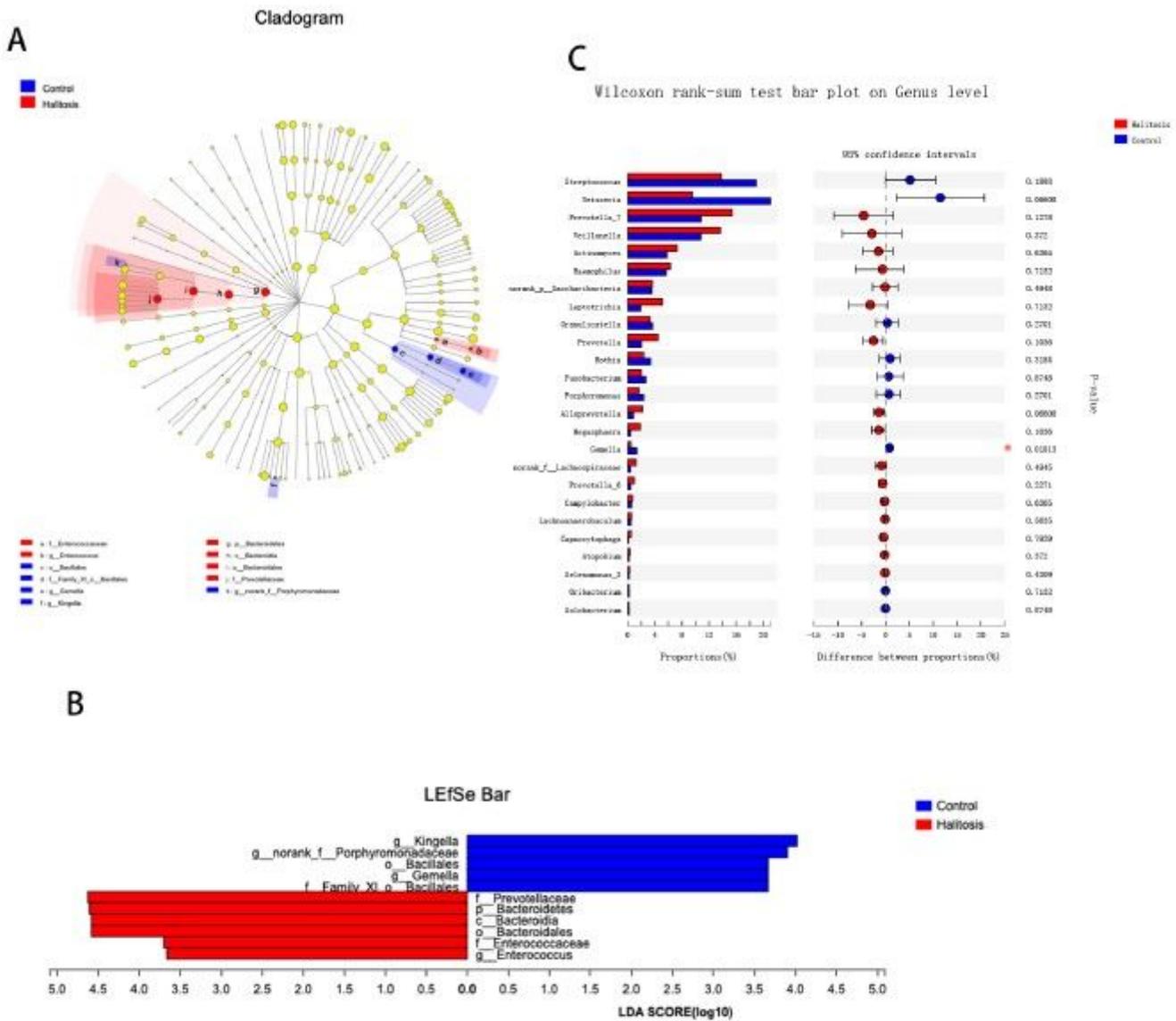


Figure 4

Comparisons of the relative abundance of tongue coating bacterial communities between the halitosis and control groups. (A) Cladogram based on the LEfSe method depicted the different microbiota structures from the phylum to genus level in the halitosis and control groups. The diameter of each circle is proportional to the taxon's abundance. (B) Linear discriminant analysis scores showed significant bacterial differences of various levels in the halitosis and control groups. (C) Comparisons of the relative bacteria abundance between the halitosis and control groups on genus level. Statistical analysis was performed by the Mann-Whitney U test. *P<0.05.

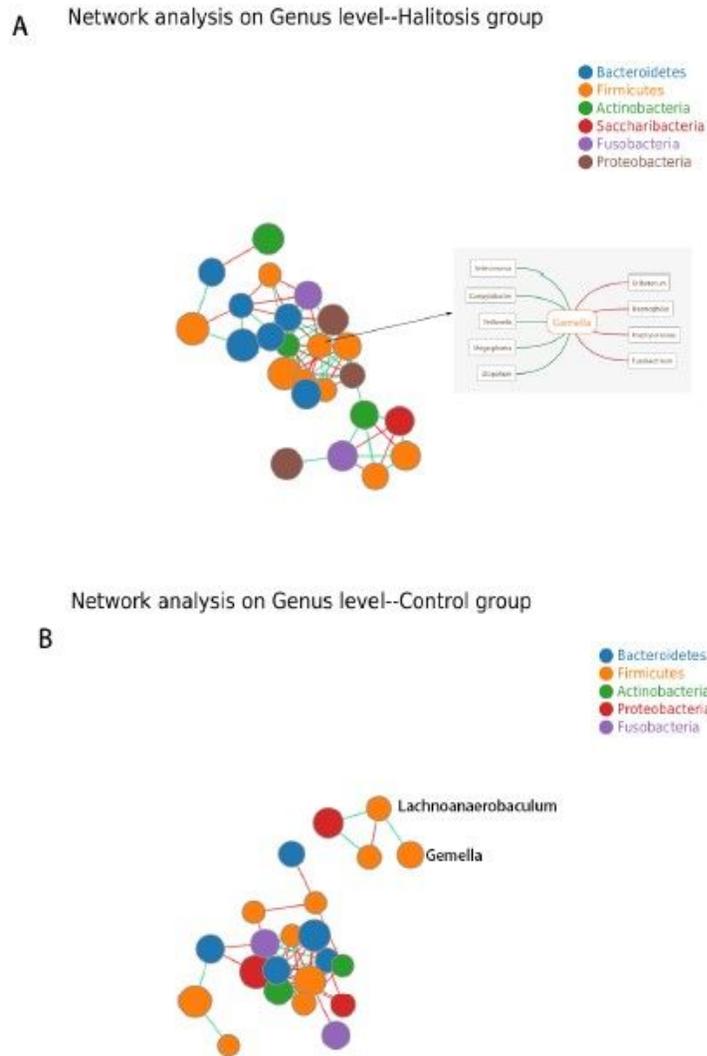


Figure 5

Microbial correlation networks of tongue coating microbiota in the halitosis and healthy groups. (A) Network of 25 most abundant bacteria at the genus level in the halitosis group. Spearman correlation coefficient was calculated and only significant correlations ($P < 0.05$) are displayed in the network. The size of the nodes indicates the abundance of species. The color of the connection indicates positive (red) and negative (green) correlations. (B) Network of 25 most abundant bacteria at the genus level in the control group.

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