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Association between tobacco use and the upper gastrointestinal microbiome among Chinese men

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Abstract

Purpose—Tobacco causes many adverse health conditions and may alter the upper gastrointestinal (UGI) microbiome. However, the few studies that studied the association between tobacco use and the microbiome were small and underpowered. Therefore, we investigated the association between tobacco use and the UGI microbiome in Chinese men.

Methods—We included 278 men who underwent esophageal cancer screening in Henan Province, China. Men were categorized as current, former, or never smokers from questionnaire data. UGI tract bacterial cells were characterized using the Human Oral Microbial Identification Microarray. Counts of unique bacterial species and genera estimated alpha diversity. For beta diversity, principal coordinate (PCoA) vectors were generated from an unweighted UniFrac distance matrix. Polytomous logistic regression models were used for most analyses.

Results—Of the 278 men in this study, 46.8% were current smokers and 12.6% were former smokers. Current smokers tended to have increased alpha diversity (mean: 42.3 species) compared

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to never smokers (mean: 38.9 species). For a 10 species increase, the odds ratio (OR) for current smoking was 1.29 (95% CI: 1.04–1.62). Beta diversity was also associated with current smoking. The first two PCoA vectors were strongly associated with current smoking (PCoA1 OR 0.66; 95% CI: 0.51–0.87; PCoA2 OR 0.73; 95% CI: 0.56–0.95). Furthermore, *Dialister invisus* and *Megasphaera micronuciformis* were more commonly detected in current smokers than in never smokers.

Conclusions—Current smoking was associated with both alpha and beta diversity in the UGI tract. Future work should consider how the UGI microbiome is associated with smoking related diseases.

Keywords

China; microbiome; tobacco; upper gastrointestinal tract

INTRODUCTION

Tobacco causes cancers at 20 organ sites and has pleotropic physiologic effects [1, 2]. It has been implicated as a cause of periodontitis [3] and there is strong evidence that a number of bacterial species are involved in the etiology of periodontitis [4]. Current smoking has been estimated to be attributable for 41.9% of periodontitis cases in the United States [5]. Periodontal disease is a risk factor for oral cancer [6] and oral bacteria, specifically *Fusobacterium nucleatum, Porphyromonas gingivalis*, and *Streptococcus mutans*, are associated with a number of systemic infections and inflammation, while *F. nucleatum* has been detected in colorectal tumors [7]. This suggests that one mechanism through which tobacco may be associated with adverse health conditions, such as cancer and periodontitis, may be through alterations in the microbiome.

The association between tobacco use and the upper gastrointestinal microbiome has not been comprehensively studied, but some research has considered associations between tobacco use and the microbiome of the respiratory and digestive system. One review of mainly culture-based studies, concluded that smoking is related to increases in periodontal and respiratory bacterial pathogens and decreases in bacteria capable of interfering with pathogen growth [8]. More recently, the availability of culture-independent methods using the 16S rRNA gene has enabled investigations of both cultivable and non-cultivable microorganisms [9]. Only a few studies have used 16S rRNA-based approaches to consider associations between tobacco use and the microbiome of the respiratory and digestive system [10–15]; although no studies have investigated associations in the upper gastrointestinal tract. Therefore, we investigated the association between tobacco use and the upper gastrointestinal microbiome ascertained using a 16S rRNA microarray in Chinese men within the Cytology Sampling Study 2 (CSS2).

MATERIALS AND METHODS

Study population

The study design for the CSS2 has been previously described in detail [16, 17]. In brief, we recruited participants in the spring of 2002 from three villages in Yaocun commune in

Linzhou, Henan Province, People's Republic of China for an esophageal cancer screening study. Eligible participants were aged 40–65, apparently healthy with no signs or symptoms of upper gastrointestinal cancer or other chronic disease, and were fit to undergo upper endoscopy. In total, 720 participants completed all portions of the study, which included a questionnaire, oral health exam [18] and endoscopy with Lugol's iodine staining and biopsy [16]. Since less than 1% of women in this study had a history of smoking, we restricted this analysis to men (N = 303). We excluded one batch of the Human Oral Microbiome Identification Microarray (HOMIM) from analysis due to inconsistent results (N = 24) and one man who did not provide information on current smoking. A total of 278 men were left for analysis. The Institutional Review Boards of the Cancer Institute of the Chinese Academy of Medical Sciences (CICAMS) and the National Cancer Institute of the United States gave approval for this study and all participants gave written informed consent.

Smoking history assessment

The participants completed a structured questionnaire including questions about cigarette and pipe smoking history. Since only 3 men had ever smoked a pipe and these men had also ever smoked cigarettes, we did not include pipe smoking in the analysis. The questionnaire assessed whether participants had ever smoked regularly for six months or longer and if so, at what age they started regularly smoking and the average number of cigarettes per day. It also assessed whether the participants smoked at the time of screening, and if not, at what age they quit smoking. Men were categorized as current, former or never smokers. Men who reported quitting smoking during the same year of interview (N = 2) or did not report the number of years since quitting (N = 2) were categorized as current smokers. We also generated quantitative smoking metrics for some analyses, such as calculating pack-years of smoking among current and former smokers and the number of years since quitting among former smokers.

Other covariates of interest

The structured questionnaire assessed information on other covariates, including age, alcohol consumption over the past year, and use of antibiotics in the past three months. Height and weight were measured during the physical examination and each participant's body mass index (BMI; kg/m^2) was calculated. An oral health and periodontal examination was conducted in this population which has been described in detail [18]. In brief, we considered the Decayed, Missing, and Filled Teeth (DMFT) score and periodontal disease status defined as 1 or more sites with attachment loss of 3 mm or greater and a pocket depth of 4 mm or greater.

Upper digestive tract biological sample collection

We randomly assigned participants to one of two esophageal balloon cytology retrieval devices used to collect upper digestive tract samples as previously described [17]. Sample collections were conducted in the local village health clinic in the morning after an overnight fast. Each participant was given 2 mL of 2% lidocaine slurry by mouth and the balloon was inserted into the back of the throat and swallowed. The balloon was expanded in the stomach and withdrawn through the esophagus. The balloon with the attached cells and saliva were

cut using sterile scissors. A cell suspension was obtained, frozen in liquid nitrogen, and stored at −80°C prior to DNA extraction.

DNA extraction and HOMIM array

Using the Gentra Puregene Cell kit (Qiagen, Valencia, California, USA), DNA was extracted from 300 μL of the cell suspension collected from the upper digestive tract. The quality and quantity of the extracted DNA was assessed using the 260:280 ratio with the Nanodrop fluorospectrometer and Picogreen assay. TaqMan assays using species-specific primers were used to ascertain the presence of human and bacterial DNA in the samples.

The Paster laboratory carried out the HOMIM hybridization assay using a previously described protocol [19, 20]. In brief, 16S rRNA-based oligonucleotide probes were printed on glass slides. The extracted DNA was amplified by PCR using 16S rRNA universal forward and reverse primers and then labeled in a second nested PCR. The labeled 16S amplicons were hybridized overnight and washed. The slides were scanned and data was extracted using GenePix Pro. The normalized median intensity score was calculated by subtracting the median background intensity for an individual feature from the median feature intensity. Using feature specific criteria, the relative intensity of each probed species/strain was determined, but the data was dichotomized to present or absent.

The HOMIM arrays were completed in batches which included up to 95 experimental samples plus quality control samples in each batch. Five experimental samples were repeated in each batch to assess technical replication between batches.

Microbial diversity metrics

We calculated alpha diversity (i.e., within participant diversity) as the number of unique species or genera. These counts were considered as a continuous variable and also categorized into tertiles based on the distribution within this population. We calculated beta-diversity (i.e., between participant diversity) using an unweighted UniFrac distance matrix for all pairs of participants with data using fast UniFrac [21] and a phylogenetic tree based on the Human Oral Microbiome Database [22]. UniFrac takes into account evolutionary differences between the microbial communities of each participant in order to determine whether the communities are distinct [23]. The unweighted UniFrac distance matrix was analyzed using the unweighted pair group method with arithmetic mean clustering algorithm. The maximal pseudo F test and minimal pseudo t^2 statistics supported the use of three clusters. However, the first cluster only contained 4 men, so we excluded it from the analysis. One major difference between cluster 2 (N = 222) and cluster 3 (N = 52) was alpha diversity. The average number of unique species for participants in cluster 2 was 43.0 (standard deviation (SD) 10.1) whereas the average for individuals in cluster 3 was 31.3 (SD 12.0). We calculated principal coordinate (PCoA) vectors from the unweighted distance matrix and included the first two PCoA vectors for analysis. The PCoA vectors were standardized by dividing the vector value by its standard deviation.

Statistical analysis

We compared demographic characteristics by the smoking categories and tested the differences using ANOVA for continuous variables and the Pearson chi-square test for categorical variables. We used polytomous logistic regression with never smoking as the reference category to estimate the odds of being a current or a former smoker, which allowed us to model a three level outcome instead of the standard dichotomous outcome from simple logistic regression. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for the alpha and beta diversity measures. For alpha diversity, we calculated the OR for an increase by 10 species or by 5 genera, and by tertiles of both species and genera. Since the continuous analysis assumes that the association between the counts of species and genera are linearly associated with the outcomes, we also used a B-spline function for these measures to allow flexibility in the shape of the association and tested for non-linearity by comparing the model with the spline to a model without the spline using the likelihood ratio test. For beta diversity, we calculated the OR for being a member of Cluster 3 versus Cluster 2 and for the first two PCoA vectors. We additionally created a permutational model, based on a previous analysis [24], which tested whether the mean similarity among current or former smokers was greater than what would be found in random selections of a population including smokers and never smokers. We created models which adjusted for potential confounders and models which excluded participants who reported taking antibiotics in the past three months since antibiotic use may alter the upper gastrointestinal microbiome.

To assess associations between average cigarettes smoked per day and pack-years of smoking for current and former smokers and years since quitting smoking for former smokers with the measures of alpha diversity, we calculated Pearson correlations between pack-years of smoking, years since quitting and counts of species and genera. For the measures of beta diversity, we created linear regression models with average cigarettes smoked per day, pack-years of smoking, or years since quitting as the outcomes and cluster or PCoA vector as the predictors.

Polytomous logistic regression models for all individual species with a prevalence greater than 0% and less than 100% were calculated. We used a Bonferroni adjusted alpha for significance to account for multiple testing ($P < 0.00018$). Analyses were conducted using SAS 9.3 except for the B-spline and permutation analyses which were conducted using R.

RESULTS

Of the 278 men in this study, 130 (46.8%) were current smokers and 35 (12.6%) were former smokers. Individual characteristics were generally similar across smoking categories. For example, 10.8% of current smokers, compared to 11.4% and 13.3% of former and never smokers, reported to have taken antibiotics in the past 3 months ($P = 0.83$). However, former smokers tended to be older than current or never smokers ($P = 0.041$; Table 1).

Current smokers tended to have increased counts of unique species (mean: 42.3; SD: 11.7) and genera (mean: 24.3; SD: 6.1) compared to never smokers (species mean: 38.9; species SD: 11.8; genera mean: 22.5; genera SD: 6.6), while former smokers had similar counts of unique species (mean: 38.7; SD: 10.4) and genera (mean: 21.9; SD: 5.5) as never smokers.

For every increase in 10 detected species, the odds of being a current smoker were 1.29 (95% CI: 1.04, 1.62) times the odds of being a never smoker. Similarly, for every increase in 5 detected genera, the OR for current smokers was 1.27 (95% CI: 1.03, 1.56) compared to never smokers. The analysis of species and genera counts by tertiles was similar to the continuous analysis for current smokers, but did not reach statistical significance. Adjustment for potential confounders and the exclusion of men who reported taking antibiotics in the past 3 months slightly strengthened the associations for current smokers. For former smokers, no significant differences were observed for any of the measures of alpha diversity compared to never smokers (Table 2). Including a spline function did not improve the fit of the polytomous logistic regression model for either species ($P = 0.28$) or genera ($P = 0.51$), which suggests that a linear model was adequate. When the analyses were restricted to current and former smokers, no correlations were detected between pack-years of smoking or average cigarettes smoked per day with either counts of unique species or genera. Similarly, when the analyses were restricted to former smokers, no correlations were detected between years since quitting with counts of unique species or genera (results not shown).

Compared to never smokers, the measures of beta diversity were associated with being a current smoker and some were associated with being a former smoker. In unadjusted analyses, the odds of being a current smoker were reduced for men in Cluster 3 with an OR of 0.55 (95% CI: 0.28, 1.08) compared to never smokers. This association was strengthened after adjustment for potential confounders (OR 0.49; 95% CI: 0.24, 1.01) and became statistically significant after exclusion of participants who reported taking antibiotics in the past 3 months (OR 0.41; 95% CI: 0.19, 0.89). No associations were detected between former smokers and Cluster 3. The first two PCoA vectors were associated with current smoking (PCoA1 OR 0.66; 95% CI: 0.51, 0.87; PCoA2 OR 0.73; 95% CI: 0.56, 0.95) and the second PCoA vector was associated with former smoking (PCoA2 OR 0.59; 95% CI: 0.40, 0.86) in unadjusted analyses, although the association with former smoking was no longer significant in adjusted analyses (PCoA2 OR 0.69; 95% CI: 0.46, 1.04; Table 2). The permutational model detected a greater mean similarity between current smokers ($P = 0.001$) compared with never smokers, but not for former smokers ($P = 0.539$) after adjustment. No associations were detected between average cigarettes smoked per day, pack-years of smoking or years since quitting with cluster. Significant inverse associations were observed between the second PCoA vector and average cigarettes smoked per day (linear regression coefficient = −2.4; $P = 0.040$) and pack-years of smoking (linear regression coefficient = −4.9; $P = 0.018$) but no associations were detected for the first PCoA vector. No association was detected between years since quitting and either PCoA vector (results not shown).

A total of 140 species, out of the 210 species represented in the HOMIM array, had variability (i.e. a population prevalence greater than 0% and less than 100%) that could be examined in polytomous logistic regression models. Two species, *Dialister invisus* and *Megasphaera micronuciformis*, were more commonly detected in current smokers compared to never smokers. *D. invisus* was detected in 30.8% and 6.2% of current and never smokers, respectively ($P = 0.000011$) and *M. micronuciformis* was detected in 63.8% and 33.6% of current and never smokers, respectively ($P = 0.000004$). No significant differences were observed comparing former smokers with never smokers (Figure 1).

DISCUSSION

In this study, we found that current cigarette smokers tended to have increased alpha diversity of their upper gastrointestinal tract microbiota compared to former and never smokers. Measures of beta diversity were also associated with smoking history, particularly among current smokers. The observed associations with alpha and beta diversity were generally similar or strengthened after adjustment for potential confounders and after exclusion of men who had taken antibiotics in the past 3 months. In species-specific analyses, after Bonferroni correction, two species on the HOMIM array were significantly associated with current smoking status *D. invisus* and *M. micronuciformis*.

To our knowledge, no previous studies have considered the association of tobacco use with the upper gastrointestinal microbiome, but previous work has considered the effect of smoking on various sites in the respiratory and digestive systems. Similar to what we found in the upper gastrointestinal tract, many of the previous studies found that smokers had increased alpha diversity compared with non-smokers. For instance, in a population of 19 healthy smokers and 45 non-smokers, increased alpha diversity was detected in oral wash samples for smokers, but not in bronchoalveolar lavage samples [13]. Another study detected more heterogeneity in the microbiota of 29 smokers in the upper respiratory tract compared to 33 non-smokers [10]. A cross-sectional study of 88 patients with periodontitis found, at the genus level, smoking status was associated with the oral microbiome in multivariable-adjusted models, but not unadjusted analyses [12]. In a longitudinal study analyzing the change in the presence and levels of bacterial microbiota in the subgingival plaque after dental cleaning within a group of 11 smokers and 11 previous smokers with periodontitis, no differences were observed in the presence of microbiota, but the quantity of microbiota among smokers tended to be higher [11]. In a study of 15 current smokers and 15 non-smokers, smokers had increased alpha diversity in both the subgingival and marginal plaque after dental cleaning [14]. However, at least one other study did not detect increased alpha diversity in smokers. Within a group of people with chronic periodontitis, no differences were observed in the number of species or uncultivated phylotypes between 15 current and 15 never smokers [15]. Additional work is needed to determine the mechanism through which smoking affects the microbiota throughout the upper gastrointestinal tract.

One possibility for the mechanism through which current smokers may have increased alpha diversity may be related to the immunosuppressive nature of tobacco. Tobacco smoking has been observed to affect the peripheral immune system through various changes, including a decrease in the activity of natural killer cells, increase in white blood cell counts, and a higher susceptibility to infection [25]. In a study which ascertained plasma antibodies to specific oral bacteria in 395 cancer-free participants, current smokers tended to have lower antibody levels to oral bacteria than never smokers [26]. Similarly, in a sample of 8,153 participants from NHANES III, smokers tended to have lower antibody levels to nine specific periodontal bacteria, but had higher titers to *Micromonas micros* [27]. This smoking related immunosuppression could permit novel bacteria to colonize the upper gastrointestinal tract which could explain the increased alpha diversity we detected in this study. It is also possible that metabolic advantages are conferred to certain taxa in a smoky environment. Exposure to cigarette smoke has been observed to increase biofilm formation

by specific bacteria [28, 29]. One study found that exposure to cigarette smoke condensate increased biofilm formation of *Streptococcus pneumoniae* and decreased bioactivity of pneumolysin, the pro-inflammatory toxin of *S. pneumoniae* [29]. These findings suggest that cigarette smoke may promote colonization and persistence of specific bacterial taxa in the human body.

Both *D. invisus* and *M. micronuciformis* are gram-negative, anaerobic bacteria [30, 31] which may have a competitive advantage in the smoky environment. Another mechanism that may lead to increased alpha diversity among smokers may be due to exposure to novel bacteria in cigarettes. One study found that cigarettes made in the European Union contained 15 different classes of bacteria [32]. Both the *Megasphaera* and *Dialister* genera were detected in cigarettes, although only in 20% and 5% of samples, respectively [32]. The presence of these genera in cigarettes could be related to the higher prevalence of *D. invisus* and *M. micronuciformis* detected in current smokers in our study, although cigarettes purchased in China may contain different bacteria and it is unknown whether the bacteria found in cigarettes are viable and able to colonize the upper gastrointestinal tract.

There are some limitations of this study. First, the HOMIM array was only able to detect the bacterial taxa represented on the microarray, so previously undetected species or species with a low prevalence not included on the microarray would not be detected. However, a relatively high correlation has been observed between the HOMIM array and 16S rRNA pyrosequencing at the phylum level and for the most common taxa at the genus level [33], so it is likely that our findings would be similar using 16S rRNA sequencing. We also only evaluated the presence or absence of each species on the HOMIM array and were unable to compare the abundance of each taxon. In addition, we did not assess the association between smoking with other microbiota, like fungi or viruses. One recent study suggested that smokers have higher levels of oral fungi than non-smokers [34] and future work may also wish to consider this potential association. Finally, this study may not be generalizable to populations other than Chinese men since we excluded women due to the low prevalence of smoking among women (< 1%) and only considered the microbiome of men in Linzhou, China. It is unlikely that smoking has a differential effect on upper gastrointestinal microbiota based on sex, but we could not directly test it in our study. And alterations induced by tobacco on the upper gastrointestinal microbiota could differ between populations both due to the types of tobacco used and due to other influences of the environment and genetics.

There are also strengths of this study. Use of the HOMIM array allowed primarily species level identification of the microbiota, whereas other methodologies typically identify at the genus level. Also, this was a relatively large sample compared to previous studies including sufficient numbers of men in each of the smoking categories. However, there was less power and precision for former smokers compared to current smokers due to smaller numbers. We also were able to assess the effect of several potential confounders in adjusted models and the exclusion of participants reporting the use of antibiotics.

In conclusion, we observed an association between current tobacco smoking and both alpha and beta diversity in the upper gastrointestinal tract using a 16S rRNA microarray in this

population of Chinese men. Specific individual bacterial species, *D. invisus* and *M. micronuciformis*, were also highly associated with current smoking. Extensive additional research is needed to investigate the association between smoking and other microbiota, whether higher diversity is associated with smoking related diseases, such as periodontal disease or esophageal squamous cell carcinoma.

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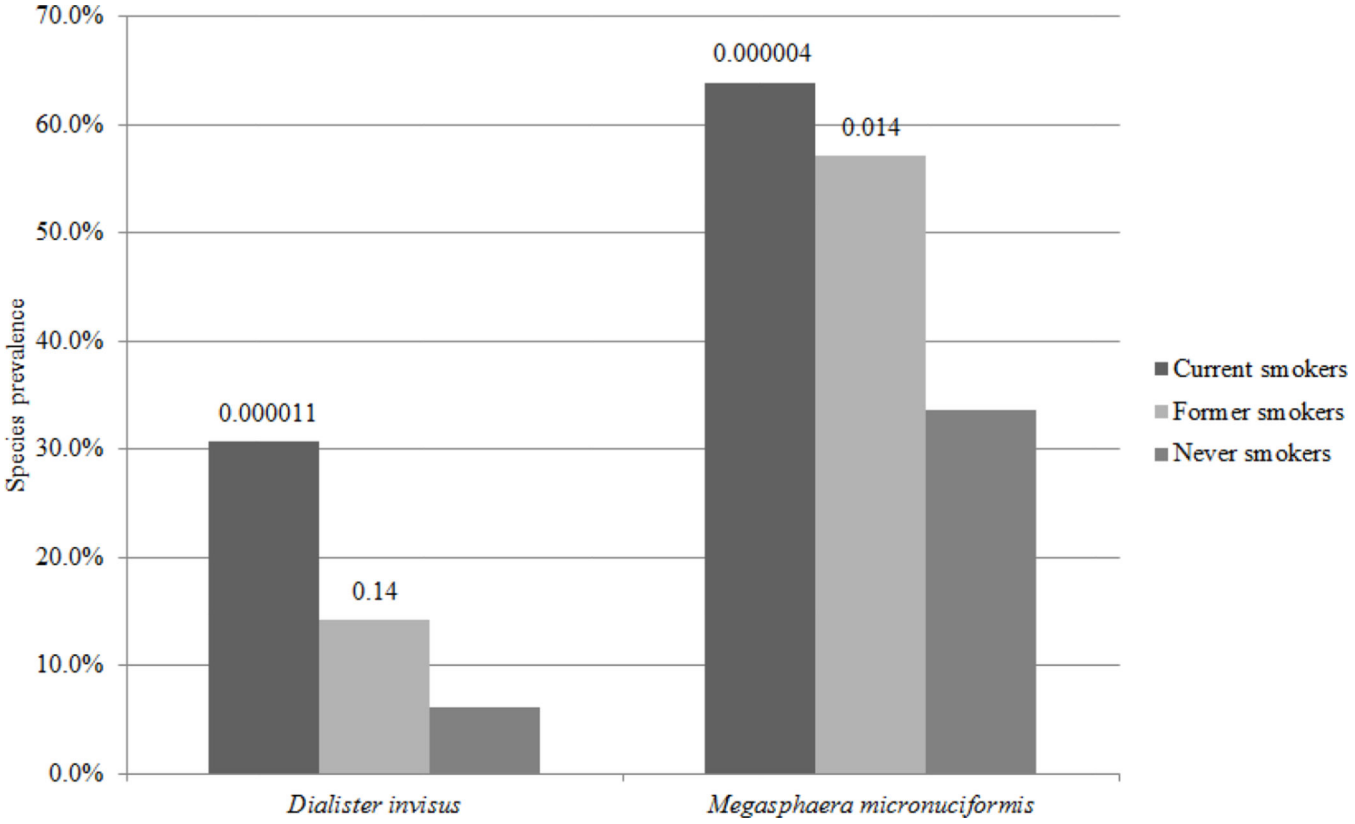

Figure 1.

Prevalence of Species by Smoking Status Among Men in Linzhou, China, 2002 a

[a] Statistical significance, compared to never smokers, set as $P < 0.00018$ after Bonferroni correction for 280 comparisons

Table 1

Demographic Characteristics by Smoking Status for Men in Linzhou, China, (N = 278)[a]

Characteristics	Current smoker N = 130	Former smoker N = 35	Never smoker N = 113	*P* value
Age (years)	55.6 (4.8)	57.7 (5.6)	55.4 (4.6)	0.041
Ever drink alcohol in the past year	25 (19.2%)	2 (5.7%)	18 (15.9%)	0.16
Body mass index (kg/m^2)	22.8 (2.8)	23.7 (2.8)	23.0 (2.6)	0.24
Decayed, Missing, and Filled Teeth score	11.4 (9.9)	11.2 (10.6)	8.6 (8.7)	0.065
Periodontitis[b]	51 (48.1%)	15 (55.6%)	43 (41.8%)	0.38
Any antibiotics in the past 3 months	14 (10.8%)	4 (11.4%)	15 (13.3%)	0.83
Pack-years of smoking[c]	24.0 (24.2)	20.0 (25.9)	-	0.43
Average cigarettes per day[c]	14.9 (12.8)	13.7 (16.8)	-	0.69
Years since quitting	-	7.2 (6.8)[d]	-	-

[a]Continuous variables are presented as mean (SD) and categorical variables are N (%) and differences by smoking status were tested using ANOVA for continuous variables and the Pearson chi-square test for categorical variables.

[b]Data was missing from 42 men who were edentulous

[c]Data was missing for 2 current and 7 former smokers

[d]Range from 1 to 28 years since quitting

Table 2

Associations Between Measures of Alpha and Beta Diversity With Smoking History Among Men in Linzhou, China

	Unadjusted				Adjusted[b]				Excluding antibiotics[c]			
	Current OR	95% CI	Former OR	95% CI	Current OR	95% CI	Former OR	95% CI	Current OR	95% CI	Former OR	95% CI
Alpha diversity												
Increase 10 species	1.29	1.04, 1.62	0.98	0.70, 1.37	1.33	1.06, 1.68	1.09	0.76, 1.56	1.36	1.06, 1.74	1.11	0.75, 1.64
Species (tertiles)												
<35	Ref		Ref		Ref		Ref		Ref		Ref	
35–45	1.05	0.56, 1.96	0.64	0.26, 1.63	1.06	0.56, 2.01	0.88	0.33, 2.36	1.27	0.64, 2.50	0.92	0.32, 2.66
≥46	1.43	0.76, 2.68	0.83	0.33, 2.07	1.49	0.78, 2.85	1.17	0.43, 3.19	1.63	0.82, 3.27	1.33	0.46, 3.85
Increase 5 genera	1.27	1.03, 1.56	0.93	0.68, 1.26	1.31	1.06, 1.62	1.02	0.72, 1.42	1.30	1.03, 1.63	1.02	0.71, 1.48
Genera (tertiles)												
<20	Ref		Ref		Ref		Ref		Ref		Ref	
20–25	0.95	0.49, 1.82	1.56	0.62, 3.88	1.02	0.52, 2.01	2.25	0.82, 6.16	1.06	0.52, 2.19	2.05	0.70, 5.99
≥26	1.48	0.80, 2.72	0.81	0.30, 2.22	1.63	0.86, 3.07	1.24	0.41, 3.71	1.55	0.79, 3.03	1.18	0.37, 3.76
Beta diversity												
Cluster 3[a]	0.55	0.28, 1.08	1.18	0.49, 2.84	0.49	0.24, 1.01	1.04	0.39, 2.76	0.41	0.19, 0.89	1.45	0.51, 4.16
PCoA vectors												
1	0.66	0.51, 0.87	0.83	0.57, 1.22	0.62	0.47, 0.82	0.81	0.54, 1.23	0.64	0.47, 0.85	0.88	0.56, 1.37
2	0.73	0.56, 0.95	0.59	0.40, 0.86	0.73	0.55, 0.97	0.69	0.46, 1.04	0.80	0.59, 1.08	0.73	0.47, 1.14

Note: Reference group is never smokers; PCoA: Principal coordinate

[a] Compared to Cluster 2 and excludes 4 men who were in Cluster 1

[b] Adjusts for age, BMI, DMFT score, balloon type, alcohol consumption, and village

[c] Excludes 33 men who reported taking antibiotics in the past 3 months; adjusts for all confounders mentioned in B