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The association between smoking and gut microbiome in Bangladesh

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Abstract

Introduction: Epidemiological studies that investigate alterations in the gut microbial composition associated with smoking are lacking. This study examined the composition of the gut microbiome in smokers compared with non-smokers.

Methods: Stool samples were collected in a cross-sectional study of 249 participants selected from the Health Effects of Arsenic Longitudinal Study (HEALS) in Bangladesh. Microbial DNA was extracted from the fecal samples and sequenced by 16S rRNA gene sequencing. The associations of smoking status and intensity of smoking with the relative abundance or the absence and presence of individual bacterial taxon from phylum to genus levels were examined.

Results: The relative abundance of bacterial taxa along the *Erysipelotrichi*-to-*Catenibacterium* lineage was significantly higher in current smokers compared to never smokers. The odds ratio comparing the mean relative abundance in current smokers with that in never smokers was 1.91 (95% confidence interval [CI] = 1.36 to 2.69) for the genus *Catenibacterium* and 1.89 (95% CI = 1.39 to 2.56) for the family *Erysipelotrichaceae*, the order *Erysipelotrichale*, and the class *Erysipelotrichi* ((FDR-adjusted p-values = 0.0008 to 0.01). A dose-response association was observed for each of these bacterial taxa. The presence of *Alphaproteobacteria* was significantly greater comparing current with never smokers (OR = 4.85, FDR-adjusted p-values = 0.04).

Conclusions: Our data in a Bangladeshi population are consistent with evidence of an association between smoking status and dosage with change in the gut bacterial composition.

Implications: This study for the first time examined the relationship between smoking and the gut microbiome composition. The data suggest that smoking status may play an important role in the composition of the gut microbiome, especially among individuals with higher levels of tobacco exposure.

Key Words: HEALS; Microbial composition; Smoking dosage; Smoking status; Relative abundance

INTRODUCTION

Despite the known and deadly risks of cigarette smoking¹, the prevalence of tobacco smoking has continued to rise in developing countries such as Bangladesh and other South Asian countries². Tobacco use is a risk factor for 6 of the 8 leading causes of death in the world, according to the World Health Organization³. The majority of the world's current smokers now live in low to middle-income countries, with South Asia being one of the largest areas on the globe for tobacco production and consumption⁴, making the Bangladeshi population of interest to study the effects of tobacco use.

The microbiota has emerged as having a potential role in smoking-related pathogenesis. The gut microbiota is composed of 10^{13} to 10^{14} microorganisms involved in physiological function such as digestion and metabolism, of which the majority are bacteria colonized from birth⁵. Modifications of the gut microbiota have been associated with chronic conditions such as obesity and inflammatory bowel disease⁶. Even with a neutralized diet, the intestinal microbial composition of smokers and non-smokers is different⁷. Several studies in humans have reported that smoking is related to differences in the microbiome measured in saliva⁸, sputum⁹, subgingival¹⁰, upper GI¹¹, throat¹², middle meatus¹³ and bronchial wash¹⁴ samples. Mouse and rat models in the gut microbiota have shown significant changes in response to nicotine and smoke exposure¹⁵. Although a few studies have investigated whether the gut microbiome differs by smoking status¹⁶⁻¹⁸, these studies included mostly Crohn's disease patients or had a small sample size. Epidemiological studies in the general population exploring the effect of smoking on the composition of the gut microbiome are lacking.

In this study, we examined the relationship between cigarette/bidi smoking and the gut microbiome in 250 participants, recruited from a large prospective cohort study, who provided fecal samples for 16s rRNA gene sequencing and completed lifestyle questionnaires.

METHODS

Study Population

Data was collected for this study as part of the Health Effects of Arsenic Longitudinal Study (HEALS), which is an ongoing, prospective cohort study located in Arai hazar, Bangladesh. Details of the study were previously published and will be summarize briefly here¹⁹. Between October 2000 and May 2002, an original cohort consisting of 11,746 married adults (to reduce loss to follow-up) between the ages of 18-75 years old were recruited from a well-defined 25 km² geographical area. An expansion cohort of an additional 8,287 participants was recruited between 2006 and 2008. Trained physicians performed in-person visits biennially to collect demographic and lifestyle data using a standardized questionnaire¹⁹. The overall participation rate, estimated by the proportion of subjects who agreed to participate among the potential participants invited to the study, was 97%. Cohort participants received medical treatment at a field clinic that was set up for this purpose and to assist in follow-up and ancillary studies. The Ethical Committee of the Bangladesh Medical Research Council and the Institutional Review Boards of Columbia University and the University of Chicago approved all study procedures. Informed consent was obtained from all of the study participants.

The study presented here was conducted to assess the relationship between smoking and the gut microbiome. Between February 2015 and November 2016, HEALS participants were recruited from

the six villages surrounding the clinic to obtain fecal sample collection. Four hundred participants between the ages of 25 and 50 years old free from any major illness were randomly selected. The distribution of demographics or lifestyle variables was not significantly different from the overall HEALS participants (data not shown). A total of 328 of the 400 fulfilled the eligibility requirements including absence of antibiotic use in the previous month and willingness to come to the clinic to provide stool samples and complete lifestyle questionnaires. Of the 328 eligible participants, 300 were recruited and 250 of these participants provided fecal samples.

Fecal Specimen Collection

Stool from a single bowel movement was collected in an empty ThermoFisher Scientific vial (Waltham, MA, USA) given to participants by a senior research officer at the field clinic for fecal sample collection. Immediately stored in a -20°C freezer, samples were kept frozen until processing at the field laboratory for DNA extraction.

Assessment of Tobacco Smoking Variables

Detailed information on status of smoking was collected around the time of stool sample collection (never, former, and current). Information on number of sticks per day was asked together regarding smoking cigarettes and bidis. Bidis are filterless locally produced thin cigarettes filled with tobacco and wrapped in leaves. Cigarettes and bidis are often sold individually in Bangladesh, however to facilitate comparison with other studies, we calculated packs per day as the number of sticks smoked per day divided by 20. Time since quitting smoking was calculated as the difference between the age at smoking cessation and the age at the time of the study visit. Current smokers of cigarettes and bidis were categorized as current smokers and past smokers of any tobacco products were classified as former smokers.

DNA Extraction and 16S rRNA Gene Sequencing

Total DNA was extracted from fecal samples at the field laboratory using the MOBIO PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The 16S rRNA gene was amplified targeting the hypervariable V3-V4 region using universal primer set 347F 5'-GGAGGCAGCAGTRRGAAT and 803R 5'-GACTACHVGGGTATCTAATCC. Agencourt AMPure XP (Beckman Coulter Life Sciences, IN) was used to purify PCR products and Agilent 4200 TapeStation (Agilent Technologies, CA) was used to quantify PCR products. Performed polymerase chain reaction (PCR) amplification and sequencing methods are described in further detail previously²⁰. The Illumina MiSeq 300-cycles (2x300bp) reagent kit (Illumina, Inc., San Diego, CA) was used to pool and sequence Amplicon libraries at equimolar concentrations. One sample was not sequenced further and was excluded for failing PCR amplification.

Bioinformatics and Quality Control

QIIME (Quantitative Insights into Microbial Ecology) 1.8.0²¹ was used to process sequencing data. Once demultiplexed, sequencing reads with low quality scores of <25 were removed. ChimeraSlayer²² was used to take out chimeric sequences.

Pre-processed sequences were clustered into operational taxonomic units (OTUs) at 97% identity using UCLUST²³ against the Greengenes database 13.8 with the most abundant sequence in each OTU selected as a representative. Greengenes database 13.8 and Ribosomal Database Project (RDP) Classifier 2.2²⁴ was used to assign taxonomy for each OTU from phylum to genus levels. QIIME was used to estimate several alpha diversity measures at the OTU level based on rarefied sequence count (10,000 sequences per sample). By calculating the number of observed OTUs and Chao1 richness estimator, microbial richness, a measure of the number of taxa in each sample (or the

abundance of microbes), was examined²⁵. The Shannon diversity index was used to assess evenness, a measure of the relative number of different taxa in each sample (or the distribution of the different microbes),²⁶.

Statistical Analyses

Descriptive analysis was conducted to compare demographic, lifestyle, and alpha diversity measures by smoking status using chi-square tests for categorical variables and t-tests for continuous variables.

The number of reads for each taxon was divided by the total number of reads from all taxa within each individual sample to calculate relative abundance at the levels of phylum, class, order, family, and genus.

The association between smoking status and beta diversity of the gut microbiome measured by unweighted Unifrac distance²⁷, weighted Unifrac distance²⁸ and Bray-Curtis distances matrices²⁹ was evaluated using the microbiome regression-based kernel association test MiRKAT³⁰ adjusting for sex, age, BMI, betel quid use and education. The omnibus test (optimum MiRKAT) which simultaneously considers the above listed distances matrices was conducted to report the overall result, since each individual distance matrix measurement just captures a specific association pattern.

To evaluate the association between smoking status and the relative abundance of gut bacterial taxa, we used beta regression adjusting for sex, age, BMI, betel quid use and education, using the R package 'betareg'³¹. Beta regression was considered because this model is useful in microbiome studies where the continuous non-zero mean relative microbial abundance is the variable of interest and is restricted to the interval (0, 1)³². Bacterial taxa with zero abundances were imputed as 10^{-6}

because the logarithm of zero is undefined. The regression coefficient in the beta regression is the log odds of the mean relative abundance of the specific taxa in smokers compared to non-smokers. The exponent of the coefficient is the odds ratio of mean relative abundance comparing the two groups. We also used logistic regression models to assess the association between smoking status and the presence/absence of the gut bacterial taxa, adjusting for sex, age, BMI, betel quid use and education. To test this association, the abundance was re-coded as 1 if it was greater than 0, i.e., the taxon was present in the sample. Logistic regression model adjusting for sex, age, BMI, betel quid use and education was then used to fit the data. Only taxa with a prevalence of >10% and <90% were evaluated. The Benjamini-Hochberg procedure was performed for all p-values at each taxonomic rank to control the false discovery rate³³. For bacterial taxa that were nominally significantly associated with smoking status, for either relative abundance or presence/ absence, we further assessed their association with packs of cigarettes smoked. Because smoking was more prevalent in men (76.5%) than in women (13.5%), sensitivity analyses were also conducted in men only. However, the results were similar and therefore sex-specific results were not presented to preserve the power of the analyses. Bacterial taxa that were differentially abundant in current smokers, former smokers, and with smoking dosage, as compared with never smokers, at either nominal or FDR-adjusted level, were plotted on a taxonomic tree (cladogram) using GraPhlAn³⁴. Since the study was originally designed to collect arsenic data, we performed a sensitivity analysis adding arsenic levels as an additional potential confounder in the beta regression model for the taxa that were significant in current compared to never smokers.

RESULTS

Sequencing Results

From the 249 samples, approximately 8.6 million quality-filtered sequencing reads were obtained with an average of 34,520 reads per sample that were clustered into 108,987 OTUs. There was a mean of 3,297 (SD = 1,295) OTUs and a range of 38-8909 OTUs per sample and the OTUs were

classified into 19 phyla, 39 classes, 78 orders, 142 families, and 273 genera. Taxa with a relative abundance of less than 0.01% or without a name at each taxonomic level were removed, which left a total of 8 phyla, 16 classes, 24 orders, 40 families, and 54 genera in the final analyses.

Characteristics of the Study Participants

Characteristics of the study population are shown in **Table 1**. Mean age of the study participants was 48.6 ± 7.9 (mean \pm SD). Average BMI for this lean population was 21.5 ± 4.1 kg/m². Participants had an average of 2.4 years of formal education. Twenty-five percent of study participants were current smokers (n=62) and 14% were former smokers (n=36), while 61% never smoked (n=151). An average of $0.50 \pm .31$ packs of cigarettes/bidis per day were smoked by current smokers. Former smokers similarly consumed 0.47 ± 0.49 packs per day. Current and former smokers were predominantly male (88.7% and 63.9% respectively), slightly older and leaner than those who never smoked and were more likely to have ever used betel quid. Current smokers were less educated compared to never smokers. Blood pressure was not associated with smoking status, nor were alpha diversity measurements.

Alpha diversity measurements compared by smoking status also did not exhibit significant differences (**Table 1**). The association between smoking status and microbiome beta diversity measured by Unifrac distance, weighted Unifrac distance and Bray-Curtis was not significant (**Table 2**).

Associations of Smoking with Microbial Composition

The relative abundances of 14 taxa were nominally significantly associated with smoking status when comparing current smokers and never smokers (**Table 3**). Bacterial taxa along the

Erysipelotrichi-to-*Catenibacterium* lineage were significantly enriched among current smokers compared with never smokers after correction for multiple comparisons. The odds ratios comparing the mean relative abundance in current smokers with that in never smokers was 1.91 for genus *Catenibacterium* (FDR-adjusted $p = 0.01$), 1.89 for the family *Erysipelotrichaceae* (FDR-adjusted $p = 0.002$), 1.89 for order *Erysipelotrichales* (FDR-adjusted $p = 0.001$), and 1.89 for class *Erysipelotrich* (FDR-adjusted $p = 0.0008$). Bacterial taxa along the *Coriobacteriia*-to-*Collinsella* lineage were also enriched among current smokers in comparison to never smokers; however, the FDR-adjusted p -values were not significant.

The presence/absence of five taxa were nominally significant in current smokers compared to never smokers (**Table 4**), with the class *Alphaproteobacteria* remaining significant after multiple comparisons correction (FDR-adjusted $p=0.04$). Current smokers were 4.85 times more likely to have this class present in their microbiome than never smokers.

For bacterial taxa that were nominally significantly associated with smoking status for either relative abundance or presence/ absence, we further assessed their association with packs of cigarettes smoked. All of these four taxa in the *Erysipelotrichi*-to-*Catenibacterium* lineage exhibited a dose-response relationship (FDR-adjusted $p = 0.01$ for *Catenibacterium*, FDR-adjusted $p = 0.004$ for *Erysipelotrichi*) with increasing mean relative abundance as packs per day of cigarettes/bidis increased (**Table 5**). An increase in one pack per day was associated with an odds ratio of 1.88-1.90 for mean relative abundance for these specific taxa. The family *Peptostreptococcaceae*, the genera *Slackia*, and *Collinsella* also had increasing mean relative abundance with increasing smoking dosage with odds ratios of 1.67, 2.75 and 1.73 associated with an increase of 1 pack per day (FDR-adjusted $p = 0.04$, 0.0004, 0.03), respectively. The presence of the genera *Slackia* and *Mitsuokella* were also

related to increased smoking dosage and were 28.5 and 20.7 more likely to be present with an increase of 1 pack per day respectively (FDR-adjusted $p=0.001, 0.048$) (**Table 5**).

In former smokers compared to never smokers, the relative abundances of 4 taxa, including 3 that remained significant after adjusting the p-values for multiple comparisons testing in current smokers (the family *Erysipelotrichaceae*, order *Erysipelotrichales*, and class *Erysipelotrichi*) were nominally associated with smoking status (**Supplemental Table 1**). A total of 12 taxa had higher prevalence among former smokers relative to never smokers at the nominal level (**Supplemental Table 2**), although none of the associations remained significant after correcting for multiple comparisons.

For the four taxa that were significantly associated with current smoking (class *Erysipelotrichi*, order *Erysipelotrichale*, family *Erysipelotrichaceae*, and genus *Catenibacterium*) after adjusting for multiple comparisons (three of which were nominally significant in the former smokers), there was also a dose-response relationship between their relative abundance and the intensity of smoking.

However, there was no correlation between the time since quitting and the relative abundance of these four taxa using linear regression adjusting for sex, age, BMI, education and betel quid use.

However, our sample size was limited for this analysis. Future studies with a larger sample size are needed to confirm the findings.

Figure 1 shows an association map of the taxa that were significantly related to smoking status and smoking dosage (packs per day). The nodes on the tree first branch into each phylum, which are shown by differently colored sections, then class, order, family and genera. The red highlighted taxa are those whose abundance was associated with smoking. The orange highlighted taxa represent those for which their presence/absence was associated with smoking. The taxa for which both abundance and presence/absence were associated with smoking are highlighted in blue.

Sensitivity Analysis

There were no significant differences in arsenic levels between the current smokers, former smokers and control group. Adding arsenic as an additional potential confounder into the beta regression model for the relative abundance of the 4 taxa along the *Catenibacterium*-to-*Erysipelotrichi* lineage that were significantly different between current and never smokers, after adjusting for multiple comparisons, did not change the results. In this model, the odds ratio comparing the mean relative abundance in current smokers with that in never smokers was 1.89 (95% confidence interval [CI] = 1.34 to 2.66) for the genus *Catenibacterium* (p-value = 0.0002) and 1.89 (95% CI = 1.39 to 2.56) for the family *Erysipelotrichaceae*, the order *Erysipelotrichales*, and the class *Erysipelotrichi* (p-values < 0.0001).

DISCUSSION

In a Bangladeshi population, we found that the relative abundance of bacterial taxa along the *Erysipelotrichi*-to-*Catenibacterium* lineage, including the genus *Catenibacterium*, the family *Erysipelotrichaceae*, order *Erysipelotrichales*, and class *Erysipelotrichi* were significantly higher in current smokers compared to never smokers, with each taxon exhibiting a dose-response relationship with packs of cigarettes smoked per day. The class *Alphaproteobacteria* was significantly more prevalent in current compared to never smokers (FDR adjusted $p = 0.04$). Genus *Slackia*, genus *Collinsella*, and other bacterial taxa along the same lineages, including family *Coriobacteriaceae*, order *Coriobacteriales*, and class *Coriobacteriia* were also enriched among current smokers in comparison to never smokers; however, the FDR-adjusted p-values were not significant. The data suggest a critical role of smoking in gut microbiome composition.

Data from this study suggests effect of cigarette smoking on the relative abundance of certain bacterial taxa in the gut remained even after smoking cessation. Of all the bacterial taxa in the gut that had significantly higher levels or had a significantly higher proportion of presence at the nominal level in current smokers compared with never smokers, the levels or presence of five bacterial taxa were also nominally significantly higher in former smokers, compared with never smokers. These taxa included class Alphaproteobacteria, class Erysipelotrichi, order Erysipelotrichale, family Erysipelotrichaceae, and genus Slackia (comparing results in Table 3 vs. Supplemental Table 1, in Table 4 vs. Supplemental Table 2). The association comparing former smokers and never smokers was weaker than that comparing current smokers and never smokers, suggesting that the effect, although lasting after quitting smoking, may diminish overtime. All other taxa that were nominally significant in the current vs never smokers are not seen in former smokers.

Several explanations have been proposed for the dysbiosis created by smoking, the simplest being cigarette exposure directly causes specific bacteria to enter the gut ³⁵. Other hypotheses include immunomodulation ^{15,36}, oxidative stress ³⁶⁻³⁸ or molecular changes to the cellular tight junctions and mucin composition of the gut ^{15,36}.

Epidemiologic studies on the association between cigarette smoking and gut microbiome measured in stool samples is limited. Several studies in humans have reported that smoking is related to differences in the microbiome measured in saliva ⁸, sputum ⁹, subgingival ¹⁰, upper GI, throat ¹², middle meatus ¹³ and bronchial wash ^{11,14} samples. Given variations in the gut microbiome between communities, it is difficult to directly compare across studies. Nonetheless, we found some similarities to other studies. Gram-positive class *Coriobacteriia* was nominally significant in our study in current vs never smokers, and was identified as enriched in current smokers in the oral microbiome ^{8,39}. This class has been implicated in oral diseases such as periodontitis, halitosis, and endodontic infections ⁶ and these diseases have also been associated with smoking ^{40,41}.

Several studies have investigated cigarette smoking in relation to the gut microbiome in patients with Crohn's disease. One study showed a decrease in richness, genus and species diversity and reduced relative abundance of *Collinsella*, *Enterobacter* and *Gordonibacter*¹⁷. Our study found a significant increase in *Collinsella* among current smokers, in contrast to the findings from this study, however we also found a dose response with *Collinsella* rendering the increase likely to be associated with smoking. *Collinsella* is well suited to colonize mucosal surfaces, metabolizes amino acids, and may directly interact with the host⁶. Higher abundance of *Collinsella* has been related to type 2 diabetes^{42,43} and symptomatic atherosclerosis⁴⁴. A study evaluating the effect of smoking cessation showed a decrease in *Bacteroidetes* (*Prevotella* spp. and *Bacteroides* spp.) and the classes *Alphaproteobacteria* and *Betaproteobacteria* after smoking cessation¹⁸. Interestingly, we found that the class *Alphaproteobacteria*, a gram-negative class in the phylum *Proteobacteria*, was 4.85 times more prevalent in current smokers than those who never smoked in our study. *Alphaproteobacteria* has been directly detected in cigarette samples³⁵.

Our finding of the higher abundance of the *Erysipelotrichi*-to-*Catenibacterium* lineage and the higher prevalence/abundance of the genus *Slackia* in current smokers are highly significant and novel. The genus *Catenibacterium* is a Gram-positive, nonspore-forming and anaerobic genus from the family *Erysipelotrichidae*, which produces short chain fatty acids as products of glucose fermentation. *Catenibacterium* has been associated with higher fat intake in a study of fecal samples in Seoul & Jeju Island in S. Korea⁴⁵. The class *Erysipelotrich* was found to be increased in murine gut in high-fat diets⁴⁶ and related to systemic inflammation⁴⁷. The family *Erysipelotrichaceae* has been linked to dyslipidemic phenotypes and systemic inflammation⁴⁸. The genus *Slackia* has been suggested to be involved in lipid and xenobiotic metabolism⁴⁹, pro-inflammatory, and enriched in prediabetic individuals. Xenobiotic degradation pathways are crucial

in bacterial upregulation to detoxify cigarette smoke. A metagenomics study found an increase in genes involved in xenobiotic metabolism in smokers⁵⁰. It is likely that an increase in tobacco exposure would lead to an increase in bacteria involved in the xenobiotic degradation of the toxins associated with smoking, and our study found a positive correlation between the genus *Slackia* and the amount of cigarettes/bidis smoked per day and also an increased likelihood for this genus to be present with increasing tobacco exposure (28.5 times more likely per 1 pack of cigarettes/bidis), which may explain the increase found in current smokers in our study. Given the many toxicants found in cigarette smoke, it is not surprising to find the higher prevalence and/or abundance of these inflammatory bacteria in smokers. However, whether their enrichment could contribute to smoking-related diseases warrants future studies.

A strength of this study is that it is the first study of the gut microbiome in South Asians, and includes participants who are free from frequent use of medications, supplements and antibiotics that disrupt the microbiome since it was conducted in rural community lacking basic health care services. Further, our findings align with other studies of the effect of smoking on the bacterial microbiome in other areas of the body and provide evidence of a dose-response relationship solidifying the results that certain taxa are more abundant in smokers. Limitations of the study include the cross-sectional design, instead of prospective, however it is not expected to be biased as participation in the study was not likely dependent on both the composition of the gut microbiome and smoking status. Longitudinal studies to evaluate the effect of smoking and smoking cessation on gut microbiome composition would be helpful to determine causality. The smoking dosage was based on the number of packs smoked per day since cotinine was not measured as part of the study. However, the questionnaire used to assess smoking dosage was standard and analyses using the data on all-cause mortality, prostate cancer mortality and multiple myeloma mortality, amongst others, generated similar data consistent with the literature^{4,51-53}. Lastly, 16s rRNA gene sequencing only has

resolution to the genus level, and this lack prevents direct functional profiling. Metagenomic sequencing studies to evaluate the relationship of the gut microbiome and smoking are needed to better understand these mechanisms.

Our data suggest evidence of an association between cigarette/bidi smoking and the relative abundance of the genus *Catenibacterium*, family *Erysipelotrichaceae*, order *Erysipelotrichales*, and class *Erysipelotrichi*, with a dose-response indicating increased smoking exposure leads to an increase in relative abundance, and to the presence of the class *Alphaproteobacteria*. These changes in microbiome composition in association with smoking may be involved in the pathogenesis of several diseases. Further studies are required to investigate the underpinnings of bacterial dysbiosis related to smoking, the impact of smoking on the metagenomic content of the gut microbiome, and whether smoking-related gut microbial and/or metagenomic changes may explain smoking-related disease mechanisms.

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Declaration of Interests

Authors have no competing interests to declare. All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The text of this article was not reviewed by the sponsor. The manuscript is not under review by another journal.

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Figure legend

Figure 1. The association mapping results of current smokers vs. never smokers, former smokers vs. never smokers, and smoking dosage (packs per day) on the taxonomic tree (generated using GraPhlAn³⁴). The nodes on the tree from inner to outer circles are the phylum, class, order, family and genus rank. The corresponding annotations are written in the reverse order.

Taxa whose abundance is associated with smoking status are highlighted in red; taxa whose presence/absence is associated with smoking status are highlighted in orange; taxa where both presence/absence and abundance is associated with smoking status are highlighted in blue. Small circles represent nominal P -value < 0.05 ; larger circles represent adjusted P -value < 0.05 (FDR=5%).

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Table 1. Distribution of population characteristics and alpha diversity metrics by smoking status

	Overall (n = 249)	Smoking Status			P*	P†
		Never	Former	Current		
		(n = 151)	(n = 36)	(n = 62)	Former	Current
BMI, kg/m ²	21.5 ± 4.1	22.5 ± 4.2	20.8 ± 3.4	19.6 ± 3.3	0.03	<0.001
Missing	-	1	-	-		
Male, %	41.0	15.9	63.9	88.7	< 0.001	< 0.001
Female, %	59.0	84.1	36.1	11.3	< 0.001	< 0.001
Age, years	48.6 ± 7.9	46.4 ± 7.5	54.5 ± 5.8	50.5 ± 7.8	< 0.01	< 0.01
Education, years [#]	2.4 ± 3.3	2.7 ± 3.6	1.8 ± 3.0	1.8 ± 1.1	0.15	0.03
Ever betel quid use, %	50.6	43.1	69.4	58.1	<0.01	0.046
Packs per day	0.19 ± 0.34	0	0.47 ± 0.49	0.50 ± .31	<0.001	<0.001
Systolic blood pressure, mmHg	112.0 ± 17.4	112.1 ± 17.8	116.9 ± 20.1	108.8 ± 14.2	0.15	0.15
Diastolic blood pressure, mmHg	71.1 ± 11.5	71.3 ± 11.3	72.3 ± 12.4	69.8 ± 11.5	0.63	0.40
Observed OTUs	3297 ± 1295	3209 ± 1331	3497 ± 1045	3394 ± 1336	0.23	0.36
Chao1 richness estimator	9943 ± 3587	9693 ± 3695	10623 ± 3068	10156 ± 3589	0.16	0.40
Shannon diversity index	4.5 ± 0.5	4.5 ± 0.6	4.5 ± 0.5	4.5 ± 0.5	0.62	0.83
Simpson diversity index	0.92 ± 0.04	0.92 ± 0.04	0.92 ± 0.04	0.92 ± 0.04	0.72	0.87

Data are presented as mean ± standard deviation (SD).

*P values were computed with the t-test or chi-square in comparison to never smokers.

[†] $n = 248$ (missing data for one person).

[#] Assessed at baseline.

Table 2. P-values of the association tests between microbiome diversity and smoking status using MiRKAT^{*}

Smoking Status	Unweighted	Weighted	Bray-Curtis	Omnibus [†]
	Unifrac	Unifrac		
Former vs. Never	0.6869	0.6586	0.7917	0.8480
Current vs. Never	0.0941	0.5342	0.2413	0.2210
Smoking dosage (pack)	0.2241	0.8524	0.6630	0.3630

^{*}The model has been adjusted for sex, age, BMI, betel quid use, and education.

[†]The omnibus test simultaneously considers unweighted Unifrac, weighted Unifrac and Bray-Curtis distances.

Table 3: Odds ratio of mean relative abundances comparing current smokers with never smokers

Taxonomy	Odds Ratio* (95% CI)	P-value	FDR adjusted P-value [†]
Class <i>Erysipelotrichi</i>	1.89 (1.39, 2.56)	0.0001	0.0008
Class <i>Coriobacteria</i>	1.46 (1.03, 2.06)	0.03	0.25
Order <i>Erysipelotrichales</i>	1.89 (1.39, 2.56)	0.0001	0.0012
Order <i>Coriobacteriales</i>	1.46 (1.03, 2.06)	0.03	0.35
Order <i>Turicibacterales</i>	1.48 (1.01, 2.17)	0.04	0.35
Family <i>Erysipelotrichaceae</i>	1.89 (1.39, 2.56)	0.0001	0.002
Family <i>Mogibacteriaceae</i>	1.52 (1.05, 2.19)	0.02	0.34
Family <i>Coriobacteriaceae</i>	1.46 (1.03, 2.06)	0.03	0.34
Family <i>Peptostreptococcaceae</i>	1.44 (1.02, 2.03)	0.04	0.34
Family <i>Turicibacteraceae</i>	1.48 (1.01, 2.17)	0.04	0.34
Genus <i>Catenibacterium</i>	1.91 (1.36, 2.69)	0.0002	0.01
Genus <i>Collinsella</i>	1.61 (1.13, 2.30)	0.01	0.21
Genus <i>Turicibacter</i>	1.48 (1.01, 2.17)	0.04	0.50
Genus <i>Rothia</i>	1.43 (1.01, 2.05)	0.047	0.50

*Odds Ratio of the mean relative abundance of taxa comparing current smokers to never smokers adjusting for age, sex, BMI, education, and betel quid use using beta regression with logit transformation based on continuous variable of relative abundance of taxa, replacing zeros with 10^{-6} . A total of 142 bacterial taxa were tested. Only the ones with nominal significance were listed.

[†]P values were corrected for multiple testing using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) \leq 5%.

Table 4: Odds ratio of the presence/absence of bacterial taxa comparing current smokers with never smokers

Taxonomy	Odds Ratio* (95% CI)	P-value	FDR adjusted P- value [†]
Class <i>Alphaproteobacteria</i>	4.85 (1.53, 15.39)	0.01	0.04
Order <i>GMD14H09</i>	0.30 (0.11, 0.82)	0.02	0.19
Family <i>Christensenellaceae</i>	3.62 (1.00, 13.09)	0.0499	0.63
Genus <i>Slackia</i>	2.89 (1.12, 7.41)	0.03	0.27
Genus <i>Mitsuokella</i>	3.84 (1.08, 13.67)	0.04	0.27

*Odds Ratio of the presence probability of taxa comparing current smokers to never smokers adjusting for age, sex, BMI, education, and betel quid use using logistic regression based on the dichotomous variable for presence/absence of taxa with a prevalence of >10% and <90%. A total of 142 bacterial taxa were tested. Only the ones with nominal significance were listed.

[†]P values were corrected for multiple testing using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) \leq 5%.

Table 5. Odds ratio of mean relative abundances or presence/absence of bacterial taxa associated with one increase in packs smoked.

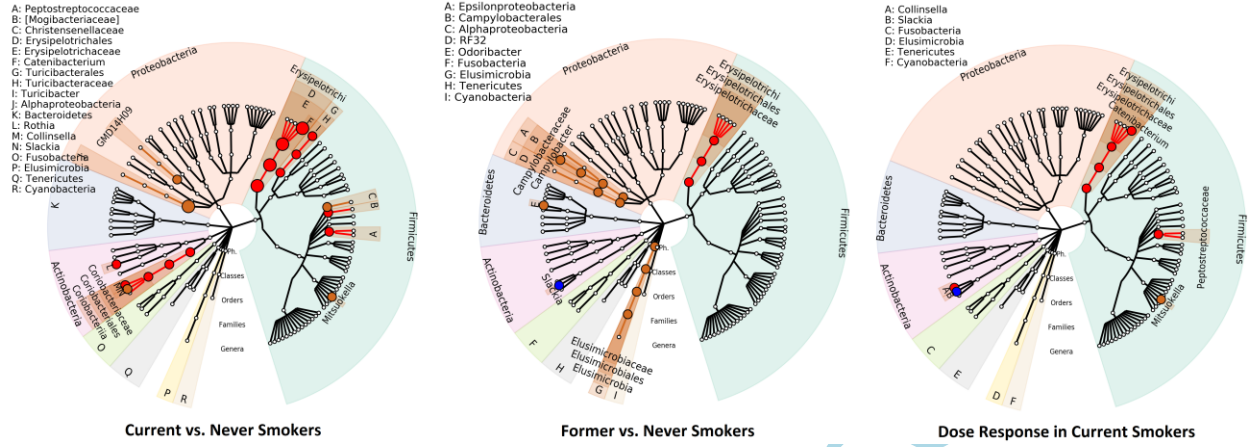
Taxonomy	Odds Ratio* (95% CI)	P-value
Abundance association test [†]		
Class <i>Erysipelotrichi</i>	1.88 (1.22, 2.89)	0.004
Order <i>Erysipelotrichales</i>	1.88 (1.22, 2.89)	0.004
Family <i>Erysipelotrichaceae</i>	1.88 (1.22, 2.89)	0.004
Family <i>Peptostreptococcaceae</i>	1.67 (1.02, 2.69)	0.04
Genus <i>Slackia</i>	2.75 (1.57, 4.76)	0.0004
Genus <i>Catenibacterium</i>	1.90 (1.17, 3.03)	0.01
Genus <i>Collinsella</i>	1.73 (1.05, 2.89)	0.03
Presence/Absence association test [#]		
Genus <i>Slackia</i>	28.5 (3.9, 208.51)	0.001
Genus <i>Mitsuokella</i>	20.7 (1.03, 411.58)	0.048

*Odds Ratio of the mean relative abundance of taxa (abundance association test) or the mean presence probability of taxa (presence/absence association test) comparing smokers to never smokers adjusting for age, sex, BMI, education, and betel quid use.

[†]Beta regression with logit transformation based on the continuous variable of relative abundance of taxa, replacing zeros with 10^{-6} .

[#]Logistic regression based on the dichotomous variable for presence/absence of taxa with a prevalence of >10% and <90%.

Figure 1



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