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Cigarette smoking and oral microbiota in low-income and African-American populations

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Abstract

Background—Cigarette smoking is a common risk factor for diseases and cancers. Oral microbiota is also associated with diseases and cancers. However, little is known about the impact of cigarette smoking on the oral microbiota, especially among ethnic minority populations.

Methods—We investigated cigarette smoking in relationship with the oral microbiota in a large population of predominately low-income and African-American participants. Mouth rinse samples were collected from 1616 participants within the Southern Community Cohort Study, including 592 current-smokers, 477 former-smokers and 547 never-smokers. Oral microbiota was profiled by 16S ribosomal RNA gene deep sequencing.

Results—Current-smokers showed a different overall microbial composition from former-smokers ($p=6.62\times10^{-7}$) and never-smokers ($p=6.00\times10^{-8}$). The two probiotic genera, *Bifidobacterium* and *Lactobacillus*, were enriched among current-smokers when compared with never-smokers, with Bonferroni-corrected p values ($P_{\text{Bonferroni}}$) of 1.28×10^{-4} and 5.89×10^{-7},

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respectively. The phylum *Actinobacteria* was also enriched in current-smokers when compared with never-smokers, with a median relative abundance of 12.35% versus 9.36%, respectively, and with a $P_{\mathrm{Bonferroni}}=9.11\times10^{-11}$. In contrast, the phylum *Proteobacteria* was depleted in current smokers ($P_{\mathrm{Bonferroni}}=5.57\times10^{-13}$), with the relative abundance being almost three times that of never-smokers (7.22%) when compared with that of current-mokers (2.47%). Multiple taxa within these two phyla showed differences in abundance/prevalence between current-smokers and never-smokers at $P_{\mathrm{Bonferroni}}<0.05$. The differences in the overall microbial composition and abundance/prevalence of most taxa were observed among both African-Americans and European-Americans. Meanwhile, such differences were not observed between former-smokers and never-smokers.

Conclusion—Smoking has strong impacts on oral microbial community, which was recovered after smoking cessation.

INTRODUCTION

The human mouth nourishes over 2000 types of microbes, which collectively compose the oral microbiota.[1] Well-balanced oral microbiota maintains oral and systemic health,[2] while dysbiosis of oral microbiota may lead to diseases.[3–5]

Cigarette smoking is a common risk factor for many diseases. Various toxicants in cigarette smoke directly contact with oral microbes; thus, long term exposure to smoking toxicants may affect the microbial ecology in oral cavity due to antibiotic effects and oxygen deprivation.[6] Studies have shown the impact of cigarette smoking on the oral microbiota,[7–11] though the results were inconsistent across studies. For example, in two previous studies investigating subgingival[9] and oral wash samples,[10] current-smokers showed a lower microbial diversity than non-smokers. However, in a subsequent study, such a difference was not observed in any of the eight oral sites investigated.[11] In addition, most of these studies focused on European-ancestry populations. Studies among ethnic minority populations, for example, African-Americans, are lacking.

In the study presented here, we investigated the impact of cigarette smoking on the oral microbiota using data from 1616 participants (1058 African-Americans and 558 European-Americans) within the Southern Community Cohort Study (SCCS), including 592 current-smokers, 477 former-smokers and 547 never-smokers.

METHODS

Study population

Launched in 2002, the SCCS was designed to investigate health disparities among low-income populations, with the majority of the study participants being African-American. Detailed descriptions of the SCCS can be accessed elsewhere.[12] Briefly, this study took 7 years to recruit over 85 000 middle-aged adults (40–70 years old) from 12 southeastern US states. In total, ~34 100 participants donated mouth rinse samples during the enrolment. The SCCS was reviewed and approved by review boards at the Vanderbilt University Medical Center and the Meharry Medical College. Written informed consent was provided by all involved individuals.

During enrolment, all participants were requested to complete a comprehensive questionnaire that was designed to collect individuals' personal information, including smoking status. After recruitment, follow-ups were performed through record linkage and surveys via mail or telephone. Major health outcomes were ascertained via linkage with state cancer registries and/or from National Death Index mortality records. Participants included in the present study were selected from four nested case-control studies for incident cases (ascertained during the first follow-up) of upper aerodigestive tract cancer, type 2 diabetes, lung cancer and colorectal cancer (n=1864). All participants were free of diseases when mouth rinse samples were collected. After excluding individuals who did not report a smoking history or disclosed a history of antibiotics usage during the year before their mouth rinse sample donation, the current study included 1616 subjects.

DNA extraction and 16S ribosomal RNA (rRNA) gene sequencing

The Qiagen's QIAmp DNA kit (Qiagen, Germantown, Maryland, USA) was used to extract DNA from mouth rinse samples. The NEXTflex 16S rRNA gene V4 Amplicon-Seq Kit (Bioo Scientific, Austin, Texas, USA) was used to construct the sequencing libraries of the 16S rRNA gene V4 domain. Sequencing was conducted using the Illumina MiSeq 300 (paired-end 150 bp) at the Vanderbilt Technologies for Advanced Genomics Core or using the Illumina Hiseq (paired-end 250 bp) at BGI Americas (Cambridge, Massachusetts, USA). For both sequencing batches, on each 96-well plate, two additional duplicated quality control samples were sequenced. All duplicate samples showed similar microbial profiles: the coefficient of variability for the Faith's Phylogenic Diversity (PD) index (a measurement of microbial community diversity) among the duplicate samples was 0.3%.

Sequencing data processing and quality controls

Raw data from two sequencing batches were processed together by QIIME,[13] using the closed-reference operational taxonomic unit calling strategy. Taxonomy assignment was conducted using the Human Oral Microbiome Database[14] (HOMD) as reference. In total, 100 153 658 reads (mean±SD = 102 302±77 432; range = (5323–854 744)) were obtained for the 956 samples from the first batch and 30 506 499 reads (mean±SD = 47 741±11 628; range = (20 428–91 660)) were retained for the 660 samples from the second batch.

Statistical analysis

The alpha diversity of each sample was measured by the Faith's PD index. The associations of alpha diversity with potential confounders, including age, sex, race, body mass index (BMI), alcohol consumption, total energy intake, oral health status, disease status at the first follow-up and sequencing batch were estimated through a linear regression analysis. The differences of beta diversity among the three smoking groups were assessed by using MiRKAT[15] V.0.02, based on the weighted UniFrac distance matrix. We also evaluated the differences of beta diversity between current-smokers and non-smokers (including former-smokers and never-smokers).

Cigarette smoking has been associated with weight loss,[16] and recently, multiple animal studies and human clinical trials have reported associations between weight loss and several probiotic bacteria, mainly belonging to the genera *Bifidobacterium* and *Lactobacillus*.[17–20]

Hence, we compared the prevalence of these two genera, along with the species belonging to them, between current-smokers and never-smokers, between former-smokers and never-smokers, and between current-smokers and non-smokers, through logistic regression analyses.

For other taxa, we focused on four taxonomic levels: phylum, family, genus and species. Similar with the analyses for probiotic taxa, differences of these taxa between current-smokers and never-smokers, between former-smokers and never smokers and between current-smokers and non-smokers were investigated. Based on the relative abundance among never-smokers, taxa were categorised as ‘common taxa’ (with a median abundance of ≥0.1%) or ‘rare taxa’ (with a median abundance of <0.1%). For common taxa (five phyla, 15 families, 16 genera and 28 species), relative abundance was normalised by arcsine-square-root transformation, and a linear regression analysis was performed for each taxon to estimate the association of smoking status with the arcsine-square-root transformed taxon relative abundance. For rare taxa, in addition to those probiotic taxa, analyses were limited to those with a prevalence among never-smokers of >30%, including three phyla, 16 families, 35 genera and 98 species. After grouping participants into carriers and non-carriers, a logistic regression analysis was conducted for each taxon to investigate smoking status in association with taxon prevalence.

Among all of the analyses described above, adjustments were made in regression models for potential cofounders, including age, sex, race, BMI, alcohol consumption, total energy intake, oral health status, disease status at the first follow-up and sequencing batch. For each of these covariates, missing data were indicated with a dummy variable and included in regression analyses. Given the intrinsic correlations among taxa from different taxonomic levels, not all association tests were independent. Following Galwey’s method,[21] we estimated the number of independent tests for common taxa and rare taxa (including probiotic taxa) separately using the function ‘meff’ of the R package ‘poolR’ (https://rdrr.io/github/ozancinar/poolR/). Among the 64 common taxa and the 152 rare taxa included in the statistical analyses, the independent tests were estimated to be 25 and 69, respectively. For the associations with a Bonferroni-corrected $p<0.05$, that is, $p<2.00\times10^{-3}$ for common taxa and $p<7.25\times10^{-4}$ for rare taxa, we further performed stratified analyses by race, as well as by sequencing batch, to evaluate the heterogeneity between African-Americans and European-Americans, and between the first and the second sequencing batch. All analyses were carried out using R V.3.3.1.

RESULTS

Characteristics of the study participants

The general profile of study participants’ characteristics is shown in table 1. In total, 1616 individuals were included in this study, including 36.6% current-smokers, 29.5% former-smokers and 33.9% never-smokers. Among the African-Americans, 39.1% were current-smokers, 24.9% were former-smokers and 36.0% were never-smokers. Among the European-Americans, a higher percentage of participants were former-smokers (38.4%), with 31.9% being current-smokers and 29.7% never-smokers. Current-smokers tended to have the lowest BMI and never-smokers had the highest BMI. Overall, the study participants had a very low socioeconomic status. Specifically, ~64% of the current-smokers had an annual household income of less than US$15 000. Only 65.8% of the study participants had oral health status data, and the majority of them had poor oral health. Specifically, current-smokers had the worst oral health, with ~90% having tooth loss, while ~80% of the non-smokers had tooth loss. We found associations of alpha diversity (Faith's PD index) with race, age, alcohol drinking, tooth loss and sequencing batch at p<0.05.

Current-smokers showed a different overall composition when compared with never-smokers and former-smokers

Differences in beta-diversity (weighted UniFrac matrices) were observed between current-smokers and never-smokers (p=6.00×10^{-8}), between current-smokers and former-smokers (p=6.62×10^{-7}) and between current-smokers and non-smokers (p<2.20×10^{-16}). Consistently, differences between current-smokers and never-smokers, between current-smokers and former-smokers and between current-smokers and non-smokers, were observed among African-Americans (p values of 9.72×10^{-4}, 6.93×10^{-3} and 3.55×10^{-4}, respectively), European-Americans (p values of 3.51×10^{-4}, 6.85×10^{-5} and 5.15×10^{-7}, respectively), the first sequencing batch (p values of 9.81×10^{-5}, 4.67×10^{-5} and 4.14×10^{-6}, respectively), and the second sequencing batch (p values of 9.72×10^{-4}, 9.09×10^{-5} and 1.83×10^{-5}, respectively). However, between former-smokers and never-smokers, no difference was observed either for either combined analyses, or for stratified analyses by race or sequencing batch.

Probiotic bacterial taxa were enriched among current-smokers

At the genus level, both *Bifidobacterium* and *Lactobacillus* were more prevalent among current-smokers (85.6% and 89.4%) than among never-smokers (67.3% and 73.5%), with Bonferroni-corrected p values ($P_{Bonferroni}$) of 1.59×10^{-4} and 1.81×10^{-4}, respectively (table 2). In addition, one species of *Bifidobacterium* and six species of *Lactobacillus* were also enriched in current-smokers when compared with former-smokers and never-smokers. For example, *Bifidobacterium longum* was observed among 67.6% of current-smokers but only 39.7% of never-smokers ($P_{Bonferroni}=1.80\times10^{-9}$). The prevalence for *Lactobacillus crispatus* was almost two-fold in current-smokers (61.2%) when compared with that in never-smokers (34.4%), with a $P_{Bonferroni}=1.80\times10^{-8}$. Further, all these nine taxa (two genera and seven species) were also significantly more prevalent among current-smokers than among non-smokers. When comparing the former-smokers and never-smokers, none of these probiotic taxa showed a difference.

Actinobacteria were enriched and *Proteobacteria* were depleted among current-smokers

As shown in table 3, the phylum *Actinobacteria* was enriched in current-smokers, with a median relative abundance of 12.4% in current-smokers and 9.4% in never-smokers ($P_{Bonferroni}=3.24\times10^{-11}$). Within *Actinobacteria*, nine common taxa showed a higher abundance in current-smokers than in never-smokers at $P_{Bonferroni}<0.05$, including two families, three genera and four species (table 3 and online supplementary figure S1). Among them, *Rothia mucilaginosa* showed the strongest enrichment with a $P_{Bonferroni}=1.25\times10^{-8}$. In addition to these common taxa, two rare taxa within *Actinobacteria*, *Bifidobacteriaceae* and *Actinomyces lingnae_(NVP)*, showed a higher prevalence in current-smokers than in never-smokers at $P_{Bonferroni}$<0.05 (table 4 and online supplementary figure S2). All of these *Actinobacteria* taxa showed a significant differential abundance/prevalence between current-smokers and non-smokers ($P_{Bonferroni}$<0.05). However, when comparing the former-smokers and never-smokers, none showed a difference (tables 3 and 4).

On the other hand, the phylum *Proteobacteria* was depleted in current-smokers, with the median relative abundance decreased to less than one-third, that is, 2.5% in current-smokers and 7.2% in never-smokers ($P_{Bonferroni}=7.58\times10^{-20}$). In this phylum, nine common taxa showed a lower abundance in current-smokers at $P_{Bonferroni}$<0.05 (table 3 and online supplementary figure S1). Among them, *Neisseriaceae* was the most representative taxon, with the median relative abundance decreased from 1.06% in never-smokers to only 0.06% in current-smokers, which corresponded to a ~18 fold change ($P_{Bonferroni}=1.13\times10^{-23}$). Similarly, within this phylum, seven rare taxa also showed a lower prevalence in current-smokers at $P_{Bonferroni}$<0.05 (table 4 and online supplementary figure S2). For example, the prevalence of *Neisseria oralis* decreased approximately three-fold, which was present in 53.2% of never-smokers but only 19.9% in current-smokers ($P_{Bonferroni}=1.20\times10^{-20}$). Similar to *Actinobacteria*, all of these *Proteobacteria* taxa were also less abundant or prevalent in current-smokers when compared with non-smokers ($P_{Bonferroni}$<0.05), while no such differences were observed between former-smokers and never-smokers (tables 3 and 4).

Taxa in the phyla *Bacteroidetes*, *Firmicutes* and *Spirochaetes* were also associated with smoking status

Multiple taxa within *Bacteroidetes* and *Firmicutes* also showed a different abundance between current-smokers and never-smokers, as well as between current-smokers and non-smokers (table 3 and online supplementary figure S1). For example, in *Bacteroidetes*, *Prevotella sp. oral taxon 313* was more abundant while *Flavobacteriaceae* was less abundant among current-smokers. In *Firmicutes*, *Megasphaera*, *Megasphaera micronuciformis* and *Streptococcus sp. oral taxon 057* were more abundant among current-smokers, while *Gemella*, *Streptococcus oligofermentans* and *Streptococcus sp. oral taxon 070* were more abundant among non-smokers. Within these two phyla, a differential prevalence of seven rare taxa was found between current-smokers and non-smokers (table 4 and online supplementary figure S2). In addition, a rare species of the phylum *Spirochaetes*, *Treponema denticola*, was more prevalent among current-smokers.

Consistent associations of smoking status and oral microbiota between ethnic groups and between sequencing batches

A substantial proportion of the significant associations identified in analyses of all participants (tables 2–4) were consistently observed when stratified by ethnic group or by sequencing batch (online supplementary tables S1–S6). Generally, the associations were much stronger among African-Americans and the first sequencing batch. For example, the probiotic species *Lactobacillus oris* showed a higher prevalence among current-smokers than among never-smokers, with p values of 7.08×10^{-5} in African-Americans and 1.13×10^{-3} in European-Americans (online supplementary tables S1), and p values of 2.19×10^{-6} for the first batch and 6.55×10^{-3} for the second batch (online supplementary table S4). Another example is the common taxa *S. oligofermentans*, which showed higher relative abundance in current-smokers than in never-smokers with p values of 9.33×10^{-13} in African-Americans and 6.23×10^{-8} in European-Americans (online supplementary table S2), and 1.90×10^{-14} in the first sequencing batch and 1.21×10^{-6} in the second batch (online supplementary table S5). However, we also found that some bacterial taxa showed stronger associations among European-Americans than among African-Americans, for example, *Lactobacillus fermentum* (online supplementary table S1), *R. mucilaginosa* (online supplementary table S2) and *Prevotella nanceiensis* (online supplementary table S3). In addition, several taxa showed stronger associations among the second batch than among the first batch, for example, *B. longum* (online supplementary table S4), *Neisseria pharynges* (online supplementary table S5) and *Kingella denitrificans* (online supplementary table S6). However, a formal test of multiplicative interaction failed to show statistical significance.

DISCUSSION

In this study, we found that among both European-Americans and African-Americans, cigarette smoking impacts overall oral microbial composition, as well as the abundance/ prevalence of multiple microbial taxa, especially for those belonging to the probiotic genera *Bifidobacterium* and *Lactobacillus*, and those within the phyla *Actinobacteria* and *Proteobacteria*. However, these changes may be recovered after smoking cessation.

In addition to smoking status, race, age, alcohol drinking, tooth loss and sequencing batch were also associated with oral microbial richness. The associations of race, age, alcohol drinking and tooth loss with the oral microbiota are consistent with previous studies.[22–26] We also found significant differences in overall microbial composition between current-smokers and never-smokers, and between current-smokers and non-smokers, but not between current-smokers and former-smokers, which are consistent with results from previous studies.[9,10]

Cigarette smoking has been associated with weight loss or lower BMI, and smoking cessation has been associated with weight gain or higher BMI.[27,28] In the present study, current-smokers also showed a lower BMI when compared with non-smokers. Interestingly, we found that two probiotic genera, *Bifidobacterium* and *Lactobacillus*, together with seven species belonging to them, were more prevalent among current-smokers when compared with never-smokers and former-smokers. Similar results were reported by two previous studies.[9,10] For example, in one study, *Bifidobacterium*, *Lactobacillus* and *B. longum* showed a higher abundance among current-smokers.[10] In the other, *L. fermentum*, *L. gasseri* and *L. reuteri* were enriched in current-smokers.[9]

The phylum *Actinobacteria*, along with 11 taxa belonging to it, was enriched in current-smokers. Among them, *Actinobacteria*, *Atopobium* and *R. mucilaginosa* were consistently reported to be enriched in oral wash samples of current-smokers.[10] *Actinomyces odontolyticus* was observed to be enriched in subgingival samples of current-smokers.[9] The phylum *Proteobacteria* and 16 taxa within it were depleted among current-smokers. Many of them, including *Proteobacteria*, *Neisseriaceae*, *Burkholderiaceae*, *Neisseria*, *Aggregatibacter*, *Lautropia*, *Kingella*, *Cardiobacterium* and *Neisseria subflava* were consistently reported to be depleted in current-smokers.[10] The depletion of *Neisseria* in current-smokers was also reported in other three studies.[8][29][30] Several in vivo studies suggest that cigarette smoking can inhibit growth of *Neisseria* species.[31][32]

Of the bacterial taxa that were enriched in current-smokers in the present study, several had been associated with risks of various diseases. For example, the common taxa *Actinomyces graevenitzii* was suggested to be involved in pulmonary abscesses in two independent case reports.[33][34] Other examples include the probiotic taxa such as *Bifidobacterium* and *Lactobacillus*, *B. longum*, *L. fermentum* and *L. reuteri*, which were reported be associated with a decreased risk of obesity prevalence.[20] There might be a potential link across smoking, oral probiotic taxa and obesity. *Actinobacteria* and *Actinomyces* were reported to be associated with a decreased risk of type 2 diabetes in our previous study of oral microbiome and type 2 diabetes.[35] *T. denticola*, a well-recognised oral pathogen, was found to be associated with a series of periodontal diseases[36] and an increased risk of colorectal cancer.[5]

Of the bacterial taxa that were enriched in non-smokers, several had been associated with decreased risks of cancers. For example, the common taxa *Neisseriaceae* and *Neisseria* were previously associated with a decreased risk of esophageal adenocarcinoma.[37] Several rare taxa, including *P. nanceiensis*, *Lachnoanaerobaculum umeaense* and *Lachnospiraceae_[G-2]*, were found to be associated with a decreased risk of esophageal adenocarcinoma or squamous cell carcinoma.[37] Another rare taxa, *Kingella*, was associated with a decreased risk of head and neck squamous cell cancer.[4]

Strengths of the present study include a large sample size, which provides higher statistical power compared with previous studies. In addition, most of previous studies focused on European-ancestry populations, while in the present study, the majority of participants were African-American and most of them have low socioeconomic status. Our results not only replicated a considerable proportion of previous findings but went further to compare the associations between African-Americans and European-Americans. Most associations identified in the overall analyses were consistently observed in both ethnic groups and both sequencing batches. Although generally the associations were slightly stronger among African-Americans and the first batch, these differences are not unexpected given the larger sample sizes of these two subsets. The main limitation is that 16S rRNA gene sequencing was used to assess the oral microbiome, which is limited in assessing the species level microbial profile and microbial pathways. Further research using the shotgun metagenomic sequencing technique is needed.

In summary, we demonstrated that among both African-Americans and European-Americans, cigarette smoking has strong impacts on the oral microbial community, which could probably be recovered by smoking cessation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is already known on this subject

- Cigarette smoking has an important impact on the human oral microbiota. However, previous studies were limited by small sample sizes and lack of replication.
- Most of the previous studies only focused on European-ancestry populations, hence the information regarding ethnic minority populations is lacking.

What this study adds

- This investigation gives us information regarding smoking and oral microbiota in low-income and African-American populations.
- We demonstrate that, among both European-Americans and African-Americans, cigarette smoking has considerable impacts on the oral microbial community structure and abundance/prevalence of multiple bacterial taxa, which could probably be recovered by smoking cessation. The associations of cigarette smoking with bacterial taxa has little heterogeneity between African-Americans and European-Americans.

Table 1

Characteristics of participants in two combined studies from the Southern Community Cohort Study

Characteristic	Group	Current-smokers (n=592)	Former-smokers (n=477)	Never-smokers (n=547)
Age (years)*		53.18±7.90	59.18±8.49	55.78±8.88
Sex (%)				
	Female	220 (37.16)	210 (44.03)	351 (64.17)
	Male	372 (62.84)	267 (55.97)	196 (35.83)
Race (%)				
	African-American	414 (69.93)	263 (55.14)	381 (69.65)
	European-American	178 (30.07)	214 (44.86)	166 (30.35)
Body mass index (BMI)*		26.86±6.41	30.32±6.95	31.17±7.43
Annual household income (US$) (%)				
	<15 000	375 (63.67)	194 (41.45)	216 (40.45)
	≥15 000 and <25 000	120 (20.37)	78 (16.67)	92 (17.23)
	≥25 000 and <50 000	64 (10.87)	90 (19.23)	102 (19.1)
	≥50 000 and <100 000	25 (4.24)	80 (17.09)	89 (16.67)
	≥100 000	5 (0.85)	26 (5.56)	35 (6.55)
Alcohol consumption† (%)				
	None	177 (30.31)	243 (52.71)	324 (60.34)
	Light	187 (32.02)	131 (28.42)	156 (29.05)
	Moderate	87 (14.90)	59 (12.80)	34 (6.33)
	Heavy	133 (22.77)	28 (6.07)	23 (4.28)
Tooth loss (%)				
	None	32 (10.53)	66 (19.58)	91 (21.51)
	One to 10	128 (42.11)	152 (45.10)	218 (51.54)
	>10, not all	79 (25.99)	68 (20.18)	67 (15.84)
	All	65 (21.38)	51 (15.13)	47 (11.11)

*For age and BMI, mean±SE were presented.

†Alcohol drink, Light, <1 drink/day; Moderate, 1–2 drink/day; Heavy, >2 drink/day.

BMI, body mass index.

Table 2

Higher prevalence of probiotic bacterial taxa among current-smokers than among never-smokers and former-smokers

	Prevalence			P value* ($P_{Bonferroni}$†)		
Probiotic taxa	**Current-smokers (n=592)**	**Former-smokers (n=477)**	**Never-smokers (n=547)**	**Current-smokers versus never-smokers**	**Former-smokers versus never-smokers**	**Current-smokers versus non-smokers‡**
Phylum *Actinobacteria*						
Genus *Bifidobacterium*	85.64%	71.07%	67.28%	2.30×10^{-6} (1.59×10^{-4})	0.88 (1.00)	2.09×10^{-7} (1.44×10^{-5})
Species *Bifidobacterium longum*	67.57%	45.07%	39.67%	2.61×10^{-11} (1.80×10^{-9})	0.32 (1.00)	2.91×10^{-12} (2.01×10^{-10})
Phylum *Firmicutes*						
Genus *Lactobacillus*	89.36%	73.38%	73.49%	2.62×10^{-6} (1.81×10^{-4})	0.46 (1.00)	1.15×10^{-7} (7.91×10^{-6})
Species *Lactobacillus crispatus*	61.15%	35.43%	34.37%	2.60×10^{-10} (1.80×10^{-8})	0.72 (1.00)	3.85×10^{-13} (2.66×10^{-11})
Species *L. fermentum*	57.60%	39.83%	35.65%	2.09×10^{-6} (1.44×10^{-4})	0.56 (1.00)	4.59×10^{-7} (3.16×10^{-5})
Species *L. gasseri*	72.47%	56.39%	53.02%	6.51×10^{-6} (4.50×10^{-4})	0.95 (1.00)	7.51×10^{-7} (5.18×10^{-5})
Species *L. oris*	43.75%	26.83%	20.84%	1.09×10^{-8} (7.54×10^{-7})	0.04 (1.00)	3.46×10^{-8} (2.39×10^{-6})
Species *L. panis*	42.23%	26.83%	25.41%	3.74×10^{-7} (2.58×10^{-5})	0.81 (1.00)	2.33×10^{-7} (1.61×10^{-5})
Species *L. reuteri*	41.55%	23.27%	20.84%	1.65×10^{-8} (1.14×10^{-6})	0.99 (1.00)	5.36×10^{-10} (3.70×10^{-8})

*P values were calculated by logistic regression. Sequencing batch as well as other covariates (age, sex, race, BMI, alcohol consumption, oral health and disease status at the first follow-up and total energy intake) were adjusted for.

†Bonferroni correction, adjusted for 69 independent tests.

‡Non-smokers includes former-smokers and never-smokers.

BMI, body mass index.

Table 3

Individual taxa showing a differential relative abundance between current-smokers and never-smokers

Taxa	Median relative abundance			P value* ($P_{Bonferroni}$†)		
	Current-smokers (n=592)	**Former-smokers (n=477)**	**Never-smokers (n=547)**	**Current-smokers versus never-smokers**	**Former-smokers versus never-smokers**	**Current-smokers versus non-smokerst‡**
Phylum *Actinobacteria*	12.35%	10.25%	9.36%	1.29×10^{-12} (3.24×10^{-11})	0.44 (1.00)	1.10×10^{-17} (2.75×10^{-16})
Family *Actinomycetaceae*	3.08%	2.51%	2.48%	1.65×10^{-4}(4.11×10^{-3})	0.29 (1.00)	3.62×10^{-7} (9.06×10^{-6})
Genus *Actinomyces*	3.05%	2.40%	2.42%	1.28×10^{-4} (3.19×10^{-3})	0.25 (1.00)	2.06×10^{-7} (5.14×10^{-6})
Species *Actinomyces graevenitzii*	0.36%	0.15%	0.16%	9.36×10^{-10} (2.34×10^{-8})	0.53 (1.00)	6.71×10^{-15} (1.68×10^{-13})
Species *Actinomyces graevenitzii*	1.44%	0.95%	0.94%	2.26×10^{-6} (5.64×10^{-5})	0.91 (1.00)	2.73×10^{-9} (6.82×10^{-8})
Genus *Rothia*	6.64%	5.50%	4.81%	2.76×10^{-9} (6.91×10^{-8})	0.27 (1.00)	1.77×10^{-12} (4.43×10^{-11})
Species *Rothia mucilaginosa*	5.64%	4.65%	3.87%	5.02×10^{-10} (1.25×10^{-8})	0.11 (1.00)	2.66×10^{-13} (6.64×10^{-12})
Family *Coriobacteriaceae*	0.13%	0.09%	0.12%	1.24×10^{-5} (3.11×10^{-4})	0.18 (1.00)	2.39×10^{-8} (5.97×10^{-7})
Genus *Atopobium*	0.13%	0.09%	0.12%	1.98×10^{-5} (4.95×10^{-4})	0.17 (1.00)	3.90×10^{-8} (9.75×10^{-7})
Species *Atopobium parvulum*	0.11%	0.08%	0.10%	2.21×10^{-5} (5.52×10^{-4})	0.15 (1.00)	2.22×10^{-8} (5.54×10^{-7})
Phylum *Proteobacteria*	2.47%	6.22%	7.22%	3.03×10^{-21} (7.58×10^{-20})	0.76 (1.00)	1.91×10^{-21} (4.77×10^{-20})
Family *Neisseriaceae*	0.06%	1.01%	1.06%	4.52×10^{-25} (1.13×10^{-23})	0.88 (1.00)	1.91×10^{-24} (4.78×10^{-23})
Genus *Neisseria*	0.05%	0.87%	1.01%	2.74×10^{-24} (6.86×10^{-23})	0.92 (1.00)	6.28×10^{-24} (1.57×10^{-22})
Species *Neisseria pharyngis*	0.01%	0.09%	0.11%	4.81×10^{-15} (1.20×10^{-13})	0.96 (1.00)	2.77×10^{-13} (6.94×10^{-12})
Species *N. subflava*	0.03%	0.43%	0.62%	3.95×10^{-22} (9.87×10^{-21})	0.75 (1.00)	2.50×10^{-21} (6.24×10^{-20})
Family *Pasteurellaceae*	1.71%	3.85%	4.30%	1.40×10^{-14} (3.49×10^{-13})	0.80 (1.00)	3.76×10^{-15} (9.39×10^{-14})
Genus *Aggregatibacter*	0.06%	0.10%	0.16%	3.71×10^{-5} (8.43×10^{-4})	0.46 (1.00)	2.71×10^{-4} (6.77×10^{-3})
Genus *Haemophilus*	1.39%	3.44%	3.89%	1.63×10^{-14} (4.08×10^{-13})	0.90 (1.00)	2.18×10^{-15} (5.46×10^{-14})
Species *Haemophilus parahaemolyticus*	0.16%	0.51%	0.48%	2.55×10^{-15} (6.37×10^{-14})	0.36 (1.00)	4.66×10^{-16} (1.16×10^{-14})
Species *H. paraphrohaemolyticus*	1.21%	2.81%	3.03%	9.59×10^{-13} (2.40×10^{-11})	0.66 (1.00)	3.82×10^{-13} (9.55×10^{-12})
Phylum *Bacteroidetes*						
Species *Prevotella sp. oral taxon 313*	4.45%	2.90%	2.97%	5.57×10^{-5} (1.39×10^{-3})	0.37 (1.00)	3.57×10^{-6} (8.93×10^{-5})
Family *Flavobacteriaceae*	0.04%	0.10%	0.10%	6.67×10^{-5} (1.67×10^{-3})	0.15 (1.00)	5.51×10^{-5} (1.38×10^{-3})
Phylum *Firmicutes*						
Genus *Gemella*	1.30%	1.90%	2.34%	4.30×10^{-15} (1.07×10^{-13})	0.02 (0.49)	1.16×10^{-11} (2.90×10^{-10})

Taxa	Median relative abundance			P value* ($P_{Bonferroni}$†)		
	Current-smokers (n=592)	Former-smokers (n=477)	Never-smokers (n=547)	Current-smokers versus never-smokers	Former-smokers versus never-smokers	Current-smokers versus non-smokerst‡
Species *Streptococcus oligofermentans*	0.13%	0.43%	0.42%	1.60×10^{-20} (4.00×10^{-19})	0.62 (1.00)	6.42×10^{-23} (1.60×10^{-21})
Species *Streptococcus sp. oral taxon 057*	10.36%	9.01%	8.07%	1.59×10^{-10} (3.99×10^{-9})	0.07 (1.00)	3.46×10^{-9} (8.64×10^{-8})
Species *Streptococcus sp. oral taxon 070*	23.23%	22.69%	23.67%	6.93×10^{-5} (1.73×10^{-3})	0.43 (1.00)	1.28×10^{-4} (3.19×10^{-3})
Genus *Megasphaera*	0.26%	0.10%	0.12%	2.05×10^{-8} (5.13×10^{-7})	0.43 (1.00)	7.29×10^{-12} (1.82×10^{-10})
Species *Megasphaera micronuciformis*	0.24%	0.10%	0.11%	1.95×10^{-7}(4.88×10^{-6})	0.40 (1.00)	1.52×10^{-10} (3.81×10^{-9})

*P values were calculated by logistic regression. Sequencing batch as well as other covariates (age, sex, race, BMI, alcohol consumption, oral health and disease status at the first follow-up and total energy intake) were adjusted for.

†Bonferroni correction, adjusted for 25 independent tests.

‡Non-smokers includes former-smokers and never-smokers.

BMI, body mass index.

Table 4

Individual taxa showing a differential prevalence between current-smokers and never-smokers

Taxa	Prevalence			P value* ($P_{Bonferroni}$†)		
	Current-smokers (n=592)	Former-smokers (n=477)	Never-smokers (n=547)	Current-smokers versus never-smokers	Former-smokers versus never-smokers	Current-smokers versus non-smokers‡
Phylum *Actinobacteria*						
Family *Bifidobacteriaceae*	95.44%	89.94%	84.64%	7.50×10^{-5} (5.17×10^{-3})	0.15 (1.00)	1.81×10^{-4} (0.01)
Species *Actinomyces lingnae_(NVP)*	92.40%	88.05%	86.65%	4.52×10^{-6} (3.12×10^{-4})	0.44 (1.00)	1.00×10^{-6} (6.91×10^{-5})
Phylum *Proteobacteria*						
Family *Burkholderiaceae*	42.74%	63.31%	69.84%	1.50×10^{-17} (1.04×10^{-15})	0.05 (1.00)	5.83×10^{-18} (4.02×10^{-16})
Genus *Lautropia*	41.55%	62.68%	69.47%	4.82×10^{-19} (3.33×10^{-17})	0.04 (1.00))	1.29×10^{-19} (8.88×10^{-18})
Genus *Kingella*	67.23%	79.87%	85.19%	3.01×10^{-8} (2.08×10^{-6})	0.08 (0.21)	1.20×10^{-8} (8.29×10^{-7})
Species *Kingella denitrificans*	24.83%	45.28%	46.62%	6.30×10^{-10} (4.35×10^{-8})	0.65 (1.00)	2.30×10^{-11} (1.59×10^{-9})
Species *K. elongata*	61.99%	76.94%	46.62%	1.68×10^{-7} (1.16×10^{-5})	0.35 (1.00)	5.08×10^{-9} (3.51×10^{-7})
Species *Neisseria oralis*	19.93%	43.19%	53.20%	1.74×10^{-22} (1.20×10^{-20})	5.14×10^{-4} (0.04)	1.00×10^{-22} (6.96×10^{-21})
Genus *Cardiobacterium*	34.46%	56.81%	59.41%	1.40×10^{-11} (9.67×10^{-10})	0.43 (1.00)	2.03×10^{-12} (1.40×10^{-10})
Phylum *Bacteroidetes*						
Species *Prevotella nanceiensis*	80.74%	86.16%	89.40%	7.11×10^{-5} (4.91×10^{-3})	0.30 (1.00)	6.68×10^{-5} (4.61×10^{-3})
Species *Capnocytophaga sputigena*	43.07%	60.80%	62.52%	5.17×10^{-8} (3.57×10^{-6})	0.97 (1.00)	1.20×10^{-8} (8.28×10^{-7})
Phylum *Firmicutes*						
Family *Lactobacillaceae*	90.37%	76.73%	77.15%	4.45×10^{-5} (3.07×10^{-3})	0.43 (1.00)	2.99×10^{-6} (2.06×10^{-4})
Genus *Enterococcus*	63.18%	71.28%	74.59%	2.51×10^{-6} (1.73×10^{-4})	0.26 (1.00)	4.27×10^{-6} (2.95×10^{-4})
Genus *Lachnospiraceae_(G-2)*	51.69%	52.83%	61.61%	1.34×10^{-4} (9.22×10^{-3})	5.30×10^{-3} (0.36)	7.97×10^{-4} (0.05)
Species *Lachnoanaerobaculum umeaense*	72.97%	81.76%	82.82%	4.45×10−4 (0.03)	0.92 (1.00)	3.80×10^{-5} (2.62×10^{-3})
Species *Eubacterium infirmum*	62.16%	71.70%	77.70%	4.15×10^{-5} (2.88×10^{-3})	0.02 (1.00)	7.28×10^{-5} (5.02×10^{-3})
Phylum *Spirochaetes*						
Genus *Treponema denticola*	69.93%	49.06%	54.30%	4.19×10^{-4} (0.03)	0.25 (1.00)	3.15×10^{-5} (2.17×10^{-3})

* P values were calculated by logistic regression. Sequencing batch as well as other covariates (age, sex, race, BMI, alcohol consumption, oral health and disease status at the first follow-up and total energy intake) were adjusted for.

† Bonferroni correction, adjusted for 69 independent tests.

[‡]Non-smokers includes former-smokers and never-smokers.

BMI, body mass index.