Influence of cigarette smoking on oral microbiota in patients with recurrent aphthous stomatitis

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► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jim-2021-002119).

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Accepted 20 October 2021
Published Online First 25 November 2021

To cite: Wang X, Luo N, Mi Q, *et al*. *J Investig Med* 2022;**70**:805–813.

ABSTRACT

Recurrent aphthous stomatitis (RAS) is a common recurrent ulcerative disease of the oral mucosa which is closely related to oral microbial composition. However, the specific effect and the mechanism of smoking in RAS are unclear. In this study, 16S rRNA sequencing technology was used to compare the differences in saliva microbial community between 28 non-smoking healthy controls (NSctrl), 31 non-smoking RAS patients (NSras), and 19 smoking RAS patients (Sras). The results showed that the bacterial community diversity in patients with RAS (NSras and Sras) was lower than that of NSctrl. The microbial community in smoking-associated RAS is less diverse and distinct from that of non-smokers. The RAS groups have higher abundance of *Veillonella*, *Rothia*, and *Sneathia* and lower abundance of *Bacteroidales*, *Bacteroides*, *Wolinella*, *Moryella*, *Pyramidobacter*, and *Christensenellaceae* at the genera level. A significantly different abundance of *Anaerovorax*, *Candidatus Endomicrobium*, *Lactococcus*, *Sneathia*, *Veillonella*, and *Cloacibacterium* was observed between the Sras and the NSras group. Notably, there was a significant difference in many species from the genus *Prevotella* and *Treponema* between the NSras and the Sras group. Further, the relative abundance of several taxa is correlated with smoking age or frequency, including *Megasphaera*, *Haemophilus*, *Leptotrichia*, and *Rothia* at the genera level, and *Prevotella melaninogenica*, *Prevotella salivae*, *Megasphaera micronuciformis*, *Haemophilus parainfluenzae*, *Alloprevotella tannerae*, *Actinomyces naeslundii*, *Lautropia mirabilis*, and *Capnocytophaga sputigena* at the species level. Among patients with RAS, smoking aggravated the pathways of respiration and human pathogens. Our results suggest that smoking is closely related to changes in the oral microbiota, which may contribute an opposite effect to the pathogenesis of RAS. This study provides new insight and theoretical basis for the cause and pathogenesis of RAS and better prevention and treatment.

Significance of this study

What is already known about this subject?
- Recurrent aphthous stomatitis (RAS) is a common and recurrent ulcerative disease of the oral mucosa.
- There is a certain relationship between change in the oral microbial flora and occurrence of recurrent oral ulcers.
- Smoking is negatively related to the occurrence of RAS.

What are the new findings?
- The microbial community of smoking-associated RAS is less diverse and distinct from that of non-smokers.
- Smoking significantly changed the relative abundance of RAS-related microbiota.
- Among patients with RAS, smoking aggravated the pathways of respiration and human pathogens.

How might these results change the focus of research or clinical practice?
- Significant changes in the oral microbial structure may contribute to the negative correlation between smoking and RAS.

INTRODUCTION

Recurrent aphthous stomatitis (RAS) is a common and recurrent ulcerative disease of the oral mucosa.[1] It has the characteristics of periodicity, long disease course, and recurrent attacks.[2] The pathogenesis of the disease is complex and has many inducing factors, such as bacterial infection, mental stress, trace element deficiency, body immunity decline, etc. The incidence of RAS in China is increasing year by year.[3] Although RAS is not a fatal disease, it can also cause physiological and psychological discomfort, especially in patients with recurrent oral ulcer. At present, there is no radical drug for treatment of RAS, and only a variety of drugs have been developed to promote its recovery. Interestingly, many studies have reported that tobacco may be closely related to the lower incidence of RAS, but the mechanism is not yet clear.[4–6]

The latest evidence shows that there is a certain relationship between change in the oral microbial flora and occurrence of recurrent oral ulcers. The decrease in several common oral bacteria, such as *Streptococcus*, *Veillonella*, *Neisseria*, and Gram-negative bacilli, has been linked to recurrent oral ulcers.[7–9] Smoking is a cause of oral dysbiosis that affects the structure of the oral microbial. Yang *et al*[10]

investigated and studied the relationship between smoking and oral microbiota in 1616 African Americans (592 current smokers, 477 former smokers, and 547 never smokers) using 16S rRNA gene sequencing technology to analyze the oral microbiota. The analysis shows that smoking has a greater impact on the oral microbial community and that the flora has significant differences. After quitting smoking, the oral microbial community returns to normal, similar to the oral microbial community of non-smoking population. Existing literature shows that smoking is negatively related to the occurrence of RAS. Studies by Mohamed and Janakiram,[11] Axéll *et al*,[4] and others have shown that the incidence of RAS in the smoking population is significantly lower than in the non-smoking population. Vaziri *et al*[12] found that using tobacco leaves as the main raw material to make mouthwash is effective in treating recurrent oral ulcers. Given that smoking may have an impact on the oral environment and the microbiome itself, it may have an important impact on host–microbe interactions. However, little is known about the effects of smoking on RAS-associated microbiota.

In this study, we employed the V3+V4 region of salivary micro-organism 16S rRNA gene sequencing between smoking and non-smoking RAS population and healthy population to conduct an oral microbial study. We aimed to analyze the difference in microbial community in the saliva and to explore the impact of smoking on the diversity of bacterial communities associated with RAS.

MATERIALS AND METHODS

Consent and permissions

Written informed consent was obtained from all subjects.

Study population

The RAS group met the following standards: (1) the episodes of aphthous ulcers should occur at least once a month; (2) subjects who had received antibiotics or steroid or drugs for treatment of ulcers within the last month and patients with xerostomia (unstimulated whole salivary flow rate <0.1 mL/min) were excluded; (3) subjects who had excessive consumption of alcohol (alcohol intake more than three times weekly) were excluded; and (4) subjects with other types of oral mucosal diseases or systemic diseases that involve RAS were also excluded.

A questionnaire survey was used to collect comprehensive demographic and lifestyle information to determine detailed data about the research subjects, including smoking status (never, current), smoking amount, smoking duration, ulcer frequency, recovery and living habits, etc, and follow-up. The final sample size was 78 patients and the distribution of the groups was as follows:

- Non-smoking healthy controls (NSctrl): n=28.
- Non-smoking RAS patients (NSras): n=31.
- Smoking RAS patients (Sras): n=19.

The clinical characteristics of the 78 patients with RAS and the control group are given in table 1. No significant differences in age and body mass index were found among the three groups. All enrolled subjects have no other inflammation disorders, including chronic hypertension, diabetes mellitus, and autoimmune disorders.

Table 1 Descriptive characteristics of the study population

	NSctrl (n=28)	NSras (n=31)	Sras (n=19)	P value
Age (years)	36.64±10.00	31.55±9.36	31.89±8.21	0.085
Sex (male/female)	14/14	15/16	11/8	0.797
Body mass index	20.86±0.37	21.37±1.03	21.48±0.86	0.736
Medical history				
Chronic hypertension	0	0	0	0
Diabetes mellitus	0	0	0	0
Autoimmune disease	0	0	0	0
Smoking age (years)				
<10			9	
11–20			7	
>20			3	
Frequency (per day)				
<10			9	
11–20			9	
>20			1	

P value was computed by one-way analysis of variance with Bonferroni test. NSctrl, non-smoking healthy control; NSras, non-smoking RAS patients; RAS, recurrent aphthous stomatitis; Sras, smoking RAS patients.

Saliva sample collection, genomic DNA extraction, and PCR amplification

Saliva sample collection followed the following standards: the subjects did not eat or smoke within 60 min before saliva sample collection and no oral cleaning measures were taken within 12 hours. At the time of sampling, the subjects collected 7 mL of non-irritating saliva in a 15 mL sterile centrifuge tube and immediately placed in dry ice for later use.

After sampling, the genomic DNA of the sample was isolated using the Genomic DNA Purification from Saliva Kit (New England BioLabs, Carlsbad, California, USA), and the diluted genomic DNA was used as a template. Based on the selection of sequencing region, the specific primers with Barcode, Phusion High-Fidelity PCR Master Mix with GC Buffer and high efficiency and high fidelity enzyme were used for PCR. According to the concentration of PCR products, an equal amount of samples was mixed, and the PCR products were purified by electrophoresis after full mixing. The products were recovered by the GeneJET Gel Recovery Kit. Then the library was constructed with Ion Plus Fragment Library Kit (Thermo Scientific, USA). After Qubit quantification and library detection, the library was sequenced by Ion S5 XL from ThermoFisher. The data were deposited in the Genome Sequence Archive of the National Genomics Data Center (https://ngdc.cncb.ac.cn/), the Beijing Institute of Genomics (China National Center for Bioinformation), and the Chinese Academy of Sciences, under accession number CRA005099.

Data processing and analysis

Quantitative Insights Into Microbial Ecology (QIIME) quality-controlled process was employed to filter and collect clean reads of high quality from raw sequencing data. UPARSE (V.7.0.1001)[13] was used to cluster the effective reads with more than 97% similarity into operational

taxonomic units (OTUs). Species annotation analysis was performed using the Mothur method and small sub-unit rRNA database of SILVA132[14] to obtain the taxonomic information and community composition at each taxonomic level. The threshold 0.8–1 was applied for annotation of the genera at each taxonomic rank. The OTUs with the highest frequencies were selected as representative sequences. The MUSCLE software (V.3.8.31)[15] was used for fast multisequence alignment to obtain the phylogenetic relationships of all OTU sequences. The data of each sample were then homogenized to calculate the alpha diversity (Chao1 index and Shannon diversity index) using QIIME software (V.1.9.1). Rarefaction curves were constructed using the rarefaction curve tool of R software (V.2.15.3). Calculation of unweighted UniFrac distance for beta diversity analysis was performed by QIIME software (V.1.9.1).

Quality control

Blind quality control samples were used in each data set of all sequencing batches. The quality control samples have good reliability. SPSS V.22.0 removed the abnormal samples from the Chao1 index and Shannon index by three times the SD.

Statistical analysis

A one-way analysis of variance with Bonferroni test and χ^2 test were used to examine the significance of differences in pairwise comparisons of the samples. Linear discriminate analysis (LDA) effect size (LEfSe) by non-parametric factorial Kruskal-Wallis test based on LEfSe software was used to compare the relative abundance of the bacterial groups in different categories of smoking status. We also tested the differences in microbial diversity between the samples using the Metastats (permuted t-statistics or Fisher's exact test). Spearman's correlation coefficient was used to test the correlation between smoking-related variables (the number of cigarettes per day and smoking age) and the relative abundance of the selected taxa. All statistical tests were double-tailed, and p values less than 0.05 (or false discovery rate (FDR) q less than 0.05) were considered statistically significant. All analyses were performed using SPSS V.22.0.

RESULTS

Differences in oral microbial diversity among the three groups

The rarefaction curves for the RAS groups stabilized at <500 OTUs, showing greater bacterial diversity than that observed for NSctrl (565 OTUs at 48082 sequences). The Sras group had similar bacterial diversity to the NSras group (approximately 497 OTUs vs 508 OTUs at 48082 sequences) (figure 1A). These results were confirmed by Chao richness and Shannon diversity indexes for the different groups (figure 1B). Principal coordinates analysis (PCoA) showed that subjects from each study group tended to cluster together, with significant differences among the three groups (UniFrac distance, p=0.037) (figure 1C).

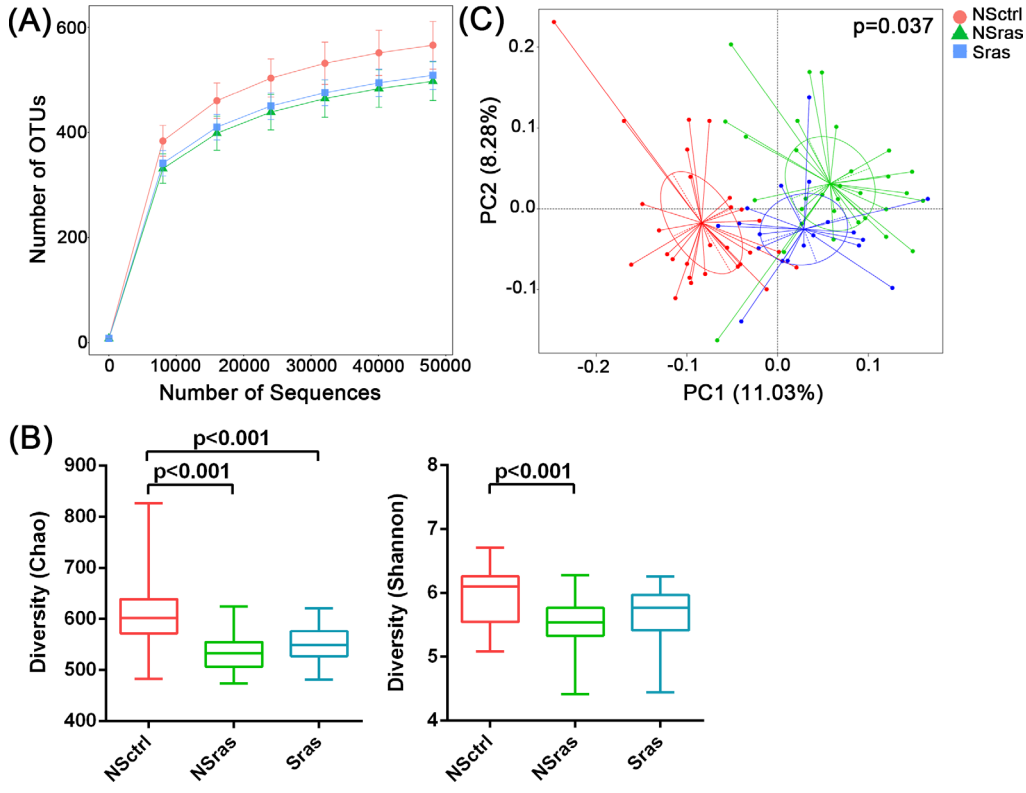

Figure 1 Bacterial diversity and community structure. (A) Rarefaction curves of all the saliva samples by study group. (B) The microbiota from Sras showed statistically significantly lower diversity and evenness similar to NSras. (C) PCoA of all 78 saliva samples according to bacterial composition, including NSctrl, NSras, and Sras. PCoA was performed with weighted UniFrac analysis with clustering at 97% sequence identity. NSctrl, non-smoking healthy controls; NSras, non-smoking RAS patients; OTUs, operational taxonomic units; PC, principal component; PCoA, principal coordinates analysis; RAS, recurrent aphthous stomatitis; Sras, smoking RAS patients.

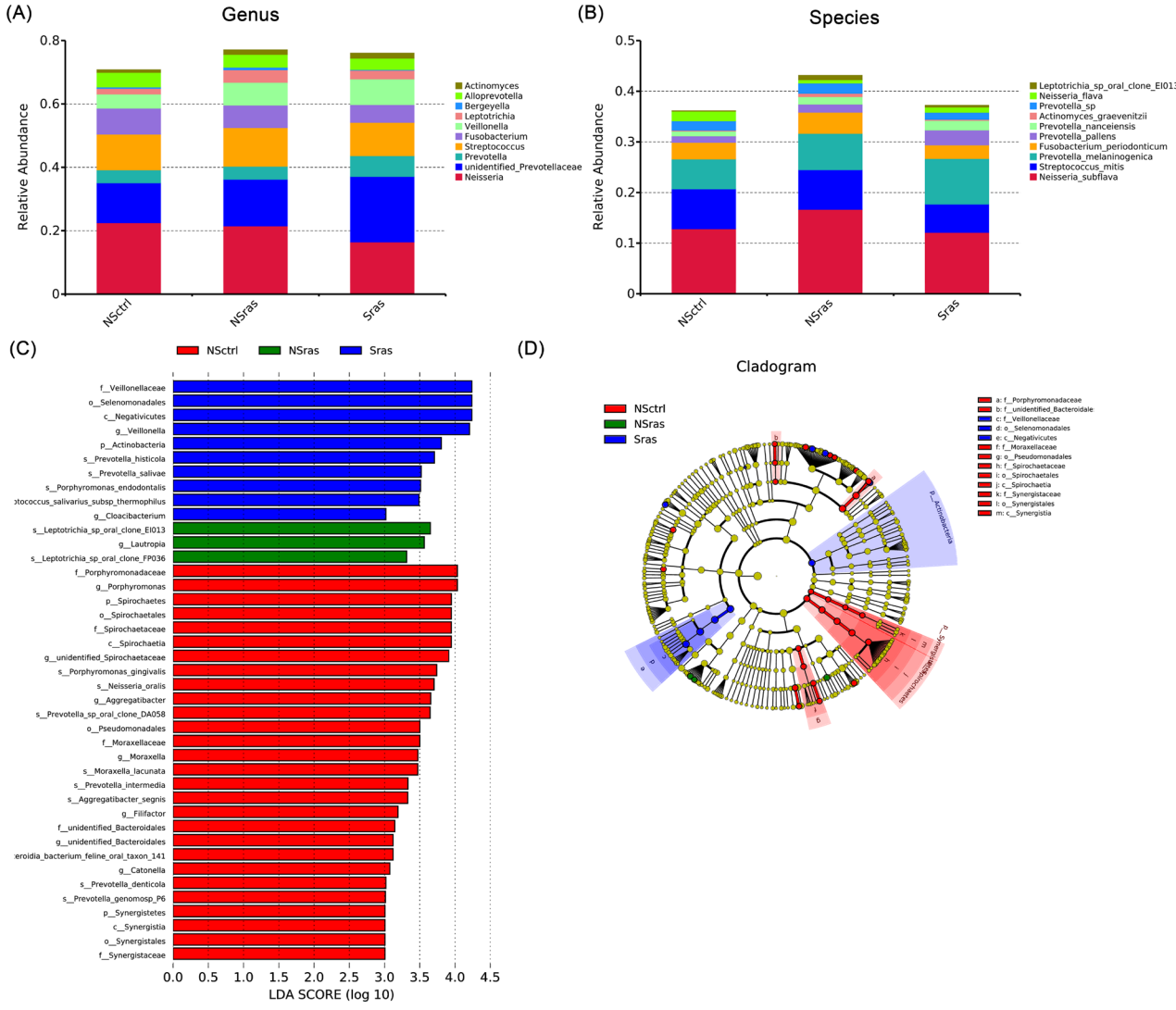

Figure 2 Multivariate analyses to identify the genera and species which presented statistically significant differences among the three study groups. (A) Genera abundance and (B) species abundance of each group. (C) LEfSe analysis by non-parametric factorial Kruskal-Wallis rank-sum test was performed to distinguish among the NSctrl, NSras, and Sras groups. LDA score higher than 3 was displayed from the phylum to the species level. (D) Evolutionary cladistics (phylogenetic distribution). LDA, linear discriminate analysis; LEfSe, LDA effect size; NSctrl, non-smoking healthy controls; NSras, non-smoking RAS patients; RAS, recurrent aphthous stomatitis; Sras, smoking RAS patients.

Microbiota composition and taxonomy in the three groups

A total of 3363 OTUs were detected, which were classified into 11 phyla, 41 classes, 74 orders, 133 families, and 278 genera. The common dominant phyla were Bacteroidetes (33.3%), Proteobacteria (29.3%), Firmicutes (24.6%), Fusobacteria (8.30%), and Actinobacteria (2.19%). In all samples, a total of 115 bacterial genera were identified. The main genera consists of characteristic oral bacteria, including *Neisseria* (21.8%), *Prevotella* (16.2%), *Streptococcus* (10.1%), *Veillonella* (7.5%), *Fusobacterium* (5.3%), and *Leptotrichia* (5.5%).

At the genus level, the predominant genera in non-smokers was *Neisseria* (22.52% in the NSctrl group, 21.52% in the NSras group, and 16.44% in the Sras group). The most predominant genera in Sras was *Prevotellaceae* (20.73%) (figure 2A). At the species level, *Neisseria subflava* has the highest content among the three groups (NSctrl accounted for 12.84%, NSras accounted for 16.67%, and Sras accounted for 12.12%), followed by *Streptococcus mitis*, *Prevotella melaninogenica*, and *Fusobacterium periodonticum* (figure 2B).

Significant difference in the microbiota between the NSctrl and the RAS (NSras and Sras) group

We performed Metastats analysis (permuted t-statistics or Fisher's exact test) to reveal the characteristic flora of the three groups. The results showed that there were 71 different genera between the NSctrl and the NSras group, while there were 55 different genera between the NSctrl and the Sras group ($p<0.05$; online supplemental table S1). Most genera were also identified by LEfSe analysis (the LDA score was set to >3) (figure 2C,D). After adjusting for p

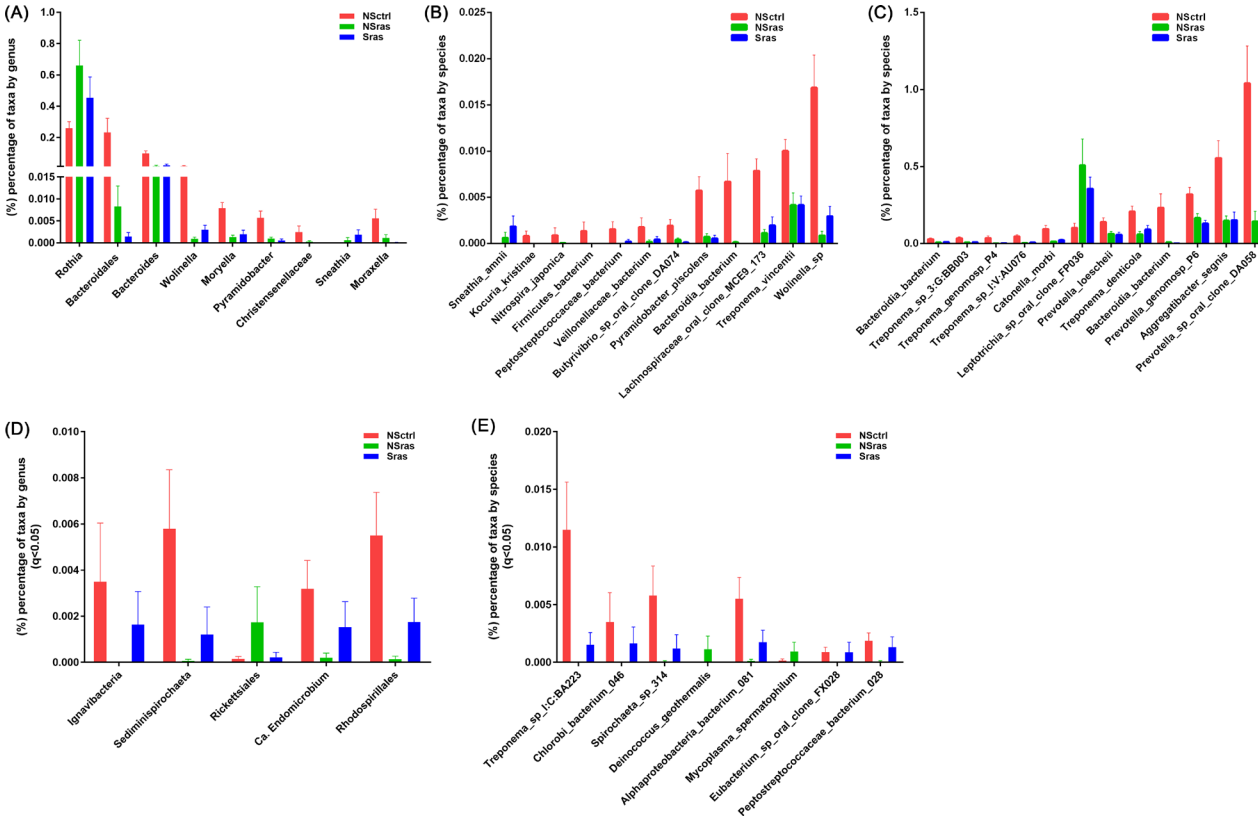

Figure 3 Significant difference in RAS-associated microbiomes in non-smokers and smokers. (A) Percentages of taxa by genera presented statistically significant differences between the NSctrl and the RAS (NSras and Sras) group (q<0.05). (B, C) Percentages of taxa by species presented statistically significant differences between the NSctrl and the RAS (NSras and Sras) group (q<0.05). After screening for differential genera or species between the NSras and the NSctrl group, the genera (D) and species (E) were identified with differential abundance between the NSras and the Sras group. NSctrl, non-smoking healthy controls; NSras, non-smoking RAS patients; RAS, recurrent aphthous stomatitis; Sras, smoking RAS patients.

value, *Bacteroidales*, *Bacteroides*, *Wolinella*, *Moryella*, *Pyramidobacter*, *Christensenellaceae*, and *Moraxella* were all observed to be different between the NSctrl and the NSras or Sras group, with higher abundance in the NSctrl group (q<0.05; figure 3A). Compared with NSctrl, the NSras and Sras groups have higher abundance of the genera *Rothia* and *Sneathia* (q<0.05; figure 3A).

There were 110 species identified between the NSctrl and the NSras group and 99 species between the NSctrl and the Sras group (p<0.05; online supplemental table S2). There were 24 co-different species identified among the three groups (q<0.05), including 12 species with <0.5% abundance (figure 3B) and 12 species with >0.5% abundance (figure 3C). LEfSe analysis (the LDA score was set to >3) also revealed high abundance of species from *Bacteroidales* and *Moraxella* in the NSctrl group, high abundance of *Leptotrichia* in the NSras group, and an abundance of *Porphyromonas endodontalis*, *Prevotella salivae*, and *Prevotella histicola* in the Sras group (figure 2C,D).

Relationship between smoking and RAS-associated microbiomes

Regarding the effects of smoking, we first screened the different classes between NSctrl and NSras and investigated whether changes were associated with smoking. Among 71 different genera between the NSctrl and the NSras group (p<0.05), 18 genera were observed between Sras and NSras (p<0.05; table 2). Comparing with the NSras group, *Rickettsiales* and *Rhodospirillales* decreased in the Sras group, while *Candidatus Endomicrobium*, *Sediminispirochaeta*, and *Ignavibacteria* increased in the Sras group (q<0.05; figure 3D).

Among 110 different species between the NSctrl and the NSras group (p<0.05), 23 species demonstrated differential abundance between the Sras and the NSras group (p<0.05; table 2). After adjusting for p value, eight species showed significant differential abundance: *Treponema sp_I:C:BA223*, *Chlorobi bacterium_COT_046*, *Spirochaeta sp_COT_314*, *Deinococcus geothermalis*, *Alphaproteobacteria bacterium_COT_081*, *Mycoplasma spermatophilum*, *Eubacterium sp_oral_clone_FX028*, and *Peptostreptococcaceae bacterium_FOT_028* (figure 3E).

The data suggest that smoking may have altered these RAS-related genera or species.

Influence of smoking habit

We further examined whether genus-level or species-level bacterial relative abundances differed according to smoking age and number of cigarettes smoked per day. We observed the relative abundance of *Megasphaera* was positively correlated with smoking age (r=0.580, p=0.009) (table 3). The relative abundance of *Haemophilus*, *Leptotrichia*,

Table 2 Identified co-different genus and species associated with RAS which showed a positive difference equal to or higher than 10% (in terms of relative abundance)

Contributing OTU	NSctrl (n=28)	NSras (n=31)	Sras (n=19)	P1 value	P2 value
Genera					
Aeromonas	0.0001±0.0001	0.0007±0.0007	0±0	0.025	0.009
Anaerovorax	0.0014±0.0011	0±0	0.0004±0.0004	0	0.021
Candidatus Endomicrobium	0.0032±0.0012	0.0002±0.0002	0.0015±0.0011	0.005	0
Deinococcus	0.0004±0.0004	0.0011±0.0011	0±0	0.03	0.001
Elizabethkingia	0±0	0.0006±0.0004	0±0	0.004	0.016
Ezakiella	0.014±0.0042	0.0007±0.0004	0.0091±0.0053	0.001	0.036
Fretibacterium	0.2809±0.0971	0.0685±0.0154	0.164±0.0369	0.002	0.025
Geobacter	0.0001±0.0001	0.0007±0.0005	0±0	0.025	0.009
Ignavibacteria	0.0035±0.0026	0±0	0.0016±0.0014	0.001	0
Lactococcus	0.0014±0.0006	0.0006±0.0003	0±0	0.037	0.016
Parascardovia	0±0	0.0008±0.0005	0±0	0.001	0.005
Phocaeicola	0.0241±0.0036	0.005±0.001	0.0161±0.0071	0.001	0.048
Rhodospirillales	0.0055±0.0019	0.0001±0.0001	0.0018±0.001	0.002	0
Rickettsiales	0.0001±0.0001	0.0017±0.0015	0.0002±0.0002	0	0
Rikenellaceae	0.1195±0.0232	0.0205±0.0052	0.0675±0.0212	0.001	0.033
Roseburia	0.0759±0.0124	0.0077±0.0021	0.042±0.0151	0.001	0.007
Sediminispirochaeta	0.0058±0.0026	0.0001±0.0001	0.0012±0.0012	0.004	0
Sneathia	0±0	0.0006±0.0006	0.0019±0.0011	0.004	0.007
Species					
Acholeplasmatales bacterium_COT_172	0.0544±0.0186	0.0015±0.001	0.0298±0.0144	0.002	0.007
Actinomyces sp_OT_414	0.0027±0.0009	0.0005±0.0002	0.0013±0.0009	0.013	0.032
Aeromonas veronii	0.0001±0.0001	0.0007±0.0007	0±0	0.025	0.009
Alphaproteobacteria bacterium_COT_081	0.0055±0.0019	0.0001±0.0001	0.0018±0.001	0.004	0
Bacteroidales OT_MCE7_164	0.0592±0.0136	0.0131±0.0034	0.0531±0.0186	0.001	0.04
Chlorobi bacterium_COT_046	0.0035±0.0026	0±0	0.0016±0.0014	0.001	0
Deinococcus geothermalis	0±0	0.0011±0.0011	0±0	0.001	0.001
Eubacterium sp_OT_FX028	0.0009±0.0004	0±0	0.0009±0.0009	0	0
Geobacter soli	0.0001±0.0001	0.0007±0.0005	0±0	0.025	0.009
Lachnospiraceae bacterium_FOT_021	0.0759±0.0124	0.0077±0.0021	0.042±0.0151	0.001	0.004
Mycoplasma spermatophilum	0.0001±0.0001	0.0009±0.0008	0±0	0.005	0.002
Peptostreptococcaceae bacterium_COT_221	0.0249±0.0085	0.0046±0.0011	0.0181±0.0068	0.004	0.033
Peptostreptococcaceae bacterium_FOT_028	0.0019±0.0007	0.0001±0.0001	0.0013±0.0009	0	0
Phocaeicola abscessus	0.0241±0.0036	0.005±0.001	0.0161±0.0071	0.001	0.035
Sneathia amnii	0±0	0.0006±0.0006	0.0019±0.0011	0.004	0.007
Spirochaeta sp_COT_314	0.0035±0.0026	0.0001±0.0001	0.0012±0.0012	0.001	0
Synergistetes bacterium_OT_363	0.0544±0.0186	0.0137±0.0031	0.0395±0.0081	0.002	0.006
Tissierella sp_feline_OT_025	0.014±0.0042	0.0007±0.0004	0.0088±0.0053	0.001	0.036
Treponema parvum	0.0029±0.0008	0.0005±0.0003	0.0014±0.0005	0.001	0.019
Treponema sp_5:C:AT040	0.1703±0.0716	0.0254±0.009	0.0605±0.015	0.003	0.044
Treponema sp_9:A:D01	0.0072±0.0016	0.0011±0.0004	0.0036±0.0012	0.002	0.05
Treponema sp_I:C:BA223	0.0115±0.0041	0±0	0.0015±0.0011	0.001	0
Treponema sp_OT_271	0.0544±0.0186	0.0015±0.001	0.0298±0.0144	0.001	0.003

P1 value: NSctrl vs NSras.
P2 value: NSras vs Sras.
NSctrl, non-smoking healthy controls; NSras, non-smoking RAS patients; OTU, operational taxonomic unit; RAS, recurrent aphthous stomatitis; Sras, smoking RAS patients.

Lautropia, *Aggregatibacter*, *Rothia*, *Bergeyella*, and *Atopobium* was correlated with smoking frequency (table 3). At the species level, the relative abundance of *P. melaninogenica*, *P. salivae*, and *Megasphaera micronuciformis* was positively correlated with smoking age (r=0.444, p=0.057; r=0.507, p=0.027; r=0.580, p=0.009) (table 3). The relative abundance of *Haemophilus parainfluenzae*, *Alloprevotella tannerae*, *Actinomyces naeslundii*, *Lautropia mirabilis*, and *Capnocytophaga sputigena* was negatively correlated with smoking frequency (r=−0.498, p=0.030; r=−0.470, p=0.042; r=−0.471, p=0.042; r=−0.752, p=0.000; and r=−0.633, p=0.004) (table 3).

Correlation between microbial function and functional signaling pathway

To explore the functional pathways of related genes in smoking-related bacterial taxa, the FAPROTAX algorithm was used to infer the bacterial gene function based on the microbial composition of the 16S rRNA gene from the prokaryotic function database. The genus count matrix was

Table 3 Correlation between genus level and number of cigarettes smoked per day or smoked time in current smokers

	Smoking time		Frequency (per day)	
Levels	r	P value	r	P value
Genera				
Haemophilus	−0.278	0.250	−0.513	0.025
Leptotrichia	0.212	0.385	−0.470	0.042
Lautropia	−0.103	0.675	−0.584	0.009
Aggregatibacter	−0.433	0.064	−0.533	0.019
Megasphaera	0.580	0.009	0.328	0.171
Rothia	−0.316	0.188	−0.549	0.015
Bergeyella	−0.344	0.149	−0.669	0.002
Actinobacillus	0.109	0.657	−0.469	0.043
Atopobium	0.262	0.279	0.465	0.045
Species				
Prevotella melaninogenica	0.464	0.045	0.120	0.623
Prevotella salivae	0.507	0.027	0.344	0.150
Haemophilus parainfluenzae	−0.363	0.126	−0.498	0.030
Alloprevotella tannerae	−0.365	0.124	−0.470	0.042
Megasphaera micronuciformis	0.580	0.009	0.328	0.171
Lautropia mirabilis	−0.237	0.329	−0.752	0.000
Capnocytophaga sputigena	−0.422	0.072	−0.633	0.004

P value is based on Spearman's correlation coefficient for ranked data.

normalized across samples using the DESeq2 size factors. The results showed that there were 20 gene pathways with different abundances among the three groups ($p<0.05$). Comparing with NSctrl, the pathways of human pathogens for pneumonia significantly decreased, while those of human pathogens for gastroenteritis significantly increased in the oral microbiota in RAS. The oral microbiota in RAS had significantly lower fumarate/nitrogen/nitrate respiration and phototrophy, photoautotrophy, and oxygenic photoautotrophy. In patients with RAS, comparing with non-smokers (NSras), the oral microbiota of smokers (Sras) had significantly less abundant genes that are involved in the pathogenesis of septicemia/pneumonia and in fumarate/nitrogen/nitrate respiration. Interestingly, compared with the NSras group, the Sras group had more genes for gastroenteritis and intestinal pathway enrichment (figure 4).

DISCUSSION

This is the first study to assess the impact of smoking on RAS and associated oral flora and highlights the importance of smoking as a clinical research factor for the microbiome. Data on the 16S rRNA gene in the saliva samples showed that smoking is significantly related to abundance of oral microbes and composition of bacteria. This is a novel observation in RAS. These changes may result in functional differences in the host–microbial interface and ultimately related to clinical progress in RAS. Based on the known effects of smoking, including changes to the immune system[16] and direct antimicrobial activity,[17] many hypotheses related to this finding can be found, such as affecting the adaptive immune cells,[18] impairing the release of the antimicrobial peptide,[19] as well as the observed changes in the composition of bacterial communities.

It is currently believed that a more diverse bacterial community represents a more stable and healthy ecosystem.[20] In fact, many oral diseases are associated with decreased bacterial diversity.[21] Consistent with previous studies,[20 22] in this study, the bacterial diversity (richness and uniformity) of patients with RAS was lower than that of healthy subjects. However, the bacterial diversity in patients with RAS increases with smoking habits. PCoA showed significantly different microbial structures among

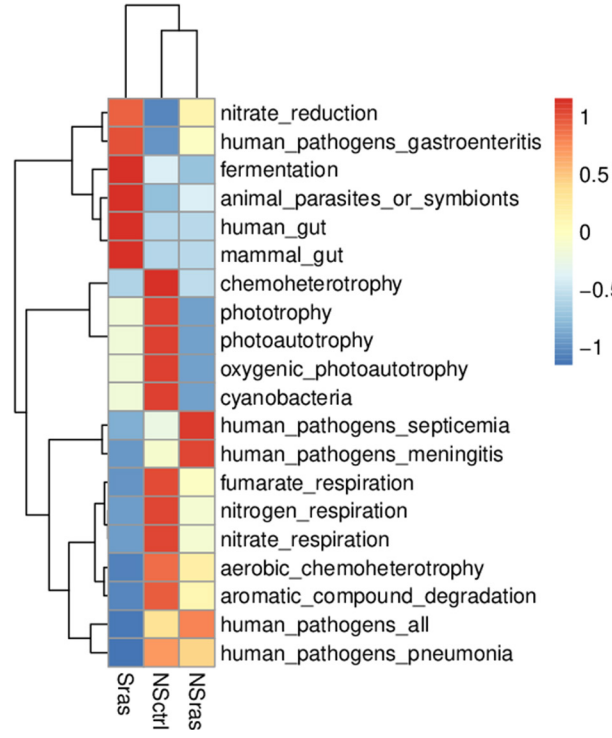

Figure 4 Annotation of gene function of the bacterial taxa related to smoking status. The FAPROTAX algorithm was used to infer the function of the bacterial genes composed of microorganisms based on 16S rRNA genes from the prokaryotic database. NSctrl, non-smoking healthy controls; NSras, non-smoking RAS patients; RAS, recurrent aphthous stomatitis; Sras, smoking RAS patient.

NSctrl, NSras and Sras. These results are consistent with the results described by Yang *et al*.[23] Using cluster analysis, Yang *et al*[23] revealed differences in the microbiome structure between the deep and shallow parts of patients with RAS. Similarly, our data show that it is important to distinguish taxa at the species level, as different species within the same genus may be associated with health as well as a disease. Most of the species related to RAS identified in this study have been previously linked to oral health by other authors using the Human Oral Microbiology Identification Chip or 16S pyrosequencing.[24 25] According to Metastats analysis (permuted t-statistics or Fisher's exact test), in the saliva of smokers or non-smokers with RAS, the relative abundance of *Rothia*, *Bacteroidales*, *Wolinella*, *Moryella*, *Pyramidobacter*, and *Sneathia* was significantly different from the NSctrl group. *Rothia* has been reported to have higher representation in the ulcerated mucosa of orally active Behçet's syndrome in comparison with a healthy case.[26] Hijazi *et al*[27] found higher levels of *Bacteroidales* in association with RAS, but this was not confirmed in our results and Yang *et al*'s data.[23] An increased abundance of *Bacteroidales* was shown in the NSras or the Sras group compared with NSctrl. In addition, *Megasphaera*, *Pyramidobacter*, and *Moryella* might be related to the pathogenesis and progression of dental caries.[28–30] The findings of this research are consistent with these studies.

This study further investigated the association between smoking and changes of oral flora in patients with RAS. Among the RAS-associated microbiome (differential abundance between NSctrl and NSras), *Rothia*, *Lautropia*, and *Leptotrichia* have been previously identified to be associated with RAS.[31–33] Moreover, 18 genera showed significant differences between the Sras and the NSras group, including *Aeromonas*, *Anaerovorax*, *Ca. Endomicrobium*, *Deinococcus*, *Elizabethkingia*, *Ezakiella*, *Fretibacterium*, *Geobacter*, *Parascardovia*, *Ignavibacteria*, *Phocaeicola*, *Rhodospirillales*, *Rickettsiales*, *Rikenellaceae*, *Roseburia*, and *Sediminispirochaeta*. They were first reported to be associated with RAS in this study. Notably, *Anaerovorax* (Metastats)[34] and *Veillonella* and *Cloacibacterium*[35] (LEfSe analysis) have been reported to be more abundant in current smokers, which is consistent with our data. At the species level, we found that NSras and Sras have different abundance of many species from the genus *Prevotella*. The abundance of *Prevotella* decreased in patients with RAS,[32] but increased in young female patients with RAS.[36] Our data support that *Prevotella* is an important genus related to RAS and that different species from *Prevotella* may contribute an opposite effect to the pathogenesis of RAS (figure 2C). For example, smoking reduced the abundance of *Prevotella shahii*, while *P. melaninogenica* and *P. salivae* were positively associated with smoking age in this study. In addition, our study showed smoking altered many species from the genus *Treponema*, such as *T. parvum*, *T. sp_5:C:AT040*, *T. sp_9:A:D01*, *T. sp_I:C:BA223*, and *T. sp_OT_271*. It is an important pathogen in the human oral cavity and is closely related to the occurrence and development of periodontal diseases.[37] Thus, we speculate that these species of *Treponema* may also be related to the inflammatory response in RAS.

We further screened the differential classes related to smoking frequency or smoking age and found that the relative abundance of *C. sputigena* was negatively correlated with smoking frequency. The present study and early studies have confirmed that these florae are highly abundant in RAS.[32 33] These data indicated that smoking frequency may be a cause of reduction in the abundance of RAS-related microbiota. Notably, *P. melaninogenica*, *P. salivae*, and *M. micronuciformis* are positively correlated with smoking years. These species have not been reported in RAS and their contribution to RAS is unclear. We cautiously speculate that smoking frequency has a more pronounced contribution to the effect of smoking on RAS.

The composition of the microbial flora is closely related to its functions. As we have found in the above results that smoking has a significant impact on the composition of the oral microbes, we speculate that its functions are also differentially enriched accordingly. We found the pathways of gastroenteritis/septicemia/meningitis diseases significantly increased, while pneumonia decreased in the oral microbiota in RAS. RAS is a system disease with a pathogenesis of infection, immunity, and metabolism disorders. The appearance of RAS could change the microenvironment of the oral mucosa with inflammation and immune activity. In addition, among patients with RAS, smoking aggravated the pathways of respiration and human pathogens. In patients with RAS, smokers had more microbiomes of human and mammal intestinal-related pathways enrichment. A few microbial species frequently found in both the mouth and the gut showed evidence of an oral–gut transmission.[38 39] Moreover, fumarate/nitrogen/nitrate respiration was observed in the Sras group compared with the NSras group. Inflammation or infection has been shown to lead to mucosal hypoxia. In the absence of oxygen, some microbiome can resort to anaerobic respiration using fumarate, nitrogen, and nitrate.[40]

Our results may have potential limitations because the number of subjects varies among the groups and there may be some non-objective conditions. Further comparative studies will be required using samples with or without consolidation to investigate whether the apparent differences between the groups were the result of consistent changes in each sample or very large changes in a small number of subsamples. Sampling and sequencing of population subgroups (races) will also need to be continued to better understand the effects of smoking on the oral microbial community.

In conclusion, the microbial community of smoking-associated RAS is less diverse and distinct from that of non-smokers. Smoking is significantly related to the structure and composition of RAS-related microbiota, specially *Anaerovorax*, *Ca. Endomicrobium*, *Lactococcus*, *Sneathia*, *Veillonella*, and *Cloacibacterium*. Notably, there was a significant difference in many species from the genus *Prevotella* and *Treponema* between the NSras and the Sras group. The relative abundance of *C. sputigena*, *H. parainfluenzae*, and *Leptotrichia* was negatively correlated with smoking frequency. Mechanismly, smoking aggravated the enrichment of different microbiota in respiration and human pathogens pathways in patients with RAS. The target microbiome will be further explored in smoking patients with RAS, which may provide new and therapeutic insight for improving RAS by regulating the oral microbiota.

Acknowledgements The authors thank the YuQi Dental Hospital for help in sampling.

Contributors Conceptualization: QG, XL, Investigation: XW, NL. Formal analysis: QM, WK. Methodology: WZ. Data curation: XW, NL. Writing - original draft: XW, NL, QG. Writing - review and editing: QG, XL. Supervision: XL. Guarantor: QG.

Funding This work was supported by the Research Foundation of China Tobacco Company (grant 110201901021(JY-08)) and the Research Foundation of China Tobacco Yunnan Industrial (grant 2018XY04).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was performed according to the Declaration of Helsinki and approved by the Biomedical Ethics Committee of the Joint Institute of Tobacco and Health (approval number: 2020-001).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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REFERENCES

1 Bilodeau EA, Lalla RV. Recurrent oral ulceration: etiology, classification, management, and diagnostic algorithm. *Periodontol 2000* 2019;80:49–60.
2 Edgar NR, Saleh D, Miller RA. Recurrent aphthous stomatitis: a review. *J Clin Aesthet Dermatol* 2017;10:26.
3 Yan H, Jin Z, Jin W, *et al*. A systematic review and meta-analysis of acupuncture treatment for oral ulcer. *Medicine* 2020;99:e21314.
4 Axéll T, Henricsson V, Henricsson V. Association between recurrent aphthous ulcers and tobacco habits. *Scand J Dent Res* 1985;93:239–42.
5 Grady D, Ernster VL, Stillman L, *et al*. Smokeless tobacco use prevents aphthous stomatitis. *Oral Surg Oral Med Oral Pathol* 1992;74:463–5.
6 Chaudhuri S, Dey S, Awasthi A, *et al*. Does tobacco addiction relate to oral mucosal changes? an epidemiological study from North India. *J Public Health* 2017;25:257–66.
7 Wang J-Z, Chen Q, Shen H. [Changes in oral microflora in patients with recurrent oral ulcers]. *Nan Fang Yi Ke Da Xue Xue Bao* 2009;29:986–9.
8 Gomes C-C, Gomez R-S, Zina L-G, *et al*. Recurrent aphthous stomatitis and helicobacter pylori. *Med Oral Patol Oral Cir Bucal* 2016;21:e187–91.
9 Radithia D, Kende SY, Parmadiati AE. Recurrent oral ulceration associated to group Aâ-Hemolytic Streptococcus reinfection in a post-rheumatic heart disease patient. *Journal of International Dental and Medical Research* 2017;10:392.
10 Yang Y, Zheng W, Cai Q-Y, *et al*. Cigarette smoking and oral microbiota in low-income and African-American populations. *J Epidemiol Community Health* 2019;73:1108–15.
11 Mohamed S, Janakiram C. Recurrent aphthous ulcers among tobacco users-hospital based study. *J Clin Diagn Res* 2014;8:ZC64.
12 Vaziri S, Mojarrab M, Farzaei MH, *et al*. Evaluation of anti-aphthous activity of decoction of nicotiana tabacum leaves as a mouthwash: a placebo-controlled clinical study. *J Tradit Chin Med* 2016;36:160–4.
13 Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;10:996–8.
14 Van de Peer Y, De Rijk P, Wuyts J, *et al*. The European small subunit ribosomal RNA database. *Nucleic Acids Res* 2000;28:175–6.
15 Edgar RC. Muscle: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 2004;5:113.
16 Sopori M. Effects of cigarette smoke on the immune system. *Nat Rev Immunol* 2002;2:372–7.
17 Belofsky G, Carreno R, Lewis K, *et al*. Metabolites of the "smoke tree", dalea spinosa, potentiate antibiotic activity against multidrug-resistant Staphylococcus aureus. *J Nat Prod* 2006;69:261–4.
18 Pavia CS, Plummer MM. Clinical implications of nicotine as an antimicrobial agent and immune modulator. *Biomed Pharmacother* 2020;129:110404.
19 Crane-Godreau MA, Maccani MA, Eszterhas SK, *et al*. Exposure to cigarette smoke disrupts CCL20-mediated antimicrobial activity in respiratory epithelial cells. *Open Immunol J* 2009;2:86.
20 Griffen AL, Beall CJ, Campbell JH, *et al*. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *Isme J* 2012;6:1176–85.
21 Willis JR, Gabaldón T. The human oral microbiome in health and disease: from sequences to ecosystems. *Microorganisms* 2020;8:308.
22 Abusleme L, Dupuy AK, Dutzan N, *et al*. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *Isme J* 2013;7:1016–25.
23 Yang Z, Cui Q, An R, *et al*. Comparison of microbiomes in ulcerative and normal mucosa of recurrent aphthous stomatitis (RAS)-affected patients. *BMC Oral Health* 2020;20:1–8.
24 Colombo APV, Boches SK, Cotton SL, *et al*. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol* 2009;80:1421–32.
25 Kistler JO, Pesaro M, Wade WG. Development and pyrosequencing analysis of an in-vitro oral biofilm model. *BMC Microbiol* 2015;15:1–10.
26 Camelo-Castillo AJ, Mira A, Pico A, *et al*. Subgingival microbiota in health compared to periodontitis and the influence of smoking. *Front Microbiol* 2015;6:119.
27 Hijazi K, Lowe T, Meharg C, *et al*. Mucosal microbiome in patients with recurrent aphthous stomatitis. *J Dent Res* 2015;94:87S–94.
28 Wang Y, Zhang J, Chen X, *et al*. Profiling of oral microbiota in early childhood caries using single-molecule real-time sequencing. *Front Microbiol* 2017;8:2244.
29 Lundmark A, Hu YOO, Huss M, *et al*. Identification of salivary microbiota and its association with host inflammatory mediators in periodontitis. *Front Cell Infect Microbiol* 2019;9:216.
30 Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett* 2014;162:22–38.
31 Seoudi N, Bergmeier LA, Drobniewski F, *et al*. The oral mucosal and salivary microbial community of Behçet's syndrome and recurrent aphthous stomatitis. *J Oral Microbiol* 2015;7:27150.
32 Y-j K, Choi YS, Baek KJ. Mucosal and salivary microbiota associated with recurrent aphthous stomatitis. *BMC Microbiol* 2016;16:1–10.
33 Stehlikova Z, Tlaskal V, Galanova N, *et al*. Oral microbiota composition and antimicrobial antibody response in patients with recurrent aphthous stomatitis. *Microorganisms* 2019;7:636.
34 Sato N, Kakuta M, Uchino E, *et al*. The relationship between cigarette smoking and the tongue microbiome in an east asian population. *J Oral Microbiol* 2020;12:1742527.
35 Jetté ME, Dill-McFarland KA, Hanshew AS, *et al*. The human laryngeal microbiome: effects of cigarette smoke and reflux. *Sci Rep* 2016;6:1–11.
36 Zhu Z, He Z, Xie G, *et al*. Altered oral microbiota composition associated with recurrent aphthous stomatitis in young females. *Medicine* 2021;100:e24742.
37 PENG R, CHENG L, PENG X. Advances in treponema denticola and oral diseases. *Journal of Prevention and Treatment for Stomatological Diseases* 2020:664–7.
38 Olsen I, Yamazaki K. Can oral bacteria affect the microbiome of the gut? *J Oral Microbiol* 2019;11:1586422.
39 Prodan A, Levin E, Nieuwdorp M. Microbiome: does disease start in the mouth. *the gut or both? Elife* 2019;8:e45931.
40 Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol* 2017;10:18–26.