

Original Article

Oral Microbiome Dynamics and Surfactant Protein A Expression in Patients with Spontaneous Intraoral Lesions

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ABSTRACT

Oral ulcers develop when the epithelial lining of the oral mucosa is compromised, often leading to pain, redness, discomfort, and bleeding. While some ulcers are triggered by local trauma, systemic illnesses, or medications, the causes of many remain unclear. This pilot study explores the composition of saliva and the oral microbiome in individuals with atraumatic pre-ulcerous and ulcerous lesions, compared to healthy controls, while accounting for three prevalent risk factors: smoking, stress, and sex. Using samples matched for age, sex, and ethnicity, we examined salivary levels of surfactant protein A (SP-A) and characterized the diversity and abundance of oral microbial populations. The study aimed to identify salivary indicators that could serve as early biomarkers for susceptibility to atraumatic oral ulcers. Our results demonstrate that SP-A levels are notably lower in female smokers than in healthy non-smoking females. Female patients with oral lesions also showed reduced SP-A compared to controls. Microbial composition was strongly influenced by both SP-A concentration and smoking status. Comparing healthy participants with those affected by lesions revealed 16 bacterial species with significant differences, all of which were modulated by SP-A and smoking. LEfSe analysis further highlighted five bacterial species as potential biomarker candidates. These findings suggest that alterations in the oral microbiome and SP-A levels are linked to risk factors for atraumatic oral ulcers. This preliminary work underscores the potential of saliva-based markers to predict ulcer susceptibility and points to possible interactions between innate immune mechanisms and microbial communities in the oral cavity.

Keywords: Pulmonary surfactant-associated protein a, Humans, Biomarkers, Oral ulcer, Microbiota

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Introduction

The mucosa, or mucosal membrane, lines the body's cavities and represents the primary interface between the internal environment and external stimuli. It serves as a crucial barrier against pathogens and chemical insults, forming a key component of the innate immune system [1]. Ulcerative lesions of the oral mucosa, or intraoral lesions, can arise in diverse medical contexts, such as autoimmune diseases, diabetes, and Sjögren's syndrome [2, 3]. Their development is influenced by multiple factors, including genetic predisposition, nutritional status, stress, hormonal fluctuations, and immune function. Clinically, oral ulcers are classified into four grades (I–IV) according to the World Health

Organization, with Grades III and IV representing severe forms marked by pronounced ulceration in the oral cavity [2, 4]. These lesions often interfere with fundamental activities like eating and speaking, significantly affecting patients' quality of life [4].

In oncology, patients receiving radiotherapy or chemotherapy for head and neck cancers frequently experience severe mucosal injury. Mucositis, a common manifestation of such treatment, affects approximately 80% of patients undergoing radiotherapy and 40% of those receiving standard chemotherapy doses [5, 6]. Beyond causing severe pain, mucositis can compromise nutrition and increase susceptibility to infections due to open sores, often

limiting the safe administration of cancer therapies [1, 7, 8].

While the mechanisms underlying severe Grade III and IV lesions in immunocompromised populations, including transplant recipients and cancer patients, are relatively well understood [2, 4, 7], the origins of milder lesions in otherwise healthy individuals remain poorly characterized [9, 10]. Most research has focused on cancer-related mucositis, leaving a knowledge gap regarding minor (Grade I–II) intraoral lesions in the general population.

Current pharmacological options for managing oral ulcers are limited. Amifostine, approved by the FDA, offers partial protection against radiation-induced mucosal damage in head and neck cancer patients [11]. Palifermin (Kepivance), the only drug approved for non-chemotherapy-associated ulcers, targets the keratinocyte growth factor (KGF) receptor on buccal mucosal cells. This interaction triggers the Ras-MAPK signaling cascade, promoting the expression of proteins essential for epithelial cell proliferation and survival [12, 13]. These treatments primarily address the downstream consequences of tissue damage, highlighting the need for preventive or alternative therapeutic strategies.

Surfactant proteins (SPs) are lectin-based molecules that play vital roles in innate immunity at mucosal surfaces. Initially identified in the lungs, SPs—including SP-A, SP-B, SP-C, and SP-D—reduce surface tension in the alveoli and function as pattern recognition molecules in immune defense [14]. SP-A and SP-D also regulate inflammatory responses [15]. In the gastrointestinal tract, surfactant proteins form hydrophobic barriers that protect tissue from acidic injury and microbial invasion [14, 16]. The oral mucosa expresses SPs that contribute to epithelial hydration and defense [17, 18]; however, their impact on oral microbiome composition and lesion prevention remains largely unexplored. Our previous work has confirmed the presence of SP-A in saliva [19], prompting this study to investigate whether salivary SP-A levels correlate with the onset and severity of oral ulcers.

Analogous to oral lesions, gastrointestinal ulcers such as peptic ulcers and ulcerative colitis are associated with microbial dysbiosis and inflammatory dysregulation, and are influenced by environmental factors including stress, sex, and smoking [20, 21]. While some studies have noted shifts in the oral microbiome in the presence of intraoral lesions, it remains unclear whether these microbial changes are causal or consequential. Moreover, the influence of risk factors such as smoking and sex on the interaction

between SP levels and oral microbial communities has not been thoroughly examined.

This pilot study focused on Grade I and II atraumatic soft tissue lesions characterized by pain, inflammation, and either localized redness or compromised mucosal integrity, occurring on the buccal mucosa, tongue, floor of the mouth, palatal tissue, attached gingiva, or oropharynx. Lesions caused by trauma or viral infection were excluded. Salivary surfactant protein levels and microbiome composition were analyzed to explore their association with lesion development, identify potential biomarkers for early detection, and provide insights into future research directions.

Materials and Methods

Study design, population, and power justification

The study population comprised individuals presenting with oral mucositis. Clinically, intraoral lesions were classified into four grades (I–IV) according to the World Health Organization (WHO) criteria for oral mucositis severity. Only lesions categorized as Grade I or II were included in this study. Grade I lesions were defined as mucosal soreness accompanied by localized erythema, while Grade II lesions involved mucosal soreness with tissue ulceration, without interfering with normal dietary intake. Both lesion types can cause discomfort significant enough to prompt patients to seek professional consultation.

Eligible lesions included any intraoral manifestation showing mucosal breakdown or localized inflammation, such as ulcerative or erythematous inflammatory lesions, occurring in the buccal mucosa, attached gingiva, floor of the mouth, tongue, palate, or oropharynx. Participants were excluded if they were under 18 years of age, had extra-oral herpetic lesions, lesions resulting from trauma (micro or macro), periodontal-related lesions, or hyperkeratotic lesions linked to smoking or mechanical irritation. There was no longitudinal follow-up, and participants completed the study upon providing saliva samples and undergoing clinical assessment.

Based on a previous investigation into salivary lipid levels in smokers [19], significant differences in SP-A levels were detected with a sample of 27 individuals. For the present study, 100 participants were screened under the inclusion criterion that they be a patient, student, or employee at UTSD between January 2018 and December 2019. Ultimately, 36 participants met all inclusion requirements. Data collection included demographic information (sex, age, ethnicity, race), smoking status, systemic health conditions, number of xerogenic medications, WHO mucositis grade, and self-reported pain levels. Associations among

demographic and health variables were assessed using cross-tabulation and Chi-square analyses. Statistical analyses were conducted using Stat-plus and GraphPad Prism software.

Ethical statement

This investigation adhered to international ethical standards, including the Declaration of Helsinki, and was authorized by the Institutional Review Board of the University of Texas Health Science Center at Houston (IRB Approval: HSC-DB-15-0742).

Clinical evaluation of UTSD patients

All participants were examined by a two-person research team, consisting of a clinician and a clinical research assistant. The clinician performed the clinical evaluation and documented all relevant findings on the study's data forms, whereas the research assistant handled the collection, labeling, and storage of biological samples. Patient pain levels were assessed using the Wong-Baker FACES pain scale [22].

To evaluate susceptibility to bruxism, the clinician scored each participant from 0 (no susceptibility) to 3 (high susceptibility) based on a series of clinical indicators. These included: reports of tooth sensitivity; presence of multiple compromised dental restorations; moderate to severe tooth wear or erosion; existence of tori, torus, or exostoses; complaints of temporal-area headaches; elevated stress scores; morning masticatory muscle discomfort; tenderness in the masseter muscles under 1 kg digital palpation; tenderness in the lateral pterygoid under ½ kg digital palpation; and tenderness of temporal tendons under ½ kg digital palpation. This assessment protocol reflects standard procedures for evaluating the head, neck, and musculoskeletal system. Pain and oral stress metrics collected through this evaluation were subsequently used as categorical metadata for downstream analysis of salivary SP-A levels and oral microbiome composition.

Saliva collection and measurements of SP-A

After obtaining written informed consent from participants, each was asked to perform an oral rinse with 10 mL of 2% citric acid for 30 seconds, then expectorate. This procedure was used to stimulate salivary secretion [23]. Following the rinse, two 0.5 mL samples of saliva were collected from each participant, immediately placed on ice, and subsequently stored at -80°C until analysis.

Salivary concentrations of SP-A were quantified in both healthy individuals and patients with oral lesions. An ELISA assay (BioVendor, LLC, Asheville, NC, Cat. No. RD191139200R) was employed to measure human SP-A levels according to previously established

methods [19]. In female participants, the presence of SP-A was further verified via Western blot. Proteins were separated by PAGE and detected using SP-A-specific antibodies (#sc-13977; Santa Cruz Biotechnology, Santa Cruz, CA). Visualization was achieved with the ECL Plus Western Blotting Detection System (#RPN2135, Amersham Biosciences Corp, Piscataway, NJ), and band intensities were quantified using a Storm 840 Phosphorimager (GE Healthcare, Piscataway, NJ).

Given that saliva is largely extracellular fluid, conventional internal controls such as beta-actin were not appropriate. Therefore, normalization was based solely on sample volume and total protein content. SP-A data did not conform to a normal distribution as determined by the D'Agostino and Pearson test, so non-parametric analyses were applied. Comparisons between two groups were performed using the Mann-Whitney test, while analyses of three or more groups employed the Kruskal-Wallis test. All statistical calculations were carried out using Stat-plus and GraphPad Prism software.

Microbiome DNA extraction from saliva

From the 36 study participants, a total of 18 individuals were selected for oral microbiome analysis. This group consisted of nine adults diagnosed with atraumatic oral lesions and nine healthy control participants carefully matched for age, sex, and ethnicity to ensure demographic comparability between the affected and unaffected groups.

For microbiome analysis, 500 µL of saliva from each participant was used for total DNA extraction. Purification was carried out using the UCP Mini Columns provided in the QIAamp DNA Microbiome Kit (Catalogue 51704, Qiagen, Hilden, Germany), following the manufacturer's instructions. The yield and quality of the extracted DNA were evaluated using both a Nanodrop 2000® spectrophotometer (Wilmington, USA) and a Qubit 1.0 fluorometer to ensure accurate quantification.

16S rRNA sequencing of oral microbiome

After extracting DNA, aliquots ranging from 100 ng to 1 µg per sample were sent to LC Sciences (Houston, TX, USA, <https://www.lcsciences.com/>) for 16S rRNA sequencing. The hypervariable V3-V4 region was targeted using primers 338F and 806R, and sequencing was carried out with paired-end reads on the Illumina MiSeq platform. During data processing, barcodes and adapter sequences were removed, paired reads were merged, and low-quality, unpaired, or chimeric sequences were filtered out. This quality control

process yielded a total of 171,563 high-quality reads across the 18 samples.

16S rRNA data analysis

The microbial composition of saliva samples was analyzed using the Microbial Genomics Diversity Module within CLC Genomics Workbench v20. Sequences were clustered into Operational Taxonomic Units (OTUs) by comparing them against the Human Oral Microbiome Database (HOMD) at a 98% similarity threshold [24]. Any sequences that did not match the database were further identified using BLAST searches against the NCBI 16S rRNA database. OTUs present in the abundance table were aligned with MUSCLE, applying a minimum count threshold of 10 to filter low-abundance features. Rarefaction was conducted by repeatedly sub-sampling OTU counts across 20 evenly spaced depth intervals ranging from 1 to 100,000 sequences, with 100 replicates at each interval to assess sampling sufficiency.

To evaluate within-sample diversity, alpha diversity indices—including observed OTUs, Chao1 (bias-corrected), Shannon entropy, and Simpson's index—were calculated, and differences across groups were tested using non-parametric methods. Between-sample diversity (beta diversity) was assessed using Bray-Curtis dissimilarity, and statistical significance was evaluated with PERMANOVA. Differences were visualized using Principal Coordinate Analysis (PCoA).

To determine which OTUs were differentially abundant between groups, non-parametric ANOVA tests were applied to the OTU abundance table, with adjustments for potential confounding factors. OTUs were considered significantly different if they appeared in at least two samples and had an FDR-adjusted *p*-value below 0.05. Microbial features potentially serving as biomarkers were identified using the Galaxy version of LEfSe, which detects taxa that are consistently overrepresented in biologically relevant categories across samples [25].

Results and Discussion

Population data

From 100 patients screened, 36 participants met the inclusion criteria and were enrolled for sampling, including 22 individuals with oral lesions and 14 healthy controls. Controls were selected to match the demographic diversity of the oral lesion group. Demographic characteristics of the study population are summarized in **Table 1**. The median age of participants was 52 years. Females comprised the

majority of the cohort (69%), and 53% identified as minorities, with 19% of these reporting Hispanic or Latino ethnicity. Twenty-five percent of participants were active smokers, 39% reported at least one systemic health condition, and 33% were taking medications associated with xerostomia.

Bruxism, assessed using the Bruxism Severity Index (BSI, where 0 indicates no clinical signs and 3 represents the most severe presentation), was present in 75% of participants, and 22% had temporomandibular disorders (TMD), as summarized in **Table 2**. Within the oral lesion group, lesion prevalence was significantly higher in women ($p = 0.04$) and in participants in their 60s ($p = 0.0001$), according to Chi-square analysis. The presence of intraoral lesions was strongly associated with pain ($p = 0.002$), with 45% of lesion patients reporting a pain score of 2 on the Wong-Baker FACES scale (**Table 2**). Notably, higher BSI scores correlated with lesion occurrence ($p = 0.01$), with only three of the 22 oral lesion patients scoring 0 on the BSI.

Although smoking was not significantly associated with oral lesion occurrence in this cohort, there were trends suggesting a potential relationship for participants in their 60s ($p = 0.07$) and for those with a BSI score above zero ($p = 0.07$).

Table 1. Demographics of enrolled subjects.

Category	Subgroup	All Patients with Oral Lesions (n=36)	%	Patients with Specific Oral Findings (n=22)	%
Gender	Male	11	31%	5	23%
	Female	25	69%	17	77%
Age (years)	20–30	3	8%	0	0%
	30–40	6	17%	2	9%
	40–50	5	14%	3	14%
	50–60	7	19%	4	18%
	60–70	9	25%	8	36%
	70 and over	6	17%	5	23%
Race and Ethnicity	African American	5	14%	4	18%
	Asian	6	17%	3	14%
	Caucasian	17	47%	9	41%
	Mixed race	1	3%	0	0%
	Hispanic/Latino	7	19%	6	27%

Demographics summary of oral lesion patients and unaffected control individuals.

Table 2. Distribution of health indicators.

Category	Subgroup	All Patients with Oral Lesions (n=36) %		Patients with Specific Oral Findings (n=22) %	
Smoking Status	Smoker	9	25%	3	14%
	Non-smoker	27	75%	19	86%
Oral Lesion Severity Scale	0 (none)	14	39%	0	0%
	1 (mild)	16	44%	16	73%
	2 (moderate)	6	17%	6	27%
Number of Systemic Diseases	None	22	61%	11	50%
	One	10	28%	8	36%
	Two or more	4	11%	3	14%
Xerostomia-Inducing Medications	None	24	67%	14	64%
	One	8	22%	5	23%
	Two or more	4	11%	3	14%
Bruxism Susceptibility Index	0 (none)	9	25%	3	14%
	1 (mild)	17	47%	12	55%
	2 or more	10	28%	7	32%
Pain Scale	0 (no pain)	24	67%	10	45%
	1 (mild)	2	6%	2	9%
	2 or more (moderate-severe)	10	28%	10	45%
Temporomandibular Disorder (TMD)	None	28	78%	15	68%
	Present (one or more signs)	8	22%	7	32%

Health status summary of oral lesion patients and unaffected control individuals.

SP-A levels

In our previous work, we observed that salivary SP-A concentrations are generally lower in women compared to men, and that female smokers exhibit lower SP-A levels than non-smoking females [19]. These patterns were also evident in the current cohort of 36 participants, with healthy female smokers showing significantly reduced SP-A levels compared to healthy female non-smokers. In this study, however, the difference in SP-A levels between males and females did not reach statistical significance, and no significant effect of smoking on SP-A was observed in male participants (**Figure 1a**). Western blot analysis was performed to validate the ELISA findings, confirming

that smoking is associated with a marked reduction of salivary SP-A in females (**Figure 1b**).

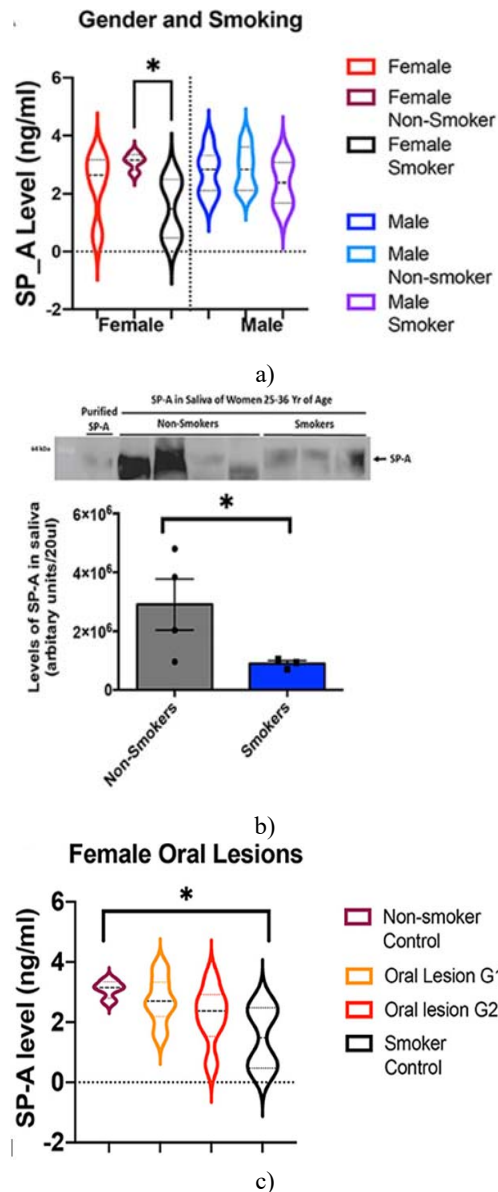


Figure 1. (a) ELISA measurements of salivary SP-A in control participants, stratified by sex and smoking status. SP-A levels are shown for all controls, followed by separate values for non-smokers and smokers. Female controls are presented on the left (n = 8; four non-smokers, four smokers) and male controls on the right (n = 8; five non-smokers, two smokers). Female non-smokers displayed significantly higher SP-A levels compared to female smokers (p = 0.03, Mann-Whitney test), while no other comparisons reached statistical significance. (b) Western blot analysis confirms that SP-A is significantly lower in female smokers versus non-smokers, with quantified band

intensities shown below the blot; an asterisk indicates a statistically significant difference. (c)

Average SP-A levels in female controls and patients with Grade I or II oral lesions, measured by ELISA, are displayed. Although SP-A levels in oral lesion patients appear lower than in controls, the overall difference did not reach significance (Kruskal-Wallis, $p = 0.09$).

We further compared SP-A concentrations in female oral lesion patients to those of smoker and non-smoker controls. While the differences were not statistically significant, a clear trend toward reduced SP-A in women with intraoral lesions was observed (**Figure 1c**). Considering the potential role of SP-A in modulating susceptibility to oral lesions in females, we also investigated whether variations in SP-A levels influence the composition of the salivary microbiome in both healthy controls and oral lesion patients.

Microbiome diversity

Given that most oral lesion patients in our cohort were female, and that females showed a trend toward lower SP-A levels, we selected a subset of 18 participants for

microbiome analysis. This subset included only one male oral lesion patient and two male controls for reference. Clustering the 16S rRNA sequences at 98% similarity yielded 383 OTUs, of which 244 were matched to the Human Oral Microbiome Database (HOMD), while the remaining 139 were identified via BLAST. When OTUs were summarized at the species level, a total of 249 distinct bacterial species were detected in the saliva of these 18 participants.

Analysis of alpha diversity revealed no significant differences between oral lesion patients and controls. Similarly, beta-diversity comparisons based on Bray-Curtis distances showed no significant distinction in microbial community composition between the lesion and control groups (FDR-adjusted $p = 0.96$). No significant effects of sex ($p = 0.32$) or race/ethnicity ($p = 0.19$) were observed.

However, metadata analysis identified significant influences of smoking ($p = 0.03$) and salivary SP-A levels ($p = 0.03$) on the microbial community (**Figure 2**). Although only three smokers were included in this sequencing subset, these findings align with prior larger studies reporting smoking-associated oral dysbiosis [26].

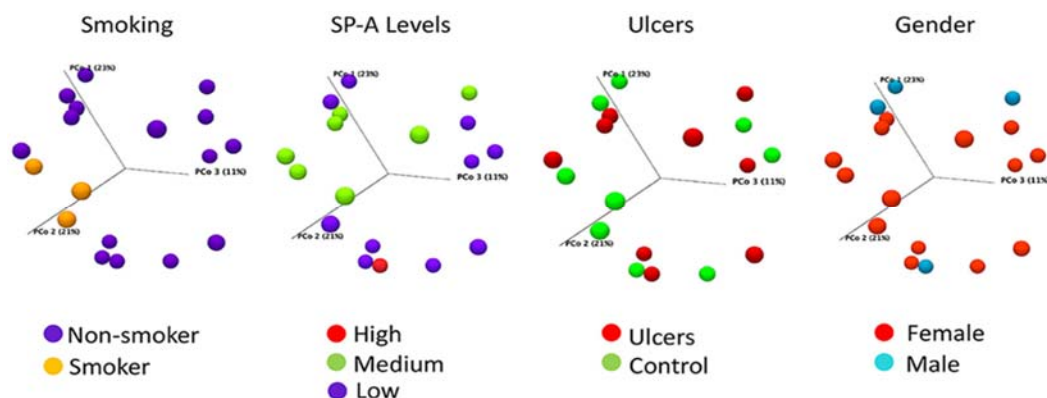


Figure 2. Principal Coordinate Analysis (PCoA) of salivary microbiome samples based on Bray-Curtis distances, with points colored according to smoking status, SP-A concentration, presence of oral ulcers, or gender. Axis 1 explains 23% of the variance, while axes 2 and 3 account for 21% and 11%, respectively. PERMANOVA analysis revealed that both smoking ($p = 0.03$) and SP-A levels ($p = 0.03$) significantly influence microbial community composition, whereas oral lesion status and gender showed no significant effect. For this analysis, SP-A concentrations were grouped into three categories: Low (0–2 ng/mL), Average (2–4 ng/mL), and High (4–8 ng/mL).

Microbiome abundance

Examination of bacterial species abundance identified 16 taxa present in at least two participants that were significantly altered in individuals with oral lesions compared to healthy controls (**Table 3**). When the analysis accounted for salivary SP-A levels, seven of these taxa no longer reached statistical significance (**Table 3**), (Block 1). Further adjustment for smoking status resulted in *Bifidobacterium dentium* losing

significance (**Table 3**), (Block 2). Controlling for both SP-A concentration and smoking removed the remaining eight significant OTUs (**Table 3**), (Block 3). These findings suggest that the differences initially attributed to intraoral lesions are largely explained by variations in SP-A levels and smoking rather than by the lesions themselves.

SP-A demonstrated a pronounced impact on the oral microbiome, with 53 species exhibiting significant

associations with SP-A levels, and 35 of these showing an FDR-adjusted p-value below 0.01 (**Table 4**). Of particular note, *Corynebacterium argentoratense* was markedly elevated in samples with low SP-A. Certain *Corynebacterium* species have previously been

implicated in cutaneous ulcer formation in susceptible hosts [27]. Overall, these results indicate that shifts in bacterial abundance are primarily driven by host factors, specifically SP-A levels and smoking, rather than the direct presence of oral lesions.

Table 3. Bacterial species displaying significant differences in oral lesion patients before and after adjusting for SP-A levels and smoking.

Block	Bacterial Species	Relative Abundance (Controls)	Relative Abundance (Oral Lesions)	Oral Lesions vs Controls Log2 Fold Change	FDR p-value	Non-Smoking Oral Lesions vs Controls Log2 Fold Change	FDR p-value	SP-A-Independent Oral Lesions vs Controls Log2 Fold Change	FDR p-value
1	<i>Campylobacter</i> sp. oral taxon 044	0.00994	0.00000	-6.64	0.030	-7.28	0.010	-	-
1	<i>Granulicatella elegans</i>	0.00760	0.00000	-6.79	0.030	-7.44	0.010	-	-
1	<i>Haemophilus pittmaniae</i>	0.05000	0.00032	-6.98	0.030	-7.61	0.010	-	-
1	<i>Haemophilus</i> sp. oral taxon 036	0.00910	0.00000	-6.50	0.030	-7.14	0.010	-	-
1	<i>Neisseria shayegani</i>	0.00636	0.00000	-6.67	0.030	-7.31	0.020	-	-
1	<i>Streptococcus rubneri</i>	0.04150	0.00234	-6.47	0.030	-7.02	0.010	-	-
1	<i>Veillonella</i> sp. oral taxon 780	0.02080	0.00000	-7.44	0.030	-8.04	0.010	-	-
2	<i>Bifidobacterium dentium</i>	0.04090	0.00000	-9.30	0.020	+8.03	0.0093	-	-
3	<i>Actinomyces israelii</i>	0.00395	0.00000	-5.87	0.040	-	-	-	-
3	Ruminococcaceae								
3	[G-1] sp. oral taxon 075	0.00431	0.00000	-5.63	0.040	-	-	-	-
3	<i>Prevotella shahii</i>	0.00000	0.00318	+5.74	0.040	-	-	-	-
3	<i>Ottowia</i> sp. oral taxon 894	0.00000	0.00555	+5.84	0.040	-	-	-	-
3	<i>Stomatobaculum longum</i>	0.00011	0.01150	+5.90	0.040	-	-	-	-
3	<i>Leptotrichia</i> sp. oral taxon 392	0.00000	0.00892	+6.37	0.030	-	-	-	-
3	<i>Capnocytophaga granulosa</i>	0.00000	0.01000	+6.76	0.030	-	-	-	-
3	<i>Capnocytophaga gingivalis</i>	0.00000	0.01060	+6.81	0.030	-	-	-	-

The “Name” column identifies the OTU that most closely matches each sequence. The next two columns show the relative abundances in the control and oral lesion groups, with the larger value indicated in blue and the smaller in green. The subsequent two columns provide the fold-change between lesion and control samples, along with the unadjusted FDR p-value. The final four columns report whether the observed

differences remain statistically significant after adjusting for potential confounders, specifically smoking and SP-A levels. The data are presented in three separate blocks: Block 1 lists OTUs affected by SP-A, Block 2 lists OTUs influenced by smoking, and Block 3 lists OTUs influenced by both SP-A and smoking.

Table 4. Bacteria sensitive to salivary SP-A levels.

Name	FDR p-value	Log2 fold change
<i>Corynebacterium argentoratense</i>	3.27E-05	-11.65
<i>Streptococcus</i> sp. oral taxon_057	4.42E-04	-10.08

<i>Stomatobaculum longum</i>	9.96E-04	-8.62
<i>Leptotrichia trevisanii</i>	1.93E-03	-8.46
<i>Prevotella albensis</i>	9.96E-04	-8.28
<i>Capnocytophaga granulosa</i>	8.56E-04	-8.26
<i>Propionibacterium propionicum</i>	9.96E-04	-8.25
<i>Capnocytophaga gingivalis</i>	1.93E-03	-8.16
<i>Catonella morbi</i>	1.36E-03	-8.01
<i>Streptococcus lactarius</i>	4.42E-04	-7.93
<i>Leptotrichia sp._oral_taxon_392</i>	1.93E-03	-7.74
<i>Lachnoanaerobaculum orale</i>	3.23E-03	-7.64
<i>Ottowia sp._oral_taxon_894</i>	4.78E-03	-7.26
<i>Prevotella shahii</i>	4.67E-03	-7.07
<i>Actinomyces odontolyticus</i>	1.93E-03	-6.93
<i>Neisseria elongata</i>	1.93E-03	-6.84
<i>Kingella denitrificans</i>	5.10E-03	-6.75
<i>Selenomonas sp._oral_taxon_137</i>	8.43E-03	-6.69
<i>Oribacterium asaccharolyticum</i>	4.93E-03	-6.44
<i>Capnocytophaga leadbetteri</i>	1.00E-02	-6.43
<i>Leptotrichia sp._oral_taxon_221</i>	1.00E-02	-6.34
<i>Treponema socranskii</i>	8.98E-03	-6.32
<i>Actinomyces sp._oral_taxon_448</i>	3.47E-03	-6.19
<i>Streptococcus anginosus</i>	4.40E-03	-6.14
<i>Cardiobacterium valvarum</i>	1.00E-02	-5.83
<i>Streptococcus vestibularis</i>	8.09E-03	-5.66
<i>Actinomyces oris</i>	8.98E-03	-5.63
<i>Selenomonas noxia</i>	1.00E-02	-5.39
<i>Actinomyces johnsonii</i>	1.00E-02	-5.06
<i>Neisseria subflava</i>	1.00E-02	-4.83
<i>Streptococcus sinensis</i>	4.42E-04	6.32
<i>Aggregatibacter sp._oral_taxon_458</i>	2.90E-03	6.89
<i>Haemophilus pittmaniae</i>	1.00E-02	6.91
<i>Haemophilus paraphrohaemolyticus</i>	7.23E-03	8.04
<i>Neisseria perflava</i>	3.27E-05	10.86

Only bacterial species with an FDR-adjusted p-value below 0.01 are included in the table. The entries are organized based on their relationship to SP-A levels, with species exhibiting an inverse correlation to SP-A indicated by negative values.

Biomarkers in microbiome data

Analysis of differential abundance revealed that the differences between oral lesion patients and controls were largely influenced by SP-A levels and smoking status. To explore potential microbial biomarkers, the dataset was analyzed using LEfSe, which applies linear discriminant analysis to identify taxa associated with specific conditions. Six species were highlighted as candidate biomarkers (Figure 3). Notably,

Capnocytophaga granulosa was detected in four oral lesion patients but was absent in all control samples. The other five species were elevated in healthy controls and not detected in lesion patients. Among these, *Bifidobacterium dentium* remained a significant discriminator between oral lesion patients and controls, independent of smoking status. No biomarkers were identified that could distinguish oral lesion status independently of SP-A levels.

Although this analysis is limited by the small sample size (18 subjects), the findings suggest that SP-A and the salivary microbiome may play important roles in oral lesion risk and warrant further investigation as potential predictive markers.

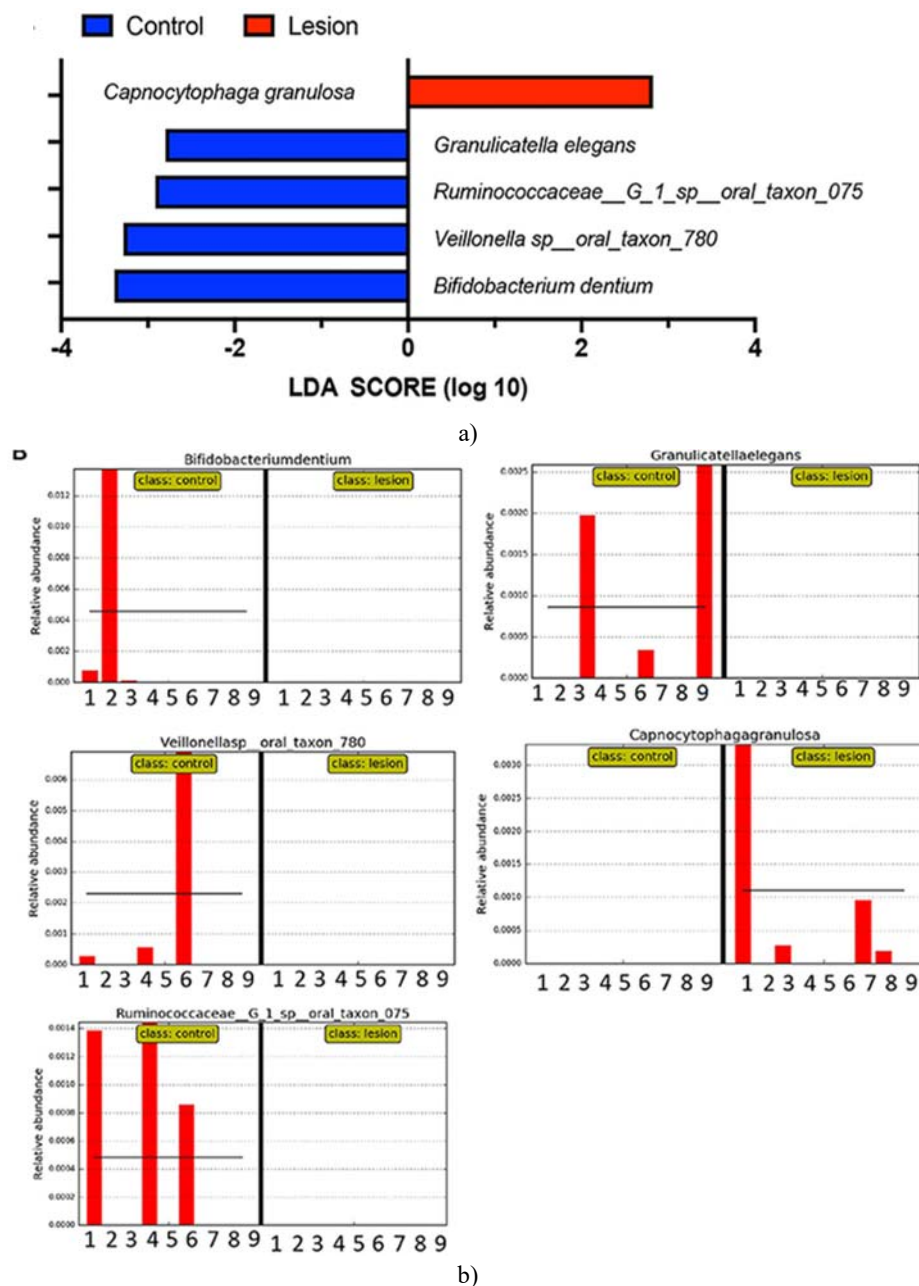


Figure 3. LefSe-based linear discriminant analysis of salivary microbiome profiles. The 18 samples were analyzed to identify OTUs most strongly associated with oral lesion status, using an LDA threshold of 2. (a)

One bacterial species, *Capnocytophaga granulosa*, was found to be enriched in oral lesion patients (highlighted in red), whereas four species were elevated in healthy controls (highlighted in blue). (b) Distribution of these candidate biomarkers across individual participants. Columns 1–9 correspond to control subjects, and columns 10–18 correspond to oral lesion patients. At least one of the four control-associated species was present in six of nine control participants, while the oral lesion-associated species appeared in four of nine oral lesion patients.

This pilot study aimed to explore potential associations among oral lesion risk factors, salivary SP-A levels, and the oral microbiome, with the goal of gaining insight into early events that may contribute to lesion development. Several limitations should be considered when interpreting these findings, including the small

sample size, the mild and early-stage nature of the lesions, high inter-individual variability in salivary microbiota, and the low number of smokers in the subset used for microbiome sequencing. Other host-related variables, such as diet, oral hygiene practices, and recent exposure to antibiotics or antimicrobial

agents, could also influence outcomes and affect the reliability of individual bacterial species as biomarkers. Despite these limitations, this is the first study to demonstrate an effect of SP-A levels on oral microbiome composition, highlighting the interaction between host innate immunity and bacterial colonization. Recognizing salivary SP-A as an immune factor that may predispose individuals to inflammation or infection could have broader implications beyond oral ulcer risk.

Saliva represents a particularly suitable sample for assessing risk because it is easily collected and reflects changes in host health. It contains bacterial communities representative of oral surfaces, which are sensitive to both local and systemic physiological conditions [28–30]. Identifying risk factors and microbial biomarkers in saliva may facilitate the development of more effective preventive strategies. In this study, oral lesions were relatively mild, with localized erythema (Grade I) or a few discrete ulcers (Grade II). Consistent with previous reports, intraoral lesions were more common in females and in individuals over 60 years of age [2, 19, 31]. Patients with lesions also reported higher levels of oral pain and displayed signs of bruxism, a behavior associated with systemic stress. These findings suggest that the Bruxism Severity Index (BSI) may warrant further investigation as a potential biomarker for oral ulcer risk.

Microbiome analysis was performed to assess whether changes in oral microbial populations are associated with lesion formation. We hypothesized that disruption of the mucosal barrier during inflammation and ulceration could alter the oral microbiome by modifying the local habitat [32–35]. Our results indicate that widespread dysbiosis is not present in mild lesions (Grade I–II); however, both SP-A levels and smoking significantly influenced microbial community structure, as shown by PERMANOVA. While some bacterial species differed between lesion patients and controls, these differences were primarily attributable to smoking, SP-A levels, or both, rather than to the lesions themselves. This suggests that microbial shifts may result from underlying risk factors rather than from changes in habitat due to the lesions, though additional research is needed to determine whether these microbial alterations precede lesion formation.

SP-A is a soluble protein with carbohydrate-recognition domains that is a key component of innate immunity, promoting the phagocytosis of bacteria in pulmonary alveoli by macrophages [36, 37]. Its role in saliva is not fully defined, but it is likely involved in protecting oral mucosa from microbial colonization. In

line with our previous findings, this study confirmed that salivary SP-A levels are significantly lower in female smokers compared to non-smokers, whereas no significant effect of smoking on SP-A was observed in males. This suggests a sex-specific regulation of SP-A production in the oral cavity. Evidence from pulmonary studies supports sex-dependent regulation of SP-A, which is influenced by hormones during lung development [38], and alveolar macrophage responses to infection are modulated by SP-A in a sex-specific manner during ozone exposure [39–41]. Our findings indicate that oral SP-A may shape microbial community composition, potentially through opsonization and macrophage-mediated clearance, although the exact mechanisms remain to be elucidated. Notably, female oral lesion patients displayed a trend toward lower SP-A levels, providing useful preliminary data for designing future studies with adequate statistical power.

SP-A-associated dysbiosis likely explains the dramatic increase in *Corynebacterium argenteum*, a species commonly present in saliva and first identified in association with tonsillitis [42]. It is also implicated in pharyngitis, upper respiratory infections, and has been isolated from blood cultures of cancer patients [42, 43]. *Corynebacteria*, in general, are opportunistic pathogens in the head, neck, and upper respiratory tract, with some species linked to cutaneous ulcer formation [27]. Future work should examine interactions between SP-A and oral *Corynebacteria* to determine whether these bacteria are sensitive to SP-A and whether they produce toxins that contribute to oral lesion development in otherwise healthy individuals.

The identification of *Capnocytophaga granulosa* as a potential biomarker for oral lesions is particularly interesting. *Capnocytophaga* spp. have been associated with inflammatory oral diseases such as periodontitis and preferentially colonize areas with necrotic cells. It is plausible that SP-A-driven dysbiosis allows opportunistic species like *Corynebacteria* to initiate inflammatory cascades that damage the mucosa, while *Capnocytophaga* serves as an indicator of ongoing cellular disruption [44–47]. Future studies with direct lesion sampling could clarify these relationships.

Conclusion

In conclusion, this pilot study demonstrates that salivary SP-A production is influenced by sex, with females showing reduced levels in the context of smoking and oral lesions. SP-A appears to modulate the oral microbiome, potentially facilitating bacterial clearance. Certain microbial species were identified as potential biomarkers of SP-A-mediated dysbiosis,

although further work is required to validate these findings and identify additional markers. These results contribute to understanding sex-specific effects of SP-A and suggest a role for SP-A and the oral microbiome in assessing lesion risk. This research provides a framework for developing simple, saliva-based chairside screening tools to evaluate oral lesion risk and host immune status [48, 49].

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Conflict of Interest: None

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