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Sample type-specific oral microbiome alterations in oral squamous cell carcinoma: a comparative analysis of saliva, dental plaque, and tongue coating

Atsumu Kouketsu

atsumu.kouketsu.c5@tohoku.ac.jp

Tohoku University Graduate School of Dentistry

Toru Tamahara

Tohoku University

Satoshi Fukase

Tohoku University Graduate School of Dentistry

Tatsuru Saito

Tohoku University Graduate School of Dentistry

Akiko Ito

Tohoku University Graduate School of Dentistry

Yutaro Higashi

Tohoku University Graduate School of Dentistry

Tsuyoshi Kurobane

Tohoku University Graduate School of Dentistry

Tomonari Kajita

Tohoku University Graduate School of Dentistry

Masaaki Miyakoshi

Tohoku University Graduate School of Dentistry

Masahiro Iikubo

Tohoku University Graduate School of Dentistry

Kensuke Yamauchi

Tohoku University Graduate School of Dentistry

Kazuki Kumada

Tohoku University

Bin Li

Tohoku University

Muneaki Shimada

Tohoku University

Miguel Reis Ferreira
King's College London
David Moyes
King's College London
Ritsuko Shimizu
Tohoku University
Tsuyoshi Sugiura
Tohoku University Graduate School of Dentistry

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Abstract

Objectives

Oral squamous cell carcinoma (OSCC)-associated microbiome alterations have been reported in various oral specimens; however, the optimal sample type for capturing disease-associated microbial changes remains unclear. This study compared bacterial communities in saliva, dental plaque, and tongue coating samples from patients with OSCC and healthy controls.

Materials and Methods

In this cross-sectional study, oral samples were collected from untreated patients with OSCC and healthy controls. After quality control and rarefaction, 47 control and 64 OSCC saliva samples, 10 control and 55 OSCC dental plaque samples, and 47 control and 61 OSCC tongue coating samples were analyzed using 16S rRNA gene amplicon sequencing. Alpha diversity, beta diversity, taxonomic composition, and genus-level differential abundance were assessed separately for each sample type.

Results

OSCC-associated microbiome alterations differed across sample types. Saliva showed significant differences between OSCC and controls across all alpha-diversity indices and beta-diversity metrics. Tongue coating also showed significant differences in multiple diversity metrics, particularly unweighted UniFrac distance, whereas dental plaque showed a more limited OSCC-associated signal. Genus-level differential abundance analysis identified the largest number of OSCC-associated taxa in saliva, followed by tongue coating.

Conclusions

OSCC-associated oral microbiome alterations showed distinct but partially overlapping patterns across sample types. Saliva most consistently captured differences between OSCC and controls, while tongue coating provided complementary information.

Clinical Relevance

Saliva appears to be a practical and informative specimen for future OSCC microbiome studies and biomarker-development research.

Introduction

Oral squamous cell carcinoma (OSCC) is the predominant malignant epithelial tumor of the oral cavity and mobile tongue and remains a major clinical challenge because of local invasiveness, functional morbidity, and regional metastasis [1]. Beyond established risk factors such as tobacco and alcohol use, local inflammatory and microbial factors have received increasing attention as potential contributors to oral carcinogenesis and tumor progression. The oral cavity harbors a complex microbial ecosystem, and

dysbiosis may influence epithelial homeostasis through inflammation, microbial metabolites, immune modulation, barrier disruption, and cancer-related signaling pathways [2, 3]. Salivary microbiome studies of oral potentially malignant disorders and OSCC have suggested associations between microbial dysbiosis and disease development [2]. However, the relationship between OSCC and the oral microbiome is likely bidirectional. Tumor lesions may reshape local microbial communities through ulceration, necrosis, bleeding, hypoxia, altered nutrient availability, tissue destruction, and changes in oral hygiene [3, 4]. Thus, OSCC-associated microbial profiles may reflect both potential microbial involvement in carcinogenesis and ecological consequences of the tumor microenvironment.

The oral cavity contains multiple ecological niches, and microbiome profiles differ substantially by sampling site. Saliva, dental plaque, and tongue coating capture distinct aspects of the oral microbial ecosystem [5]. Saliva contains microorganisms shed from multiple oral surfaces and provides a convenient, non-invasive sample for assessing broad microbial alterations [6]. Dental plaque is a structured biofilm influenced by local ecological interactions, oral hygiene, periodontal inflammation, and dentition [7]. Tongue coating forms on the tongue dorsum and retains desquamated epithelial cells, nutrients, and microorganisms; it has also been linked to oral and systemic conditions, including cancer and precancerous lesions [8]. Accordingly, these sample types provide complementary information and are not interchangeable. This is particularly relevant to OSCC, because tumor-associated microbial signatures may vary with disease-related ecological changes and the sampled niche. However, few studies have directly compared all three sample types in patients with OSCC and controls within a single analytical framework.

Although many studies have reported OSCC-associated oral microbiome alterations, the specific microbial features linked to OSCC remain inconsistent [9]. This heterogeneity may reflect biological variation and methodological factors, including sampling site, DNA extraction, sequencing platform, targeted 16S rRNA region, taxonomic database, and statistical approach [10]. Sample type may be particularly important because each specimen reflects a distinct microbial niche and may differ in its ability to capture tumor-associated changes. We therefore compared bacterial communities in saliva, dental plaque, and tongue coating from patients with OSCC and healthy controls using 16S rRNA gene amplicon sequencing. We hypothesized that saliva, by integrating microorganisms shed from multiple oral surfaces, would provide a more comprehensive representation of OSCC-associated alterations than site-restricted biofilm samples. We evaluated sample type-specific differences in alpha diversity, beta diversity, taxonomic composition, and genus-level differential abundance to determine which sample type most consistently captures OSCC-associated microbial alterations and to inform sample selection in future studies.

Materials and methods

Study design and participants

This cross-sectional observational study was conducted at Tohoku University Hospital, Miyagi, Japan, and included patients with oral squamous cell carcinoma (OSCC) and healthy controls. Saliva, dental plaque, and tongue coating samples were analyzed using 16S rRNA gene amplicon sequencing to evaluate sample type-specific oral microbiome alterations associated with OSCC.

The study protocol was approved by the Ethics Committee of Tohoku University Graduate School of Dentistry (approval no. 25091). Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki and reported according to the STROBE guidelines (Table S1).

OSCC was histopathologically diagnosed according to the World Health Organization classification, and clinical staging was determined using the Union for International Cancer Control TNM classification. Patients who had received chemotherapy or radiotherapy before sample collection, had non-oral or non-squamous cell malignancies, recent antibiotic or topical steroid use, or were unable to provide informed consent were excluded. Healthy controls had no oral cancer, oral potentially malignant disorders, oral mucosal disease, immune-related disease, nutritional disorders, or active dental infection.

Clinical variables, including age, sex, smoking, alcohol consumption, medical history, number of remaining teeth, plaque control record (PCR), and periodontal status, were recorded at the time of oral sample collection. Periodontal status was assessed by probing pocket depth, and the proportion of teeth with probing pocket depth ≥ 4 mm was calculated. Oral examinations and oral sample collection were performed by trained oral and maxillofacial surgeons. After quality control and rarefaction, the final sample numbers were: saliva, 47 controls and 64 OSCC samples; dental plaque, 10 controls and 55 OSCC samples; and tongue coating, 47 controls and 61 OSCC samples.

Oral sample collection

For patients with OSCC, oral samples were collected before surgical or oncological treatment. Unstimulated whole saliva, supragingival dental plaque, and tongue coating were collected before food intake, tooth brushing, oral rinsing, or oral treatment on the day of sampling.

Saliva was collected by passive expectoration into sterile tubes. Supragingival dental plaque was collected from the buccal surfaces of molars using sterile curettes, and tongue coating was obtained by gently scraping the dorsal tongue surface. Each sample type was placed in a separate sterile tube containing physiological saline. Samples were immediately frozen, transferred to the Tohoku University Clinical Biobank, aliquoted under standardized biobanking procedures for storage and quality control, and stored at − 80°C until DNA extraction [11]. No tumor tissue or tumor-surface specimens were collected or analyzed in this study.

DNA extraction and 16S rRNA gene sequencing

Bacterial genomic DNA was extracted separately from saliva, dental plaque, and tongue coating samples using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's

instructions.

The V3–V4 region of the bacterial 16S rRNA gene was amplified using a two-step PCR protocol with primers 341F and 805R, as described previously [5]. The expected amplicon size of approximately 540 bp was confirmed by gel electrophoresis, followed by addition of Illumina sequencing adapters and dual-index barcodes. Indexed libraries were quantified, pooled, diluted with PhiX control library, and sequenced on an Illumina MiSeq platform using paired-end chemistry. Nuclease-free water was included as a negative control from the PCR step onward and in the sequencing run.

Sequence processing and taxonomic assignment

Although sequencing was performed using paired-end chemistry, only forward reads were used for downstream sequence processing. Forward reads were processed using QIIME 2 version 2024.10.1 and DADA2 [12, 13]. The resulting ASV table was subjected to quality control and taxonomic filtering.

Taxonomy was assigned using a classifier based on the Greengenes2 database release 2024.09 [14]. The final taxonomically filtered ASV table was used for downstream diversity, taxonomic composition, and differential abundance analyses. Primary analyses were performed separately for each sample type, whereas supplementary complete-triplet analyses jointly evaluated saliva, dental plaque, and tongue coating samples obtained from the same participants.

Diversity analyses

Diversity analyses were performed separately for each sample type using a rarefied ASV table at 5,000 sequences per sample. Alpha diversity was assessed using observed features, Shannon diversity, Pielou's evenness, and Faith's phylogenetic diversity [15, 16].

Beta diversity was evaluated using weighted UniFrac, unweighted UniFrac, Bray–Curtis, and Jaccard distances and visualized by principal coordinates analysis [17–20]. Weighted UniFrac reflected abundance-weighted phylogenetic differences, unweighted UniFrac reflected presence–absence-based phylogenetic differences, Bray–Curtis reflected abundance-based compositional dissimilarity, and Jaccard reflected presence–absence-based compositional dissimilarity.

To directly compare sample types within participants, supplementary complete-triplet analyses were performed among 62 participants (9 controls and 53 patients with OSCC) for whom saliva, dental plaque, and tongue coating samples were all retained after rarefaction, yielding 186 samples. Alpha-diversity metrics were analyzed jointly across the three sample types. For beta diversity, within-participant distances were extracted for the three sample pairs: saliva–dental plaque, saliva–tongue coating, and dental plaque–tongue coating.

Taxonomic composition analysis

Taxonomic profiles were generated separately for saliva, dental plaque, and tongue coating. The ASV table was collapsed at the class and genus levels, and relative abundance was calculated for each sample. Relative abundances were calculated for control and OSCC samples within each sample type.

Class- and genus-level heatmaps were created to visualize sample type-specific compositional differences. Genus names were used when available; otherwise, the closest informative taxonomic annotation was retained. Mean relative abundance and mean difference for each taxon were summarized in a taxonomic composition table.

Differential abundance analysis

Genus-level differential abundance analysis was performed separately for saliva, dental plaque, and tongue coating using ANCOM-BC2 [21]. Genus-level count tables were compared between control and OSCC samples, with disease status as the explanatory variable. A 10% prevalence filter was applied, and Benjamini–Hochberg correction was used for multiple testing. Significance was defined as $q < 0.05$.

The direction of differential abundance was determined from the ANCOM-BC2 log fold change (natural-log scale), with positive values indicating enrichment in OSCC and negative values indicating depletion in OSCC. For Fig. 4, representative named genera with $q < 0.05$ and a maximum group-wise mean relative abundance ≥ 0.05% were selected when the ANCOM-BC2 direction agreed with group-wise mean differences; up to eight genera per sample type were shown, prioritized by abundance, q value, and absolute log fold change. Full results are provided in Table S5.

Predictive functional profiling

Functional profiles were predicted from 16S rRNA gene data using PICRUSt2 version 2.6.3 and summarized as MetaCyc pathway relative abundances [22]. Analyses were performed separately for saliva, dental plaque, and tongue coating samples. Pathways present in at least 10% of the combined control and OSCC samples within each sample type were retained and compared between groups using two-sided Wilcoxon rank-sum tests. P values were adjusted using the Benjamini–Hochberg method independently within each sample type, and significance was defined as $q < 0.05$. Rank-biserial correlation was used as an effect-size measure, with positive values indicating higher predicted abundance in OSCC.

Statistical analysis

Statistical analyses were performed using QIIME 2, R version 4.5.3, and JMP Pro version 17. Continuous variables are presented as mean ± standard deviation or median with interquartile range, and categorical variables as numbers and percentages. Clinical characteristics were compared using the Wilcoxon rank-sum test, chi-square test, or Fisher’s exact test, as appropriate.

Alpha-diversity metrics were compared between groups using pairwise Kruskal–Wallis tests, with Benjamini–Hochberg correction applied across the three pairwise comparisons within each sample type

and diversity metric. Beta-diversity differences were assessed using PERMANOVA based on rarefied distance matrices with 999 permutations, with Benjamini–Hochberg correction applied across the three pairwise comparisons within each sample type and beta-diversity metric [23]. Multivariable PERMANOVA was additionally performed separately for each sample type, adjusting for plaque control record, smoking history, alcohol consumption, and periodontal status, with disease status entered last in the sequential model. Multivariable analyses were based on complete cases, used 9,999 permutations, and applied Benjamini–Hochberg correction across the 12 disease-status tests. Multivariate dispersion was assessed using PERMDISP with 9,999 permutations; PERMDISP P values were reported without multiple-testing adjustment.

In the supplementary complete-triplet analysis, alpha-diversity metrics were analyzed using linear mixed-effects models including disease group, sample type, and their interaction as fixed effects and participant as a random intercept. Within-participant beta-diversity distances were analyzed similarly using disease group, sample pair, and their interaction. Outcome transformations and residual variance structures were selected based on model diagnostics and are detailed in Table S7. Estimated marginal means, 95% confidence intervals, effect sizes, and Benjamini–Hochberg-adjusted q values were reported. Statistical significance was defined as $q < 0.05$.

Results

Patient characteristics

The clinical characteristics of healthy controls and OSCC patients are shown in Table S2.

Alpha-diversity differences between OSCC and controls across oral sample types

Alpha-diversity differences between OSCC and control samples were assessed across the three oral sample types (Fig. 1, Table 1). In saliva, all four alpha-diversity indices differed significantly between groups, with lower Shannon diversity and Pielou's evenness but higher observed features and Faith's phylogenetic diversity in OSCC samples. In dental plaque, significant differences were limited to Pielou's evenness and Faith's phylogenetic diversity, whereas Shannon diversity and observed features were not significant after FDR correction. In tongue coating, Shannon diversity, Pielou's evenness, and Faith's phylogenetic diversity differed significantly between groups, while observed features did not remain significant after correction. Overall, OSCC-associated alpha-diversity differences were most consistently detected in saliva, followed by tongue coating, whereas dental plaque showed a more restricted pattern.

In the complete-triplet analysis of 62 participants with all three sample types available, no significant disease group × sample type interaction was detected for any alpha-diversity metric after multiple-testing correction (all $q \geq 0.216$; Supplementary Figure S3 and Table S7). Thus, the magnitude of the OSCC-associated alpha-diversity differences did not differ significantly among the three sample types.

Beta diversity revealed OSCC-associated microbial community differences across oral sample types

Beta-diversity analysis demonstrated sample-type-dependent differences in microbial community composition between OSCC and control samples (Fig. 2, Supplementary Figure S1, and Table 2). In the unweighted UniFrac PCoA, saliva and tongue coating samples showed visible separation between groups, whereas dental plaque showed less distinct separation. PERMANOVA showed significant differences across all four beta-diversity metrics in saliva, indicating that OSCC-associated community differences were detectable using both phylogenetic and non-phylogenetic distance measures. In dental plaque, significant differences were limited to Jaccard and unweighted UniFrac distances, whereas in tongue coating, Bray–Curtis, Jaccard, and unweighted UniFrac distances were significant. Overall, saliva showed the most consistent OSCC-associated beta-diversity differences, while tongue coating showed particularly strong separation based on unweighted UniFrac distance. Dental plaque exhibited a more limited pattern of community-level differences.

After adjustment for plaque control record, smoking history, alcohol consumption, and periodontal status, disease status remained significantly associated with all four beta-diversity metrics in saliva, with unweighted UniFrac and Jaccard distances in dental plaque, and with unweighted UniFrac, Bray–Curtis, and Jaccard distances in tongue coating (Table S3). PERMDISP indicated significant dispersion differences for Jaccard distances in all three sample types and for Bray–Curtis distance in tongue coating; these findings should therefore be interpreted cautiously.

In the complete-triplet analysis, significant disease group × sample pair interactions were observed for Jaccard ($q = 0.0385$) and unweighted UniFrac distances ($q = 0.0182$), but not for Bray–Curtis or weighted UniFrac distances. Within-participant distances were lower in OSCC for saliva–dental plaque and dental plaque–tongue coating based on Jaccard distance (both $q < 0.001$), and for dental plaque–tongue coating based on unweighted UniFrac distance ($q = 0.0029$), indicating greater similarity between these specimen types in OSCC (Table S7).

Taxonomic composition at the class and genus levels

We next examined class- and genus-level taxonomic composition across saliva, dental plaque, and tongue coating samples (Fig. 3; Table S4). At the class-level, *Bacilli A*, *Bacteroidia*, *Gammaproteobacteria*, *Actinomycetes*, and *Fusobacteriia* were dominant across the three oral sample types, but their relative abundance patterns differed by specimen. Descriptive compositional differences between OSCC and control samples were observed in each sample type, including increased *Bacilli A* in saliva and dental plaque and reduced *Fusobacteriia* in dental plaque.

Genus-level heatmaps further showed sample-type-specific compositional patterns. *Streptococcus* was abundant across all specimens and showed higher mean relative abundance in OSCC saliva and dental plaque samples. Other genera, including *Rothia*, *Gemella*, *Granulicatella*, *Peptostreptococcus*, *Alloprevotella*, and *Fusobacterium C*, also showed sample-dependent differences between OSCC and control groups. Overall, these descriptive taxonomic analyses suggested that OSCC-associated microbial shifts were detectable across oral sample types, although formal differential abundance testing was required to identify statistically associated genera.

ANCOM-BC2 analysis reveals prominent OSCC-associated genus-level alterations in saliva and tongue coating

Genus-level differential abundance analysis using ANCOM-BC2 revealed sample-type-dependent OSCC-associated taxonomic alterations (Fig. 4 and Table S5). Saliva showed the largest number of differentially abundant taxa, with 38 significant taxa, including 9 enriched and 29 depleted in OSCC. Genera enriched in OSCC saliva included *Streptococcus*, *Peptostreptococcus*, *Gemella*, *Granulicatella*, *Alloprevotella*, and *Corynebacterium*. In contrast, dental plaque showed only 4 significant taxa, including enrichment of *Streptococcus* in OSCC. Tongue coating showed 15 differentially abundant taxa, including 4 enriched in OSCC, such as *Haemophilus A*, *Alloprevotella*, *Corynebacterium*, and *Fusobacterium C*. Overall, saliva and tongue coating captured broader OSCC-associated genus-level alterations than dental plaque, supporting their potential utility as informative sample types for future OSCC microbiome studies.

Predicted functional alterations

PICRUSt2 identified 35 significantly different MetaCyc pathways in saliva and 36 in tongue coating, whereas none remained significant in dental plaque after multiple-testing correction (Supplementary Table S6). Polyamine biosynthesis III, ectoine biosynthesis, and the 2-methylcitrate cycle I were increased in OSCC in both saliva and tongue coating. Saliva additionally showed increases in L-carnitine degradation and N-acetylated sugar degradation, whereas tongue coating showed increased pyruvate fermentation to propanoate I and reduced L-arginine degradation II (Supplementary Figure S2).

Discussion

OSCC-associated oral microbiome alterations differed across saliva, dental plaque, and tongue coating. In sample-type analyses, saliva most consistently captured differences between OSCC and controls across alpha diversity, beta diversity, and genus-level differential abundance. Tongue coating also reflected substantial changes, whereas dental plaque showed a more restricted signal. The complete-triplet analysis showed no significant disease group × sample type interaction for alpha diversity, so OSCC-associated differences did not differ significantly among sample types. In contrast, significant disease group × sample pair interactions were detected for Jaccard and unweighted UniFrac distances, with lower within-participant distances for selected specimen pairs in OSCC. These findings suggest that sample type-specific patterns may coexist with greater presence–absence similarity across some oral niches in OSCC.

Previous studies report OSCC-associated alterations in oral microbial diversity, community structure, and taxonomic composition, consistent with our findings. The salivary differences support previous studies showing dysbiosis across oral potentially malignant disorders and OSCC and differences between individuals with and without OSCC [2, 24]. Several taxa identified here, including Streptococcus, Fusobacterium, Prevotella, Peptostreptococcus, Gemella, Granulicatella, and Alloprevotella, have also been reported in earlier OSCC studies [2, 24, 25]. Recent reviews and meta-analyses likewise indicate

shifts in oral microbial diversity and composition in OSCC [9, 26]. However, the direction and magnitude of taxonomic differences vary across studies, and no universally consistent OSCC-specific microbiome signature has been established. This inconsistency may reflect differences in sampling site, DNA extraction and sequencing protocols, targeted 16S rRNA regions, taxonomic databases, statistical methods, geographic background, oral hygiene, periodontal condition, tumor site, and disease stage [9, 26]. Unlike many studies focusing on a single specimen, often saliva, we compared saliva, dental plaque, and tongue coating within the same population and analytical framework. This enabled evaluation of both the OSCC-associated signals captured by each specimen and within-participant relationships among sample types.

The observed differences among sample types are biologically plausible. Saliva contains microorganisms shed from multiple oral surfaces and may integrate microbial changes arising from the tumor surface, oral mucosa, periodontal sites, and tongue dorsum [5, 6]. Tumor-associated ulceration, bleeding, necrosis, inflammation, hypoxia, and altered nutrient availability may further influence microorganisms released into saliva [4], potentially explaining why saliva most consistently captured OSCC-associated differences across diversity, differential abundance, and predicted functional analyses. Tongue coating also showed clear OSCC-associated microbial alterations. The tongue dorsum provides a large mucosal surface with papillary structures and functions as an important microbial reservoir [8, 27]. Its strong separation based on unweighted UniFrac distance and the second largest number of differentially abundant taxa after saliva suggest sensitivity to presence–absence changes, including low-abundance taxa.

The complete-triplet analysis further showed lower Jaccard distances between saliva and dental plaque and between dental plaque and tongue coating in OSCC, while unweighted UniFrac distance between dental plaque and tongue coating was also reduced. Because these metrics emphasize presence or absence rather than relative abundance, they may indicate greater sharing of bacterial lineages across oral niches in OSCC. This convergence could reflect increased microbial dispersal, common exposure to tumor-associated ecological conditions, or changes in mucosal and biofilm environments. Nevertheless, within-participant distances cannot determine the direction of microbial transfer or identify the anatomical source of shared taxa. Dental plaque remains a structured biofilm strongly influenced by local ecology, oral hygiene, periodontal inflammation, and dentition [7]. Thus, its limited differential abundance signal does not necessarily indicate an absence of OSCC-associated ecological change, particularly given the observed cross-site similarity and small number of control plaque samples.

Genus-level taxa identified in this study should be interpreted as ecological signatures associated with OSCC rather than direct causal agents. Several genera enriched in OSCC, including Streptococcus, Peptostreptococcus, Gemella, Granulicatella, Alloprevotella, Corynebacterium, and Fusobacterium C, have also been reported in previous OSCC, oral cancer, or oral potentially malignant disorder microbiome studies [2, 24, 25, 28, 29]. These changes may reflect tumor-associated conditions such as inflammation, mucosal disruption, bleeding, necrosis, and nutrient-rich or anaerobic microenvironments. Because this study used 16S rRNA gene amplicon sequencing, however, species- or strain-level identification and

functional inference remain limited. The observed genus-level alterations should therefore be regarded as OSCC-associated microbial patterns rather than evidence that specific bacteria directly contribute to carcinogenesis or tumor progression [10].

Predicted functional profiles also differed by sample type. Shared increases in polyamine biosynthesis, ectoine biosynthesis, and the 2-methylcitrate cycle may reflect altered microbial growth, stress adaptation, and substrate utilization in the OSCC-associated oral environment. Sample type-specific changes in carnitine, N-acetylated sugar, arginine, and fermentation pathways further suggest shifts in microbial metabolism. Such shifts could influence the local inflammatory and immune environment through microbe-derived metabolites, although host responses were not directly assessed in this study [2, 3]. These findings require caution because PICRUSt2 predicts functional potential from 16S profiles rather than directly measuring microbial genes, gene expression, or metabolite production.

These findings have implications for future OSCC microbiome and biomarker research. Approaches for early detection and risk assessment increasingly include liquid biopsy, salivary biomarkers, genetic risk stratification, and artificial intelligence-assisted diagnostic models [30–34]. Within this context, oral microbiome analysis may provide additional biological information reflecting tumor-associated ecological changes. Because saliva is non-invasive, easy to collect, and suitable for repeated sampling, it represents a practical specimen for future OSCC microbiome research, while tongue coating may provide complementary information on presence–absence-based community alterations. However, diagnostic performance was not evaluated, and the observed differences should not be interpreted as validated screening biomarkers. Future studies should develop and externally validate predictive models in larger prospective cohorts.

This study has several limitations. First, its cross-sectional design precludes causal conclusions; the observed alterations may reflect either factors contributing to OSCC development or ecological consequences of the tumor microenvironment. Second, this was a single-center study, and the generalizability of the findings to other populations, geographic regions, and clinical settings remains uncertain. Third, the small and imbalanced number of control dental plaque samples may have reduced statistical power and precision. The complete-triplet analysis included only 62 participants, including nine controls, and should therefore be regarded as supplementary and exploratory. Fourth, although multivariable PERMANOVA adjusted for plaque control record, smoking history, alcohol consumption, and periodontal status, residual confounding by other lifestyle, systemic, and oral environmental factors, including diabetes and oral infections, cannot be excluded. Significant dispersion differences for selected metrics also indicate that some PERMANOVA findings may partly reflect within-group variability. Fifth, 16S rRNA gene amplicon sequencing provides limited species- or strain-level resolution and does not directly assess microbial function or pathogenicity. Sixth, detailed subgroup analyses by tumor site, clinical stage, or histological differentiation were not performed, and no external validation cohort was included. Finally, because tumor-surface and intratumoral specimens were not analyzed, we could not determine whether the detected microbial patterns originated directly from the tumor or

reflected broader oral environmental changes. The within-participant distance analysis assessed ecological similarity but did not establish microbial transmission or source–sink relationships.

Despite these limitations, the study has several strengths. Saliva, dental plaque, and tongue coating were compared within the same study, and OSCC samples were collected before surgical or oncological treatment. All sample types were analyzed using a consistent 16S rRNA sequencing and bioinformatics workflow, allowing comparisons under a unified analytical framework. The complete-triplet analysis accounted for repeated measurements within participants and directly evaluated relationships among the three sample types. By combining alpha diversity, beta diversity, taxonomic composition, differential abundance, and predicted functional analyses, this study provides a comprehensive assessment of OSCC-associated microbial alterations across three commonly used oral specimens. These features strengthen the practical value of the findings for informing sample selection in future OSCC microbiome studies.

Conclusion

OSCC-associated microbiome alterations showed distinct but partially overlapping patterns across saliva, dental plaque, and tongue coating. Saliva most consistently captured differences between OSCC and control samples, while tongue coating provided complementary information and dental plaque showed a more restricted signal. Although the magnitude of OSCC-associated alpha-diversity differences did not differ significantly among sample types, presence–absence-based beta-diversity analyses indicated greater within-participant similarity among selected specimen pairs in OSCC. Future large-scale, multicenter, and longitudinal studies incorporating tumor-associated specimens, shotgun metagenomic analysis, source-tracking approaches, and external validation are needed to clarify the biological and clinical significance of these findings.

Declarations

Disclosure statement

The authors declare no competing interests.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used ChatGPT in order to assist with language editing and drafting support. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Author Contribution

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request, subject to institutional and ethical restrictions.

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