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RECEIVED 23 April 2026

REVISED 26 June 2026

ACCEPTED 29 June 2026

PUBLISHED 24 July 2026

CITATION

Mandalà E, Coppini M, Mauceri R,
Pizzo M, Giammanco GM, Campisi G
and Bonura C (2026) Analysis of 16S
rRNA to evaluate taxonomic
differences between salivary and
intratumoral microbiota in patients
affected by oral squamous cell
carcinoma: a single-center pilot study.
Front. Bacteriol. 5:1863255.
doi: 10.3389/fbri.2026.1863255

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Analysis of 16S rRNA to evaluate taxonomic differences between salivary and intratumoral microbiota in patients affected by oral squamous cell carcinoma: a single-center pilot study

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Background: Advancements in DNA sequencing technology have facilitated the assessment of the connection between the oral microbiome and various diseases. The aim of the present study was to investigate the differences between salivary and intratumoral microbiota composition in patients affected by oral squamous cell carcinoma (OSCC).

Methods: Unstimulated saliva and intratumoral fresh biopsy samples were collected. DNA was extracted using the QIAamp PowerFecal Pro, and next-generation sequencing was performed using the MiSeq Illumina device.

Results: A total of 12 patients were enrolled, of whom 7 were diagnosed with OSCC and five were OSCC-free, comprising the control group, including healthy patients and those affected by oral potentially malignant diseases (OPMDs). In the OSCC group, four were male and three were female, with a mean age of 58.4 ± 11.5 years; in the control OSCC-free group, four were male and one was female, with a mean age of 59.4 ± 18.3 years. The masticatory mucosa was the most affected OSCC site (42.8%), followed by the floor of the mouth (28.6%), the border of the tongue (14.3%), and the palate (14.3%). Compared to controls, the OSCC group exhibited an enrichment of bacteria such as *Fusobacterium nucleatum* in the tumor tissue, alongside a distinct salivary profile characterized by the enrichment of the *Neisseria* genus and a consequent depletion of *Streptococcus*.

Conclusions: Despite the limited sample size of this pilot study, we observed that the oral microbiota differs significantly depending on both the patient's clinical status (OSCC vs. OSCC-free) and the specific sampling site (tumor tissue vs. saliva). A potential association between oral dysbiosis and OSCC onset may provide a new target for future validation and early diagnosis.

KEYWORDS

disease progression, *Fusobacterium nucleatum*, intratumoral microbiota, next generation sequencing, squamous cell carcinoma of head and neck, tumor microenvironment

Comparative Analysis of Salivary and Intratumoral Microbiota in OSCC

1. STUDY DESIGN

Cohort:
N = 12 Patients
(7 OSCC, 5 Controls/OPMD)

Samples:
Unstimulated Saliva
Intratumoral Biopsy

Method:
16S rRNA NGS
(Illumina MiSeq)

2. KEY FINDINGS

**OSCC Microenvironment
& Salivary Dysbiosis:**

□ ***Fusobacterium***
(Intratumoral Enrichment)

□ ***Neisseria***
(Salivary Enrichment)

□ ***Streptococcus***
(Commensal Depletion)

3. CLINICAL IMPACT

**Periodontopathic Bacteria
as potential OSCC drivers in TME**

Salivary signatures mirror
dysbiotic alterations

*Targets for new
diagnostic tools*

GRAPHICAL ABSTRACT

1 Introduction

Oral cancer represents the most prevalent neoplasm within the biologically diverse group of head and neck carcinomas, currently ranking as the sixth most common malignancy globally (Badwelan et al., 2023). Driven by a rising incidence trend, approximately 377,713 new cases were recorded worldwide in 2020 (Sung et al., 2021). The vast majority of these malignancies, which represent over 90% of cases, are classified as oral squamous cell carcinoma (OSCC) arising from the mucosal epithelium (Warnakulasuriya, 2009; Gissi et al., 2019). Despite significant diagnostic and therapeutic advancements, the overall 5-year survival rate remains at approximately 50%–60% (Mitchell et al., 2018; Musella et al., 2025). This persistently high mortality rate is primarily driven by delayed detection, as a substantial proportion of patients are diagnosed at advanced clinical stages (stages III and IV) (González-Ruiz et al., 2023).

OSCC etiology is multifactorial, traditionally linked to host genetics and well-established environmental risk factors such as tobacco smoking and alcohol consumption (Lee et al., 2017; Panzarella et al., 2021). However, the oral cavity also hosts a highly dynamic ecosystem responsive to continuous environmental shifts, comprising over 700 species of bacteria, fungi, viruses, and protozoa (Lamont et al., 2018). While the concept of the

“microbiome” as a defined ecological community with distinct physicochemical properties has been recognized for decades (Marchesi and Ravel, 2015), its exact mechanistic relationship with OSCC development is still an area of active investigation.

Interest in non-invasive diagnostic methods has grown recently (Tayebi-Hillali et al., 2025). In this context, saliva analysis has provided an accessible means to study shifts in microbial populations across the oral cavity and identify potential OSCC associations. More recently, however, scientific focus has broadened to include the intratumoral microbiota, which consists of microorganisms actively residing within the tumor tissue itself. These microbes are now recognized as integral components of the tumor microenvironment (TME), exhibiting a composition that often differs profoundly from that of adjacent healthy tissues (Sepich-Poore et al., 2021). Chronic colonization by specific oral pathogens can upregulate inflammatory cytokines and dysregulate host metabolic pathways, thereby fostering a pro-tumorigenic niche (Rajeev et al., 2012).

Within the OSCC context, periodontal pathogens such as *Fusobacterium*, *Prevotella*, and *Streptococcus* are frequently enriched in both saliva and tissue samples (Karpiński, 2019). *Fusobacterium nucleatum*, an anaerobic bacterium notably linked to colorectal cancer (Castellari et al., 2012), exhibits a particularly high abundance within OSCC tissues (Mauceri et al., 2023). This

pathogen actively promotes carcinogenesis and cellular invasion by triggering chronic inflammation and exerting anti-apoptotic effects. Specifically, *F. nucleatum* stimulates OSCC proliferation via the E-cadherin/beta-catenin pathway (Rubinstein et al., 2013) and promotes immune evasion by interacting with the TIGIT inhibitory receptor on host immune cells (Gur et al., 2015). Additionally, species such as *Streptococcus anginosus* and various *Prevotella* strains (*Prevotella intermedia*, *Prevotella melaninogenica*, and *Prevotella jejuni*) are prominent in OSCC. Some of these bacteria directly contribute to oncogenesis through the production of carcinogenic metabolites, such as ethanol-derived acetaldehyde (Nieminen and Salaspuro, 2018).

Because the oral ecosystem encompasses distinct ecological niches—ranging from saliva to mucosal surfaces and deep tissues—each site harbors a unique microbial community. Consequently, comparative analyses between saliva and intratumoral samples from the same patient consistently reveal profound compositional differences (Zhao et al., 2017).

The objective of the present pilot study was to characterize the salivary and intratumoral microbiota in the OSCC group compared to OSCC-free controls, investigating the ecological discrepancies between these distinct niches. By detailing the pathogenic roles and structural shifts of specific bacterial species, we aimed to contribute to the understanding of microbe-driven carcinogenesis in the oral cavity. Following this initial ecological comparison, we subsequently stratified the microbial profiles across four distinct clinical stages. This analytical framework evaluates the translational potential of 16S ribosomal RNA (rRNA) sequencing in clinical practice, seeking to identify non-invasive salivary microbial signatures that can be utilized for early detection and risk stratification. Beyond diagnostics, comprehensively characterizing the intratumoral ecosystem provides the essential biological foundation for next-generation clinical management. Recent evidence suggests that the microbiome itself holds immense therapeutic potential as a form of microbial medicine, where actively targeting or engineering the dysbiotic tumor microenvironment using bacteria-mediated drug delivery systems represents a promising new frontier in oncology (Canowitz et al., 2026). Specifically, pioneering techniques have demonstrated that microbes can be engineered to remodel the tumor stroma following systemic administration, thereby enhancing immunotherapy efficacy (Thomas et al., 2022), or even designed as radiolytic platforms administered intratumorally to precisely deliver immunomodulating payloads upon a radiation trigger (Kamble et al., 2022).

2 Materials and methods

2.1 Ethics statement

The study protocol conformed to the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The study was approved by the institutional review board of the “Paolo Giaccone” Policlinico University Hospital in Palermo (Italy) (approval #11/2020). Written informed consent was

obtained from all participants involved in the study. The study reported was assessed following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

2.2 Population recruitment

The subjects were unselectively and consecutively recruited from the Unit of Oral Medicine at the “Paolo Giaccone” Policlinico University Hospital in Palermo (Italy) from May to September 2025.

The patients were selected based on the following inclusion criteria:

- age older than 18 years,
- patients diagnosed with OSCC confirmed by pathological findings, and
- ability to provide informed consent.

Patients were excluded if they were pregnant or nursing, had received antibiotics or periodontal therapy in the previous 3 months, were affected by severe periodontitis, had any immunocompromising conditions, or had received any previous surgical treatment, radiotherapy, and/or chemotherapy.

To confirm the diagnosis of OSCC, a histopathological examination was undertaken. An incisional biopsy was performed under local anesthesia. The section from one sample was fixed in formalin solution and sent to the pathology laboratory for histopathological OSCC diagnosis.

To serve as an OSCC-free baseline, patients diagnosed with oral potentially malignant diseases (OPMDs) and healthy patients were combined into a macro “control group”.

2.3 Samples collection

Saliva and intralesional tissue samples were collected from each subject to evaluate oral microbiota dysbiosis or to investigate the correlation between specific bacterial species and pathological conditions. According to a standardized protocol (Mauceri et al., 2022, 2023), a non-stimulated saliva sample was obtained in the morning from each participant. Patients were asked to refrain from eating, drinking, or smoking for at least 2 h before sample collection and to rinse their mouths with saline solution for 60 s. Saliva was allowed to accumulate on the floor of the mouth and then collected in sterile Falcon tubes and stored frozen at -80°C until ready to use.

The collection of fresh intratumoral samples is performed using a specific method.

Patients underwent magnetic resonance imaging for the detection of tumor depth of invasion and lesion extension (Lo Casto et al., 2022), and during an incisional biopsy, one fragment of tumor tissue was chosen, placed in a cryotube, and frozen immediately at -80°C until ready to use.

The dataset was organized into four groups: OSCC biopsy, control biopsy, OSCC saliva, and control saliva. In comparative analyses, both healthy mucosa and OPMDs are included as control samples unless otherwise specified.

2.4 Outcome measures

For each patient, the following data were recorded: demographic data, localization of OSCC lesion according to International Classification of Diseases (ICD; 10th revision); Tumor, Node, Metastasis (TNM) stage (version 7.0) according to the guidelines of American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC); smoking habits; alcohol drinking; and traumatic risk factors (i.e., incongruous prostheses or sharp cusp). Based on the TNM stage, the patients were classified as early (stage I and II) or advanced stage (stage III and IV).

2.5 DNA extraction

DNA was extracted from each saliva sample using the QIAamp PowerFecal Pro Kit (Qiagen, Cat No. 12830, Hilden, Germany). Specifically, a volume of 200 µL of saliva was processed following the manufacturer's protocol (QIAGEN, 2020).

From each intratumor biopsy before the DNA extraction, the tissue was additionally cut into small pieces and homogenized in 200 µL of lysis buffer with the addition of proteinase K, and then the solution was processed following the QIAamp® PowerFecal Pro protocol.

At the end of the extraction protocol, the microbial DNA was recovered in a 1.5-mL Eppendorf tube. The quality of the extracted DNA was determined using a Qubit dsDNA HS (High Sensitivity) assay kit (Invitrogen Co., Life Sciences, Carlsbad, CA, USA).

2.6 Library preparation and sample sequencing

The library preparation for next-generation sequencing (NGS) of the bacteria-specific 16S ribosome gene was performed utilizing a Microbiota Solution A kit targeting the hypervariable regions V1–V2–V3 (Arrow Diagnostics S.r.l., Genoa, Italy). The A kit was composed of enzyme mix 1 solution containing the enzyme mixture for the polymerase chain reaction (PCR) target, enzyme mix 2 solution containing the enzyme mixture for the PCR index, Amp Mix V1–V3 solution of degenerated oligonucleotides for amplifying the hypervariable regions V1–V2–V3 of the bacterial 16S rDNA gene, and an oligonucleotide solution for indexing amplified samples with the PCR target (index mix).

The samples and the indexed product were cleaned using AMPure XP beads to remove excess primers and primer dimers, and after fluorometric sample quantification using Invitrogen's Qubit dsDNA HS (High Sensitivity) assay kit (Invitrogen Co., Life Sciences, Carlsbad, CA, USA), samples were diluted to 4 nM and pooled. The library pool was then denatured and diluted to 20 pM, and a PhiX control was added. The pool was sequenced on the MiSeq Illumina® sequencing platform (Illumina, CA, USA) using v2 500-cycle reagent.

2.7 Bioinformatics and statistical analysis

Sequencing data were analyzed using dedicated bioinformatics software (Illumina BaseSpace Hub). The BaseSpace 16S Metagenomics app is a specialized application within the platform that performs automated taxonomic classification of 16S rRNA

gene sequencing data from the kingdom to the species level (Wu et al., 2018).

The classification workflow uses a proprietary algorithm that provides species-level classifications for paired-end reads by matching short subsequences of the reads (called words) to a set of 16S reference sequences (Wu et al., 2018).

The accumulated word matches for each read are then used to assign reads to specific taxonomic classifications (Wu et al., 2018). For reference databases, the app utilizes either an Illumina-curated version of the RefSeq RDP 16S v3 database (Cole et al., 2014; Athen et al., 2021; Delgadinho et al., 2022).

The workflow includes essential data preprocessing steps such as primer sequence removal and quality filtering, where reads with low-quality scores (average score less than 20) are filtered out using the FASTQ toolkit within BaseSpace (Kyndt, 2020).

A notable advantage of the BaseSpace 16S metagenomics application is that it does not require upfront operational taxonomic unit (OTU) clustering for taxonomic classification, as it uses longer paired-end reads for direct taxonomic assignment (Kugadas et al., 2017).

Users can access the application through the BaseSpace online portal and execute the 16S metagenomics analysis by uploading FASTQ sequences directly to the platform (Chen et al., 2016; Kato et al., 2016).

Statistical analyses and data visualization were performed using the R software environment for statistical computing (version 4.5.2; R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2024; RStudio Team, 2024). The analysis relied on the following R packages (McMurdie and Holmes, 2013) for microbiome data handling: *vegan* (Oksanen and Simpson, 2024) for diversity analysis and PERMANOVA, and *ggplot2* for visualization (Wickham, 2016). Taxonomic differential abundance between the study groups was evaluated using the non-parametric Mann–Whitney U test (Wilcoxon rank-sum test). To address the multiple testing problem inherent to high-dimensional microbiome datasets, false discovery rate (FDR) estimation was performed using the Benjamini–Hochberg step-up procedure. In the text, a dual-reporting strategy was adopted to ensure analytical transparency while maintaining narrative clarity: both nominal p-values and their corresponding FDR-adjusted q-values were explicitly reported exclusively for taxa demonstrating nominal statistical significance (nominal $p < 0.05$) or robust statistical trends. Conversely, given the pilot nature of this study and the high inter-patient heterogeneity of the oral microenvironment, substantial taxonomic shifts and structural reconfigurations that did not reach nominal statistical significance were discussed purely as quantitative biological trends without explicit p-value notation.

3 Results

3.1 Study cohort and sequencing metrics

A total of 12 patients were included, with a mean age of 58.5 ± 14.7 years (range 35–84). One subject from the control group (P4)

was identified as a biological outlier and excluded from downstream analysis. Despite the absence of clinical pathology, this subject exhibited a microbial profile clustering closely with the OSCC group. Finally, 11 subjects were included in the analysis: 7 patients diagnosed with OSCC and 4 OSCC-free controls (2 healthy patients and 2 with oral leukoplakia). The main characteristics of the included patients are reported in Table 1.

In the OSCC group, four were male (57.2%) and three were female (42.8%), with a mean age of 58.4 ± 11.5 years (range 43–83). The masticatory mucosa was the most affected OSCC site (42.8%), followed by the floor of the mouth (28.6%), the border of the tongue (14.3%), and the palate (14.3%), as reported in Figure 1.

In the control group, two patients were male (50%) and two were female (50%), with a mean age of 59.4 ± 18.3 years (range 35–84).

According to the TNM staging classification, OSCC cases were classified as follows: three patients with stage I (3/7, 42.8%), two patients with stage II (2/7, 28.6%), one patient with stage III (1/7, 14.3%), and one patient with stage IV (1/7, 14.3%).

Based on the TNM stage, four patients were classified as early (4/7, 57.2%), and three were classified as advanced stage (3/7, 42.8%).

Regarding habits of OSCC patients, three patients were smokers (3/7, 42.8%), and two were both smokers and drinkers. Regarding the potential mechanical trauma, clinical examination revealed the presence of incongruous prostheses in one patient (1/7, 14.3%).

TABLE 1 The main characteristics of the included patients.

Variable	OSCC (n = 7)	Controls (n = 4)
Sex, n (%)		
Male	4 (57.2)	2 (50.0)
Female	3 (42.8)	2 (50.0)
Age (years)		
Mean $\pm$ SD (range)	58.4 ± 11.5 (43–83)	59.4 ± 18.3 (35–84)
Primary tumor site (OSCC)		
Masticatory mucosa	3 (42.8)	—
Tongue border	1 (14.3%)	—
Floor of mouth	2 (28.6)	—
Palate	1 (14.3)	—
TNM stage (OSCC)		
Stage I	3 (42.8)	—
Stage II	2 (28.6)	—
Stage III	1 (14.3)	—
Stage IV	1 (14.3)	—
Stage grouping (OSCC)		
Early stage (I–II)	5 (71.4)	—
Advanced stage (III–IV)	2 (28.6)	—
Risk factors		
Smokers	3 (42.8)	—
Smokers + drinkers	2 (28.6)	—
Incongruous prosthesis	1 (14.3)	1 (25)

In the control group, one patient was a prosthesis wearer (1/4, 25%).

For each subject, paired saliva and tissue biopsy samples were collected, resulting in a dataset of 22 samples.

High-throughput sequencing of the 16S rRNA gene across the initial cohort of 24 samples yielded over 2 million raw reads passing filter (PF). Following taxonomic assignment via the Illumina BaseSpace Hub 16S Metagenomics app and the exclusion of the biological outlier (Patient 4), a total of 1,217,524 species-classified reads were retained for the final 22 analyzed samples. This resulted in a robust average sequencing depth of 55,342 classified reads per sample, ensuring optimal taxonomic coverage for both salivary and intratumoral matrices.

3.2 Alpha and beta diversity

Alpha-diversity analysis revealed no significant differences in species richness [Abundance-based Coverage Estimator (ACE) index] between controls and OSCC patients in either saliva ($p = 0.65$, Figure 2A) or biopsy samples ($p = 0.53$, Figure 2A). In terms of diversity and evenness (Shannon and Simpson indices), no significant differences were found between groups in saliva samples ($p > 0.05$, Figures 2B,C). However, a trend approaching significance emerged in biopsy samples for both Shannon ($p = 0.11$, Figure 2B) and Simpson indices ($p = 0.11$, Figure 2C), with OSCC patients showing higher diversity compared to controls (Figure 2; Supplementary Table 1).

Beta diversity was examined using principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity to evaluate the variation in microbial community structure (Figure 2D; Supplementary Table 2).

In saliva samples, the microbial composition of controls and OSCC patients appeared largely similar, with no distinct clustering observed (PERMANOVA, $p = 0.764$).

Conversely, in biopsy samples, a notable separation between the two groups was evident along the primary axes. Statistical analysis revealed a marginal trend approaching significance ($p = 0.058$), suggesting that the dysbiotic shifts associated with OSCC are more pronounced in the tissue microenvironment than in saliva.

3.3 Taxonomic results of oral samples

As a result of the taxonomic assignment and after excluding the biological outlier, a total of 1,217,524 successfully classified reads were retained from the 22 analyzed samples. This robust sequencing depth allowed for the identification of 1,711 unique species distributed across 737 genera.

3.3.1 Taxonomic composition at the genus level

To characterize the core microbiome of the study cohort, we analyzed the relative abundance of the most prevalent bacterial genera across all samples (saliva and biopsy). The top 10 most abundant genera collectively accounted for approximately 71.1% of the total reads, constituting the dominant microbial signature of the cohort (Figure 3; Supplementary Table 3).

Streptococcus was the predominant genus, representing nearly one-quarter of the total sequencing reads ($24.2\% \pm 13.5\%$), followed

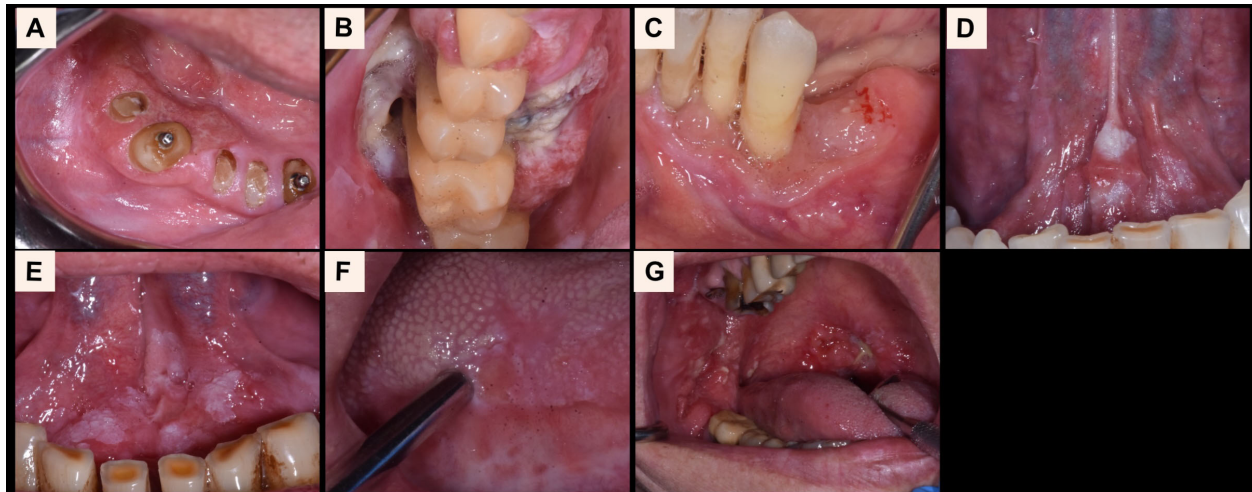


FIGURE 1

Clinical presentation of the seven oral squamous cell carcinoma (OSCC) cases included in the study. (A–C) show three patients affected by OSCC on the masticatory mucosa; (D, E) show two patients with lesions located on the floor of the mouth; (F) depicts a patient with OSCC affecting the lateral border of the tongue; and (G) shows a patient with a palatal OSCC.

by *Prevotella* ($9.7\% \pm 8.8\%$) and *Neisseria* ($8.8\% \pm 12.8\%$). Other notable contributors to the core microbiome included *Haemophilus* ($6.5\% \pm 8.0\%$) and *Actinomyces* ($5.1\% \pm 5.9\%$). The remaining top genera—*Fusobacterium*, *Granulicatella*, *Gemella*, *Porphyromonas*, and *Campylobacter*—exhibited lower mean relative abundances ranging from 3.2% to 3.7% (Figure 3).

3.3.2 Most abundant genera across sample groups

The taxonomic overview of the oral microbiome was further characterized by analyzing the distribution of the top 10 most abundant genera across the four study groups (Figure 4; Supplementary Table 4). The OSCC saliva profile showed a distinct alteration where *Streptococcus* abundance decreased ($17.2\% \pm 3.1\%$) and was nearly equaled by a substantial expansion of *Neisseria*

($17.1\% \pm 7.2\%$). In contrast, in control saliva, the community was predominantly driven by *Streptococcus* ($28.1\% \pm 7.7\%$), followed by *Prevotella* ($12.0\% \pm 4.3\%$) and *Gemella* ($5.5\% \pm 2.4\%$). Regarding tissue samples, in OSCC biopsies, while *Streptococcus* remained the most abundant taxon ($21.7\% \pm 4.7\%$), its dominance was markedly reduced, accompanied by an increased presence of periodontal pathogens such as *Fusobacterium* ($7.4\% \pm 2.1\%$) and *Prevotella* ($6.9\% \pm 2.3\%$). Conversely, control biopsies were heavily dominated by *Streptococcus* ($37.0\% \pm 7.4\%$) and *Haemophilus* ($12.8\% \pm 7.6\%$).

Building on these observations, the relative abundance of dominant dysbiotic genera was compared between OSCC patients and controls (Table 2). While the overall microbial profile appeared stable ($p > 0.05$), biopsy samples revealed distinct alterations in specific abundance levels. *Streptococcus* decreased to 21.7% in OSCC from 37.0% in control tissues, displaying a mathematical trend toward contraction (nominal $p = 0.073$, FDR $q = 0.18$).

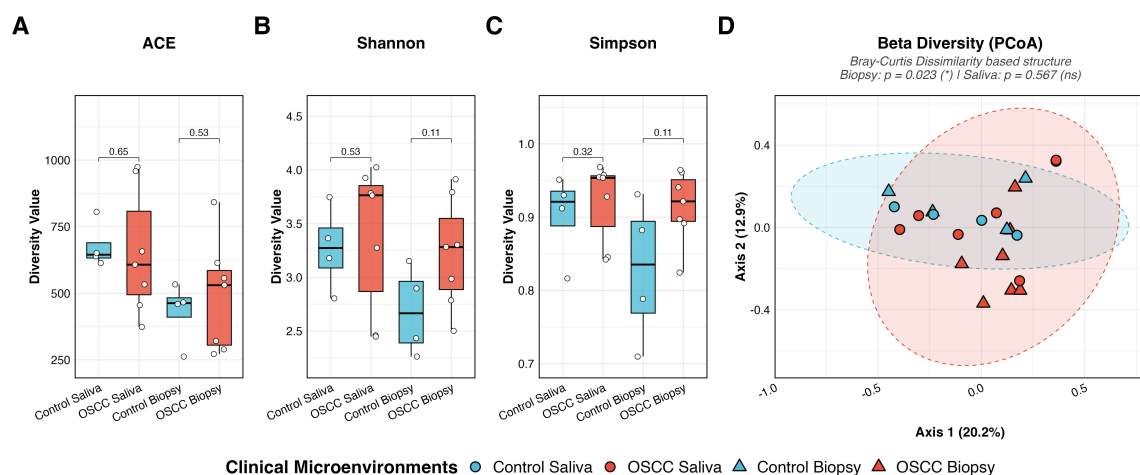


FIGURE 2

Ecological community structure by microenvironment. Boxplots representing alpha diversity metrics for (A) ACE, (B) Shannon, and (C) Simpson indices across control and OSCC saliva and biopsy groups. (D) Principal Coordinates Analysis (PCoA) plot of beta diversity.

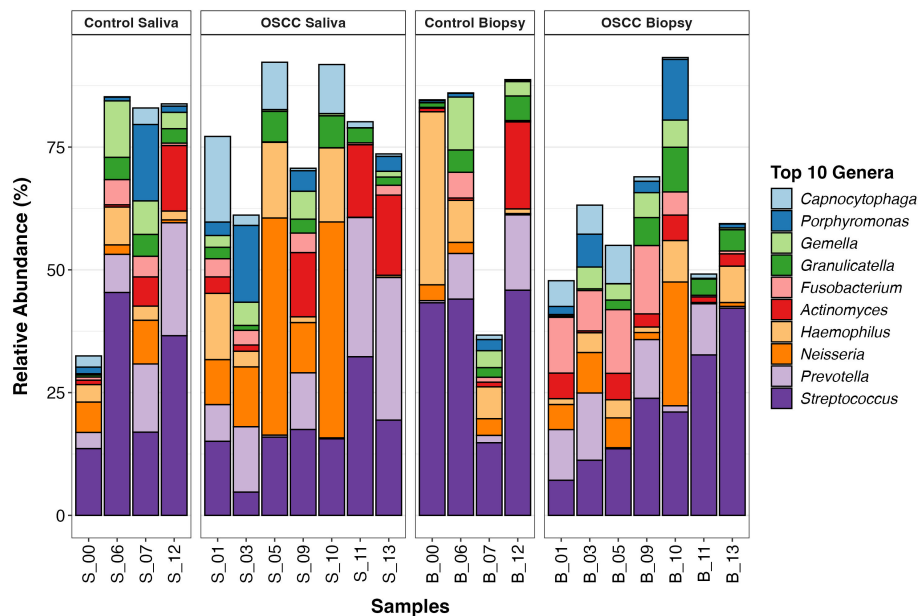


FIGURE 3
Microbiome composition (Genus Level).

Conversely, *Fusobacterium* increased to 7.4% in OSCC biopsies from 1.7% in controls, reflecting a descriptive enrichment within the neoplastic tissue (nominal $p = 0.11$, FDR $q = 0.22$). A notable upward trend was also identified in the salivary microbiome regarding the genus *Neisseria*, whose relative abundance showed a substantial increase in OSCC patients, rising to 17.1% in the OSCC group from an average of 4.4% in controls. Although the limited sample size of this pilot cohort constrained the statistical power of these high-level comparisons, the inverse relationship observed—specifically the depletion of *Streptococcus* and the concurrent enrichment of *Fusobacterium*—suggests a biologically relevant dysbiotic pattern within the tumor microenvironment that warrants

clinical consideration despite the lack of strict statistical significance after multiple testing corrections.

3.3.3 Taxonomic composition at species level

Before proceeding with species-level analyses, potential procedural contaminants (*Ralstonia*, *Kingella*, and *Leclercia*) were filtered from the dataset. Although these taxa exhibited high read counts in isolated samples, their sporadic distribution suggested that they were likely artifacts rather than true biological signals. Following this filtering, the relative abundance (%) of the top 15 most prevalent bacterial species across the four study groups (control

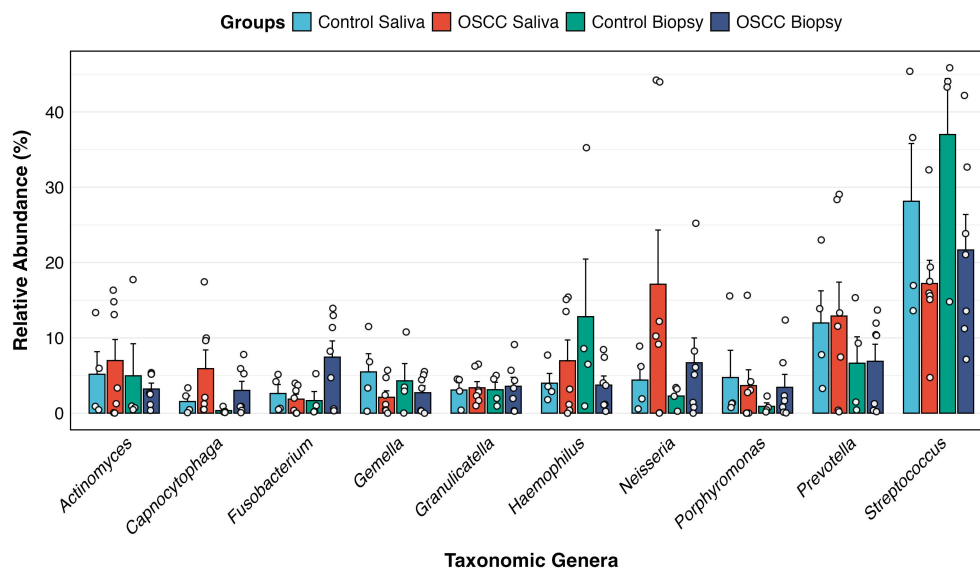


FIGURE 4
Taxonomic profile of the 10 most abundant genera across sample groups, direct comparison (Mean $\pm$ SEM).

TABLE 2 Relative abundance of *Streptococcus*, *Fusobacterium* and *Neisseria* genera in healthy controls and oral squamous cell carcinoma (OSCC) patients.

Genus	P-value saliva	P-value biopsy	Healthy saliva (%)	OSCC saliva (%)	Healthy biopsy (%)	OSCC biopsy (%)
<i>Streptococcus</i>	0.41	0.07	28.14	17.22	37.00	21.67
<i>Fusobacterium</i>	0.32	0.11	2.61	1.87	1.67	7.44
<i>Neisseria</i>	0.32	0.53	4.40	17.12	2.28	6.70

Values are expressed as mean relative abundance (%); statistical significance was evaluated using the Mann–Whitney U test.

saliva, OSCC saliva, control biopsy, and OSCC biopsy) was evaluated (Figure 5). As displayed in the heatmap, abundance levels are represented by a color gradient ranging from blue (absent/low) to red (dominant), with a white threshold set at 3% to differentiate background presence from clinically relevant abundance (capped at 30%).

This analysis successfully resolves the genus-level shifts into specific taxon dynamics. In OSCC tissues, the depletion of *Streptococcus* is driven by the loss of health-associated commensals, specifically *Streptococcus salivarius* (declining from 11.7% in control tissues to 0.4% in OSCC) and *Streptococcus oralis* (9.0% vs. 1.8%), while *Streptococcus mitis* persists as a dominant colonizer of the tumor niche (11.3% in OSCC). In saliva, the genus-level expansion of *Neisseria* is distinctly attributable to *Neisseria macacae*, which forms a specific signature of the OSCC salivary microbiome (surging from 1.1% in controls to 9.8% in patients). Furthermore, the tumor microenvironment shows clear signs of colonization by periodontal pathogens: alongside *Fusobacterium periodonticum* (increasing from 0.1% to 3.8% in biopsies), *F. nucleatum* exhibited a massive expansion, rising from a negligible 0.2% in control tissues to 4.9% in the OSCC group.

3.3.4 Dominant bacterial species across study groups

To further resolve the taxonomic alterations observed at the genus level, the distribution of the most abundant bacterial species

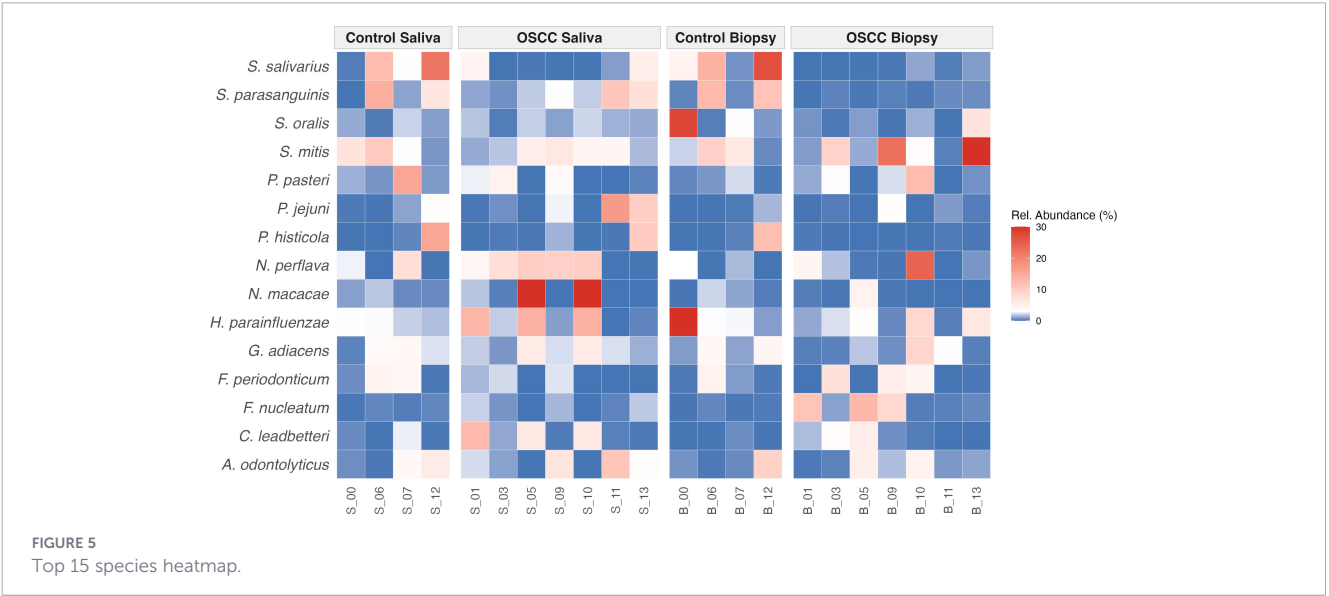
was analyzed across the four study groups (Figure 6; Supplementary Table 5).

In OSCC saliva, the microbiome exhibited a distinct shift characterized by the emergence of *N. macacae* as the most abundant species (9.8% ± 6.1%), followed by *Haemophilus parainfluenzae* (6.3% ± 2.6%) and *Neisseria perflava* (5.9% ± 1.7%). Conversely, in control saliva, the community was dominated by commensal streptococci, specifically *S. salivarius* (9.4% ± 4.9%) and *Streptococcus parasanguinis* (5.5% ± 3.3%).

Regarding tissue samples, OSCC biopsies displayed a dramatically restructured profile: typical health-associated commensals were depleted, while *S. mitis* persisted as the dominant taxon (9.7% ± 4.6%). Furthermore, the tumor niche was marked by the specific enrichment of the periodontal pathogen *F. nucleatum* (4.9% ± 2.2%), which established itself as the second most abundant species in the neoplastic tissue. In contrast, control biopsies showed a diverse profile driven by *S. salivarius* (11.5% ± 5.8%), *H. parainfluenzae* (10.4% ± 8.2%), and *S. oralis* (8.0% ± 6.7%). Statistical significance for these comparisons is detailed in Table 3.

3.4 Focus on abundance differences between OSCC and control saliva

Analysis of the 10 most abundant salivary species revealed a marked compositional shift between the study groups. The salivary microbiome of OSCC patients was characterized by the expansion of specific bacteria (Figure 7; Supplementary Table 6), primarily within



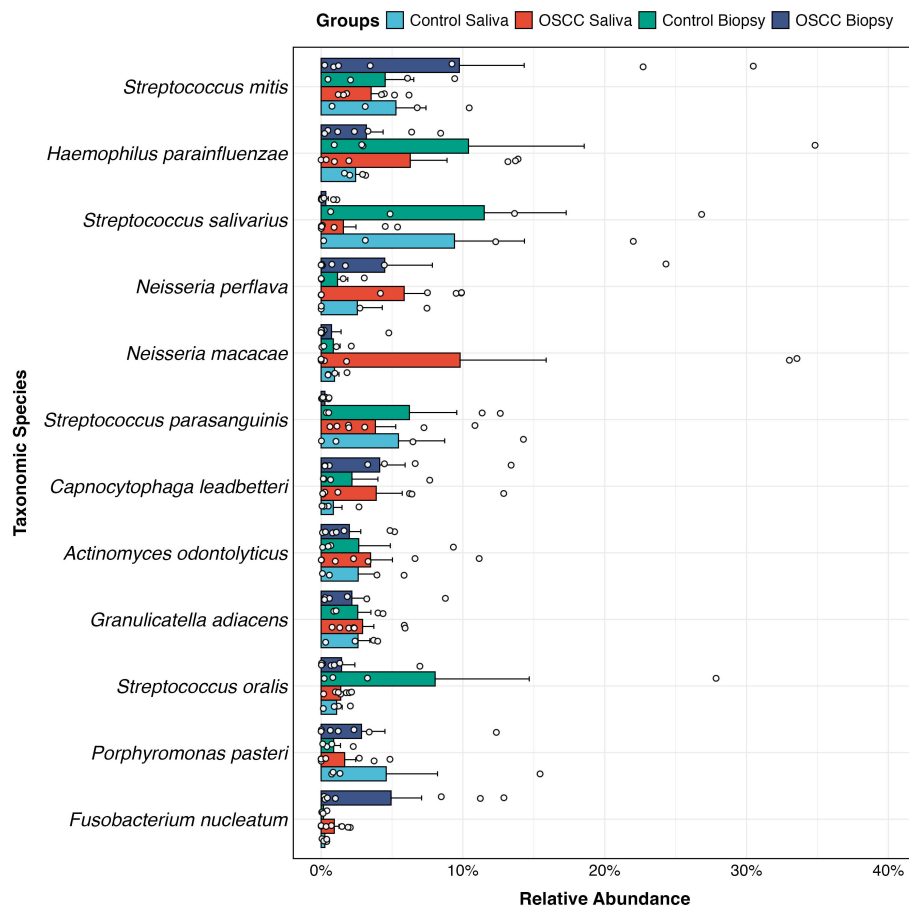


FIGURE 6
Relative abundance of dominant bacterial species across study groups.

the genus *Neisseria*. *N. macacae* was identified as the dominant species in the OSCC group ($9.8\% \pm 6.1\%$), exhibiting a 10-fold increase relative to controls ($0.9\% \pm 0.3\%$). Concurrently, upward trends were observed for *N. perflava* (5.9% vs. 2.6%) and *H. parainfluenzae* (6.3% vs. 2.4%), alongside a fourfold increase in *P. jejuni* (4.3% vs. 1.1%). In contrast, the control salivary profile was defined by the prevalence of commensal streptococci, specifically *S. salivarius*, which decreased to $1.6\% \pm 0.9\%$ in the OSCC patients from $9.4\% \pm 4.9\%$ in the controls. Other health-associated taxa, including *Porphyromonas pasteri* and *S. parasanguinis*, showed similar patterns of depletion. While these alterations indicate a distinct biological restructuring of the salivary bacteriome in OSCC, statistical significance was limited by high inter-individual variability.

3.5 Focus on abundance differences between OSCC and control biopsy

In contrast to the salivary findings, the analysis of tissue biopsies revealed a statistically robust restructuring of the microbiome (Figure 8; Supplementary Table 7), characterized by the replacement of health-associated commensals with a tumor-associated consortium. The most significant alteration was the dramatic enrichment of *F. nucleatum*, which exhibited a 29-fold increase in abundance, rising

to become a hallmark of the OSCC microenvironment (mean relative abundance $4.9\% \pm 2.2\%$ in OSCC vs. $0.2\% \pm 0.1\%$ in controls; nominal $p = 0.012$, FDR $q = 0.05$). The tumor niche was also dominated by *S. mitis*, which, unlike other streptococci, persisted and expanded to become the most abundant species in OSCC samples ($9.7\% \pm 4.6\%$ vs. $4.5\% \pm 2.0\%$ in controls). Furthermore, *N. perflava* showed a distinct upward trend, increasing nearly fourfold in the tumor tissue (4.5% vs. 1.2% in controls).

Conversely, a specific set of commensals was systematically depleted in the neoplastic environment compared to the control tissue microbiome. *S. salivarius* was virtually eradicated in OSCC tissues, dropping from its status as the most abundant species in control biopsies ($0.3\% \pm 0.2\%$ vs. $11.5\% \pm 5.8\%$; nominal $p = 0.006$, FDR $q = 0.05$). A similar significant depletion within the tumor was observed for the core symbiont *S. oralis* (1.4% vs. 8.0% in controls; nominal $p = 0.015$, FDR $q = 0.05$). Additionally, *S. parasanguinis* exhibited a strong downward taxonomic shift, displaying a robust biological trend approaching significance after multiple testing correction (0.3% vs. 6.2% in controls; nominal $p = 0.024$, FDR $q = 0.06$). Finally, *H. parainfluenzae* was reduced to 3.2% in the tumor group from $10.4\% \pm 8.2\%$, where it acted as a major constituent of the control gingival mucosa. This further confirms the dysbiotic shift toward a pathogen-enriched community from a homeostatic one.

TABLE 3 Statistical comparison of the relative abundance of dominant bacterial species between healthy controls and oral squamous cell carcinoma (OSCC) patients.

Species	Biopsy P-value Control VS OSCC	Saliva P-value Control VS OSCC
<i>Streptococcus salivarius</i>	0,006 *	0,109
<i>Fusobacterium nucleatum</i>	0,012 *	0,527
<i>Streptococcus oralis</i>	0,015 *	0,210
<i>Streptococcus parasanguinis</i>	0,024 *	0,415
<i>Capnocytophaga leadbetteri</i>	0,180	0,230
<i>Porphyromonas pasteri</i>	0,292	0,482
<i>Neisseria macacae</i>	0,350	0,776
<i>Neisseria perflava</i>	0,367	0,214
<i>Haemophilus parainfluenzae</i>	0,444	0,189
<i>Granulicatella adiacens</i>	0,790	0,787
<i>Actinomyces odontolyticus</i>	0,794	0,684
<i>Streptococcus mitis</i>	1,000	0,680

Comparisons were performed separately for saliva and biopsy samples using the Mann-Whitney U test. Statistically significant differences ($p < 0.05$) are indicated by an asterisk.

3.6 Exploratory 16S reference-sequence profiling of *F. nucleatum*

To explore whether the *F. nucleatum* enrichment in OSCC tissues was driven by a generalized increase or the potential dominance of specific sequence variants, we analyzed the total relative load and reference-sequence profiles at the single-patient level (Figure 9). The relative abundance analysis confirmed substantial heterogeneity within the tumor group: while the mean proportion was elevated, specific patients (P5 and P1) exhibited *F. nucleatum* relative abundances exceeding 11%–12% of the total microbiome, compared to

<0.5% in the controls. Exploratory profiling mapping to the 16S reference database suggested that this increase was not uniform but predominantly associated with specific reference sequences. In the majority of OSCC patients (five out of seven), the tumor niche was largely dominated by a 16S sequence variant corresponding to the reference sequence ID: NR_117842.1. This specific variant accounted for an average of 72.2% of the total *Fusobacterium* population within these tumor biopsies. Notably, in patients with the highest relative burden (P5 and P9), this sequence match displayed a marked predominance, representing >99% of the *F. nucleatum* reads detected. A distinct dominant reference sequence (NR_117840.1) was observed in P1, suggesting that while the ecological niche may be conserved, the specific sequence variant colonizing the tissue may vary between individuals. However, these preliminary observations must be interpreted with caution. It is important to acknowledge that standard 16S rRNA amplicon sequencing lacks the necessary resolution for definitive strain-level characterization. Robust strain-level inference and the confirmation of true clonal expansion would require validation through shotgun metagenomics, isolate sequencing, targeted strain-resolved assays, or comparable methods.

3.7.1 Stage-specific microbial dynamics

To characterize the stage-specific microbial dynamics across the complete continuum of OSCC development, we evaluated the relative abundances of both key opportunistic pathogens (*Fusobacterium*, *Prevotella*, and *Capnocytophaga*) and primary health-associated commensals (*Streptococcus*) utilizing a four-stage clinical stratification model. Tissue biopsies were categorized into a non-malignant control macro-group (CTRL; $n = 4$)—comprising strictly healthy mucosa (normal; $n = 2$) and leukoplakia lesions (OPMD; $n = 2$)—and a malignant macro-group (OSCC; $n = 7$), further stratified by clinical progression into early-stage (early; $n = 5$) and late-stage (advanced; $n = 2$) carcinomas.

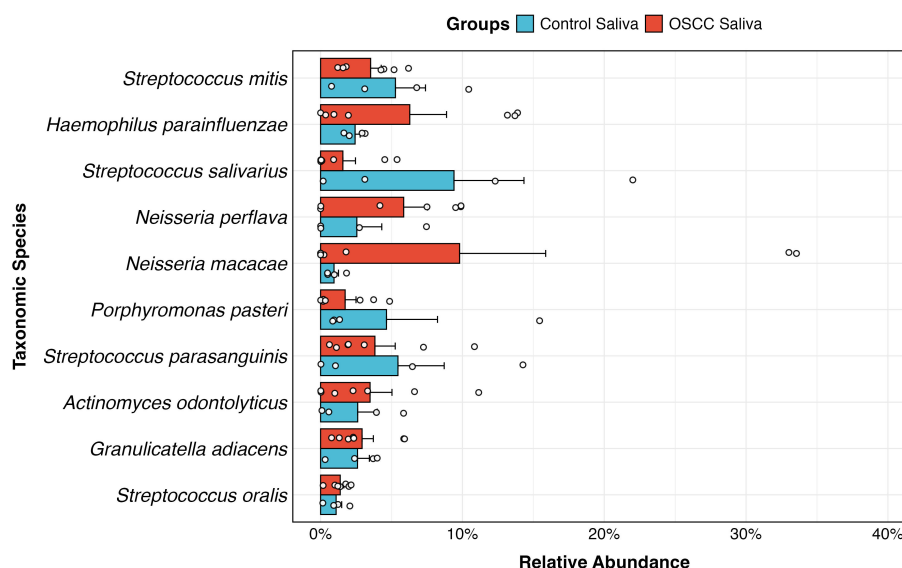
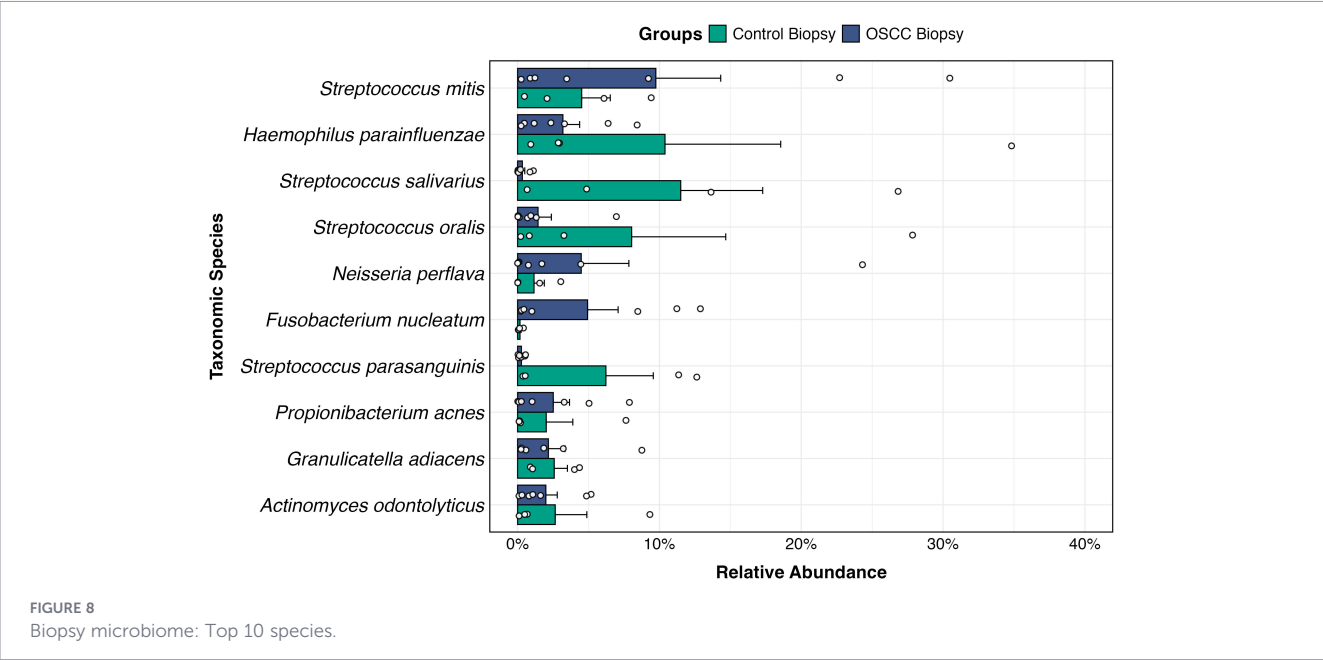


FIGURE 7
Saliva microbiome: Top 10 species.



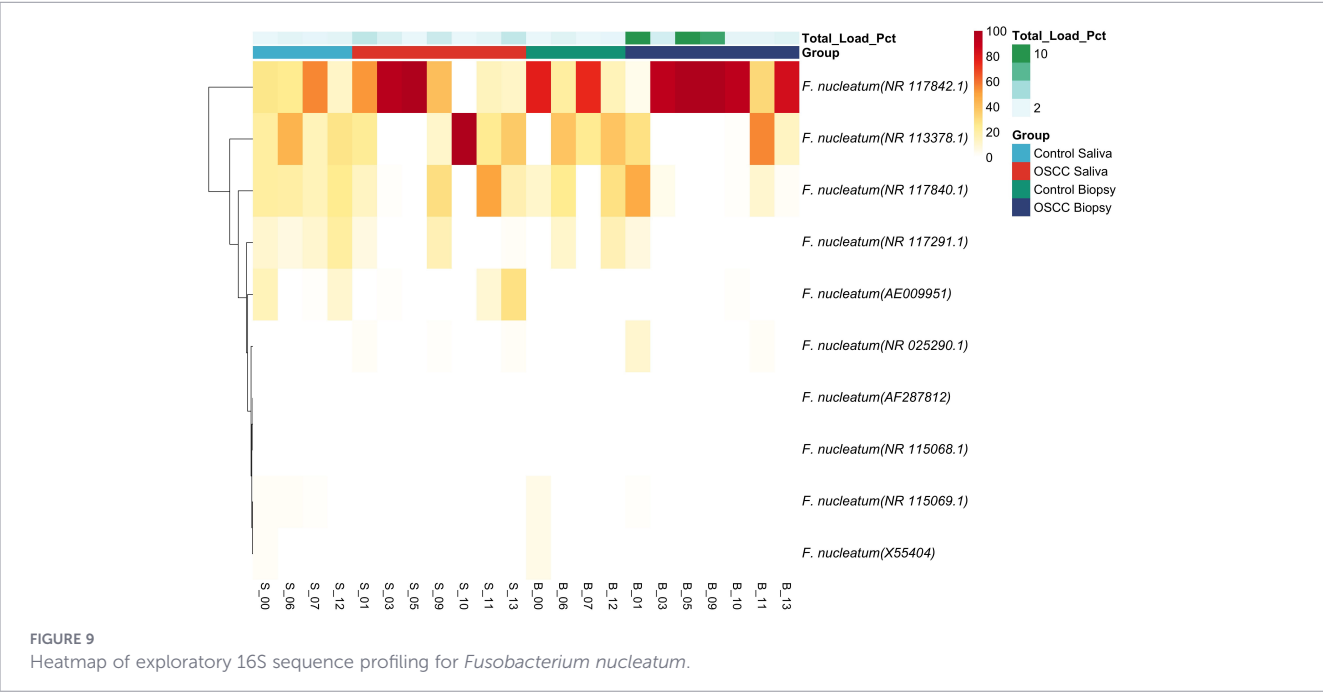
3.7.2 Stage-specific microbial distribution in biopsies

Determining the stage-specific distribution of bacteria revealed a significant enrichment of the *Fusobacterium* genus during the early stages of malignancy. In non-malignant control tissues (CTRL), *Fusobacterium* abundance remained at baseline levels (normal range, 0.26%–1.00%; OPMD, 0.18%–5.24%). The transition to early-stage OSCC was associated with a substantial increase in relative abundance (early range, 0.57%–13.92%). Species-level analysis identified *F. nucleatum* as the primary associated factor of this trend, with relative abundances increasing from <0.41% in CTRL to peaks of 12.89% in early OSCC, followed by a decline in

advanced tumors (range, 0.27%–1.01%). This targeted early enrichment was formally significant (nominal $p = 0.020$, FDR $q = 0.036$ for the CTRL vs. OSCC macro-groups).

Conversely, *Prevotella* exhibited a different colonization pattern. While displaying high variability across the CTRL macro-group (range, 0.44%–15.45%) and early-stage lesions (range, 0.36%–14.69%), this genus consistently established a dominant presence in advanced-stage tumors (T3/T4), with relative abundances stabilizing at 10.44% and 16.51%.

Capnocytophaga remained uniformly depleted in CTRL tissues (max, 0.90%). Malignant progression was characterized by heterogeneous increases, reaching localized peaks of 7.79% in the early OSCC



cohort and 5.91% in the advanced cohort, without reaching the generalized dominance observed for *Fusobacterium* or *Prevotella*.

The analysis of the *Streptococcus* genus indicated a stage-progressive contraction of health-associated commensals. In CTRL tissues, *Streptococcus* maintained a dominant presence (range, 14.84%–45.88%). Upon malignant transformation, genus-level levels exhibited a notable contraction (early range, 9.81%–42.23%; advanced, 14.59%–33.16%).

Species-level refinement demonstrated that this depletion was selective. *S. mitis* displayed persistent but heterogeneous presence across all stages (overall range, 0.25%–30.47%). In contrast, a statistically robust eradication was observed for *S. salivarius* (nominal $p = 0.024$, FDR $q = 0.036$) and *S. parasanguinis* (nominal $p = 0.042$, FDR $q = 0.042$).

S. salivarius relative abundance declined from CTRL levels (max, 26.85%) to <1.11% in OSCC tissues, while *S. parasanguinis* plummeted from 12.63% to negligible levels (range, 0.03%–0.56%).

Collectively, these data characterize a staged ecological transition within the tumor microenvironment. This process is defined by the significant erosion of foundational commensals (specifically *S. salivarius* and *S. parasanguinis*) and a biphasic colonization by pathogens, marked by an initial peak of *F. nucleatum* in early malignancy followed by *Prevotella* stabilization in late-stage tumors. These stage-specific distribution patterns are summarized in Figure 10.

3.7.3 Stage-specific microbial distribution in saliva

To parallel the dynamics of the intratumoral microbiome, salivary microbial profiles were evaluated across different disease stages, focusing on the depletion of health-associated commensal taxa.

The *Streptococcus* genus, a dominant component of the salivary ecosystem, exhibited relative abundances ranging from 13.71% to 45.39% in the CTRL group and 12.02% to 36.24% in the OSCC group. Species-level refinement identified *S. salivarius* as a primary indicator of this commensal contraction. In CTRL samples, *S. salivarius* relative abundance reached peaks of 22.03%, followed by a substantial depletion in early-stage carcinoma (range, 0.01%–5.39%) and near-total eradication in the advanced-stage cohort (range, 0.01%–1.58%).

A successional pattern was observed for the *Neisseria* genus and its representative species. *Neisseria* maintained stable baseline levels in CTRL samples (range, 0.57%–8.90%) but was characterized by high distributional variance upon malignant transformation, with relative abundances fluctuating between 0.01% and 44.23% in the OSCC macro-group. This trajectory was further characterized through the analysis of the *N. macacae* species. While maintained at 0.49%–1.83% in the CTRL group, this taxon exhibited significant inter-patient heterogeneity in early-stage lesions (range, 0.00%–33.56%) before reaching near-complete depletion in advanced-stage patients (range, 0.00%–0.24%). Collectively, the systematic loss of salivary commensals mirrors the dysbiotic shifts observed in matched tissue biopsies. Although the limited sample size of this pilot cohort constrained the formal statistical significance for certain pairwise comparisons ($p > 0.05$), the observed distributional patterns demonstrate a global reorganization of the salivary

ecosystem during OSCC progression. These dynamics are visually summarized in Figure 11.

4 Discussion

The etiology of oral cancer is multifactorial, with emerging evidence pointing to oral dysbiosis as a key factor in pathogenesis (Abbas and Javid, 2025). Current evidence suggests that this dysbiosis is not merely a consequence of the tumor but actively drives inflammation and immune evasion (McIlvanna et al., 2021; Chiscuzzi et al., 2025; Zang et al., 2025). Therefore, to understand the microbial contribution to carcinogenesis, it is necessary to dissect the intricate composition of the oral microbiome. In this context, our study highlights that this dysbiosis is not uniform but rather site-specific, identifying distinct microbial signatures in tumor tissue compared to saliva.

4.1 Microbial alterations in tumor vs. OSCC-free tissues

The transition from health to disease in our cohort appeared to be characterized by a dual mechanism: pathogen colonization and commensal depletion. We observed a marked expansion of *F. nucleatum* within the tumor tissue. As described in recent reviews (McIlvanna et al., 2021; Pignatelli et al., 2023; Chiscuzzi et al., 2025), *F. nucleatum* acts as a pivotal architect of the tumor microenvironment, promoting immune evasion and inflammation.

In tumor tissues, we also observed a substantial restructuring of the microbiome characterized by the enrichment of anaerobic pathogens and the concomitant depletion of commensal species. While our finding of *F. nucleatum* overrepresentation in OSCC biopsies is consistent with the theoretical “driver-passenger” model, we acknowledge that this association is exploratory. This aligns with recent findings by Kaliamoorthy et al. (2023), who demonstrated a strong association between intratumoral *F. nucleatum* load and cancer staging (Zang et al., 2025).

Crucially, this pathogenic shift is mirrored by a significant depletion of *Streptococcus* species (e.g., *S. mitis* and *S. oralis*). Streptococci are primary colonizers that maintain oral homeostasis through the production of bacteriocins and hydrogen peroxide (Herrero et al., 2016; Radaic and Kapila, 2021). Without these commensal sentinels, the mucosal barrier becomes vulnerable, potentially allowing opportunistic anaerobes to establish their dominance; in this way, their significant reduction in tumor tissue represents a functional loss of “colonization resistance”, potentially creating a permissive niche for the invasion of pro-inflammatory anaerobes such as *Fusobacterium*. The depletion of *Streptococcus* populations we observed is well supported by the current body of literature. Indeed, a comprehensive systematic review (Mauceri et al., 2022) confirmed that the depletion of health-associated *Streptococcus* species is one of the most consistent salivary and mucosal signatures across multiple independent OSCC cohorts worldwide. This generalized loss further supports our hypothesis that the breakdown of colonization may be a key factor in the establishment of the tumor microbiome.

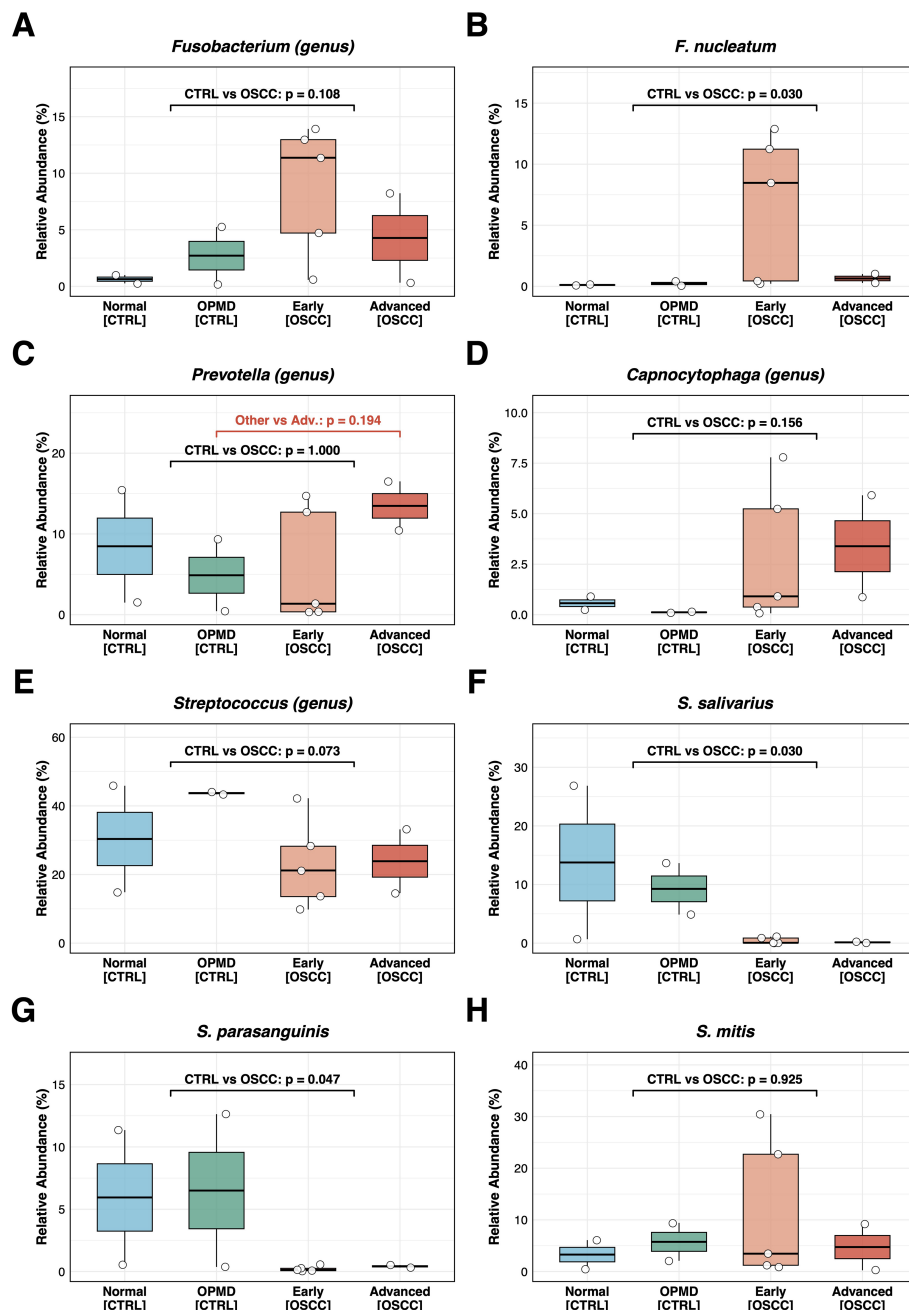


FIGURE 10

Stage specific Microbial Distribution in Biopsies. Panels show: (A) *Fusobacterium* genus; (B) *Fusobacterium nucleatum*; (C) *Prevotella* genus; (D) *Capnocytophaga* genus; (E) *Streptococcus* genus; (F) *Streptococcus salivarius*; (G) *Streptococcus parasanguinis*; and (H) *Streptococcus mitis*.

4.2 Microbial alterations in tumor vs. OSCC-free saliva

While tissue samples showed a dominance of anaerobes, saliva exhibited a distinct dysbiotic signature. Interestingly, we observed an upward trend in the abundance of *N. macacae* in OSCC patients. Although *Neisseria* is often considered a commensal, its specific enrichment in the cancer salivary bacteriome is consistent with findings by Yang et al. (2021), who identified *N. macacae* as a discriminator for OSCC. This finding supports the hypothesis that

N. macacae abundance warrants further investigation as a potential salivary biomarker candidate for non-invasive screening, distinct from the intratumoral pathogen load.

4.3 Intra-patient heterogeneity: microbial differences between tissue and saliva in OSCC patients

Our paired analysis indicates that saliva is not a perfect mirror of the tumor microenvironment.

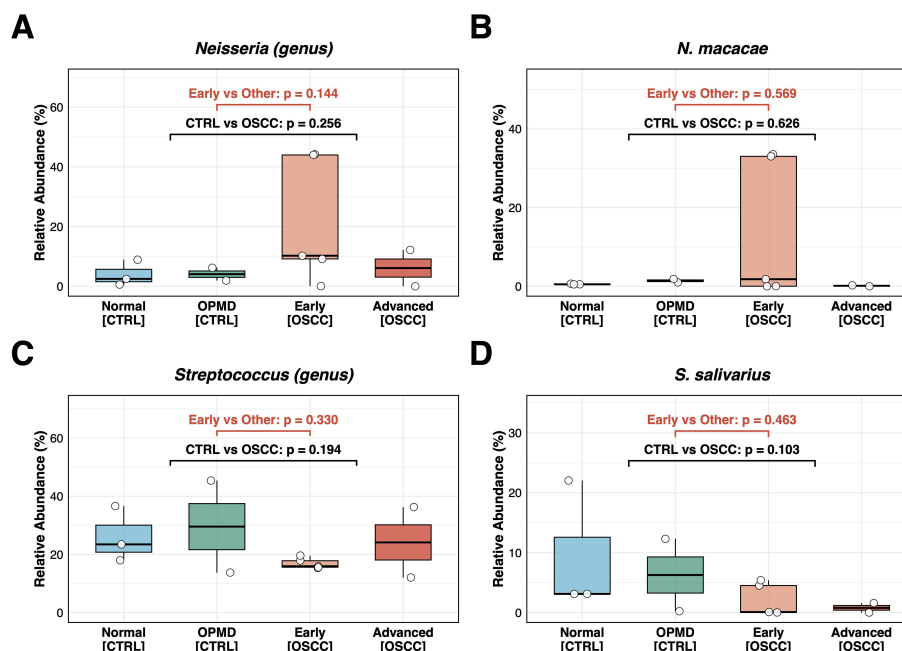


FIGURE 11

Stage specific Microbial Distribution in Saliva. (A) *Neisseria* genus; (B) *Neisseria macacae*; (C) *Streptococcus* genus; and (D) *Streptococcus salivarius*.

Indeed, our observation that tumor tissue possesses a dysbiotic profile distinct from that of saliva is strongly supported by recent literature.

As noted by Zeng et al. (2022), the tumor creates a specific “tumor space” that selects for bacteria capable of adhering to malignant cells and surviving hypoxia. This could explain why *F. nucleatum* is abundant in biopsies but less dominant in the saliva of the same patients. Conversely, the salivary signature reflects a broader ecosystem shift. In support of this, Yang et al. (2021) similarly reported a significant discrepancy between tissue and salivary microbiota in OSCC patients, suggesting that saliva acts as a distinct ecological niche rather than a direct mirror of the tumor microenvironment.

4.4 Enrichment of specific *F. nucleatum* subspecies in OSCC

F. nucleatum acts as a pivotal architect of the tumor microenvironment, promoting immune evasion and inflammation.

Our investigation (Figure 9) went beyond generic taxonomic profiling to highlight a notable pattern: the marked dominance of specific *F. nucleatum* strains within the tumor niche.

While the total bacterial load varied among patients, the structural shift in the OSCC tissue was primarily characterized by the dominance of a single genotype. In the majority of cases (5/7), including those with the highest burden (e.g., P5), we identified a sequence corresponding to NR_117842.1, which maps to the reference type strain ATCC 25586 (*F. nucleatum* subsp. *nucleatum*).

This finding is potentially of high biological relevance. As demonstrated by Han (2015), the ATCC 25586 strain is the model organism for *Fusobacterium* pathogenicity, known to express high levels of the FadA adhesin. This virulence factor binds to E-

cadherin on epithelial cells, activating β -catenin signaling and fueling cell proliferation. The observation that this specific strain reached near-total dominance (>99% of the *Fusobacterium* population) in specific tumors suggests an active selection process driven by the malignant microenvironment, rather than passive colonization due to poor hygiene.

Furthermore, the detection of a distinct dominant genotype (NR_117840.1, subsp. *vincentii*) in P1 indicates that while the specific strain may vary between individuals (inter-patient heterogeneity), the functional requirement for a hyper-virulent, adhesive phenotype remains a conserved feature of the OSCC microbiome. This aligns with recent reviews (Chiscuzzu et al., 2025) emphasizing that tumor-associated strains are characterized by enhanced adhesive properties and immune-suppressive capabilities (e.g., Fap2-TIGIT binding), which protect the tumor from natural killer (NK) cell cytotoxicity (McIlvanna et al., 2021).

Although standard 16S sequencing lacks definitive strain-level resolution, the dominance of sequence variants mapping to highly virulent genomes strongly suggests targeted ecological selection within the tumor. This provides a clear rationale for future shotgun metagenomics or Whole Genome Sequencing (WGS) to validate these strain-specific dynamics.

4.5 Clinical mapping

Interestingly, clinical mapping across a four-stage disease continuum indicated a highly dynamic, stage-specific microbial reorganization during OSCC progression. In strictly healthy mucosal tissues and OPMD leukoplakia lesions, *F. nucleatum* was consistently maintained at relatively low baseline levels (median <1% and peak abundance <5.3%). While previous studies have noted initial microbial dysbiosis within oral potentially malignant disorders such

as leukoplakia (Amer et al., 2017; Deo et al., 2025), our data suggest that the OPMD microenvironment alone may be insufficient to sustain massive *Fusobacterium* colonization. Rather, coinciding with frank malignant transformation, its relative abundance increased markedly during the early stages of OSCC (T1/T2), reaching up to 13.9% of the total intratumoral microbial community. This early microbial shift is remarkably consistent with the recent stage-specific profiling by Pratap Singh et al. (2023), who similarly identified *Fusobacterium* as a hallmark of early-stage OSCC rather than merely a late-stage opportunistic colonizer of necrotic tissue.

Mechanistically, this immediate colonization upon malignant transition can be explained by the altered surface topography of the host tissue. Although dysplastic cells in leukoplakia begin to undergo morphological changes, it is during the structural breakdown of early-stage malignancy that the overexpression of Gal-GalNAc sugars on the surface of oral epithelial cells peaks, providing highly specific binding targets for the fusobacterial Fap2 adhesin (McIlvanna et al., 2021; Pignatelli et al., 2023; Chiscuzzu et al., 2025). Once anchored to the nascent tumor, this high fusobacterial burden actively orchestrates a localized pro-inflammatory and immunosuppressive environment, heavily compromising clinical outcomes even in early-stage patients (Desai et al., 2022). This foundational role is consistent with the broader paradigm of microbe-driven carcinogenesis (Kostic et al., 2012; Zang et al., 2025). Furthermore, while recent large-scale cohorts have linked intratumoral *Fusobacterium* to advanced stages and poor prognosis (Zeng et al., 2022; Pratap Singh et al., 2023; Zang et al., 2025), our findings provide a potential temporal nuance: the active recruitment of these hyper-virulent variants may represent an early event in tumor progression, potentially differentiating established early malignancies from OPMD lesions.

However, our data reveal a fascinating microbial shift as the tumor progresses into advanced stages, aligning well with the “driver-passenger” model recently conceptualized by Chiscuzzu et al. (2025) and Zang et al. (2025). In advanced-stage (T3/T4) biopsies, *Fusobacterium* levels displayed high heterogeneity and localized depletion, being heavily replaced by strict anaerobes, most notably *Prevotella* (which consistently dominated advanced tissues with relative abundances ranging from 10.4% to 16.5%). *F. nucleatum* acts as an active “driver” in the initial phases of oncogenesis, establishing the malignant niche. As the tumor expands and the microenvironment becomes highly hypoxic and morphologically disrupted, the niche is subsequently colonized by strict anaerobic “passengers” such as *Prevotella*, which thrive in the deep necrotic core.

It is important to acknowledge that the present work was designed as a pilot study. Consequently, the relatively small sample size—particularly within the advanced-stage subgroup—represents a limitation that restricts the statistical power needed for broad epidemiological generalizations. Nevertheless, the magnitude of the *Fusobacterium*-to-*Prevotella* shift observed across disease stages, coupled with the distinct microbial signature distinguishing leukoplakia from early OSCC, provides a preliminary biological signal that strongly supports the hypothesis about highly dynamic, stage-specific microbial evolution during oral carcinogenesis.

4.6 Limitations

This study possesses some limitations that must be acknowledged. First, given the pilot design and the small sample size, our findings are inherently hypothesis-generating. The limited statistical power reduces the ability to detect subtle compositional shifts and necessitates caution regarding the generalizability of the results. Furthermore, our statistical framework relied primarily on univariate comparisons, which may not fully capture the complex, multidimensional interactions within the microbial ecosystem.

Second, the cohort structure and available clinical data introduce specific constraints. The inclusion of OPMD cases within the non-OSCC comparator macro-group, while functionally adopted to establish a non-malignant baseline, adds intrinsic biological heterogeneity to the control dataset. Additionally, the lack of systematic periodontal indices for the enrolled patients precludes a precise assessment of baseline dental health. Given the established relationship between periodontitis, oral dysbiosis, and OSCC, along with potential unmeasured confounding from smoking and alcohol consumption habits, residual confounding cannot be entirely excluded.

Methodologically, the use of 16S rRNA amplicon sequencing inherently offers limited taxonomic resolution. As previously noted, this prevents definitive strain-level inference and functional profiling, which would require more advanced approaches such as shotgun metagenomics or isolate sequencing.

Finally, the cross-sectional design precludes causal inferences regarding the relationship between microbial shifts and OSCC development. Future longitudinal studies on larger, multicenter cohorts are required to determine whether the observed dysbiotic patterns represent active drivers of carcinogenesis, early biomarkers, or secondary consequences of tumor progression.

5 Conclusions

In conclusion, our findings suggest that OSCC is characterized by a specific, compartment-dependent dysbiotic signature. Within the tumor tissue, *F. nucleatum* emerges as a primary microbiological biomarker, with its dominant colonization potentially contributing to a localized pro-inflammatory niche. Furthermore, the correlation of the specific strain ATCC 25586 (NR_117842.1) with OSCC progression highlights how strain-level resolution may provide targeted predictions. Building upon these initial results, our future investigations will focus on the functional profiling of *F. nucleatum* within early-stage lesions to further explore its active carcinogenic pathways. Additionally, we aim to monitor intra-patient microbial dynamics through longitudinal follow-up samplings, tracking both OPMD lesions and established OSCC cases to assess microbial shifts during malignant transformation and post-treatment.

This localized intratumoral dysbiosis appears to be systemically mirrored in the salivary microenvironment, characterized by a selective depletion of core health-associated streptococci

concomitant with altered dynamics of *N. macacae*. To refine the reliability of this non-invasive indicator, subsequent studies will evaluate these salivary ratios on an expanded cohort, allowing for a more accurate assessment of baseline inter-patient heterogeneity. Concurrently, a systematic evaluation of the ecological discrepancies between tissue and saliva matrices, mapped on both intra- and inter-patient bases, remains a priority to fully understand shedding dynamics.

By stratifying these profiles across four distinct clinical stages, this pilot study provides preliminary temporal resolution. The data indicate that the microbial shift defining oral carcinogenesis may not emerge gradually from OPMD lesions but rather coincides with early-stage malignant transformation, driven by a significant enrichment of *F. nucleatum*. Moreover, these stage-specific dynamics suggest a potential ecological succession within the tumor micro-environment: while *Fusobacterium* may be strongly associated with the early malignant niche, it could subsequently be outcompeted by strictly anaerobic taxa, such as *Prevotella*, in advanced T3/T4 tumors.

The integration of these spatial and temporal signals supports the conceptual development of a multi-marker translational panel. While further validation in larger independent cohorts is strictly necessary, our current data support investigating a combinatorial diagnostic approach: evaluating the *Streptococcus/Neisseria* imbalance ratio in saliva as a potential biomarker candidate, followed by the quantification of intratumoral *F. nucleatum* load for preliminary clinical correlation. Together, these microbial signatures offer a promising, foundational dataset for future studies (Scheme 1) for future applications of microbiome-based precision medicine in oral oncology.

Data availability statement

The raw 16S rRNA gene sequencing data generated and analyzed during the current study are available in the EMBL-EBI European Nucleotide Archive (ENA) repository under the Study accession number PRJEB115488. A detailed list of individual biosample and run accession numbers is provided in [Supplementary Table S7](#).

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the “Paolo Giaccone” Policlinico University Hospital in Palermo (Italy) (approval #11/2020). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

EM: Conceptualization, Writing – original draft, Investigation, Software, Visualization, Formal analysis, Validation, Writing – review & editing, Methodology, Data curation. MC: Conceptualization, Investigation, Resources, Project administration, Writing – review & editing, Methodology. RM: Project administration, Methodology, Investigation, Writing – review & editing, Conceptualization. MP: Writing – review & editing, Methodology, Investigation, Software. GG: Resources, Conceptualization, Project administration, Writing – review & editing, Funding acquisition, Supervision. GC: Writing – review & editing, Resources, Supervision, Project administration, Conceptualization. CB: Funding acquisition, Data curation, Project administration, Conceptualization, Writing – review & editing, Supervision.

Funding

The authors declared that financial support was not received for this work and/or its publication.

Acknowledgments

The authors wish to express their deepest gratitude to the Arrow Diagnostics team for their outstanding support. Special thanks go to Gabriele Bivona, Roberta Carpine, and Maurizio Caruso, whose dedication and commitment significantly contributed to the realization of this work.

Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fbri.2026.1863255/full#supplementary-material>

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