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Microbial signatures in head and neck squamous cell carcinoma: an *in silico* study

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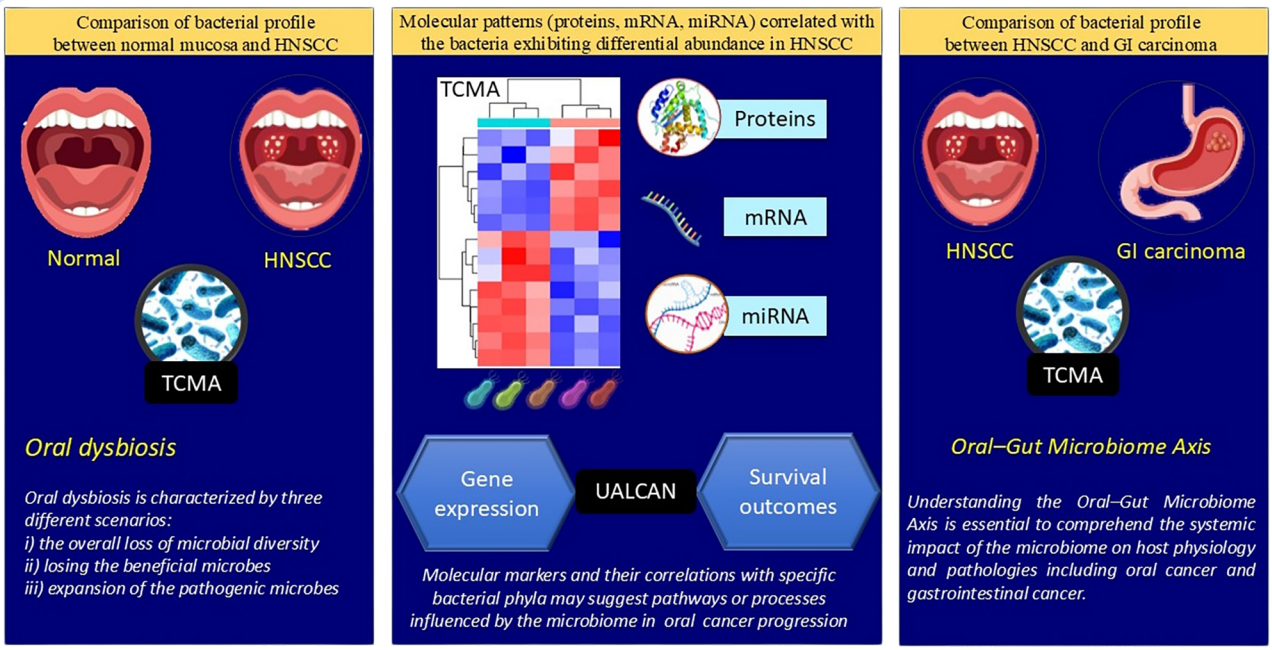
Abstract

Objectives: The oral cavity harbors a plethora of bacterial species. Dysbiosis of oral and gut microbiota is associated with several oral and systemic pathologies, such as cancer, obesity, diabetes, atherosclerosis and gastrointestinal diseases. Imbalance in the oral-gut microbial axis has been associated with head and neck squamous cell carcinoma (HNSCC). This study aims to analyze the bacterial profile of HNSCC across various taxonomic units, investigate molecular patterns associated with prevalent bacterial phylum in HNSCC, and compare the bacterial profile in HNSCC and gastrointestinal (GI) carcinoma using computational analysis. Methodology: The microbe-host transcriptomic, proteomic, and epigenetic analyses of HNSCC and GI carcinomas were performed using The Cancer Microbiome Atlas (TCMA) database. The differential expression of the host's mRNA transcripts and proteins associated with tumor microbiome were analyzed using The University of Alabama at Birmingham Cancer data analysis (UALCAN) and Clinical Proteomic Tumor Analysis Consortium (CPTAC) websites. Results: A decrease in Actinobacteria and an enrichment of Flavobacteria at the class level, Neisseriales, Pasteurellales, and Campylobacterales at the order level, *Pasteurellaceae*, *Flavobacteriaceae*, *Campylobacteraceae*, and *Peptoniphilaceae* at the family level, and *Hemophilus*, *Porphyromonas*, and *Leptotrichia* at the genus level were observed in HNSCC compared to the normal mucosa. RICTOR protein, mRNA transcripts (HIST1H2BB, SCARNA11, TBC1D21 gene), and hsa-miR-200a-5p miRNA were significantly correlated with prevalent bacterial species in HNSCC. A major increase in Actinobacteria, Fusobacteria, and Spirochaetes was observed in HNSCC compared to GI carcinoma. Conclusion: The oral-gut microbial dysbiosis, as reflected by the differential abundance of bacterial species in oral and GI carcinomas, suggests the implication of tumor microbiome and their genomic interactions with the host in carcinogenesis.

Keywords: Microbiome. *In silico*. Oral squamous cell carcinoma. HNSCC. UALCAN. TCMA.



Abbreviations: HNSCC - Head and Neck Squamous Cell Carcinoma; GI carcinoma - Gastrointestinal carcinoma; mRNA - messenger RNA; miRNA - microRNA
TCMA - The Cancer Microbiome Atlas; UALCAN - University of Alabama at Birmingham CANcer data analysis

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Introduction

The oral microbiome comprises bacteria, fungi, viruses, archaea, and protozoa living in the oral cavity. Approximately 700 bacterial species have been identified, making it the second-largest bacterial community with the second-highest level of alpha diversity (microbial diversity within individuals) in the human body, following the gut.[1] In a healthy host, the oral microbiome maintains balanced symbiotic/commensal relationships, described as ''microbial homeostasis" or ''eubiosis." This promotes beneficial commensalism without harming the microorganisms or the host. It is also essential in the development of natural oral physiology and defense mechanisms. Inter-species and host-microbial interactions regulate the microbial composition and can impact the health of the host.[2] The oral microbiome can shift this balance from commensalism to an unbalanced parasitic/pathogenic disease state, known as an ''unbalanced microbiome" or ''dysbiosis".[1,2] The shift in the oral microbiota from eubiosis to dysbiosis can provoke various pathological conditions, including carcinogenesis.[2]

Dysbiosis is characterized by loss of microbial diversity, loss of beneficial microbes, and expansion of the pathogenic microbes as an isolated or simultaneous process,[1,3] being associated with several intraoral and systemic diseases. The role of microorganisms in the former, such as dental caries, gingivitis, periodontitis, and oral candidiasis, is well established. The number of microorganisms ingested daily is about one hundred billion (10^{11}) to one trillion (10^{12}). The oral microbiota has unrestricted access to the gastrointestinal tract and other organ systems, which explains its impact on systemic diseases such as Alzheimer's disease, diabetes, pregnancy complications, and several types of cancer, including oral, gastrointestinal, lung, breast, prostate, and uterine cancer.[2,4] Oral microbiota-mediated carcinogenesis includes sustaining proliferative signaling, the ability to evade growth suppressors and induce angiogenesis, resisting cell death, limitless replicative potential, and activation of invasion and metastasis.[4]

Head and neck squamous cell carcinoma (HNSCC) varies worldwide and is generally correlated with tobacco-derived carcinogens and excessive alcohol consumption. Oral squamous cell carcinoma (OSCC) constitutes around 90% of HNSCC and is often preceded by oral potentially malignant disorders (OPMDs) with varying risks of malignant transformation to OSCC. Oral cancer is the 16th most common malignancy, accounting for 389,485 new cases, and is the 15th leading cause of mortality globally.[5] Oral bacteria community dynamics have been linked to OSCC staging in studies, raising the possibility of using bacteria as OSCC diagnostic markers. Salivary microbiome dysbiosis and cytokines influence OSCC through inflammation.[6] *Fusobacterium nucleatum, Porphyromonas gingivalis,* and *Prevotella intermedia* are strongly associated with OSCC; other bacterial genera demonstrated in OPMDs and OSCC include *Actinomyces, Clostridium, Enterobacteriaceae, Fusobacterium, Haemophilus, Porphyromonas, Prevotella, Streptococcus spp. (S. infantis, S. mitis, S. gordonii)* and *Veillonella*. Several periodontal disease-associated bacterial species, namely *Porphyromonas gingivalis, Tannerella forsythia,* and *Prevotella intermedia*, are correlated with an increased risk of gastrointestinal (GI) cancer.[7]

Characterizing oral microbiome is essential to understand oral health and systemic diseases. *In silico* studies serve as prediction models and can give insight to initiate experimental studies. Recently, *in silico* studies analyzed the mRNA expression and mutation profiles of the target genes in human cancer, including HNSCC, colorectal, pancreatic, hepatic, and lung cancers.[8-12] Using *in silico* analysis, we investigated the microbial profile of oral bacteria in HNSCC across different taxonomic units, the molecular patterns (differential expression of protein, mRNA, microRNA, and DNA methylation sequences) in the host associated with the oral bacteria during carcinogenesis, and compared the bacterial prevalence in HNSCC and GI carcinoma to increase the understanding of the oral-gut microbial axis in carcinogenesis. This study also discusses the role of oral dysbiosis in the xenobiotic metabolism of carcinogens, which are crucial in the development of OPMDs and OSCC.

Methodology

Data source

Computation (*in silico*) analysis unraveling the bacterial profile and molecular signatures in HNSCC employs two comprehensive, user-friendly, and interactive websites: The Cancer Microbiome Atlas (TCMA) and the University of Alabama at Birmingham

Cancer database (UALCAN).[13,14]

TCMA is a database of decontaminated and tissue-resident microbial profiles of The Cancer Genome Atlas (TCGA). It provides a collection of curated microbial compositions of oropharyngeal, esophageal, gastrointestinal, and colorectal tissues in healthy controls and carcinoma. It is a resource for performing multi-omic, pan-cancer analyses of host-microbe interactions that enable the identification of diagnostic and prognostic species. TCMA facilitates a matched microbe-host transcriptomic, proteomic, and epigenetic analysis that identifies associations between microbes and gene expression patterns and pathways of the host.[13]

UALCAN is a website that enables researchers to analyze gene expression using the TCGA database on about 20,500 protein-coding genes in 33 different tumor types. It provides interactive graphs and plots depicting gene expression profiles and their influence on patient survival.[14]

Study workflow and selection criteria

In this study, the TCMA data repository was used to analyze the prevalence of bacteria in HNSCC and its association with the host molecular characteristics. The prevalence of bacteria in HNSCC was compared to that of normal tissue in the following taxonomic units: phylum, class, order, family, and genus.

The phyla predominant in HNSCC were correlated with the host molecular characteristics, which include RPPA (reverse-phase protein array) proteins, mRNA and miRNA sequences. Interactive heat maps showed the correlation between each bacterial phyla and the host molecular features. The molecular traits (proteins/mRNA/miRNA) with the highest and lowest correlation values with each bacterium were screened. RPPA proteins, mRNA, and miRNA sequences that demonstrated a moderate and strong correlation with the bacterial phyla in the TCMA database were selected. Their gene expression and the effect of the gene on HNSCC survival outcomes were studied in the UALCAN database.

TCMA also facilitated the comparison between the relative abundance of bacteria in HNSCC and GI carcinoma (esophageal carcinoma, adenocarcinoma, colon adenocarcinoma, and rectal adenocarcinoma).

The proportion test was done to compare various microbiomes in tumor tissue and normal tissue using STATA (ver.12), with $p<0.05$ representing statistical significance. Oral microbial dysbiosis involving xenobiotic metabolism in OPMDs and oral and gastrointestinal carcinogenesis were reviewed from the literature. The study workflow is summarized in the graphical abstract**.**

Results

The TCMA database included microbiome compositions of 177 HNSCC samples (155 tumors and 22 tumor-adjacent tissue as normal samples) at phylum, order, and genus levels. Table 1 summarizes demographic details of the samples. The TCMA database was used to extract 11 phyla, 38 orders, and 221 genera of microbial taxa in each sample.

Differential bacterial profiling of HNSCC tumor tissue and normal samples (Figure 1)

A relative abundance of obligate, anaerobic, Gram-negative bacilli belonging to the phylum Fusobacteria (Class: Fusobacteriia, Order: Fusobacteriales; Family: *Fusobacteriaceae*, Genus: *Fusobacterium*), and the phylum Bacteroidota (synonym: Bacteroidetes)

Table 1- Clinicopathological characteristics of normal mucosa and HNSCC tissue samples

Clinicopathological characteristics	Normal	HNSCC
Gender [(n (%)]		
Male	15 (68.2)	113 (72.9)
female	7 (31.8)	42 (31.8)
Age (median range)	60 (29-87)	60 (19-90)
Site of biopsy [n (%)]		
Tongue base	1 (4.5)	7 (4.5)
Mouth floor	0 (5.2)	8 (5.2)
larynx	2 (9.1)	22 (14.2)
Tumor stage [n (%)]		
Stage I	-	10 (6.5)
Stage II	-	23 (14.8)
Stage III	-	23 (14.8)
Stage IV	-	73 (47.1)
Not reported	-	26 (16.8)
Histologic grade [n (%)]		
Grade 1	-	13 (8.4)
Grade 2	-	93 (60.0)
Grade 3	-	44 (28.4)
Grade 4	-	1 (0.6)
Grade X	-	4 (2.6)
Total	**22**	**155**

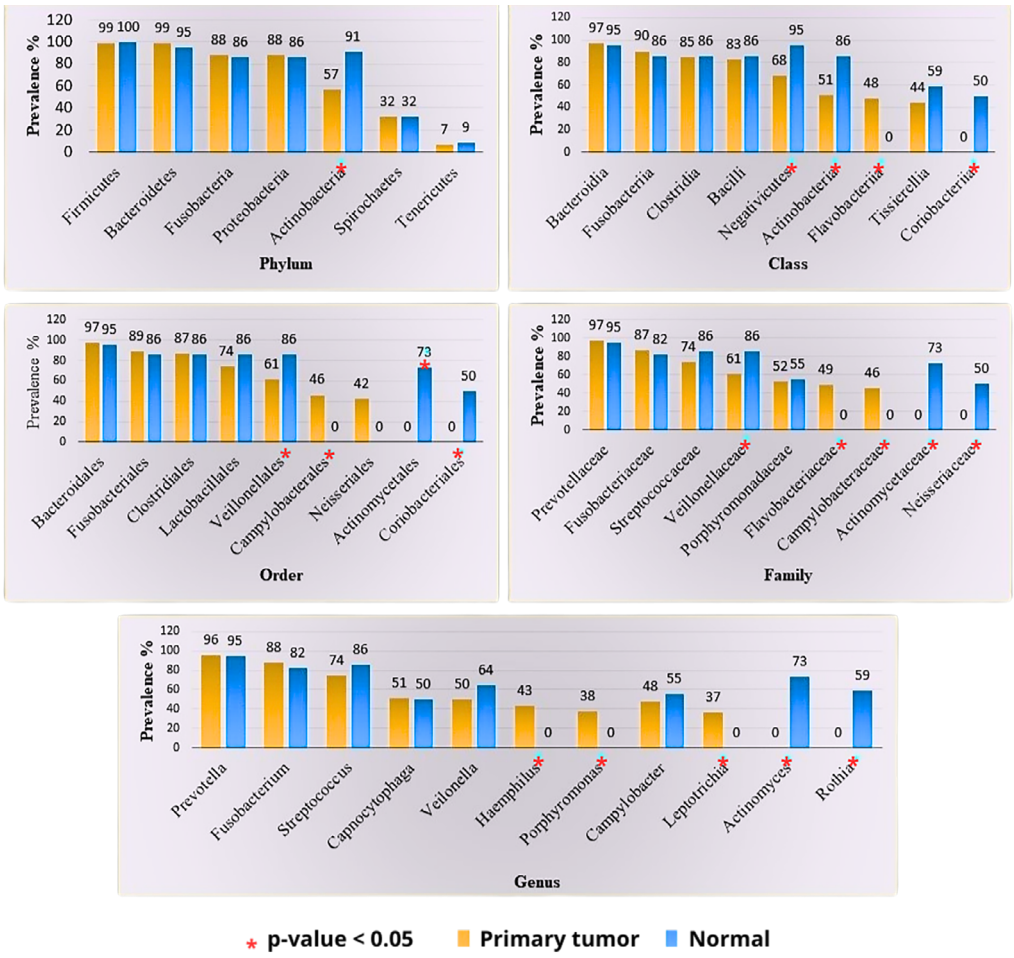


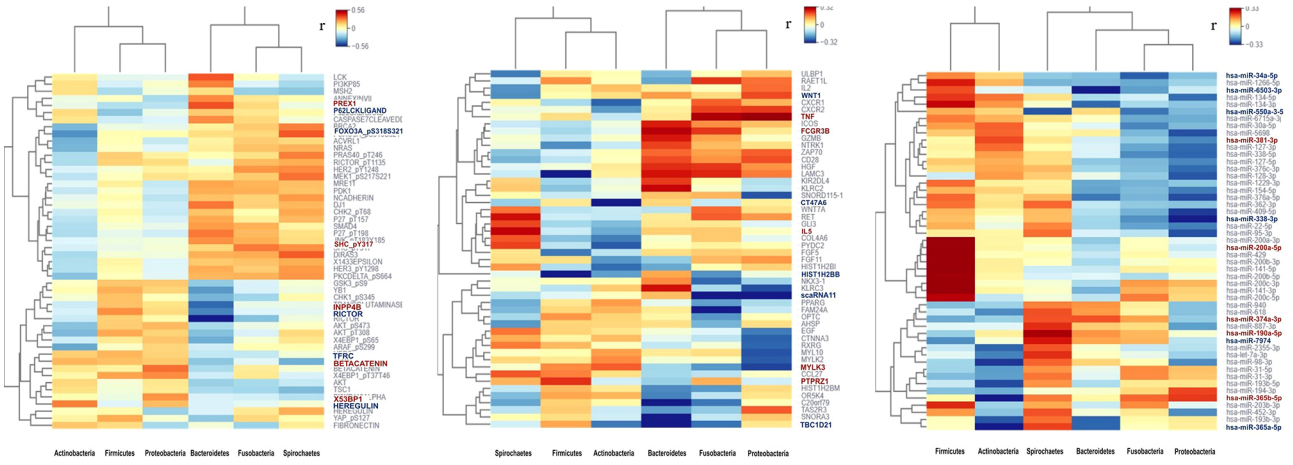
Figure 1- Prevalence of bacteria across various taxonomic units in HNSCC and normal mucosa.

(Order: Bacteroidales, Family: *Prevotellaceae* and Genus: *Prevotella*) was observed in both HNSCC and normal mucosa. At the order level, Campylobacterales were enriched in the tumor tissue compared to the normal tissue; Veillonellales and Coriobacteriales were significantly abundant in the normal tissue ($p<0.05$). At the family level, Flavobacteriaceae and Camplyobacteraceae were prevalent in tumor tissue, whereas Neisseriaceae was more prevalent in normal tissue ($p<0.05$). At the genus level, *Hemophilus, Porphyromonas,* and *Leptotrichia* were significantly recurrent in tumor tissues, whereas *Actinomyces* and *Rothia* were relatively abundant in the adjacent normal tissue ($p<0.05$). Actinobacteria was lower in tumors compared to normal tissue in all taxonomic units, from phylum to genus ($p<0.05$).

Molecular patterns (proteins, mRNA, miRNA) correlated with bacteria exhibiting differential abundance in HNSCC (Figure 2; Table 2)

RICTOR protein was negatively correlated (r=-0.56; moderate correlation) with Bacteroidetes. The mRNA transcripts of the HIST1H2B gene (r=-0.72; strong correlation), SCARNA11 gene (r=-0.45; moderate correlation), and TBC1D21 gene (r=-0.59; moderate correlation) were negatively correlated with Firmicutes, Proteobacteria, and Bacteroidetes respectively. The hsa-miR-200a-5p showed a moderate positive correlation (r=0.44) with Firmicutes. hsa-miR-365-5p (r=0.25), hsa-miR-374a-5p (r=0.22), and hsa-miR-365b-5p (r=0.20) exhibited a weak positive correlation with Proteobacteria, Bacteroidetes, and Fusobacteria respectively. The hsa-miR-365a-5p (r=-0.35) and hsa-miR-338-3p (r=-0.36) demonstrated a weak negative correlation with Actinobacteria and Proteobacteria in HNSCC, respectively.

The UALCAN database demonstrated the gene expression of molecular patterns significantly associated with the bacterial phyla abundant in HNSCC and the effect of differential gene expression (DGE) on HNSCC survival outcomes (Figure 3). Upregulation of RICTOR and HIST1H2B gene was observed in HNSCC (Median: 5.1, 0.1, respectively; transcripts per million) compared to normal mucosa (Median: 3.6, 0, respectively; transcripts per million). Similarly, hsa-miR-200a expression was greater in HNSCC (Median:



A: Proteins correlated with bacterial phlya

B: mRNA correlated with bacterial phyla

C: miRNA correlated with bacterial phlya

In the above heat maps (A,B,C), each row represent the protein/ mRNA/ miRNA and each column represents the bacterial phylum predominant in HNSCC. The colors denote the changes in expression of the molecular patterns. The grids presenting in varying hues of red and blue demonstrate upregulation and downregulation of protein/ mRNA/ miRNA, respectively. Molecular traits texted in red and blue signify those with the highest and lowest correlation values with each bacterium. The Pearson correlation coefficient (r) gives a measure of any linear trend between phylum and the molecular patterns. The value of r ranges between –1 and 1; 0.1-1 denotes the level of upregulation; -0.1 to -1 denotes the level of downregulation.

Figure 2- Correlation of bacterial phylum and host molecular features (Proteins, mRNA, miRNA) in HNSCC

Table 2- The molecular patterns (proteins, mRNA, and miRNA) correlated with the bacteria exhibiting differential abundance in HNSCC.

Predominant Phylum		Actinobacteria	Firmicutes	Proteobacteria	Bacteroidetes	Fusobacteria	Spirochaetes
Host RPPA proteins	Positively correlated proteins	X53BP1 r= 0.28	INPP4B r= 0.26	Beta-catenin r= 0.31	PREX1 r= 0.35	SHC_pY317 r= 0.29	FOXO3A r= 0.34
	Negatively correlated proteins	FOXO3A r= - 0.37	P62LCKL ligand r= - 0.26	Heregulin r= - 0.24	RICTOR r= - 0.56	RICTOR r= - 0.28	TFRC r= - 0.31
Host mRNA-seq	Positively correlated mRNA-seq	MYLK3 r= 0.20	PTPRZ1 r= 0.27	TNF r= 0.36	FCGR3B r= 0.30	TNF r= 0.31	IL5 r= 0.29
	Negatively correlated mRNA-seq	CT47A6 r= - 0.33	HIST1H2BB r= - 0.71	scaRNA11 r= - 0.45	TBC1D21 r= - 0.55	scaRNA11 r= - 0.40	WNT1 r= - 0.22
Host miRNA-seq	Positively correlated miRNA-seq	hsa-miR-381-3p r= 0.22	hsa-miR-200a-5p r= 0.44	hsa-miR-365b-5p r= 0.23	hsa-miR-374a-3p r= 0.21	hsa-miR-365b-5p r= 0.19	hsa-miR-190a-5p r= 0.32
	Negatively correlated miRNA-seq	hsa-miR-365a-5p r= - 0.36	hsa-miR-7974 r= - 0.22	hsa-miR-338-3p r= - 0.36	hsa-miR-6503-3p r= - 0.33	hsa-miR-34a-5p r= - 0.27	hsa-miR-550a-3-5p r= - 0.28

The table shows the correlation of specific molecular patterns (proteins, mRNA, and miRNA) with various bacterial phyla showing differential abundance in head and neck squamous cell carcinoma (HNSCC). Each column represents a different bacterial phylum (Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Spirochaetes), and each row represents a molecular category (host proteins, mRNA, and miRNA). Underlined molecular patterns are associated with HNSCC/ OSCC.
Absolute values of r (Pearson's correlation coefficient)*:
0-0.19: very weak, 0.2-0.39: weak, 0.40-0.59: moderate, 0.6-0.79: strong, and 0.8-1: very strong
> 0 denotes positive correlation; < 0 denotes negative correlation
*Schober P, Boer C, Schwarte LA. Correlation Coefficients: Appropriate Use and Interpretation. Anesth Analg. 2018;126(5):1763-1768. doi: 10.1213/ANE.0000000000002864.

872.6; reads per million) compared to normal mucosa (Median: 578.1; reads per million). However, UALCAN had insufficient data on scaRNA11 and TBC1D21 for analysis.

Comparison of bacterial profile in HNSCC and GI Carcinoma

At the phylum level, Actinobacteria, Fusobacteria, and Spirochaetes were increased in HNSCC compared to GI carcinoma ($p<0.05$) (Figure 4).The prevalence of Bacteroidetes, Firmicutes, Proteobacteria, and Tenericutes did not vary significantly between

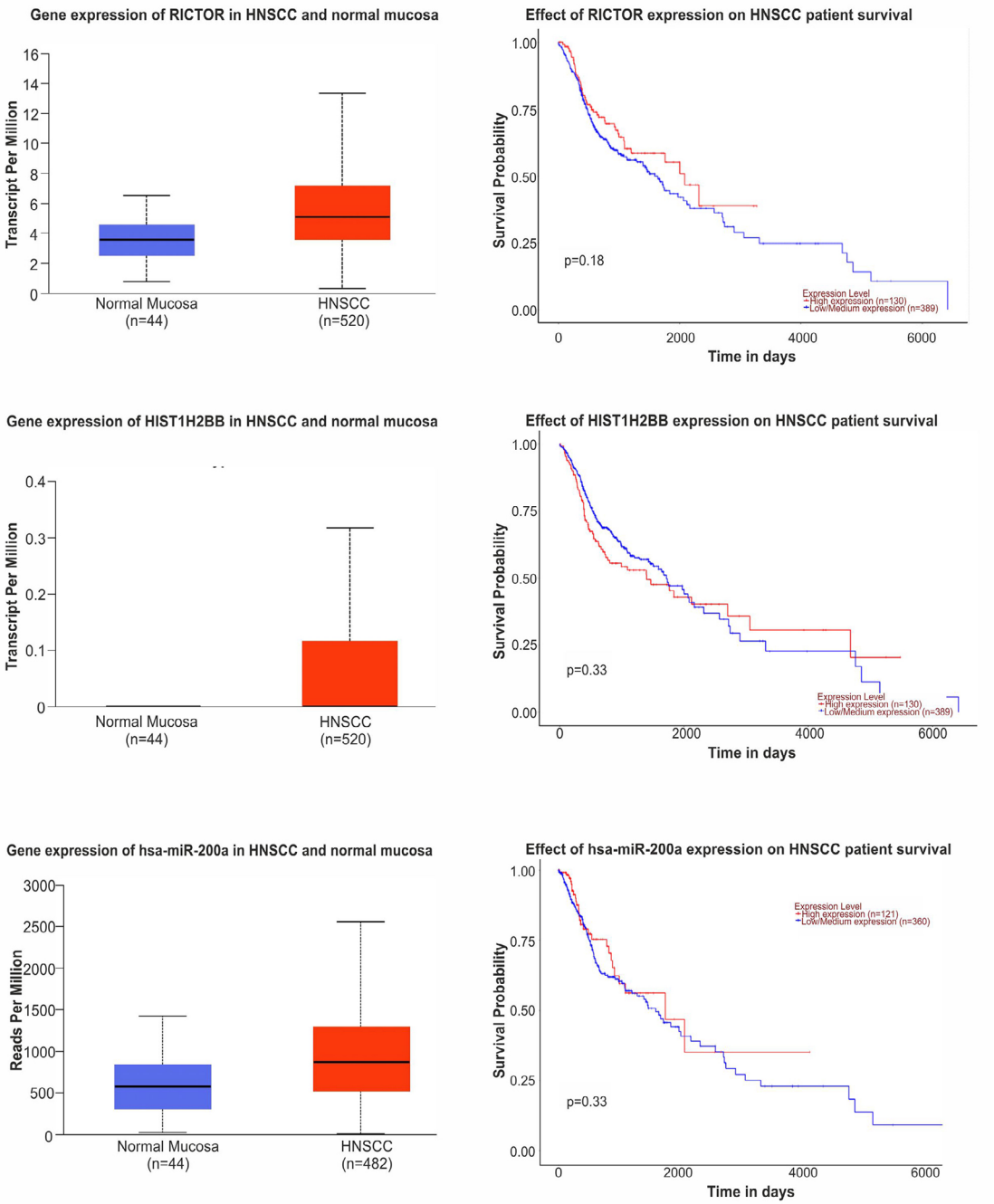


Figure 3- Gene expression of RICTOR, HIST1H2BB, and miR-200a-5p in HNSCC and their effect on survival outcomes.

both groups. *Alistipes*, *Blautia*, *Faecalibacterium*, *Parabacteroides*, *Prevotella*, *Roseburia*, *Ruminococcus*, *Streptococcus*, Verrucomicrobia, and Thermotogae were enriched in GI carcinoma compared to HNSCC. Supplementary Table 1 summarizes the difference in the bacterial prevalence in other taxonomic units between HNSCC and GI carcinoma.

Discussion

The human microbiota is a complex, diverse, and abundant population of symbiotic microorganisms that inhabit many sites in the human body, including the skin, oral cavity, and gastrointestinal tract (GIT; gut). The oral cavity is the entry of all microorganisms into the human body through the GIT. The microbiota of the oral cavity and the GIT are the largest microbial ecosystems in the human body with the predominance of the bacterial microbiota. The bacterial diversity of the oral-gut microbial axis and their complex interactions have been implicated in the pathogenesis of oral and gastrointestinal diseases, including cancer development and progression.[15,16] In this study, we analyzed the bacterial profile involving 11 taxa with the associated host molecular patterns in 155 HNSCC cases

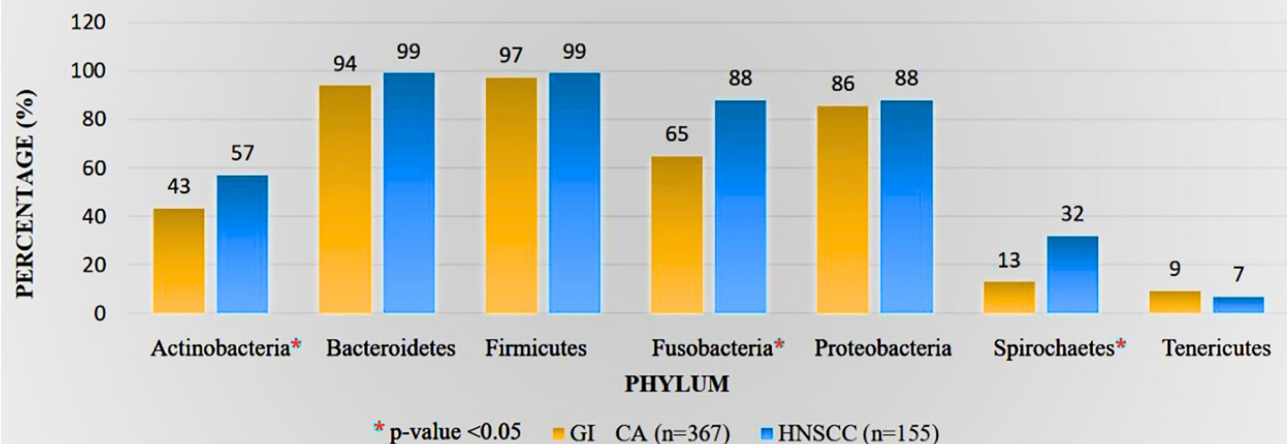


Figure 4- Comparison of bacterial profile between HNSCC and GI carcinoma.

and a portion of their adjacent normal tissues from 22 cases. The differential abundance of the bacteria in the oral cavity was compared to that of GIT to understand the role of the oral-gut microbial axis in oral and GI carcinogenesis.

In this study, an abundance of obligate, anaerobic, Gram-negative bacilli included in the Fusobacteria phylum (Class: Fusobacteriia, Order: Fusobacteriales; Family: *Fusobacteriaceae*, Genus: *Fusobacterium*), and the Bacteroidota phylum (synonym: Bacteroidetes) (Order: Bacteroidales, Family: *Prevotellaceae* and Genus: *Prevotella*) was observed in both HNSCC and normal mucosa.

In oral cancer, *F. nucleatum* causes double-stranded DNA breaks and promotes GLUT1 upregulation and lactic acid accumulation. Its lipopolysaccharides can stimulate inflammatory cytokines, leading to a proinflammatory environment and promoting tumor progression.[17] In colorectal cancer, the Fap2 protein of *F. nucleatum* interacts with the TIGIT receptor (T cell immunoreceptor with Ig and ITIM [immunoreceptor tyrosine-based inhibitory motif] domains) present on natural killer (NK) cells. It inhibits their cytotoxicity and, subsequently, contributes to tumor evasion. The protein Fad A of *F. nucleatum* binds to E-cadherin on colorectal cancer cells and induces β -catenin signaling, leading to regulation of inflammatory and oncogenic responses.[18]*F. nucleatum* regulates the polarization of macrophage to M2 phenotype by secreting IL-6 and activating IL-6/STAT3/c-MYC signaling. *Bacteroides fragilis* induce the production of IL-8 by activating E-cadherin/β-catenin/NF-kB signaling pathway.[19]*Bacteroides fragilis* toxin (BFT) secreted by *B.fragilis* degrades E-calmodulin, causing alterations in signaling pathways that lead to upregulation of spermidine oxidase, which in turn promotes irreversible DNA damage and may eventually lead to carcinogenesis.[20] *P. intermedia* treatment significantly stimulates tumor growth, invasion, angiogenesis, and metastasis, affects levels of inflammatory cytokines, and alters M2 macrophages and regulatory T cells (Tregs) infiltration in the tumor microenvironment. It stimulates tyrosine kinase receptors that modulate cell proliferation, migration, and differentiation associated with disease progression.[21]

We also observed an enrichment of Flavobacteria at the class level, Neisseriales, Pasteurellales, and Campylobacterales at the order level, *Pasteurellaceae*, *Flavobacteriaceae*, *Campylobacteraceae*, and *Peptoniphilaceae* at the family level, and *Hemophilus*, *Porphyromonas*, and *Leptotrichia* at the genus level were observed in HNSCC compared to the normal mucosa. *Pasteurella multocida* produces a toxin that modifies heterotrimeric G-proteins and activates Rho GTPase, focal adhesion kinase, cyclooxygenase-2, β-catenin signaling, and calcium signaling pathways involved in carcinogenesis.[22] *Porphyromonas gingivalis* has virulence factors such as gingipains, outer membrane vesicles (OMVs), E-cadherin, toxin, and proteolytic enzymes. *P. gingivalis* can also convert ethanol to acetaldehyde, a carcinogenic intermediate.[23]*Leptotrichia hofstadii* was abundant in stage III oropharynx cancer.[24] Various studies have explored the differential abundance of the oral microbiota across various taxonomic units. Figure 5 summarizes important observations from some of these studies.[25-42]

An enrichment of Actinobacteria (phylum), Negativicutes, Coriobacteria (class), Veillonellales, Actinomycetales, Coriobacteriales, Micrococcales (order), *Veillonellaceae, Neisseriaceae, Actinomycetaceae, Micrococcaceae* (family),

Author/ Year	Study Objective	Study Groups	Study Samples	Technique	Study findings
Makinen et al., 2023[25]	To investigate possible changes in the salivary microbiome profiles of OSCC patients before and after cancer treatment and to compare these changes with the profiles of healthy controls.	99 OSCC patients, 101 healthy controls	Paraffin-stimulated whole saliva samples	Amplification of 16S rRNA targeting V4 hypervariable regions with Illumina MiSeq	At baseline, the OSCC patients showed a higher relative abundance of zOTUs classified as Streptococcus anginosus, Abiotrophia defectiva, and Fusobacterium nucleatum. The microbial profiles differed significantly between OSCC patients and healthy controls.
Dou et al., 2022[26]	To investigate the association between tumor microbiota and outcomes of HNSCC patients.	129 primary tumors of HNSCC	Paraffin-embedded tumor tissue	Amplification of 16S rRNA targeting V4 hypervariable regions with Ion Plus Fragment Library Kit, p16 IHC, and Peritumoral inflammatory infiltrate examination	A reduced richness and enriched abundances of genera Schlegelella and Methyloversatilis in tumor microbiota of HNSCC patients with poor prognosis. However, a richer tumor microbiota with greater abundances of genera Bacillus, Lactobacillus, and Sphingomonas was characterized in the patients with favorable prognosis.
Yang et al., 2021[27]	To investigate the association between the microbiota and risk of oral squamous cell carcinoma (OSCC)	23 OSCC, paired normal precancerous tissue (NPT) and paired normal tissue from the same patient	Saliva and tissue samples; non-stimulated saliva collected from 19 out of the 23 OSCC	Amplification of the 16S rRNA gene V3–4 region followed by bioinformatic analysis	The phylum Campilobacterota was significantly enriched in tumor tissue (TT). The genera Veillonella and Granulicatella were highly enriched in NPT, and the genera Campylobacter, Gemella, Filifactor, and Catonella were highly enriched in TT. The species Rothia mucilaginosa, Granulicatella adiacens, Streptococcus sanguinis, and Veillonella rogosae were highly enriched (proportion difference >0.5%) in NPT, and Aggregatibacter segnis, Campylobacter showae, and Granulicatella morbillorum were highly enriched in TT.
Yost et al., 2018[28]	a small pilot study of community-wide metatranscriptome analysis to profile mRNA expression in the entire oral microbiome in OSCC to reveal molecular functions associated with this disease	15 samples, including four tumor sites from OSCC subjects, four tumor-adjacent sites from OSCC subjects, four sites from healthy patients who matched the locations of the tumor sites and three buccal sites in healthy tumor-free subjects that matched the locations of tumour-adjacent samples	Oral swab	Meta-transcriptome analysis using two methods: NOISeq and GFOLD	Fusobacteria, Selenomonas spp., Capnocytophaga spp. and members of the genera Dialister and Johnsonella were significantly more active in the tumour sites, while the genus Bacillus and the species Porphyromonas catoniae, Kingella denitricans, Capnocytophaga gingivalis, Neisseria elongata, bacterium MGEHA from the candidate division SR1, Veillonella sp. oral taxon 780, Aggregatibacter segnis and Streptococcus downei were more active in the healthy control sites
Yang et al., 2018[29]	To profile the oral microbiota and identified bacteria biomarkers associated with OSCC	51 healthy individuals and 197 OSCC patients	Oral rinse	16S rRNA V3V4 amplicon sequencing, followed by bioinformatics	The abundance of Fusobacteria increased significantly with the progression of oral cancer from the healthy controls (2.98%) to OSCC stage 1 (4.35%) through stage 4 (7.92%). At the genus level, the abundance of Fusobacterium increased, while the number of Streptococcus, Haemophilus, Porphyromonas, and Actinomyces decreased with cancer progression. Fusobacterium periodonticum, Parvimonas micra, Streptococcus constellatus, Haemophilus influenza, and Filifactor alocis were associated with OSCC, and they progressively increased in abundance from stage 1 to stage 4. The abundances of Streptococcus mitis, Haemophilus parainfluenzae, and Porphyromonas pasteri were inversely associated with OSCC progression.
Lim et al., 2018[30]	To characterize the oral microbiome fluctuation associated with oral cavity cancer (OCC) and oropharyngeal cancers (OPC).	Normal healthy controls (n = 20), high-risk individuals with poor oral hygiene and/ or oral diseases (n = 11) and OCC and OPC patients (n = 31, HPV-positive; n = 21, HPV-negative).	Oral rinse samples	16S rRNA gene amplicon sequencing on the MiSeq platform	Rothia, Haemophilus, Corynebacterium, Paludibacter, Porphyromonas, and Capnocytophaga are found in significantly lower abundance in oral rinse samples from OCC and OPC patients while Oribacterium is significantly higher. While Actinomyces, Parvimonas, Selenomonas, and Prevotella have a significantly higher abundance in OCC compared with OPC; HPV has a positive correlation on the abundance of Haemophilus and Gemella. Pathogenic and/or opportunistic bacteria such as Actinomyces, Actinobacillus, Lautropia, Fusobacterium and Aggregatibacter are significantly more abundant in high-risk individuals.
Zhao et al., 2017[31]	To unravel the connections underlying oral bacterial dysbiosis and oral squamous cell carcinoma (OSCC), cancer lesion samples and anatomically matched normal samples	40 OSCC and matched controls	Oral swabs	Illumina sequencing and bioinformatics analysis of 16S rRNA gene amplicons.	Bacteroidetes, Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, the top five most abundant phyla, comprised 98.62% of all sequences. Bacteroidetes was the most abundant phylum, accounting for 37.6% of sequences. Synergistetes, SR1, and Chloroflexi, were less than 0.1%. At genus level, Prevotella, Neisseria, Streptococcus, Fusobacterium, and Haemophilus were the five most abundant genera, comprising 22.46%, 13.67%, 8.17%, 6.95%, and 5.74% of sequences, respectively.
Lee et al., 2017[32]	To investigate the microbiota differences between normal individuals, epithelial precursor lesion patients, and cancer patients	125 cases of OSCC; 124 cases of epithelial precursor lesion with dysplasia, hyperplasia, or hyperkeratosis; and 127 normal controls with no malignancy	Saliva	Next-generation sequencing and bioinformatics	Microbial community changes (Bacillus, Enterococcus, Parvimonas, Peptostreptococcus, and Slackia) in the saliva might represent a convenient marker for predicting, detecting, and prognosis oral cancer, especially the epithelial precursor lesion-cancer transition.

Figure 5- The differential abundance of the oral microbiota across various taxonomic units in HNSCC and OPMD.

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Author/ Year	Study Objective	Study Groups	Study Samples	Technique	Study findings
Al-hebshi et al., 2017[33]	To characterize the species composition and the functional potential of the bacteriome associated with OSCC.	20 OSCC and matched controls	Tissue biopsies and Deep epithelial swabs	Amplification of the V1-3 region of the 16S rRNA gene using standard PCR conditions. Sequencing of the indexed library was performed employing the v3 2×300bp chemistry on a MiSeq platform	Fusobacterium nucleatum subsp. polymorphum was the most significantly overrepresented species in the tumors, followed by Pseudomonas aeruginosa and Campylobacter sp. Oral taxon 44, while Streptococcus mitis, Rothia mucilaginosa, and Haemophilus parainfluenzae were the most significantly abundant in the controls.
Guerrero-Preston et al., 2016[34]	To compare the saliva microbiome in DNA isolated from oropharyngeal carcinoma and OSCC patients and normal epithelium controls to characterize the HNSCC saliva microbiota and examine their abundance before and after surgical resection.	25 patients with no history of cancer and 19 HNSCC patients.	Saliva	16S rRNA V3-V5 marker gene approach	Abundances of Firmicutes, Proteobacteria, and Bacteroidetes, with less frequent presence of Actinobacteria and Fusobacteria in HNSCC before surgery. At lower taxonomic levels, the most abundant genera were Streptococcus, Prevotella, Haemophilus, Lactobacillus, and Veillonella, with lower numbers of Citrobacter and Neisseraceae genus Kingella.
Schmidt et al., 2014[35]	To investigate changes in the microbiome associated with oral cancers	83 oral cancer, carcinoma in-situ and precancer	Oral swab	16S rDNA sequencing of hypervariable region amplicons	In cancer samples, abundance of Firmicutes (especially Streptococcus) and Actinobacteria (especially Rothia) was significantly decreased relative to contralateral normal samples from the same patient. Significant decreases in abundance of these phyla were observed for pre-cancers but not when comparing samples from contralateral sites (tongue and floor of mouth) from healthy individuals.
Pavlova et al., 2013[36]	To analyse the enzymes involved in ethanol metabolism in Streptococcus gordonii V2016	Two groups of oral streptococcal strains were analyzed. First group: 14 laboratory strains of Streptococcus sanguinis and Streptococcus gordonii. Second group: 38 clinical strains isolated from the saliva of 12 healthy volunteers.	Oral Bacterial strains	To detect acetic acid production from ethanol by oral Streptococcus purple broth was used. Construction of adh mutants in S. gordonii V2016 by recombinant DNA techniques. ADH and ALDH were determined by a specific enzyme activity gel assay (zymogram).	S. gordonii V2016 expressed three primary alcohol dehydrogenases, AdhA, AdhB and AdhE, which all oxidize ethanol to acetaldehyde, but their preferred substrates were 1-propanol, 1- butanol and ethanol, respectively. Two additional dehydrogenases, S-AdhA and TdhA, were identified with specificities to the secondary alcohol 2-propanol and threonine, respectively, but not to ethanol. Mutants with adhE deletion showed greater tolerance to ethanol than the wild-type and mutant with adhA or adhB deletion, indicating that AdhE is the major alcohol dehydrogenase in S. gordonii.
Pushalkar et al., 2011[37]	To investigate the association of oral bacteria in oral squamous cell carcinoma and compare with adjacent non-tumor mucosa	10 squamous cell carcinoma with adjacent non-tumor mucosa sampled 5 cm distant from the same patient	Oral tissue samples	Culture-independent 16S rRNA approaches, denaturing gradient gel electrophoresis (DGGE) and cloning and sequencing,	The clonal analysis indicated that six phyla, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Actinobacteria and uncultivated TM7 (Bacteria from the Saccharibacteria phylum) in non-tumor and tumor libraries. Bacterial species, Streptococcus sp. oral taxon 058, Peptostreptococcus stomatis, Streptococcus salivarius, Streptococcus gordonii, Gemella haemolysans, Gemella morbillorum, Johnsonella ignava and Streptococcus parasanguinis were highly associated with tumor site whereas Granulicatella adiacens was prevalent at non-tumor site. Streptococcus intermedius was present in 70% of both non-tumor and tumor sites.
Katz et al., 2011[38]	to investigate the presence of P. gingivalis in specimens from squamous cell carcinoma patients.	10 squamous cell carcinoma of gingiva; 5 non-neoplastic gingival tissues	Paraffin-embedded tissues	Immunohistochemistry	Staining for P. gingivalis was higher in normal gingival tissues and gingival carcinoma (more than 33% in carcinoma samples). The staining intensity was also significantly enhanced in the malignant tissue by 2 folds compared to specimens stained for the non-invasive S. gordonii.
Meurman et al., 2008[39]	To summarize the observations on acetaldehyde production by oral microbiota and its suggested role in alcohol-related carcinogenesis.	Review: Several gastrointestinal microbial species possess the enzyme alcohol dehydrogenase (ADH), responsible for alcohol metabolism in the liver. In oral microbiota, Streptococci viridans and Candida possess ADH. Ethanol can be detected in the mouth hours after consuming alcoholic beverages. ADH-containing microorganisms in the mouth present a risk for carcinogenic acetaldehyde production, with subsequent potential for the development of oral cancer, particularly among heavy drinkers.			
Sasaki et al., 2005[40]	To elucidate the frequency of S. anginosus infection in oral cancer tissues	42 squamous cell carcinoma, two lymphoma, two rhabdomyosarcoma, 3 leukoplakia	Tissue specimens, dental plaque, and saliva samples	Microbial culture, PCR assay, and pulse field gel electrophoresis	S. anginosus DNA was frequently detected in 19 of 42 cases of squamous cell carcinoma and not in other cancer types (lymphoma and rhabdomyosarcoma) or leukoplakia samples. S. anginosus was solely detected in dental plaque and not in the saliva of S. anginosus-positive squamous cell carcinoma cases. The genotype of S. anginosus isolated from cancer tissue was identical to that of dental plaque in the same patients.
Mager et al., 2005[41]	To determine if the salivary counts of 40 common oral bacteria in subjects with an oral squamous cell carcinoma (OSCC) lesion would differ from those found in cancer-free (OSCC-free) controls.	229 OSCC-free and 45 OSCC subjects	Unstimulated saliva samples	Checkerboard DNA-DNA hybridization to evaluate 40 common oral bacteria	Capnocytophaga gingivalis, Prevotella melaninogenica and Streptococcus mitis, were elevated in the saliva of individuals with OSCC
Nagy et al., 1998[42]	To investigate the microbial contents of the biofilms, present on the surfaces of oral squamous cell carcinomas	21 OSCC patients and from contiguous healthy mucosa	Biofilm	Microbial culture using aerobic and anaerobic complete and selective media	The median number of anaerobic and aerobic colony-forming units was significantly higher than for the normal mucosa. The species isolated in increased numbers at tumor sites were Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces, Clostridium (anaerobes), Haemophilus, Enterobacteriaceae and Streptococcus spp. (aerobes).

Figure 5- The differential abundance of the oral microbiota across various taxonomic units in HNSCC and OPMD.

Actinomyces, Rothia, and *Atopobium* (genus) in the normal mucosa was observed in this study. This supports the protective role of actinobacteria, which is well documented in the literature.[33,35,43] Actinobacteria are Gram-positive bacteria with high Guanine and Cytosine content in their genetic makeup. Actinobacteria produce a wide range of natural bioactive compounds and secondary metabolites due to their environmental diversity and metabolic potential. They are diverse groups of microorganisms that are ubiquitous in the aquatic and terrestrial environment. The natural compounds and metabolites from Actinobacteria produce a range of anti-cancer, immunosuppressive, anti-parasitic, and anti-viral agents.[44] Consistent with the observations of this study, Al-Hebshi, et al.[33] (2017) and Schmidt, et al.[35] (2014) report that *Rothia sp*., belonging to the phylum Actinobacteria, is associated with relative abundance in healthy controls and is decreased in OPMDs and OSCC. In contrast with the beneficial role of Actinobacteria, pancreatic head carcinoma is associated with phylum Actinobacteria (*Rothia*, *Actinomyces*, *Corynebacterium*).[35]*Neisseria elongate* has been associated with pancreatic cancer. Karpiński (2019)[45] and Muto, et al.[46] (2000), in an *in vitro* study, reported that *Neisseria* can be a regional source of carcinogenic acetaldehyde and may play an essential role in alcohol-related carcinogenesis in humans. They demonstrated the 100-fold higher ability of *Neisseria* to produce acetaldehyde compared to *Streptococcus sp*., *Stomatococcus sp*., or *Moraxella sp*.

The oral microbiome forms a close symbiotic relationship with human host cells in the oral cavity. The molecular patterns in the host substantially influence the differential expression of microorganisms. A negative correlation was observed between the RICTOR protein and Bacteroidetes. The gene and protein expression of RICTOR was upregulated in HNSCC with poor survival outcomes in patients showing high RICTOR expression. The tumor-promoting role of the RICTOR protein and the probiotic nature of Bacteroidetes correlate with the observations of this study. Rapamycin-insensitive companion of mTOR (RICTOR), a subunit of mTOR, is a critical regulator of the PI3K/AKT pathway and plays an essential role in tumors driven by receptor tyrosine kinase (RTK) alterations. Inhibition of RICTOR protein and blocking RTK co-activation can serve as an independent or combined therapeutic target.[47] RICTOR is a scaffold protein for Integrin-Linked Kinase (ILK), a β1-integrin interacting protein with kinase activity. The mTOR-independent ILK/RICTOR complex was detected in several cancer cell lines and is involved in Transforming Growth Factor (TGF) beta-1-mediated EMT.[48] RICTOR promotes tumor progression regulating the tumoral microenvironment by increasing cell proliferation and survival, decreasing apoptosis in cancer cells, facilitating angiogenesis, and remodeling the stroma. Overexpression of RICTOR is positively associated with tumor progression and poor survival in colorectal cancer, hepatocellular carcinoma (HCC), endometrial carcinoma, pituitary adenoma, and pancreatic ductal adenocarcinoma.[47] Using next-generation sequencing, it was shown that RICTOR up-regulation strengthens mTORC2 activity, promoting cell growth and motility. Conversely, RICTOR down-regulation suppresses cell proliferation and tumor formation.[49] *Bacteroides*, the predominant genus in the phylum Bacteroidetes, comprise Gram-negative bacteria with a rod-shaped morphology. In healthy adults, *Bacteroides* comprise 20-80% of gut microbiota, constituting the predominant flora. *Bacteroides spp*. possess polysaccharide utilization loci that enable them to metabolize polysaccharides that are not easily absorbed in the intestine, providing energy to adjacent bacteria and helping maintain the balance of gut microbiota.[50]

The mRNA sequence of Histone 1 H2b (HIST1H2B) was negatively correlated with Firmicutes. Oncohistones have emerged as a new field in cancer epigenetics research. Oncohistone mutations are clustered mono-allelic missense mutations that often affect only one of the histone genes (human histones are polygenic; all four histones are encoded by at least 15 genes), the expression of which exhibits oncogenic features. Garciaz, et al.[51] (2019), reported that HIST1H1D plays a role in acute myeloblastic leukemia blast cell lineage differentiation. Histone 2 mutations have been reported in pancreatic cancer, glioblastoma, prostate cancer, and lung cancer.[52] Zhao and Dai[53] (2021) reported that HIST3H2A might regulate the progression of tumor immunity in pancreatic cancer by modulating the JAK-STAT pathway.[53]

Similarly, a negative correlation was observed between the mRNA sequence of scaRNA11 and TBC1D21 and Proteobacteria and Bacteroidetes, respectively. Small Cajal body-specific RNAs (scaRNAs) guide post-transcriptional modification of spliceosomal

RNA and have defined roles in tumorigenesis. scaRNA11 is one of the 20 upregulated genes associated with RNA-binding protein HuR in thyroid cancer cells.[54] Tian, et al.[55] (2022) report that TBC1 domain family member 2 (TBC1D2) is overexpressed in ovarian cancer and contributes to tumor metastasis via epithelial cadherin (E-cadherin) degradation. However, the role of scaRNAs and TBC1D21 is not elucidated in HNSCC.

Hassan, et al.[8] (2023) in an *in silico* analysis of DGE in colorectal cancer, identified STAT3 and HNRNPA2B1 as key hub proteins in colorectal cancer (CRC). Raj, et al.[9] (2024) demonstrated negative correlation between c-MET and immune cell infiltration, suggesting c-MET might have a role in immune suppression in the TCGA HNSCC dataset using different *in silico* tools. Abu-Shahba, et al.[10] (2023) reported that patients with high expression of POU2F1 or low expression of PPARA exhibited low survival probability and vice versa ($p \leq 0.05$) in HCC. Gene expression analysis in lung squamous cell carcinoma showed a significant downregulation of DEL-1 and IL-6 and upregulation of CXCL13, suggesting the role of differential expression of these genes in lung carcinogenesis.[11] Computational analysis using the Gene Expression Omnibus (GEO) database (GSE172096 dataset) revealed that transmembrane protein (TMEM2), associated with higher-risk groups in pancreatic adenocarcinoma was strongly correlated with familial adenomatous polyposis (FAP) gene, a cancer-associated fibroblast (CAF) marker.[12]

Firmicutes, also known as Bacillota, are Gram-positive bacteria. Zhang, et al.[56] (2020) and Li, et al.[57] (2021) report an abundance of Firmicutes in oral cancer and healthy controls, similar to the findings of this study. However, Yang, et al.[29] (2018) and Guerrero-Preston, et al.[34] (2016) reported a higher abundance of Firmicutes in the tissue samples and saliva rinses of OSCC patients. The hsa-miRNA-2005p showed a moderate positive correlation with Firmicutes in this study. miRNAs (microRNAs, miRs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. miRNA dysregulation is often reported in carcinogenesis. miR-200 family members act by targeting several mRNAs associated with cancer cell proliferation.[58] miR-200a targets Cyclin-Dependent Kinase 6 (CDK6) in melanoma and thus causes cell-cycle arrest and decreases cancer cell proliferation. A downregulation of miR-200a and miR-125a has been reported in oral cancer patients. It is considered that a decrease in miR-200a promotes the epithelial-mesenchymal transition (EMT) of tumor cells.[59]

Oral microbiota dysbiosis is associated with oral cancer development by various mechanisms. Inflammatory cytokines and matrix metalloproteinases promote development and progression of tumors. The oral bacteria produce oxygen and nitrogen-reactive species, and oncogenic metabolites (e.g., nitrosamines) induce genetic damage to oral mucosal cells. There is an alteration of the epithelial barriers predisposing to OPMDs. Several epigenetic alterations (e.g., alteration of onco-miR or DNA methylation phenomena) are implicated in oral dysbiosis associated with tumorigenesis.[7]

The use of tobacco, with or without areca nut and alcohol use, has a substantial contribution to oral dysbiosis associated with the development of OPMDs and OSCC. A loss of commensal bacteria with a protective role, such as *Neisseria*, and a reduced response to *Porphyromonas gingivalis* is associated with tobacco use. A decrease in *Lactobacilli*, a commensal of the oral mucosa that can break down salivary acetaldehyde (carcinogen) production, is observed in alcohol consumers.[60] Amer, et al.[61,62] (2017, 2020) reported that smokers had reduced levels of *Neisseria sp*., *Fusobacterium nucleatum*, and *Leptotrichia*, and alcohol consumers showed increase in *Campylobacter* species and *Rothia mucilaginosa* with a decrease in acetaldehyde-dehydrogenase-producing *Streptococci*. Dysregulation in the xenobiotic metabolism contributes to higher exposure to this carcinogenic metabolite, which promotes the development of oral leukoplakia and its malignant transformation in alcohol consumers.[63] Areca nut chewers with oral lesions (leukoplakia and submucous fibrosis) had significantly elevated levels of *Oribacterium*, *Actinomyces*, and *Streptococcus*, including *Streptococcus anginosus*.[64]

In healthy conditions, physical and chemical barriers (e.g., gastric and bile acids) segregate the oral cavity from the gastrointestinal tract. In the absence of these barriers, under pathological conditions, the oral microbiota can translocate to the intestines and modulate the gut microbiota, contributing to the development of gastroenterological diseases and cancer. The oral-gut microbiome axis and its involvement in tumorigenesis gained importance in cancer research.[15] Oral microorganisms, such as *Fusobacterium*, *Parvimonas*, and *Peptostreptococcus*,

have been found in the intestines of CRC patients, suggesting the role of the oral-gut microbiome axis in colorectal carcinogenesis. *Porphyromonas gingivalis*, a key pathogen in periodontitis, promoted the proliferation of the infected CRC cells with *F.nucleatum*. The salivary microbiota of HCC patients was enriched with *Haemophilus*, *Porphyromonas* and *Filifactor* species. In mouse models of pancreatic cancer, oral administration of *P. gingivalis* accelerated the progression of pancreatic ductal adenocarcinoma and promoted EMT.[16] In this study, the normal mucosa adjacent to the carcinoma showed an abundance of Firmicutes in HNSCC patients. In contrast, Bacteroidetes and Firmicutes were abundant in the normal mucosa adjacent to GI carcinoma. There was no significant difference in the prevalence of Bacteroidetes, Firmicutes, Proteobacteria, and Tenericutes between the HNSCC and GIT carcinoma, suggesting overlapping microbial signatures in HNSCC and GIT carcinoma.

Computational approaches are valuable tools for researchers as they provide access to comprehensive data collection and facilitate short-term, cost-effective analyses.[65] Inherent to any *in silico* study, it has limitations. The alpha and beta diversities of microbial communities involved in carcinogenesis need further exploration. The heterogeneity in microorganisms among varied geographic locations mandates meta-genomics analysis using next-generation sequencing technologies that can demonstrate bacterial profiles and relationships between microbial diversity, genetic variation, and oral diseases.

Conclusion

Characterization of oral and gastrointestinal microbial dysbiosis might provide new biomarkers useful for diagnosing oral and gastrointestinal carcinomas. While proteomic and genomic biomarkers are subjected to the individual's biological variations, the oral microbiome is relatively conserved among unrelated individuals. A decrease in Actinobacteria and an enrichment of Flavobacteria at the class level, Neisseriales, Pasteurellales, and Campylobacterales at the order level, *Pasteurellaceae*, *Flavobacteriaceae*, *Campylobacteraceae*, and *Peptoniphilaceae* at the family level, and *Hemophilus*, *Porphyromonas*, and *Leptotrichia* at the genus level were observed in HNSCC compared to the normal mucosa. RICTOR protein, mRNA transcripts such as HIST1H2B, SCARNA11, TBC1D21 gene, and hsa-miR-200a-5p miRNA were significantly correlated with the bacterial species predominant in HNSCC. The oral microbiome and its association with host molecular signatures can serve as tumor biomarkers and oncotherapy targets, enabling early diagnosis and treatment of head and neck cancers.

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Conflict of interest

The authors declare no conflict of interest.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available in the SciELO Data repository - https://doi.org/10.48331/scielodata.LEAKQN.

Authors' contributions

Kavitha, Loganathan: Conceptualization (Equal); Data curation (Equal); Formal analysis (Equal); Investigation (Equal); Methodology (Equal); Writing—original draft (Equal); Writing—review & editing (Equal).**Kuzhalmozhi, Manogaran:** Data curation (Equal); Formal analysis (Equal); Writing—review & editing (Equal).**Priyadharsini, Vijayashree Priyadharsini:** Conceptualization (Equal); Formal analysis (Equal); Methodology (Equal); Supervision (Equal); Validation (Equal); Writing—review & editing (Equal). **Kumar, A. Arun:** Data curation (Equal); Formal analysis (Equal); Writing—review & editing (Equal). **Umadevi, Krishna Mohan Rao:** Formal analysis (Equal); Methodology (Equal); Supervision (Equal); Validation (Equal). Writing—review & editing (Equal). **Kannan, Ranganathan:** Conceptualization (Equal); Formal analysis (Equal); Methodology (Equal); Project administration (Equal); Supervision (Equal); Writing—review & editing (Equal).

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