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Review

The oral microbiome and its role in oral squamous cell carcinoma: a systematic review of microbial alterations and potential biomarkers

Angela Crispino[1*], Silvia Varricchio[1*], Aurora Esposito[2], Alessandra Marfella[1], Dora Cerbone[1], Angelica Perna[2], Giulio Petronio Petronio[2], Stefania Staibano[1**], Francesco Merolla[2**], Gennaro Ilardi[1**].

[1] *Department of Advanced Biomedical Sciences, Pathology Unit, University of Naples "Federico II", Naples, Italy;*
[2] *Department of Medicine and Health Sciences "V. Tiberio", University of Molise, Campobasso, Italy*
**These authors equally contributed*
***Co-senior authors*

Summary

Background. Oral squamous cell carcinoma (OSCC) is one of the most common cancers worldwide. Despite advances in diagnosis and treatment, the incidence of OSCC is increasing, and the mortality rate remains high. This systematic review aims to examine the potential association between the composition of the oral microbiota and OSCC.

Materials and methods. This study's protocol was developed according to the PRISMA guidelines. Several search engines, including Medline-PubMed, Scopus (via Elsevier), and Google Scholar, were used to identify original studies that analyzed differences in the oral microbiome between OSCC patients and controls. Twenty-seven studies were identified that reported significant differences in microbial abundance between OSCC and controls.

Results. The systematic review highlights a complex relationship between the oral microbiome and the pathogenesis of OSCC. Significant changes in the microbial composition were identified, with a predominance of phyla such as *Bacteroidetes and Fusobacteria*, which are associated with inflammatory mechanisms facilitating tumor progression. A remarkable variability in microbial profiles emerged based on the different stages of the disease and the types of samples analyzed, demonstrating the complexity of the oral microbial ecosystem.

Conclusion. Although alterations in the oral cavity microbiome composition are evident in patients with OSCC, identifying a specific pattern remains challenging. However, the integration of advanced analytical techniques, such as artificial intelligence, could overcome this problem, allowing the identification of crucial biomarkers and improving the understanding of the role of the microbiome in carcinogenesis. This approach could transform microbiome analysis into a useful tool for screening and monitoring patients with OSCC.

Key words: oral cancer, oral microbiota, OSCC, systematic review

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Correspondence
Francesco Merolla
francesco.merolla@unimol.it

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Introduction

Globally, head and neck cancers rank sixth in terms of malignancy [1]. They are extremely aggressive tumors despite their rarity. As a result, the tumor is thoroughly examined by the scientific community, looking at any factor that might affect its course and be relevant to diagnosis, prognosis, and treatment. The primary issue with HNSCC is its delayed diagnosis, which contributes to low quality of life (QOL), significant impairments, and poor 5-year survival [2,3]. Due to field cancerization, the latter frequently experience recurrence or develop second primary tumors [4].

HNSCC not only puts physical health at risk but also mental health, being the second cancer with the highest risk of suicide. Survivors of this cancer face comorbidities related to survival and lifestyle. It causes disfigurements and functional disorders, including swallowing, speech, and taste, which substantially impact patients' quality of life. Psychological distress and impaired QOL are probably the main factors underlying suicide [5]. Among cancer sites associated with suicide, HNSCC is ranked in the top four [6].

The fight against HNSCC has become increasingly urgent in recent years, as the Global Cancer Observatory (GCO) revealed a worryingly increasing trend. In 2020, the number of reported cases of HNSCC reached 377,713 worldwide, with a significant concentration in Asia [7]. However, future predictions are even more disturbing: the GCO indicates that the incidence of HNSCC could increase by 40% by 2040, resulting in increased mortality rates [7]. These data reflect a growing challenge for the medical and scientific community to find new diagnostic and therapeutic strategies to address this serious public health threat.

A crucial aspect emerges more clearly in the panorama of oncology research: the decisive role of the oral microbiota in carcinogenesis and the development of different forms of cancer. This awareness has roots in the last two decades, during which the scientific community has identified and analyzed the distinctive characteristics of cancer, "The Hallmarks of Cancer", outlining fundamental traits that guide its development [8,9]. In parallel, oral microbiota studies have revealed a close correlation between oral microbiota-mediated carcinogenesis, which satisfies or induces most hallmarks [10]. Interestingly, these tumors are not limited to the oral cavity alone, but oral microbiota-associated primary tumors have been observed in the esophagus, stomach, pancreas, and colon/rectum [11].

The oral cavity hosts a huge collection of microbes, including bacteria, fungi, viruses, and bacteriophages, and is considered one of the largest microbiological reservoirs in the human body [12]. While the microbiome provides an important homeostatic mechanism, the alteration of its balance, or dysbiosis, compromised by factors such as tobacco smoking, psychological stress, poor dietary habits, and chronic periodontitis, promotes carcinogenesis through several mechanisms, including damage to host DNA and chronic inflammation [13].

Studies suggest that alterations in the microbiota may also contribute to the development of tumors, including HNSCC. In particular *P. gingivalis* and *F. nucleatum* have both been shown to be able to induce the production of inflammatory cytokines (IL-1, IL-6, IL-17, IL-23, TNF-α and MMP-8, -9, -13), as well as cell proliferation and cellular invasion, in OSCC with various mechanisms [14,15,16,17] Further studies conducted on OSCC have demonstrated that microbes other than bacteria present in the microbiota can contribute to the development of cancer. In the oral cavity, persistent fungal infections (mainly *Candida spp.*) and human papillomavirus (HPV) infection and virus infection (Epstein-Barr EBV) may be involved in the formation of oncogenic mutations, leading to the development of carcinoma [18]. Despite the various evidence present in the literature, the relationship between oral microbiome and OSCC remains complex and not fully explained. The lack of standardized analytical protocols makes establishing specific microbial models for OSCC difficult, limiting the early identification of oral cancer. Thus, this study aims to conduct an up-to-date systematic review of available oral microbiome composition studies in OSCC, identify current news of correlation with carcinoma, and outline directions for future research. In particular, the effect of the presence or absence of key bacteria and fungi such as *Candida* on carcinoma development will be compared, while outlining future research directions better to understand these interactions and their potential therapeutic implications.

Materials and methods

Search strategy

A systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria [19]. We conducted a comprehensive search of the following databases: Medline-PubMed and Scopus (via Elsevier), to find original full-text research publications published between 2019 and 2024. Boolean operators (AND, OR, NOT) and different combinations of phrases (Tab. I) were used in the search according to the specifications of each database. In addition, the reference lists of the included studies were manually searched for further relevant publications. The search was completed in September 2024.

Eligibility criteria

Table I. The search strategy used.

Query:	((microbiota) AND (oral cavity)); ((microbiome)AND (oral squamous cell carcinoma OR OSCC)); ((oral microflora OR oral microbes OR oral bacteria OR oral microorganism) AND (OSCC));((oral pathogen) AND (OSCC)); ((oral dysbiosis AND (OSCC OR oral squamous cell carcinoma)); ((dysbiosis) AND (Inflammation) AND (OSCC OR oral squamous Cell Carcinoma)).

For our review, we selected studies published in English as inclusion criteria to investigate the relationship between oral squamous cell carcinoma (OSCC) and the oral microbiome. Only clinical studies conducted in humans, in which the microbiological content of all samples was assessed, were considered. In addition, studies that used healthy tissue samples from the same OSCC patient as a control group were accepted for inclusion. Exclusion criteria included articles presented at conferences or congresses, systematic reviews, and meeting abstracts, as well as studies based on experiments conducted on animals, research focused on tumors other than OSCC, and in vitro studies. The study design was performed according to the PECO (Population, Exposure, Control, and Outcome) method, in which:

- **P (population):** represents patients with OSCC.
- **E (exposure):** indicates alterations in the composition of the microbiota in the oral cavity.
- **C (control):** includes both healthy tissue samples from the same patient with OSCC and healthy tissue samples from individuals without pathology.
- **O (result):** concerns the microbial diversity and relative abundance of various oral bacteria, including *Candida spp.*, which could be considered a risk factor for OSCC.

Study selection and data collection process

Once the eligibility criteria had been established, two reviewers proceeded with the independent selection of articles. To improve the efficiency of data collection, Rayyan software [20] was used, a tool designed to facilitate systematic reviews by removing duplicates and allowing a rapid initial assessment. All articles identified through the search strategy were collated in a central Rayyan database. The reviewers conducted a preliminary examination of the titles and abstracts to identify potentially relevant papers, excluding those not meeting the inclusion criteria. Subsequently, the full version of the selected articles was examined to assess their final eligibility. Each reviewer independently evaluated each article.

Study risk of bias assessment

The quality of the included studies was assessed using the Newcastle-Ottawa Scale (NOS) [21]. The NOS is a scoring system that assesses eight elements grouped into three categories: selection of participants (maximum 4 points), comparability of groups (maximum 2 points), and ascertainment of the outcome (for cohort studies) or exposure (for case-control studies) of interest (maximum 3 points). Two reviewers independently assessed the risk of bias, and disagreements were resolved by discussion with a third reviewer. Studies with a score above 7 were considered good.

Data Analysis

Data were collected from selected studies and recorded using a pre-designed Excel sheet for extraction. The following parameters were noted:

- Study characteristics: publication year, first author name, name of the country where the study was conducted.
- Study population characteristics: number of cases and controls, age and sex of the study population.
- Methodologies used: type of samples, sample collection methods, DNA extraction methods, DNA amplification, sequencing platforms, and database.
- Results: microbial abundance, genera, species and phyla detected.

The collected data provided an overall picture of the correlations between the oral microbiome and OSCC.

Results

Study selection

The initial search strategy identified a total of 4,025 studies. We identified and removed 3,017 duplicate studies using Rayyan. After this process, 1.008 articles were assessed through title and abstract analysis. Following this screening phase, 236 articles were excluded because they did not meet the inclusion criteria. Subsequently, 772 studies were examined in detail by applying the eligibility criteria. Of these, 745 studies were excluded for the following reasons: non-English language (n = 46), conference or congress abstracts and systematic reviews (n = 434), in vitro experiments (n = 132), and animal studies (n = 133). Qualitative analysis was mainly conducted through descriptive analysis using Microsoft Excel. Finally, 27 studies were subjected to a qualitative risk of bias assessment; of these, 27 passed the analysis and were included in our review. The PRISMA flowchart in Figure 1 illustrates the detailed study selection process.

Study characteristics

The characteristics of the selected studies are detailed in Table II. Overall, the studies came from various countries, with significant representation from the United States, China, and India. Of the 27 included studies, 3 were from the United States of America (USA) [22,23,24] ; 10 from China [25,26,27,29,30,31,32,33,34,35,36] ; six from India [29,37,38,39,40]; 1 from Poland [41]; 1 from Colombia [42]; 1 from Spain [43]; 1 from Turkey [44]; 2 from Finland [45,46]; one from Egypt [47] and 1 from Japan [48].

The number of participants varied significantly from study to study, with samples ranging from a minimum of 10 to a maximum of 228 subjects. The mean ag-

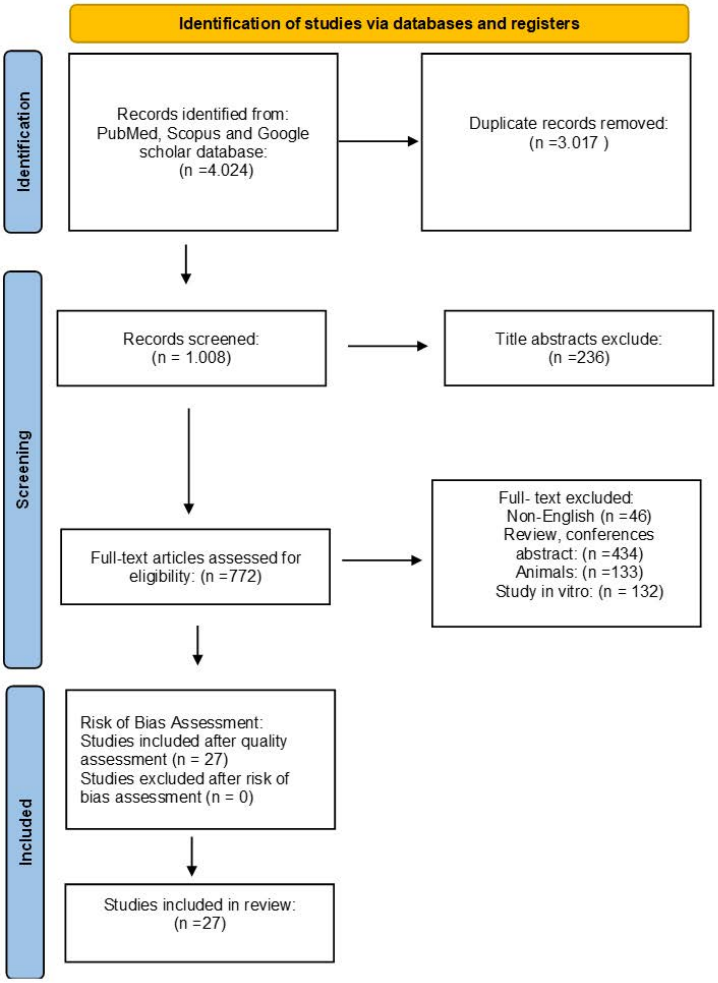

Figure 1. The flowchart summarizes the selection process for the articles included.

Table II. Analysis of case characteristics.

Author, year	Country	Sample Size	Group	Average/Range of Age (Years)	Sex
Chang et al., 2019 [35]	China	OSCC HC	61 30	57.4 ± 10.4 55.4 ± 10.2	22 F 39M 12 F 18M
Ganly et al., 2019 [22]	USA	OSCC PML HC	18 8 12	59.8±10.9 66.1±17.9 44.4±15.6	7F 11M 5F 3M 9F 3M
Su et al., 2021 [25]	China	OSCC Cancers of the buccal mucosa	74 42	53.96±11.07 56.31±10.05	Only males n.d
Sarkar et al., 2021 [37]	India	OSCC	50	52.6	n.d.
Torralba et al., 2021 [41]	Poland	OSCC	18	n.d.	n.d.
Yang et al., 2021 [26]	China	OSCC	23 19	61.9 ± 12.3 62.7 ± 12.1	12 F 11M 9F 10M
Ye et al., 2021 [27]	China	OSCC	23	63.0 ± 9.6	5 F 18 M
Rai et al., 2020 [29]	India	OSCC HC	25 24	55.32 50.375	8F 16M 9F 16M

Table II. Follows from the previous page.

Author, year	Country	Sample Size	Group	Average/Range of Age (Years)	Sex
Zhou et al., 2021 [36]	China	OSCC HC	47 46	n.d.	n.d.
Zhong et al., 2021 [30]	China	OSCC OSF HC Healthy areca chewer	45 42 46 29	49.62 ± 9.28 32.62 ± 9.22 30.58 ± 10.34 26.16 ± 6.53	2F 43M 2F 40M 33F 12M 2F 27M
Erira et al., 2021 [42]	Colombia	OSCC HC	10 20	56.5 ± 15.59 56.4 ± 18.36	90% Female
Saxena et al., 2022 [23]	USA	OSCC HC	34 32	48.61 ± 12.76 32.15 ± 9.19	n.d.
Zeng et al., 2022 [31]	China	OSCC	228	52.4	n.d.
Heng et al., 2022 [32]	China	OSCC OPL HC	29 32 30	61.97±10.11 56.00±13.67 56.63±11.12	13F 16M 19F 13M 15F 15M
Liu et al., 2022 [33]	China	OSCC HC	40 10	59.25 ± 16.6 60.3 ± 8.4	13F 27M 3F 7M
Hashimoto et al., 2022 [48]	Japan	OSCC Postoperative OLK	41 20 25	67.7(28–92) 68.2 (29–85) 64.4 (29–91)	111F 30M 6F 14M 9F 16M
Oyeyemi et al., 2023 [38]	India	OSCC TA (tabacco abuse) HC	10 10 10	55.30 34.30 23.60	n.d.
Mäkinen et al., 2023 [45]	Finland	OSCC HC	99 101	68.0 (10.3) 66.4 (14.3)	n.d.
Lan et al., 2023 [34]	China	OSCC OLK HC	18 21 21	59.24 ± 12.30 54.11 ± 15.43 48.81 ± 12.38	10F 8M 17F 4M 14F 7M
Jain et al., 2023 [24]	USA	OSCC	20	n.d.	n.d.
Herreros-Pomares et al., 2023 [43]	Spain	OSCC OSCC-PVL HL PVL HC	10 8 9 12 11	72.80 ± 9.05 80.25 ± 12.42 66.33 ± 8.12 66.83 ± 12.16 67.91 ± 12.00	4F 6M 2F 6M 5F 4M 9F 3M 6F 5M
Singh et al., 2023 [51]	India	OSCC Pre-cancer HC	69 15 11	48 ± 12	24F 71M
Hafed et al., 2019 [47]	Egypt	OSCC without LNM OSCC with LNM Oral dysplasia HC	16 15 16 7	53,44 54,87 46,56 44,43	6 F 9 M 7 F 9 M 6 F 10 M 3 F 4 M
Sankari. et al., 2020 [40]	India	OSCC OPMD HC	97 200 200	n.d.	n.d.
Arya et al., 2021 [39]	India	OSCC OPMD HC	20 20 20	54 45 n.d.	3 F 17M 6 F 14 M n.d.
İlhan **et al., 2023 [44]**	Turkey	Benign Mild/moderate dysplasia Carcinoma in-situ OSCC	20 20 20 20	46.45 ± 8.16 58.7 ± 6.90 69.05 ± 5.02 61.07 ± 11.54	11 F 9 M 11 F 9 M 6 F 14M 8 F 12M
Rusanen et al; 2024 [46]	Finland	OSCC HC	30 26	64 (31–85) 30.8 (18–56)	12 F 18M 17 F 9M

*HC = Healthy control; AP = Adjacent paracancerous tissues; F = Female; M = Male; OPMD = Potentially malignant disorders; LNM = Lymph node metastasis; OLK = oral leukoplakia.

es of participants were recorded in different ranges, highlighting the prevalence of OSCC in an older age group, with values ranging from 26 to over 80 years. The studies show a predominance of male subjects, even if there is significant female participation. In addition to OSCC patients, 18 studies included healthy control groups (HC) [26,29,31,36,37,38,40,41,42,44,45,46,47,48,50,51,54,55]. In comparison, 6 studies used normal tissue adjacent to the tumor to compare the microbiota composition of tumor and non-tumor areas of the same patient [24,31,36,37,41,51]. Six studies analyzed samples from premalignant or potentially malignant lesions, such as oral leukoplakia (OLK), oral premalignant lesions (OPMD), and dysplasia. Other comparison groups included patients with buccal mucosal cancer or other head and neck cancer subtypes (2 studies), areca chewers without OSCC (1 study), and tobacco users (1 study).

DNA extraction tools and sequencing strategies in microbiomes

The samples ranged from tumor tissues to saliva, oral rinses, bacterial plaque, and oral swabs. As described in Table III, 10 articles use saliva as the main matrix for microbiome analysis, while tumor tissue and adjacent tissue samples are present in 13 studies. In addition, 5 articles use oral swabs for sample collection. Only a few studies used combinations of multiple sample types, such as tumor tissues and saliva or bacterial plaque and saliva, highlighting various approaches in oral microbiome analysis.

Different methods were used for DNA extraction in

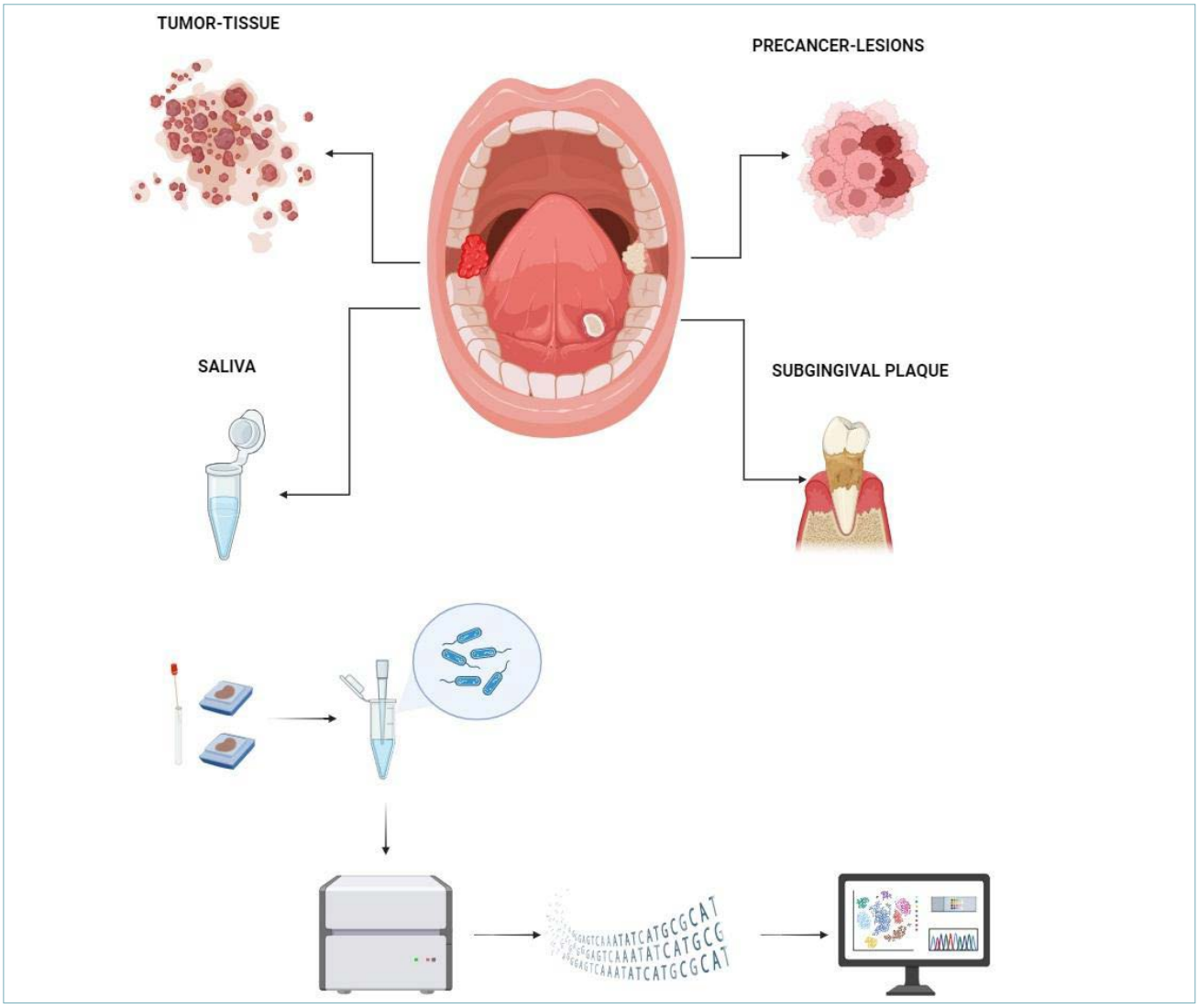

Figure 2. Summary image of typically used samples and workflow to identify the microbiome in OSCC (Created with BioRender.com).

Table III. A summary of DNA extraction, amplification, sequencing techniques, and reference databases.

Author, year	Sample Type	Extraction DNA	DNA Amplification	Amplification Sequencing	Reference database
Chang et al., 2019 [35]	Cancer tissues AP tissue Subgingival plaque Normal oral tissues	QIAampFast DNA Stool Mini Ki	V3-V4	Illumina MiSeq	NCBI
Ganly et al., 2019 [22]	Oral rinse samples between patients and normal people as a control	Modified QIAGEN DNA Extraction Method	V3-V4	454 FLX platform	Greengenes (v13.5)
Su et al., 2021 [25]	Oral swab	QIAamp® DNA Blood Mini Kit	V4	Illumina MiSeq	SILVA
Sarkar et al., 2021 [37]	Tissue cancerous lesions Adjoining normal area	DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany)	V3-V4	Illumina MiSeq	Greengenes (v13.8) RDP
Torralba et al., 2021 [41]	Paired tumor and adjacent normal tissue samples	Phenol-chloroform based DNA extraction	V4	Illumina MiSeq	HOMD
Yang et al., 2021 [26]	Tissue Saliva	TIANamp Swab DNA Kit	V3-V4	Illumina MiSeq	HOMD
Ye et al., 2021 [27]	Tissue cancer lesions Paracancerous normal tissues	DNeasy PowerSoil Kit (Qiagen, Hilden, Germany)	V3-V4	Illumina MiSeq	SILVA
Rai et al., 2020 [29]	Saliva	Qiagen DNeasy Blood and Tissue Kit	V3-V4	Illumina MiSeq	Greengenes (v13.8)
Zhou et al., 2021 [36]	Saliva, tumor tissue, Plaque, control side of healthy mucosa	E.Z.N.A.® soil DNA Kit	V3-V4	Illumina MiSeq	SILVA
Zhong et al., 2021 [30]	non-stimulating oral swabs	QIAamp® DNA Stool Mini Kit (Qiagen)	V3–V4	Illumina MiSeq	n.d.
Erira et al., 2021 [42]	Tumor tissues, Saliva Bacterial plaque Saliva Bacterial plaque	DNA Master Pure TM kit (EpicentreBiotechnologies® Madison, WI)	n.d.	Illumina MiSeq	n.d.
Saxena et al., 2022 [23]	Swab samples Saliva	Qiagen DNeasy Blood and Tissue Kit	V3	Illumina Nextera XT	Greengenes (v13.5) SILVA
Zeng et al., 2022 [31]	Saliva, Swab, Adjacent Normal Tissues, Tumor tissue, Lymph Nodes	FastDNA SPIN Kit	V4	HiSeq 2500 (Illumina Inc).	SILVA
Heng et al., 2022 [32]	Saliva, plaque swabs, buccal swabs	HiPure tissue and blood DNA kit (Magen Biotechnology Co. Ltd., Guangzhou, China).	V3-V4	Illumina NovaSeq 6000 platform	SILVA
Liu et al., 2022 [33]	Swab samples	Phenol-chloroform based DNA extraction	n.d.	Illumina NovaSeq 6000 platform	NCBI
Hashimoto et al., 2022 [48]	Saliva	Oragene® DNA kit	V4	Illumina MiSeq	HOMD
Oyeyemi et al., 2023 [38]	Saliva	Qiagen DNeasy Blood & Tissue Kit)	V3–V4	Illumina MiSeq	Greengenes (v13.8)
Mäkinen et al., 2023 [45]	Saliva	MagMini DNA Isolation Kit	V4	Illumina MiSeq	HOMD
Lan et al., 2023 [34]	Saliva	Extraction method with cetyltrimethylammonium bromide (CTAB)	n.d.	NovaSeq platform	n.d.

Table III. Follows from the previous page.

Author, year	Sample Type	Extraction DNA	DNA Amplification	Amplification Sequencing	Reference database
Jain et al., 2023 [24]	Tissue Tumor, adjacent normal tissue	AllPrep DNA/RNA Micro kit (Qiagen)	nd	Illumina, DNBSEQ-T7	SILVA
Herreros-Pomares et al., 2023 [43]	Tissue	DNAeasy kit (QiaGen, Barcelona, Spain)	V3–V4	Illumina MiSeq	SILVA
Singh et al., 2023 [51]	Peripheral blood,Tumor tissues,Adjacent normal tissues (AT),Precancerous lesion tissues	DNeasy power soil kit (Qiagen, USA)	V3–V4	Illumina HiSeq 2500 i	Greengenes (v13.8)

*HOMD: Human Oral Microbiome Database, NCBI: National Center for Biotechnology Information, NA: not available, SILVA: Silva ribosomal RNA Gene Database Project.

the various studies in this review, listed in Table IV. The most commonly used commercial kits were the DNeasy Blood and Tissue Kit (Qiagen), which was used in 4 studies [23,29,37,38] and the DNeasy PowerSoil Kit (Qiagen), which was adopted in 2 studies [27,51]. Other kits that were used include the QIAampFast DNA Stool Mini Kit [30], the Qiamp DNA Blood Mini Kit (Qiagen) [25], the modified QIAGEN DNA Extraction Method [22], and the TIANamp Swab DNA Kit [26]. Other approaches for DNA extraction include the use of the E.Z.N.A.® soil DNA Kit 36, the FastDNA SPIN Kit [31], the HiPure tissue and blood DNA kit [32], the AllPrep DNA/RNA Mini Kit [24] and the DNA Master Pure TM kit (Epicentre Biotechnologies® Madison, WI) [42]. Other approaches for DNA extraction include the use of the MagMini DNA Isolation Kit [51], the Oragene® DNA kit [48], and the DNAeasy kit (Qiagen, Barcelona, Spain) [43]. Furthermore, the use of a cetyltrimethylammonium bromide (CTAB) extraction method was reported by Lan et al. (2023) [34]. Finally, it is essential to note that extraction by the traditional phenol-chloroform method was adopted in 2 studies [33,41].

In these studies, DNA amplification was performed by targeting different hypervariable regions of bacterial 16S rRNA genes. In most studies, sequencing of the V3-V4 hypervariable regions of 16S rRNA genes was performed. Five studies used only the V4 region [25,31,41,45,48] and only one study used only the V3 region [23]. After the amplification step, the sequencing step was performed using different platforms. Although the Illumina MiSeq system is the predominant choice, less common techniques were also employed, such as 454 pyroxeqing, used by Ganly et al. (2019) [22]. Other platforms include the Illumina NovaSeq 6000 and the HiSeq 2500, used by Heng et al. (2022) [32] and Singh et al. (2023) [51], respectively. Furthermore, the DNBSEQ-T7 system was adopted in some research, as highlighted by Jain et al. (2023) [24].

Multiple reference databases were employed for sequencing alignment, including Greengenes [22,29,37,38,51], SILVA [32,33,35,40,48,54], Human Oral Microbiome Database (HOMD) [26,41,45,48], NCBI [33,35], and Ribosomal Database Project (RDP) [37].In addition, the functional composition of the metagenomes was inferred from 16S rRNA data using PICRUSt in 5 studies [10,23,27,29,31], and with PICRUSt2 software in 3 studies [22,26,32].

Assessment of risk of bias in included studies

We used the Newcastle-Ottawa Scale (NOS) to assess the methodological quality of all included studies. Table IV summarizes all the scores assigned to each of the studies. Case selection was generally well-defined in almost all studies, scoring 1 for most articles. Furthermore, case representativeness was considered good in most studies, scoring 1. Regarding comparability, most studies matched comparison groups for at least one confounder, such as age or sex, resulting in a score of 1. Studies with the highest scores on the NOS scale (8 or 9 points), such as Ganly et al. (2019) [22] and Ye et al. (2021) [27], demonstrated transparent and replicable 'exposure' with well-defined methods and well-documented matching for multiple confounders. Finally, we only considered studies with scores higher than 7 for our analysis. This choice was made to ensure the inclusion of studies with robust methodology and to reduce the risk of bias, thus improving the reliability and validity of our results.

Microbiota diversity

Microbial Diversity and Abundance

Alpha-diversity and beta-diversity were not evaluated in 14 of the 27 studies (Tab. V). Alpha diversity measures, which describe the variety of species within a single sample, were analyzed using various indices,

Table IV. Newcastle-Ottawa scale showing the quality evaluation of studies included.

Author, year	Selection			Comparability		Exposure			Total
	Definition of cases	Representativeness of the cases	Selection of Controls	Definition of Controls	Comparability of cases and controls	Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-Response rate	
Chang et al., 2019 [35]	1	1	1	1	1	1	1	0	7
Ganly et al., 2019 [22]	1	1	1	1	2	1	1	0	8
Hafed et al., 2019 [47]	1	1	1	1	1	1	1	0	7
Sankari et al., 2020 [40]	1	1	1	1	1	1	1	1	8
Su et al., 2021 [25]	1	1	1	1	1	1	1	1	8
Sarkar et al., 2021 [37]	1	1	1	1	1	1	1	0	7
Torralba et al., 2021 [41]	1	1	1	1	1	1	1	1	8
Yang et al., 2021 [26]	1	1	1	1	1	1	1	1	8
Ye et al., 2021 [27]	1	1	1	1	2	1	1	1	9
Rai et al., 2020 [29]	1	1	1	1	1	1	1	1	8
Zhou et al., 2021 [36]	1	1	1	1	1	1	1	0	8
Zhong et al., 2021 [30]	1	1	1	1	2	1	1	1	9
Erira et al., 2021 [42]	1	1	1	1	1	1	1	0	7
Arya et al., 2021 [39]	1	1	1	1	1	1	1	0	7
Saxena et al., 2022 [23]	1	1	1	1	2	1	1	1	9
Zeng et al., 2022 [31]	1	1	1	1	2	1	0	1	8
Heng et al., 2022 [32]	1	1	1	1	1	1	1	0	8
Liu et al., 2022 [33]	1	1	1	1	1	1	1	1	9
Hashimoto et al., 2022 [48]	1	1	1	1	1	1	1	1	8
Oyeyemi et al., 2023 [38]	1	1	1	1	2	1	1	0	8
Mäkinen et al., 2023 [45]	1	1	1	1	1	1	1	1	8
Lan et al., 2023 [34]	1	1	1	1	1	1	1	1	8
Jain et al., 2023 [24]	1	1	1	1	1	1	1	0	7
Herreros-Pomares et al., 2023 [43]	1	1	1	1	1	1	1	0	7
Singh et al., 2023 [51]	1	1	1	1	1	1	1	1	8
Jain et al., 2023 [24]	1	1	1	1	1	1	1	0	7
İlhan B. et al., 2023 [44]	1	1	1	1	1	1	1	1	8
Rusanen et al., 2024 [46]	1	1	1	1	1	1	1	1	8

including Shannon, Simpson, and Chao1. In contrast, beta diversity, which measures the difference between bacterial communities of distinct samples, was calculated mainly via UniFrac and Bray-Curtis distances and visualized through principal coordinate analysis (PCoA). In particular, two investigations that just looked at alpha diversity were those by Heng et al. (2022) [32] and Ye et al. (2021) [27]. According to Shannon and Simpson indices, Ye et al. (2021) [27] found that tumor samples had a much higher alpha diversity than controls. However, no significant differences were detected with the Chao1 index or the observed OTUs. Heng et al. (2022) [32] determined the differences in alpha-diversity indices using the Wilcoxon test.

In the remaining 11 studies included in the review, both alpha and beta diversity of the oral microbiome were assessed. In the work of Ganly et al. (2019) [22], no significant difference in alpha diversity was observed

Table V. Alpha- and beta-diversity of oral microbiome in OSCC patients compared with healthy controls.

Study	Indices of α–Diversity	Microbiota α-Diversity	Indices of β-Diversity	Microbiota β-Diversity
Ganly et al. 2019 [22]	Shannon index, Chao1 index, ANOVA	No differences were observed	UniFrac analysis(weighted and unweighted analyses)	Significant difference
Rai et al. 2020 [29]	Shannon index, Simpson index,Chao1 index	No differences were observed	UniFrac analysis (weighted and unweighted analyses), PCoA	Significant difference
Sarkar et al. 2021 [37]	Shannon index, Simpson index,Chao1 index	Lower diversity in OSCC	UniFrac analysis(weighted and unweighted analyses), analysis (PCoA) was performed using Bray-Curtis dissimilarity, PERMANOVA	Significant difference
Ye et al. 2021 [27]	Shannon index, Simpson index, Chao1 index, number of observed OTUs	Significant increase in tumor samples (Shannon and Simpson); no difference with Chao1 and OTU	N.D.	N.D
Zhong et al. 2021 [30]	Shannon index, Simpson index, number of observed OTUs	Lower diversity in OSF	UniFrac analysis(weighted and unweighted analyses)	No differences were observed OSCC vs normal mucosa
Saxena et al. 2022 [23]	Shannon index, Simpson index,Chao1 index,	Lower diversity in OSCC	UniFrac analysis(weighted and unweighted analyses),analysis (PCoA) was performed, using Bray-Curtis dissimilarity,PERMANOVA	Significant difference
Zeng et al. 2022 [31]	Shannon index	Lower diversity in OSCC	Bray-Curtis dissimilarity	No differences were observed
Oyeyemi et al. 2023 [38]	Shannon index, Simpson index, Chao1 index, Fisher	No differences were observed	Analysis (PCoA) was performed using Bray-Curtis dissimilarity	No differences were observed
Heng et al., 2022 [32]	Test of Wilcoxon.	No differences were observed	n.d	n.d
Herreros-Pomares et al. 2023 [43]	Shannon index, Simpson index,Chao1 index,Inverse Simpson	Lower diversity in OSCC	n.d.	Greater homogeneity between OSCC and PVL-OSCC
Makinen et al. 2023 [45]	Shannon index,Wilcoxon signed-rank test	Lower diversity follow up vs basal	PCA, permutational analysis of variance, Bray–Curtis distance,PERMANOVA	Significant difference
Lan et al., 2023 [34]	Shannon index, Simpson index, Chao1 index,ACE index,Wilcox test	Significant differences between healthy and precancerous lesions	PCA, Bray-Curtis distance, PERMANOVA	Significant difference
Singh et al. 2023 [51]	Shannon index, Chao1 index	Higher in precancerous samples	PCA based on the Bray-Curtis dissimilarity distance, UniFrac analysis	Significant change between pre-cancer and cancer

ACE: abundance-based coverage estimator; OTUs: operational taxonomic units; PCoA: principal component analysis.

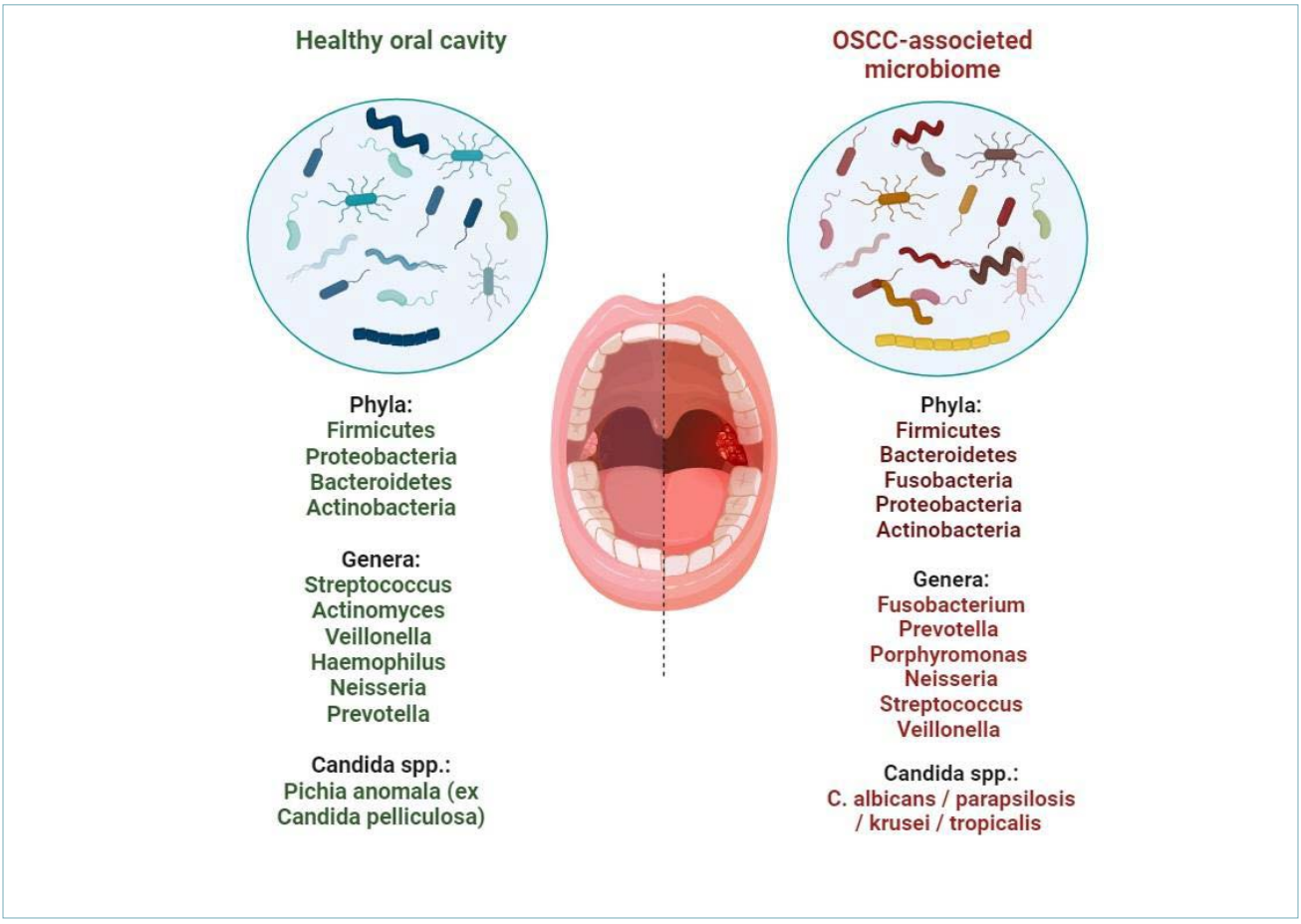

Figure 3. Healthy oral cavity and OSCC in bacteriome results.

between OSCC, pre-malignant lesions (PML), and control groups, while significant separation in beta diversity was observed, suggesting that oral microbiome composition varies substantially by disease. Similarly, Rai et al. (2020) [29] found no significant difference in alpha diversity between OSCC cases and controls. Notwithstanding, a beta diversity analysis revealed a significant separation of the groups, highlighting a change in microbial composition associated with OSCC. In contrast, Sarkar et al. (2021) [37] observed a reduction in alpha diversity in OSCC samples compared to controls, with significant differences in indices such as Chao1, Shannon and Simpson, confirming an alteration in microbial composition in cancerous lesions. Beta diversity analysis also revealed significant differences between groups, suggesting a correlation between the oral microbiome and OSCC. Makinen et al. (2023) [45] found a significant reduction in diversity in patients at follow-up compared to baseline, using the Shannon index, Bray-Curtis distance. Zhong et al. (2021) [30] noted a reduction in alpha diversity in OSF (oral submucosal fibrosis) samples. However, they did not observe significant differences between normal oral mucosa and OSCC lesions, indicating that external factors, such as alcohol or tobacco consumption, may influence microbial diversity.

Studies such as Saxena et al. (2022) [23] found reduced alpha diversity in OSCC samples, particularly concerning the Chao1 index, with a clear separation in beta diversity analyses between the OSCC and control samples. In contrast, Zeng et al. (2022) [31] observed a significant reduction in alpha diversity in OSCC samples. Nonetheless, they did not detect significant differences in beta diversity, suggesting that the oral microbiome composition may not undergo obvious changes at the beta level despite the reduction in microbial richness. However, Oyeyemi et al. (2023) [38] did not identify any significant differences in alpha and beta diversity between the groups, indicating that oral microbiome diversity may remain unaltered in certain

instances, even in oral disease. Herreros-Pomares et al. (2023) [43] showed a reduction in microbial richness in OSCC patients. Still, beta diversity analysis revealed greater homogeneity in the microbiomes of OSCC and PVL-OSCC patients, indicating less variability between individuals. The Lan et al.(2023) [34] study showed significant differences in alpha diversity between healthy control and precancerous groups, with a clear separation in the PCoA plots of beta diversity, suggesting a change in microbiome composition during disease progression, but with minimal effects of external factors such as smoking and alcohol. Finally, Singh et al. (2023) [51] observed greater richness in pre-cancer samples compared to patients with advanced stage (T4) cancer and less microbial variability in stage T4 samples, with a significant shift in microbial composition between pre-cancer and cancer microbiomes but no differences between adjacent tumor tissue samples and actual tumors. These findings illustrate the variability in methodology and conclusions between studies, emphasizing the necessity for further research to understand better the complex interactions between the oral microbiome and oral disease.

The main data regarding microbial abundance are described in detail in Table VI. Supplementary I (Table VI).

Bacteriome characterization

Taxonomical level

Most of the studies concentrated on the most abundant 5 phyla in the oral cavity: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Prevotella.*

Bacteroidetes emerged as a key phylum, with thirteen of the 27 studies identifying its predominance in cancer lesions [23,24,25,26,27,29,31,32,33,35,37,38,43,48]. Studies such as those by Erira et al. (2021) [42] confirmed these findings using next-generation sequencing on dental plaque, reporting high levels of Bacteroidetes in cancerous tissues. Chang et al. (2019) and Heng et al. (2022) similarly observed elevated levels of Bacteroidetes not only in OSCC lesions, but also in precancerous lesions, indicating that this phylum may be involved in early carcinogenesis. Zhong et al. (2021) [30] also observed an increase in Bacteroidetes in areca consumers, suggesting that this phylum could promote inflammation and tumor progression. Notably, Hashimoto et al. (2022) found elevated levels of Bacteroidetes in the saliva of OSCC patients, indicating a potential role in the disease's diagnostic specificity and progression [48]. Conversely, Zhou et al. (2021) [36] highlighted reduced levels of Bacteroidetes in tumor lesions.

Fusobacteria was frequently associated with tumor samples, with 10 studies reporting significant enrichment compared to healthy controls. Its presence was strongly linked to dysbiosis and inflammation, both of which are factors in OSCC progression [23,25,26,29,31,33,36,37,38,48].

Similarly, Firmicutes was identified as abundant in cancerous lesions by 7 studies [24,27,29,31,37,38,41].

This phylum has been correlated with both inflammatory states [36] and areca nut consumption [30], potentially exacerbating existing inflammation and fostering neoplastic progression. For example, Singh et al. (2023) [51] observed an abundance of Firmicutes of 40.90% in precancerous lesions and 28.38% in cancerous samples.

In contrast, Heng et al. (2022) [32] noted a reduction in Firmicutes in OSCC patients, with percentages of 25% in buccal swab and plaque samples and 19% in saliva, compared to healthy controls (46% in buccal swab and 42% in saliva) and leukoplakia lesions (32% in buccal swab and plaque).

Proteobacteria was less frequently mentioned but still appeared relevant, with 4 studies reporting its abundance in OSCC samples [33,34,37,43]. Notably, Yang et al. (2021) [26] observed a high abundance in salivary samples from OSCC patients. Singh et al. (2023) [51] reported that this phylum was abundant in both precancerous (22.09%) and cancerous samples (29.39%). In contrast, Herreros-Pomares et al. (2023) [43] found low levels of Proteobacteria in both OSCC and proliferative verrucous leukoplakia (PVL)-associated samples. Lastly, Actinobacteria was highlighted in both precancerous and cancerous samples. Lan et al. (2023) [34] and Singh et al. (2023) [51] observed the enrichment of Actinobacteria in patients with precancerous lesions, suggesting a potential role in malignant transformation. Jain et al. (2023) [24] and Rai et al. (2020) [29] found it high in the presence of the cancerous lesion OSCC. However, Heng et al. (2022) [32] reported a significant reduction of Actinobacteria in saliva samples from OSCC patients compared to healthy controls. Liu et al. (2022) [33] found that it was markedly reduced in patients with deep invasion. Saxena et al.(2022) [23] found an increased abundance of Actinobacteria in the contralateral healthy sites.

In several studies, Fusobacterium has emerged as one of the most abundant genera in tumor samples. Eleven out of 27 studies found that *Fusobacterium* is significantly more abundant in tumor tissues than in normal tissues [22,23,25,26,29,30,31,33,36,43,48]. Similarly, Torralba et al. (2021) [41], using a Random Forest analysis, identified *Fusobacterium* as a predictive genus of virulence factors associated with inflammatory processes and immunoevasion mechanisms. Like *Fu-*

sobacterium, Prevotella is also frequently reported to be prevalent in tumor samples. Five out of 27 studies found an increase in *Prevotella* in cancerous lesions compared to healthy controls [22,25,29,31,37] associated *Prevotella* with inflammatory processes. Torralba et al. (2021) [41] highlighted its connection to growth factors and inflammatory metabolites, suggesting its potential role in promoting tumor progression. *Porphyromonas* is another genus frequently found in tumor samples. Zeng et al. (2022) [31] and Rai et al. (2020) [29] identified it as abundant in tumor tissues, highlighting its potential involvement in the pathogenesis of oral cancer. On the other hand, it is interesting to note that nine of the analyzed studies reported *Streptococcus* spp. release in carcinoma lesions. These studies observed a higher abundance of this genus in healthy sites, suggesting that *Streptococcus* may serve as a marker of a healthy oral microbiome [22,23,25,26,27,31,32,36,43 48]. While in precancerous lesions, Singh et al. (2023) [51] found a significant abundance of *Streptococcus*, indicating a potential diagnostic role in the early stages of the disease. However, some studies present conflicting results: five of the 27 articles analyzed highlighted significant amounts of *Streptococcus* in patients with OSCC [24,29,34,42,45]. Furthermore, Herreros-Pomares et al. (2023) [43] documented high levels of *Streptococcus* not only in healthy subjects but also in those affected by proliferative verrucous leukoplakia (PVL), suggesting a possible variability in the microbial composition depending on the clinical context. Finally, three of the 27 studies analyzed reported a high abundance of *Neisseria* in tumor samples, suggesting a potential diagnostic role in the evaluation of lesions [23,29,31,32,33,38,42]. However, Ye et al. (2021) [27] and Chang et al., 2019 [35] found low levels of *Neisseria* in OSCC samples compared to healthy controls, highlighting some variability in the results. *Rothia* has been identified with high abundance in tumor samples [29,42]. Singh et al. (2023) [51] highlighted a higher abundance of *Rothia* in precancerous samples, suggesting a potential role in disease progression.

Fusobacterium nucleatum is one of the most abundant species in OSCC patients. Eight of the 27 studies analyzed highlight a significant correlation with the pathogenesis of oral cancer [23,29,33,36,41,42,45,51]. Torralba et al. (2021) [41] confirmed its role in tumor progression through virulence factors. *Prevotella* spp. is another significant genus, particularly *Prevotella intermedia*, whose presence has been documented in several studies of OSCC patients. Liu et al., 2022 [33], Heng et al., 2022 [32], and Rai et al., 2020 [29] reported its presence in tumor samples. Furthermore, Saxena et al.(2022) [23] and Zhou et al.(2021) [36] confirmed the abundance of *Prevotella intermedia* in tumor and paracancerous tissues. Herreros-Pomares et al. (2023) [43] detected an enrichment of *Prevotella nanceiensis* in OSCC samples associated with proliferative verrucous leukoplakia (PVL). Finally, Torralba et al. (2021) [41], through their metagenomic analysis, documented a high presence of several *Prevotella spp*. in samples. Rai et al.(2020) [29] reported a high presence of *Prevotella spp.* and *Porphyromonas* spp. in tumor tissues. Rai et al. (2021) [29] highlighted the association of *Streptococcus anginosus* with the early stages of oral cancer development. Furthermore, Erira et al. (2021) [42] documented a significant prevalence of several *Streptococcus spp.* in the saliva of OSCC patients. Also, among the genus Rothia (Rothia in grassetto), Singh et al. (2023) 51 identified high levels in the pre-cancerous lesions. Finally, Herreros-Pomares et al., 2023 [43] found a notably higher presence of *Campylobacter jejuni* in the microbiomes of patients with proliferative verrucous leukoplakia.

Methods Used for *Candida spp.* Identification

Unlike the bacteriome, which has been characterized mainly by sequencing techniques, the importance of the role of *Candida* spp. in the context of OSCC has led some authors to use a polyphasic approach. The techniques used to identify *Candida* spp. in the reviewed articles include phenotypic and genotypic methods (Tab. VII). CHROMagar Agar was used among the phenotypic methods, which allows rapid identification of *Candida* spp. through specific chromogenic reactions [39]. Periodic Acid-Schiff (PAS) staining was employed by Hafed et al. (2019) [47] to visualize *Candida* hyphae in tissue samples, allowing for histological identification of the fungus. Colony counting was also performed by Rusanen et al. (2024) [46], providing quantitative data on fungal presence. DNA extraction was performed by boiling lysis for cell samples [37], while tissue samples were deparaffinized and assessed with a specific kit [44]. Real-time PCR was employed to identify and quantify species using specific primers and standard curves [44]. Hafed et al. (2019) [47] performed RNA extraction with an RNeasy® FFPE kit from tissue samples and analyzed it by RT-PCR (Reverse Transcription PCR) to study gene expression. Furthermore, Sankari et al.(2020) [40] applied the restriction fragment length polymorphism (RFLP) technique to distinguish species through amplification of the ITS1-5.8SrDNA-ITS2 region.

Incidence of *Candida spp.* in OSCC Patients

We examined 5 articles that were specifically about *Candida* spp. in the current literature evaluation. The results of the five studies included in this review indicated a significantly higher prevalence of *Candi-*

Table VII. Sampling and polyphasic approach used in *Candida* spp. identification.

Author, year	Sampling methods	Detection methods
Hafed et al., 2019 [47]	Tissue biopsy	- PAS staining and Real-time PCR
Sankari et al., 2020 [40]	Saliva collecting	-DNA extraction by boiling lysis method -PCR-ITS1-5.8SrDNA-ITS2 region -Sequenced using ABI PRISM® BigDye™ Terminator e ABI 3730XL sequencer -NCBI GenBank
Arya et al., 2021 [39]	Saliva swab	Colony morphology on CHROM-agar *Candida* medium
Ilhan B. **et al., 2023** [44]	Tissue biopsy	-Colony count Fungal/bacterial DNA isolation kit (Zymo Research, Irvine, CA, USA) -Tissue Samples High Pure PCR Template Preparation Kit
Rusanen et al., 2024 [46]	Tissue biopsy	-Colony count -Immunohistochemistry

da spp. in patients with OSCC and oral potentially malignant disorders (OPMD) compared to healthy subjects. Arya et al. (2021) [39] found an increasing incidence of *Candida albicans*, *Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis* in healthy subjects, patients with OPMD, and patients with OSCC. Similarly, Sankari et al. (2020) [40] reported oral *Candida* prevalence of 72.2%, 58%, and 20.5% in patients with OSCC, OPMD, and healthy controls, respectively. Differences in the distribution of *Candida* spp. emerged between the groups, predominating *Candida krusei*, *Candida tropicalis*, and *Pichia anomala* (formerly known as *Candida pelliculosa*). In contrast, *Candida* and other pathogenic fungi such as *Acremonium exuviarum* and *Aspergillus fumigatus* were significantly increased in OSCC patients compared to healthy controls and OPMD patients [32]. During follow-up, a significant reduction in salivary flow and an increase in *Candida* spp. in saliva post-treatment was observed, suggesting that the treatment may have impaired salivary function and facilitated *Candida* spp. proliferation [45].

Discussion

Table VIII. Summary of *Candida spp.* and their associations with oral conditions.

Author, year	OSCC↑:			
Hafed et al., 2019 [47]	*Candida spp.*↑	n.d.		HC: *Candida spp.* ↓
Sankari et al., 2020 [40]	*C. krusei:* 21% *C. tropicalis:* 21% *Pichia anomala (ex C. pelliculosa):* 21% *C. famata:* 17% *C. rugosa:* 6%	OPMD (Potentially malignant oral diseases)↑ *Pichia anomala (ex C. pelliculosa):* 33% *C. krusei:* 27% *C. tropicalis:* 10% *C. famata:* 9% *C. rugosa:* 7%		HC: *Pichia anomala (ex C. pelliculosa):* 40% *C. tropicalis:* 24% *C. krusei:* 17%
Arya et al., 2021 [39]	*C. albicans* ↑ *C. krusei* ↓ *C. tropicalis* ↓ *C. parapsilosis* ↑	*C. albicans* ↑ *C. krusei* ↑ *C. tropicalis* ↑ *C. parapsilosis*		*C. albicans* ↓ *C. krusei* ↓ *C. tropicalis* ↓ *C. parapsilosis* ↓
Ilhan B. et al., 2023 [44]	*C. albicans* ↓ *C. glabrata* ↓ *C. sarcofago* ↓ *C. tropicalis* ↓ *C. parapsilosi* ↓	Mild/moderate dysplasia *C. albicans* ↑ *C. glabrata* ↑ *C. sarcofago* ↓ *C. tropicalis* ↑ *C. parapsilosi* ↑	Carcinoma in situ *C. albicans* ↓ *C. glabrata* ↓ *C. sarcofago* ↑ *C. tropicalis* ↓	Benign *C. albicans* ↓ *C. glabrata* ↑ *C. sarcofago* ↓ *C. parapsilosi* ↑
Rusanen et al., 2024 [46]	*Candida spp.* ↑			HC↑: *Candida spp.* ↓

In this systematic review, we examined 27 studies on the role of different microorganisms, including bacteria and fungi, concerning OSCC, highlighting the geographical variables, DNA extraction methods, amplification, and sequencing strategies used.

Our analysis confirms that DNA extraction is crucial to high-quality 16S rDNA sequences for reliable microbiome profiling.

Most studies included in this review indicated a preference for commercial kits due to their consistency and reduced risk of contamination. This finding supports the conclusions of Zhang et al. (2022) [52], who emphasize the reproducibility offered by standardized kits. Only a minority employed traditional extraction methods, such as CTAB or phenol-chloroform. These methods, although convenient, present greater variability and risk of contamination [53]. However, there is a preference for commercial kits despite their limitations due to potential PCR inhibitors and cost [54]. Selection of the appropriate extraction technique is crucial; recent guidelines emphasize the need for standardization to ensure reproducibility [55].

The selection of hypervariable regions for 16S rRNA amplification significantly impacts taxonomic resolution, as demonstrated by Gopinath et al.(2019) [56].

Our results showed that the V3-V4 regions were selected the most, followed by the V4 region. Indeed, studies like that by Johnson et al. [57] have highlighted the potential to produce an accurate, high-resolution taxonomy of organisms via full-length 16S sequencing [57]. In contrast, the V4 region has been employed less frequently, although it is often chosen for compatibility with platforms that support shorter reads [58]. This choice depends on the sequencing platform used; for example, the 250-bp paired-end reads of the Illumina MiSeq system fit the V3-V4 region well [59], ensuring complete coverage. Although the Illumina MiSeq is a prevalent platform, while some studies, such as that by Ganly et al. (2019) [22], have used less common platforms, including the NovaSeq 6000 and 454 pyrosequencing.

The studies analyzed in this review also employed newer platforms like DNBSEQ-T7 to explore deeper coverage. The databases used for sequence alignment exhibited notable variability, with Greengenes, SILVA, and the Human Oral Microbiome Database (HOMD) the most frequently utilized. This diversity reflects an ongoing effort to align databases with specific research requirements, affecting downstream bioinformatics analysis and taxonomic accuracy.

The different approaches highlight the challenges in drawing uniform conclusions about OSCC-related microbial communities. Our results are consistent with those of Bars-Cortina et al. (2024) [60], who compared 16S rRNA sequencing to shotgun metagenomics and found that targeted 16S regions remain superior for identifying disease-associated microbiota changes. These insights are critical because they imply that, although shotgun metagenomics offers broader functional insights, 16S sequencing retains its value for taxonomic investigations in targeted disease studies.

The analysis of the studies revealed a need for more consistency in the results about the microbial diversity associated with OSCC. Some studies have reported significant differences in alpha and beta diversity between OSCC samples and controls, thereby underscoring alterations in the microbial composition associated with the disease. For instance, several studies have documented a reduction in alpha diversity in tumor samples, as indicated by indices such as Shannon and Simpson. However, other studies have not identified any significant differences. While alpha diversity does not appear to be consistently affected by oral diseases, beta diversity frequently demonstrates a notable distinction between OSCC groups and controls, suggesting that the composition of the oral microbiome may vary considerably concerning the pathology. However, the variability of the results indicates that there may be discrepancies in the analytical methods, samples, and confounding factors that influence the outcomes.

The oral microbiome composition in OSCC patients differs markedly from that of healthy subjects, with significant alterations in several bacterial phyla and genera. The collected data show a predominance of some phyla, including *Bacteroidetes* and *Fusobacteria*, which are frequently associated with tumor lesions. For example, the phylum *Bacteroidetes* was reported to be enriched in 13 out of 27 studies, confirming a close association with cancerous lesions [23,24,25,26,27,29,31,32,33,37,38,43,49]. This phylum has been associated with inflammatory processes and tumor progression in studies of areca consumers [30]. In contrast, other studies, such as that by Zhou et al. (2021) [36], reported a reduction of *Bacteroidetes* in tumor samples. *Fusobacteria* emerges closely associated with dysbiosis and inflammatory states that promote carcinogenesis [25,36]. The role of *Firmicutes* is more controversial: while some studies report it as abundant in tumor samples [41], others, such as Heng et al. (2022) [32], observed a significant reduction in OSCC patients compared to healthy controls. In particular, *Streptococcus* spp., a key genus within the Firmicutes, shows variable behavior. Some studies, such as those by Ganly et al. (2019) [22] and Heng et al. (2022) [32], report a reduction in Streptococcus spp. in OSCC patients, suggesting a possible protective role. However, other research indicates an increased pres-

ence of *Streptococcus* spp. in precancerous stages, associating it with chronic inflammation [42,49]. For example, a significantly higher abundance of *Streptococcus infantis* was found in smokeless tobacco nonusers than in smokeless tobacco users and the contralateral buccal site of OSCC specimens than in the OSCC tumor site [23].

Furthermore, Mäkinen et al. (2023) [45] documented elevated levels of multiple *Streptococcus* spp. in smoking patients with oral squamous cell carcinoma (OSCC). Furthermore, a reduction in *Streptococcus* spp. and *Rothia* spp. levels have been documented during the development of OSCC in patients with oral potentially malignant disorders (OPMD) [51]. This duality may reflect the differing roles of *Streptococcus* spp. in the various stages of the disease. *Fusobacterium nucleatum* has been identified as one of the most strongly associated genera with tumor occurrence. Studies such those by Ganly et al. (2019) [22], Zhou et al. (2021) [36] and Zeng et al. (2022) [31] have shown a marked increase in *F. nucleatum* in tumor samples compared to controls, with direct implications for tumor progression through mechanisms including chronic inflammation and immunoevasion. Its association with virulence factors and inflammatory processes further supports the dysbiosis theory as a pathogenic mechanism in OSCC [41]. *Porphyromonas gingivalis* has been linked to a more aggressive malignant profile in patients with OSCC, with the bacterium modulating the local immune response and promoting tumor growth by activating inflammatory pathways [28]. However, Chen et al. (2021) [28] also revealed a lower incidence of relapse in patients with high *P. gingivalis* load, raising interesting questions regarding the complexity of the role of this bacterium in different stages of the disease. Chang et al. 2019 [35] demonstrated that *Fusobacterium nucleatum* and *Porphyromonas gingivalis* activate Toll-like receptors (TLRs), triggering chronic inflammatory responses that facilitate tumor progression. The authors identified an elevation in the levels of inflammatory cytokines, including interleukin-6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α). This correlation between alterations in microbial composition and elevated levels of inflammatory cytokines has also been observed by Singh et al. (2023) [51], further supporting the link between dysbiosis and inflammation in the context of OSCC. In addition, Ganly et al. [22] found significant increases in the expression of the HSP90 gene and TLR ligands 1, 2, and 4 as oral tissues progressed from health to OSCC. Specifically, genes related to heat shock protein 90 and TLR ligands for various microbial components were progressively enriched. *Prevotella* was identified as the primary contributor to this enrichment of pro-inflammatory genes, with notable additional contributions from *Alloprevotella, Fusobacterium, Veillonella,* and *Porphyromonas* 22. Liu et al. [33] further linked lipopolysaccharides (LPS) to specific bacteria in the oral microbiome, identifying *Fusobacterium nucleatum* as a key pathogenic bacterium responsible for increased LPS levels. This suggests that *F. nucleatum* plays a significant role in inflammation and the progression of OSCC, highlighting its impact on the microbial ecosystem. These findings have also been found for fungi such as *Candida*. For example, Rusanen et al. (2024) confirmed that *Candida albicans* induces chronic inflammation by activating Toll-like receptors (TRL 1-10) and the transcription factor NF-κB in OSCC patients. *Candida albicans* also produce acetaldehyde, which increases the risk of carcinogenic mutations. Its ability to metabolize ethanol into acetaldehyde in the oral environment, thanks to the expression of alcohol dehydrogenase 1, amplifies the carcinogenic effect linked to alcohol consumption [47]. In recent years, the role of interactions between members of the oral microbiota in oral tumorigenesis, progression, and metastasis has gained widespread attention. For example, acetaldehyde produced by *C. albicans* facilitates the production of phenazines by *Pseudomonas aeruginosa*, which further induces the production of acetaldehyde by *C. albicans*, and the two jointly promote oral carcinogenesis [61]. Also, Heng et al. (2022) [32], using both traditional and metagenomic methods, found a significant increase in OSCC patients. Specifically, *Candida* spp. levels were higher in buccal swabs, plaque samples, and saliva from the OSCC group compared to healthy controls (HC) (5% vs. 1%, P = 0.002; 5% vs. 1%, P = 0.007; 5% vs. 1%, P = 0.012). Additionally, levels were elevated in plaque swabs of the OSCC group compared to the oral potentially malignant lesion (OPL) group (5% vs. 1%, P = 0.008) [32].

Several studies have shown that the composition of the oral microbiome varies with the stage OSCC. Singh et al., (2023) [51] showed that specific bacterial genera, such as Fusobacterium, are more abundant in the early stages of the disease, while others, such as *Capnocytophaga*, prevail in the advanced stages. Yang et al. (2021) [26] analyzed the relationship between microbiota and clinical stages (I–IV), finding that *Treponema* spp. and *Leptotrichia* spp. were found to be more abundant in early stages (I/II), while *Prevotella* spp. and *Capnocytophaga* spp. were enriched in tumor samples. In advanced stages (III/IV), *Solobacterium moorei* and *Slackia exigua* were predominant in tumor tissues. Zeng et al. (2022) [31] correlated the clinicopathological data with the microbiome, noting that higher T-stage was associated with a higher N-stage. They suggested that Fusobacterium may have an on-

cogenic role, more frequent in T3/T4 stages and lymphatic metastasis. However, contradictions emerged, such as a higher proportion of Porphyromonas in the T1/T2 groups. These observations highlight the complexity of the interactions between oral microbiota and OSCC progression, suggesting a significant impact of specific bacteria on the disease.

Our review also highlighted significant differences in oral microbiome composition among different sample types analyzed. Heng et al. (2022) [32] showed an increase in diversity in OSCC swab samples, while saliva samples from both OSCC and OED showed a significant decrease in diversity. In particular, Yang et al. (2021) [26] reported that the phyla *Bacteroidetes* and *Proteobacteria* were significantly enriched in tumor samples (tumor-Saliva). Four genera, such as *Filifactor* and *Peptostreptococcus*, showed high abundance in tumor tissues (TT), while 9 species, including *Streptococcus oralis* and *Neisseria macacae*, predominated in TS samples. Similarly, Erira et al.(2021) [42] demonstrated that dental plaque, saliva, and tumor tissue had significantly different microbiome compositions, highlighting how the oral microenvironment affects the distribution of bacterial species. In tumor tissue, bacteria such as *Fusobacterium nucleatum, Porphyromonas gingivalis, Peptostreptococcus, Prevotella,* and *Parvimonas micra* are abundant and are associated with inflammatory processes and carcinoma progression.

In contrast, dental plaque shows a higher microbial diversity, predominating anaerobic bacteria such as *Streptococcus, Veillonella,* and *Actinomyces*, which are not identified as key species in tumor tissues. Finally, saliva has a less diverse bacterial composition than dental plaque, but more heterogeneous than tumor tissue. The detected species, such as *Streptococcus* and *Haemophilus*, reflect the recirculation of bacteria in oral biofilm, with a lower representation of those directly associated with tumor progression. These results highlight the importance of the analyzed sample in understanding the dynamics of the oral microbiome concerning OSCC.

The reliability of microbiome studies depends largely on the molecular biology techniques used downstream. Most studies sequenced the V3-V4 regions, although some chose the V4 and V3 regions. Experimental studies have concluded that the choice of 16S rRNA region type for amplification can significantly influence the size of distinct taxa [56].

Analysis of DNA extraction methodologies and sequencing techniques has revealed a variety of approaches, each with its advantages and disadvantages. The predominant use of commercial kits such as the DNeasy® Blood and Tissue Kit and the QIAamp-Fast® DNA Stool Mini Kit suggests a necessary standardization to facilitate comparison between studies. Additionally, the type of 16S rRNA region chosen for amplification can significantly influence the results [25]. Metagenomics represents a more comprehensive approach than 16S rRNA alone as it allows for identifying fungi and viruses, reducing the bias due to genetic amplification. Recent studies, such as that by Torralba et al.(2021) [41], have used metagenomics and metaproteomic analyses to analyze saliva from patients with OSCC. This combination of methodologies offers a more integrated view of the microbial composition and functional dynamics within the oral microenvironment. Despite promising results, most of the included studies have significant limitations. As they are mainly case-control studies, there is a high risk of bias due to confounding factors. However, variables such as age, smoking habits, alcohol consumption, and chewing were considered, and only a few studies adequately adjusted for these factors in the design or analysis phases.

Another methodological issue that emerged in this review is the need for more standardization in microbiome analysis techniques, which made it difficult to compare results between studies. Differences in DNA extraction protocols, sequencing methods, and bioinformatics analysis contributed to this heterogeneity. This highlights the need to integrate more precise and standardized techniques.

Artificial intelligence (AI) can overcome many challenges of analyzing and managing large volumes of complex data, offering advanced solutions beyond the limits of traditional techniques Thanks to its ability to learn from data, AI can detect hidden patterns, improve predictions, and support evidence-based decisions. In our review, we explored studies such as those by Ganly et al., 2019 [22] who used Support Vector Machine (SVM) and Random Forest models, and Sarkar et al., 2021 [37] with Random Forest to analyze complex datasets, obtaining excellent predictive results. Zhou et al. (2021) [36], for example, achieved a diagnostic accuracy of 95.7% in gene sequencing of oral tissues and predicted OSCC with 100% accuracy using Random Forest. Torralba et al. (2021) [42] also took an innovative approach, combining metagenomics and metaproteomics to identify the presence of bacteria and characterize the proteins expressed in the samples. Next, they applied Random Forest analysis to distinguish distinctive markers between tumor and non-tumor tissues.

This is a promising first approach. An alternative methodology would be to utilize AI in the context of Digital Pathology (DP), specifically in analyzing digital slides. A recent example of bacterial identification using AI is

the detection of *Helicobacter pylori (HP)* in intestinal biopsies [62]. The study was conducted with the assistance of DP, utilizing deep learning algorithms to analyze digitized histological images (WSI), which resulted in a notable enhancement in diagnostic efficiency compared to traditional methods. The researchers demonstrated that AI, when applied to low-resolution images (20X), can identify *HP* infection in intestinal tissue samples with remarkable accuracy while simultaneously reducing the number of false positives and negatives. This demonstrated that the integration of DP and AI accelerates the examination of intricate specimens and yields outcomes analogous to those obtained through conventional microscopy. This validates the efficacy of AI in authentic clinical contexts. While there are currently no studies exploring the use of this approach in the context of oral biofilm, preliminary evidence suggests that similar techniques can be used to identify bacterial species in the intestine. This perspective opens the way to possible future applications of AI in analyzing the OSCC-associated microbiome, offering a unique opportunity to improve diagnostic accuracy and understand microbial interactions in the tumor context.

Conclusion

The present systematic review elucidates the complex relationship between the oral microbiome and the pathogenesis of OSCC. Our findings indicate that OSCC is associated with specific alterations in microbial composition, characterized by a predominance of specific phyla, such as *Bacteroidetes* and *Fusobacteria*, which appear to facilitate tumor progression through inflammatory mechanisms. The diversity of microbial profiles observed across different stages of OSCC and between various types of samples underscores the intricate nature of the oral microbial ecosystem. Moreover, incorporating sophisticated analytical techniques, including AI, presents promising avenues for future research, enabling the identification of crucial biomarkers and enhancing our comprehension of the microbiome's role in cancerogenesis. Addressing the methodological limitations identified in this review is crucial for establishing a robust framework for future studies. In conclusion, these insights could pave the way for innovative diagnostic and therapeutic strategies targeting the oral microbiome in the context of OSCC.

Conflicts of interest statement

Any current or prospective financial, personal, or other conflicts of interest that might influence or skew their study are completely disclosed by the authors.

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Authors' contributions

AC: Performed the analysis, Wrote the paper; SV: Performed the analysis; AE: Collected the data; AM: Collected the data; DC: Collected the data; AP: Revised the manuscript; GPP: Revised the manuscript; SS: Conceived and designed the analysis; FM: Conceived and designed the analysis, revised the manuscript; GI: Performed the analysis, revised the manuscript.

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