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The oral microbiota change in oral cancer – a preliminary study

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Abstract

Introduction and Objective. The relationship between oral bacteria and oral squamous cell carcinoma (OSCC) is not yet fully understood. This study aimed to profile the microbiota composition at tumour sites versus adjacent normal tissues in the oral mucosa of 10 OSCC patients using a qPCR Array.

Materials and Method. We recruited 10 OSCC patients (8 men and 2 women, aged 40 to 89 years) from the Department of Oral Surgery at the Medical University of Lublin. The diagnosis of OSCC was confirmed through clinical presentation and histopathology. Bilateral swabs were taken for further examination. The study identified 93 microorganisms using the Oral Disease Microbial DNA qPCR Array.

Results. From the 20 samples analyzed, we retrieved 868 species/genes across 37 genera, representing 90 different microbial species. The relative abundance of *Fusobacterium nucleatum* was significantly higher in OSCC samples ($P < 0.05$). Receiver operating characteristic (ROC) curve analysis identified *F. nucleatum* as a potential OSCC biomarker ($p = 0.007$). Correlation analysis revealed distinct ecological relationships within the bacterial communities of OSCC samples, consistent with the observed bacterial diversity.

Conclusions. Notably, associations centred around *Fusobacterium* spp. were prominent in cancer samples, further suggesting a potential role for this genus in OSCC development. The differences in oral bacterial profiles between tumour and non-tumour tissues may serve as diagnostic markers.

Key words

microbiota, biomarker, oral squamous cell carcinoma, Fusobacterium nucleatum

INTRODUCTION

Oral, head, and neck cancers represent a significant global health concern. Lifestyle factors such as tobacco smoking, excessive alcohol consumption, unhealthy diets, and lack of physical activity, significantly increase the risk of developing cancers in these regions. Environmental factors, including exposure to UV radiation, HPV and EBV infection, and workplace chemicals, also contribute to the occurrence of these cancers. Antibiotic misuse and the resulting changes in oral microbiota may further increase the risk of developing certain types of cancers. Head and neck cancers remain a major medical issue, with persistently high incidence and mortality rates [1–6].

The oral environment, due to its unique anatomical and functional characteristics, creates specific conditions for the development of microorganisms. However, maintaining symbiosis is challenging due to various local factors, such as temperature changes (hot foods), microtrauma to the mucosa (teeth, dentures), poor oral hygiene, and inflammation [7]. These factors, alongside others previously mentioned, can promote the development of dangerous pathogens, particularly anaerobes.

In epidemiology, a high clinical stage at diagnosis is critical. The dynamic nature of malignant changes complicates the early diagnosis of oral cavity cancers. In many cases, cancerous diseases have a long-standing background, with many patients developing cancer from untreated and undiagnosed, potentially malignant oral lesions. [8]. Low awareness of self-examination in this area also significantly contributes to the under-diagnosis of pre-malignant lesions and early-stage cancers [4].

While the risk factors for oral cavity cancers are well established, the role of oral microbiota – a component of the oral cavity – has not been definitively determined in the context of these cancers. The role of infectious agents in cancer development gained increased attention after Helicobacter pylori was recognized in the early 1990s as a causative agent of gastric cancer [9]. Bacteria constitute the majority of the oral microbiota, with fungi and viruses to a lesser extent. Numerous oral bacterial species have been implicated in oral cancer development [10, 11]. It is suspected that the presence of certain bacterial strains within the oral microbiome may correlate with the risk of malignant transformation in the oral cavity.

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OBJECTIVE

The aim of the study is to determine whether the oral microbiota is a modulating factor in the local propensity for potentially malignant and cancerous lesions, or whether it undergoes modifications as a result of developing cancer. To this end, the differences in the microbiota of patients diagnosed with malignant oral cavity tumours were examined, and compared to healthy controls. Additionally, the study aimed to identify potential differences within the oral cavity of the same patients by comparing both the affected and healthy sides. Changes in oral microbiome composition, particularly an increase in pathogenic bacteria, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, have been associated with oral squamous cell cancer (OSCC). These bacteria can induce chronic inflammation, promote DNA damage, and alter cellular signalling pathways that contribute to carcinogenesis [12]. The oral microbiome can also interact with the host's genetic background, with the potential to influence immune response genes, such as those involved in cytokine production (e.g., IL-6, TNF-α), potentially leading to a pro-tumourigenic environment [13].

MATERIALS AND METHOD

Selection of patients. Ten patients with OSCC of the oral mucous (8 men and 2 women, aged between 40 – 89), were recruited from the Department and Chair of Oral Surgery at the Medical University in Lublin, eastern Poland. The diagnosis criteria of OSCC were confirmed by clinical presentation and histopathological examination – all patients were diagnosed with OSCC. The study was conducted with the approval of the Ethics Committee at the Medical University in Lublin (Protocol No. KE/0245/191/10/22).

During the first visit by each patient, the diagnosis of planoepithelial cancer in the oral mouth was confirmed by biopsy. Bilateral swabs were taken for further examination. All the patients diagnosed presented poor oral hygiene in advanced clinical stages, but in the case of patients in the lower clinical staging of T1, the hygiene was at an even higher level (Fig. 1 and 2).

Sampling procedure. *B*ilaterally mucosal swabs were taken from each patient with OSCC – 20 oral tissue samples (10-paired samples) were obtained from non-tumour and tumour sites. The swab samples were stored in SLB (A&A Biotechnology, Poland) until RT-PCR could be performed.

Real-time PCR analysis. DNA extraction was carried out using the Genomic DNA purification method with the QIAamp DNA Mini Kit (Qiagen, Germantown, USA), following the manufacturer's protocol. The extracted DNA was analyzed using the Oral Disease Microbial DNA qPCR Array (Qiagen, Germantown, USA). Real-time PCR assays were conducted with a Light Cycler 96 system (Roche, Basel, Switzerland), targeting the 16S rRNA gene. The assays employed PCR amplification with the use of primers and hydrolysis-probe detection to enhance specificity. Each Microbial DNA qPCR Array plate was designed to analyse one sample at a time for 93 species (NCBI Tax ID)/genes. Pan-bacteria assays, capable of detecting a wide range of bacterial species, were included as positive controls for bacterial DNA detection. For relative profiling, host genomic DNA and the overall bacterial load were quantified. The analyses enabled sample input normalization through the ΔΔCT method.

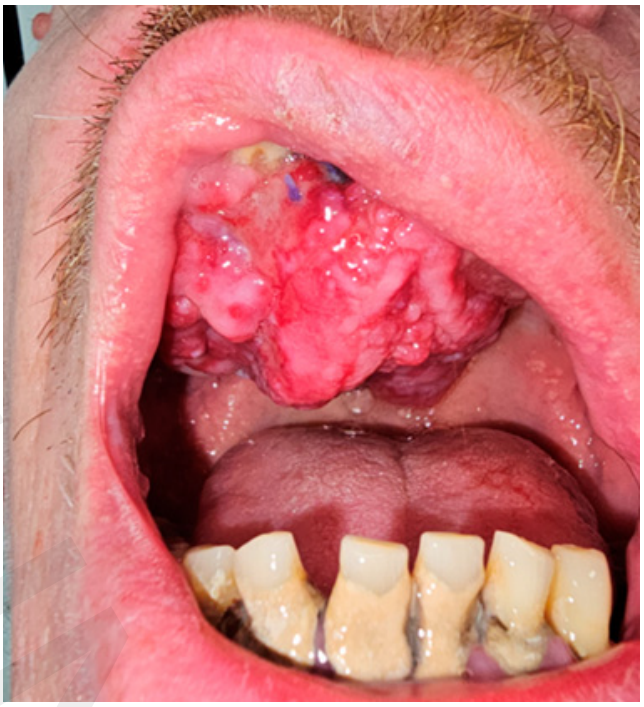

Figure 1. Patient with advanced oral squamous carcinoma. Poor hygiene of the mouth due to advancement of the tumour

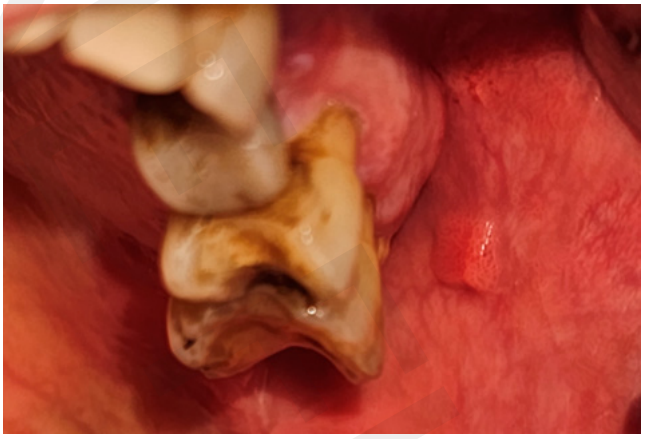

Figure 2. Patient with the oral verrucous squamous carcinoma of T1

Statistical analysis. *S*tatistical analysis was performed with Tibco Statistica 13.3 (StatSoft, Palo Alto, CA, USA). The values of the parameters were presented as medians, minimum and maximum values. The normal distribution of continuous variables was tested using the Shapiro-Wilk test; the Mann-Whitney U-test and Kruskal-Wallis tests were used for independent variable comparisons among two or more groups; Pearson correlation was performed among the most abundant bacterial genera and species. Receiver Operating Curve (ROC) analysis was utilized to evaluate the diagnostic performance of the predictive bacterial biomarkers.

RESULTS

The clinical characteristics of the study patients are listed in Table 1. Two oral microbiota samples (one from the OSCC lesion and one from the healthy/control site) from each patient were collected for analysis. The diagnosis criteria of OSCC were confirmed by clinical presentation and pathologic diagnosis and all patients were diagnosed with OSCC.

Table 1. Clinical characteristics of the patients

Characteristics		Total	Male	Female
Age	Mean	64.0±15.4	64.5±15.7	62.0±19.8
	Median (range)	67.5 (40–89)	67.5 (40–89)	62.0 (48–76)
Gender	Male	8 (80%)	8 (100%)	0
	Female	2 (20%)	0	2 (100%)
Tumour stage	T1	2 (20%)	1 (12.5%)	1 (50%)
	T2	3 (30%)	2 (25%)	1 (50%)
	T3	5 (50%)	5 (62.5%)	0
Site of lesion	Hard palate	1 (10%)	1 (12.5%)	0
	Floor of the mouth	2 (20%)	2 (25%)	0
	Alveolar process of maxilla	2 (20%)	2 (25%)	0
	Alveolar process of mandible	1 (10%)	0	1 (50%)
	Oral mucous of the cheek	2 (20%)	1 (12.5%)	1 (50%)
	Lower lip	1 (10%)	1(12.5%)	0
	Retromolar triangle	1 (10%)	1 (12.5%)	0
Drinking		10 (100%)	8 (100%)	2 (100%)
Smoking		10 (100%)	8 (100%)	2 (100%)
HPV positive		6 (60%)	5 (62.5%)	1 (50%)

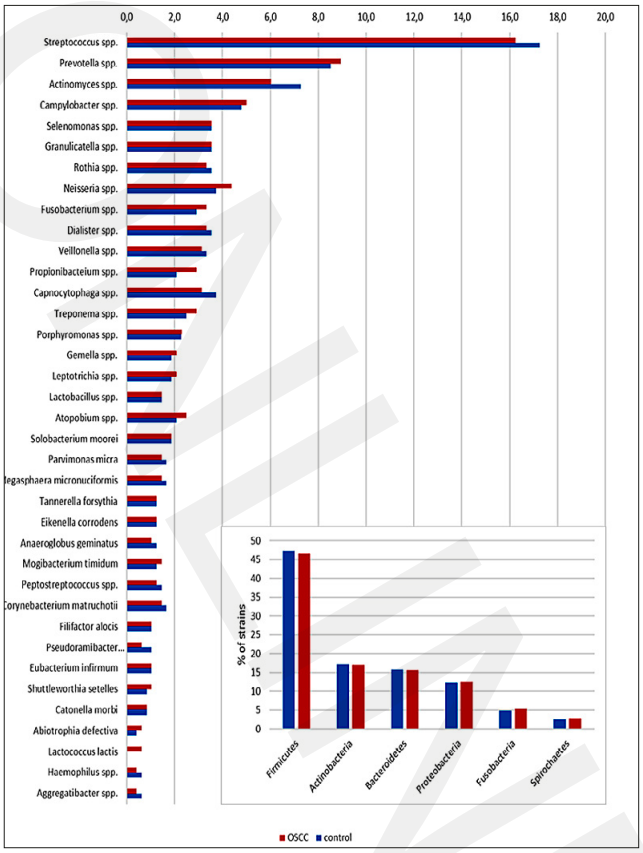

Figure 3. Distribution of microorganisms in the oral samples from OSCC patients obtained by molecular methods

Quantitative analysis of 93 microorganisms was performed using Oral Disease Microbial DNA qPCR Array (Qiagen, USA) in real-time PCR. From the 20 samples studied, 868 species/genes from 37 different genera of 90 various microbial species were retrieved. In one sample, 3–67 (mean 43.4) species/genes were detected. Of the major reported phyla, Firmicutes (46.6% vs. 47.3%), Actinobacteria (17.1% vs. 17.3%), Bacteroidetes (15.6% vs. 15.8%), Proteobacteria (12.5% vs. 12.3%) and Fusobacteria (5.4% vs. 4.8%), were detected in tumour and non-tumour sites, respectively (Fig. 3).

The bacterial communities in the cancer lesions and the controls were analyzed at different taxonomic levels. The richness and diversity of bacteria were not significantly higher in tumour sites in comparison to the control tissues. However, cancer tissues were enriched in nine genera: *Neisseria* spp., *Fusobacterium* spp., *Propionibacterium* spp., and *Atopobium* spp. *Rothia* spp., *Campylobacter* spp., *Lactococcus* spp., *Prevotella* spp. and *Treponema* spp.

Microbial profiles showing bacterial composition and relative abundance of swab samples are presented in Figure 4. A non-parametric Mann-Whitney test was performed to confirm differences in abundance among the cancer and normal healthy samples for each bacterial taxon. The relative abundance of *Fusobacterium nucleatum* was at a significantly high level among the OSCC cancer samples ($P < 0.05$). Receiver operating characteristic (ROC) curve analysis was subsequently performed with the putative OSCC cancer marker of *Fusobacterium nucleatum* (p=0.007). As revealed by the area under the curve [AUC = 0.785, 95% confidence interval (CI): 0.58–0.99], this model exhibited a robust and statistically significant diagnostic accuracy (Fig. 5). The statistical analysis of relative abundance for each bacterial taxon among patients with different tumour stages in the cancer samples significant differences were observed for *Prevotella loescheii* (p=0.019), *Neisseria mucosa* (p=0.047), *Eikenella corrodens* (p=0.041). However, when analyzing the bacterial abundance from patients in different tumour stages and in samples taken from healthy tissue, statistical differences were found for *Streptococcus sanguinis* (p=0.044), *Selenomonas infelix* (p=0.046), *Rothia* spp. (p=0.049), *Neisseria mucosa* (p=0.025), *Eikenella corrodens* (p=0.025), and *Capnocytophaga ochracea* (p=0.032).

Next, the ΔΔCT method was used for the relative profiling and comparison between oral microbiota profiles of the OSCC and control groups. Microbial DNA qPCR Array correlated increased amounts of (Fig. 6). At the species level, the abundances of *Actinomyces israeli, Dialister invisus, Fusobacterium nucleatum, Parvimonas micra, Peptostreptococcus stomatis, Selenomonas infelix, Solobacterium moorei*, were significantly increased at tumour sites, suggesting a potential association between these bacteria and OSCC.

Correlation analysis among the abundance of genera present in OSCC and healthy samples revealed a sharp difference in relationships (Fig. 7). Among bacteria identified in OSCC samples, a significant correlation was observed between *Eubacterium* spp., *Filifactor* spp., and *Fusobacterium* spp. and their mutual correlations with *Anaeroglobus* spp., *Campylobacter* spp., *Mogibacterium* spp., *Porphyromonas* spp, *Propionibcaterium* spp., *Tannerella* spp., *Treponema* spp., and *Veillonella* spp. In the control samples, such correlations were detected only for *Porphyromonas* spp., *Mogibacterium* spp., *Tannerella* spp. and *Anaeroglobus*

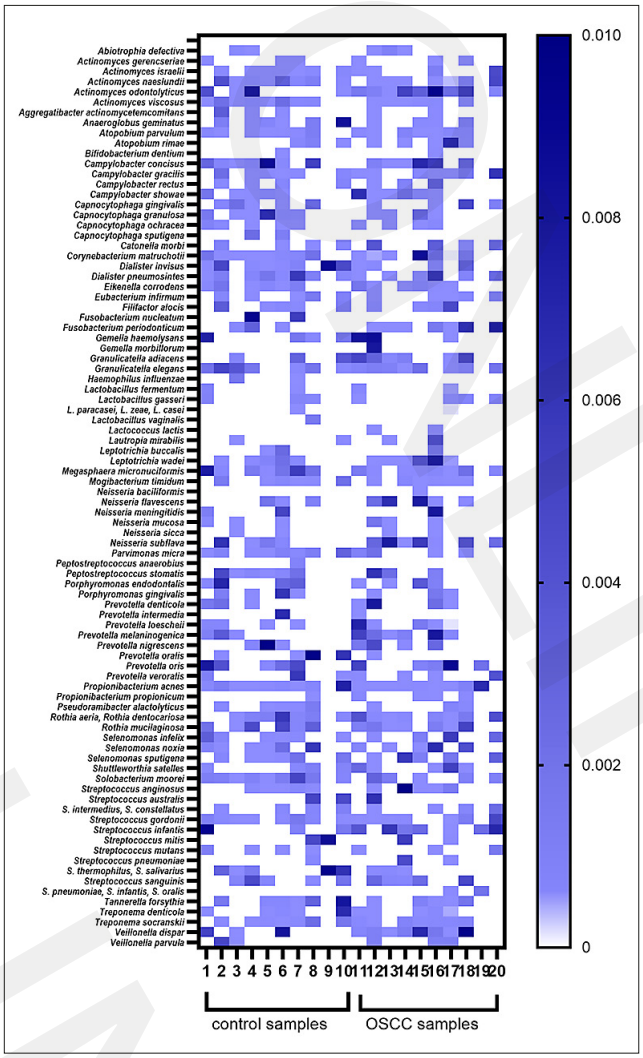

Figure 4. Heat map showing the relative abundance of species/genera genes across samples

spp., as well as *Fusobacterium* spp., *Eubacterium* spp., and *Porphyromonas* spp. Moreover, in control samples, *Rothia* spp. were significantly correlated with *Aggregatibacter* spp., *Eikenela* spp., *Filifactor* spp., *Gemella* spp., *Haemophilus* spp., and *Pseudomonas* spp.

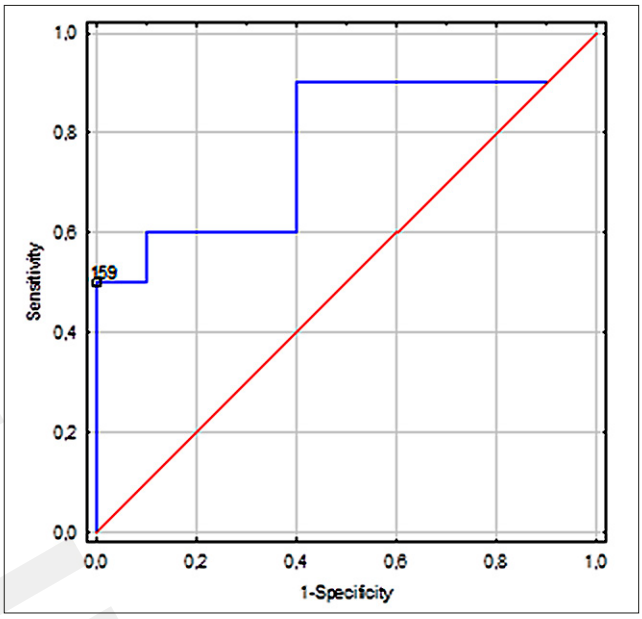

Figure 5. Receiver Operating Curve (ROC) analysis for OSCC cancer with the predictive bacterial biomarker of *Fusobacterium nucleatum*

DISCUSSION

Oral cancer is a highly complex disease influenced by various factors, including an imbalance in the oral microbiota. To better understand the development of oral cancer, it is crucial to conduct an in-depth study of the oral microbiota. A linkage between oral microbiome and OSCC cancer has been reported in previous studies [14,15,16,17,18]. The current study investigates the oral microbiota composition of paired tumour/healthy swab samples from patients with OSCC. Firmicutes was the most abundant phylum observed, which is consistent with previous reports [19,20]. However, other studies have identified Bacteroidetes as the most abundant phylum [15]. In the current study, a significant increase was observed in Fusobacteria in cancer lesions, which aligns with the findings of other researchers [21]. In particular, the higher abundance of Fusobacteria in cancer samples was statistically significant in the current study. Among the highly abundant genera identified, many are associated with periodontitis, including *Fusobacterium, Prevotella,*

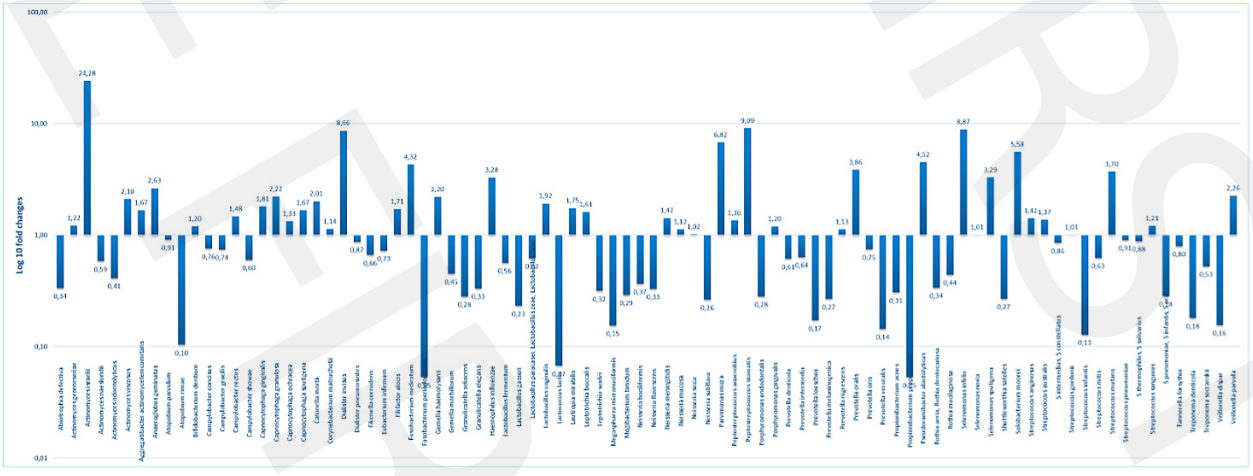

Figure 6. Accurate profiling of pathogenic and commensal microbes in non-tumour tissue and tumour lesion in OSCC patients. Foldchange in microbial species abundance (non-tumour/tumour tissue) was calculated by the ΔΔCT method using Pan Bacteria 1 genomic DNA to normalize. An at least 5–10-fold increase or decrease in relative abundance may be considered significant

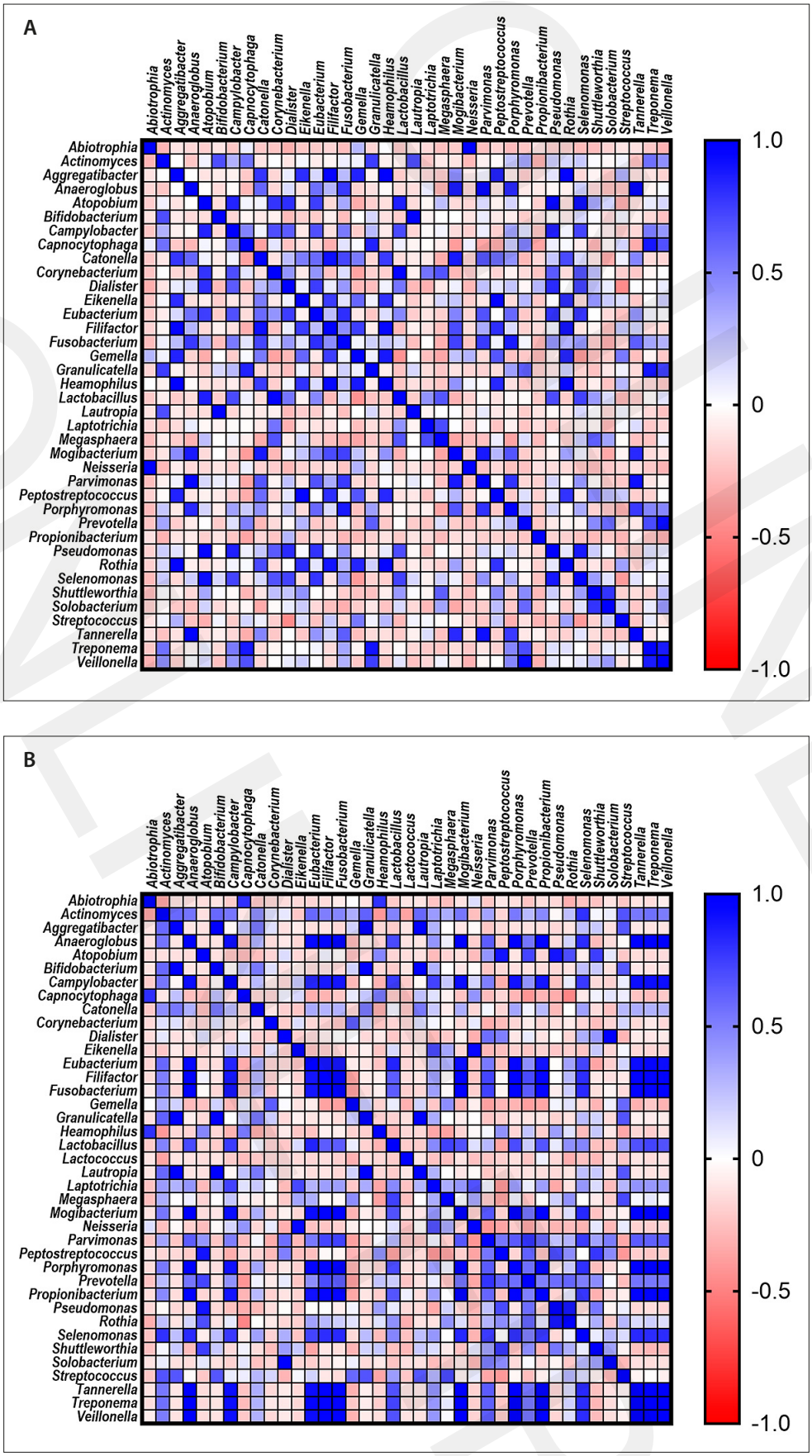

Figure 7. Pearson correlations among the most abundant bacterial genera identified by Microbial DNA qPCR Array. (A) Correlations in control samples. (B) correlations in OSCC samples

Gemella, Neisseria, Dialister, Peptostreptococcus, Filifactor, Peptococcus, Catonella, and *Parvimonas*, all of which were enriched in OSCC samples. This is consistent with previous findings [15,18,22].

Additionally, as noted by Pushalkar et al., *Streptococcus* spp. was the most abundant genus across all samples in the presented analysis [22]. However, similar to other studies, a non-significant decrease was observed in the abundance of *Streptococcus* spp. and *Rothia* spp. in cancer lesions [20,21]. Among oral bacteria, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* may have the greatest potential correlation with oral cancer. Recent studies by Binder Gallimidi et al. [23] indicated that *P. gingivalis* and *F. nucleatum* promote oral cancer progression through direct interactions with oral epithelial cells via Toll-like receptors. In the current study, similar to Zhao et al. [15], *P. gingivalis* did not differ in abundance between groups, whereas *F. nucleatum* was significantly enriched in cancer lesions. Al-Hebshi et al. [12] also identified *F. nucleatum* subsp. *polymorphum* as the most significantly over-represented species in OSCC biopsies. Additionally, Chang et al. [24] found a correlation between *F. nucleatum* and subgingival plaques, noting its higher prevalence in cancerous tissues compared to healthy tissues. Another study using polymerase chain reaction (PCR) confirmed that *F. nucleatum* was much more common in OSCC tissues than in healthy tissues [18,25].

Bacteria cohabit in complex interaction networks, and disturbances in these interactions can lead to disease. Correlation analysis in the presented study revealed specific ecological relationships within the bacterial communities of OSCC samples, consistent with the observed bacterial diversity. Associations centred around *Fusobacterium* spp. emerged in the cancer samples, which suggests that this genus plays a role in OSCC development, particularly given its significant increase in cancer samples. *Fusobacterium* spp. tends to co-adhere with other species in oral biofilms, forming bridges between early and late colonizers [26].

Socransky's theory classifies periodontal bacteria into colour-coded complexes based on their role in disease progression. The red and orange complexes are associated with severe periodontitis, while the yellow, green, purple, and blue complexes are less pathogenic or even beneficial [27]. In the current study, *Fusobacterium* spp. *Filifactor* spp. and *Eubacterium* spp. formed a positively related group correlated to members of the periodontal 'red complex' (e.g., T. *forsythia, T. denticola* and *P. gingivalis*), and 'orange complex' (e.g., *Campylobacter gracilis, C. rectus, C. concisus*, *Parvimonas micra, P. denticola, F. peridonticum, P. intermedia, P. oralis, S. constellatus*). *F. nucleatum* was also positively correlated with members of the yellow (e. g., *A. israeli, A. neaslundii, S. sanguinis, S. mutans*) and purple complexes (e.g., *V. parvula*), which are early colonizers that precede the multiplication of the predominantly gram-negative orange and red complexes.

Overall, the findings of the current study support the importance of the *Porphyromonas–Fusobacterium–Tannerella* triumvirate, which has a diagnostic value of 'specific plaque hypothesis' [28], and suggests a critical role for *Fusobacterium* in increasing OSCC bacterial diversity. Further evaluation of the role *of Fusobacterium* spp. in OSCC may require additional studies.

A central debate in current research is whether *F. nucleatum* plays an active role in the development of neoplastic changes in epithelial cells or whether it is merely a passive passenger that colonizes the tumour due to the favourable conditions within its micro-environment. Traditionally, *F. nucleatum* has been recognized as a bridging organism, facilitating the formation and structure of multi-species biofilms. Recent studies, however, suggest that it may have more direct, active roles. The possibility that *F. nucleatum* functions as a carcinogen is plausible, given its demonstrated ability to promote inflammation and suppress local immune responses [12,29].

The most likely reason of becoming carcinogenic for a microorganism commonly found in the mouth throughout life depends on shifts within the oral microbiome, influenced by host factors such as genetics, oral hygiene practices, nutrition, aging, and exposure to risk factors like tobacco and alcohol [30,31]. Recent research has shown that tobacco use, regardless of the method, alters the oral environment in ways that favour anaerobes, such as *Fusobacterium* spp., potentially contributing to its pathogenic behaviour [30]. Other researchers have confirmed changes in the overall oral microbiota on the cancer lesion side compared to the control [15]. These changes may result from the tumour structure and development, including surface irregularities, recesses, and necrotic foci. The altered surface structure of the tumour, particularly at the advanced clinical stage (Fig. 1), can easily become colonized due to its complexity. The protective properties of saliva may also be insufficient, making the irregular surface more susceptible to colonization of various bacterial species.

In the current study, significant differences in the relative abundance of *Actinomyces israeli*, *Dialister invisus*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Peptostreptococcus stomatis*, *Selenomonas infelix*, and *Solobacterium moorei* were observed between cancer and control samples. However, it is suspected that the physiological component of saliva in the oral cavity may obscure potential differences, despite significant morphological differences between the tumour surface structure and the clinically unchanged mucous membrane, as well as varying conditions for the growth of specific bacterial strains. The immunological properties of saliva influenced by immunoglobulins (especially IgA), lysozyme, lactoferrin, peroxidase, cytokines, and mucins, work together to maintain oral health by neutralizing pathogens, preventing their colonization, and modulating the immune response [32].

Limitations of the study. The small sample size (n=10) is a is and the findings should be interpreted with caution. Nevertheless, the consistent trends observed in this small cohort study suggest potential directions for further exploration.

In the study, each analysis was based on PCR amplification of a species-specific genetic region of the relevant microbe. Assays for detecting bacterial species target the 16S rRNA gene and were designed using the GreenGenes database for 16S sequences. The limitation stems from the technique used which, while easier and quicker, detects 93 genera/species-specific genes compared to the more comprehensive next generation sequencing (NGS) technique. As a result, some species reported in other studies using NGS technique were not detected. This restricted coverage could have impacted the identification of potentially relevant bacterial species not included in the array. Consequently, the microbial diversity within the samples may have been under-represented. This

variability in methods can make it challenging to compare trends in the microbiota composition associated with OSCC. However, variability between studies using complex NGS workflows is also inevitable and may arise from differences in the sequencing regions targeted (e.g., V4-V5, V4, V3-V5, V1-V3), sample types, selection of control tissues, and the number of samples included, potentially leading to inconsistent results [15–17,20]. Despite these challenges, the same taxa tend to be detected in samples, albeit with different relative abundances. Nevertheless, to gain more detailed insights into bacterial communities coexisting in cancer lesions, whole-metagenome sequencing is warranted in future studies.

Recent studies suggest that there are significant differences in the composition of the oral microbiota between urban and rural populations. These differences are influenced by various factors, including diet, oral hygiene practices, and access to healthcare. In a study conducted in Indonesia, researchers found that urban participants had a higher relative abundance of Prevotella and Leptotrichia, while rural individuals had more Streptococcus species and a greater proportion of Firmicutes bacteria [33]. Another study on dental plaque microbiota showed that urban populations tended to have greater microbial diversity, possibly due to more varied diets rich in protein and fibre. In contrast, rural diets were often less diverse, potentially contributing to reduced microbiota diversity [34]. These microbial differences may have clinical relevance. For example, a higher abundance of Prevotella in urban populations has been associated with inflammatory conditions, whereas a dominance of Streptococcus in rural groups may reflect differences in caries risk or oral pH environment.

CONCLUSIONS

The oral microbiota is increasingly recognized as a comorbidity factor in oral squamous cell carcinoma (OSCC). The results of the current study contribute to the understanding of the relationship between oral bacterial communities and the development of oral cancer. Notably, the bacterial profiles differed significantly between tumour sites and normal tissues in OSCC patients, suggesting that these microbial differences could serve as potential diagnostic markers. *Fusobacterium nucleatum* was found to be significantly elevated in cancerous lesions, and emerged as a central component of the bacterial communities in these samples. This supports the hypothesis that *F. nucleatum* plays a role in OSCC formation and progression, highlighting its potential as an indicator of the disease.

While both urban and rural populations face risks for oral cancer, the nature and prevalence of these risks differ. Rural areas often contend with traditional risk factors and limited healthcare acess, whereeas urban areas may face emerging lifestyle-related risks, but benefit from better healthcare infrastructure [35].

Differences in the oral microbiota between rural and urban populations should be taken into account in comparative studies, particularly in relation to the risk of developing oral cancer. Such studies require further analysis in larger groups, with consideration of microbiota differences as a potential factor in the development of oral cancers.

Declaration of authenticity of figures. All figures submitted have been created by the authors, who confirm that the images are original with no duplication, and have not been previously published in whole or in part.

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