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Posted Date: 27 August 2024

doi: 10.20944/preprints202408.1912.v1

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Article

Intratumoral Microbes in Oral Squamous Cell Carcinoma: Focus on *Treponema denticola*, *Lactobacillus casei*, and *Candida albicans*

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Subtitle: **Potential Microbial Biomarkers for OSCC**

Abstract: Aim: In this study, we aimed to explore the oral bacteria and fungi that can help discern oral squamous cell carcinoma (OSCC) and investigate the correlations between multiple key pathogens. **Methods:** Twelve participants (8 females and 4 males; mean age, 54.33 ± 20.65 years) were prospectively recruited into three groups: Group 1: healthy control, Group 2: patients with stomatitis, and Group 3: patients with OSCC, with 4 individuals in each group. Unstimulated whole saliva samples from these participants were analyzed using real-time PCR to assess the presence and abundance of 14 major oral bacterial species and *Candida albicans*. **Results:** The analysis revealed significant differences for certain microorganisms, namely *Treponema denticola (T. denticola)*, *Lactobacillus casei (L. casei)*, and *Candida albicans*. *T. denticola* was most abundant in the OSCC group (5,358,692.95 ± 3,540,767.33), compared to the stomatitis (123,355.54 ± 197,490.86) and healthy control (9,999.21 ± 11,998.40) groups. *L. casei* was undetectable in the healthy control group, but was significantly more abundant in the stomatitis group (1,653.94 ± 2,981.98) and even higher in the OSCC group (21,336.95 ± 9,258.79) ($p = 0.001$). A similar trend was observed for *C. albicans*, with DNA copy numbers rising from the healthy control (464.29 ± 716.76) to the stomatitis (1,861.30 ± 1,206.15) to the OSCC group (9,347.98 ± 5,128.54) ($p = 0.006$). The amount of *T. denticola* was positively correlated with *L. casei* ($r=0.890$, $p<0.001$) and *C. albicans* ($r=0.724$, $p=0.008$). *L. casei*'s DNA copy number was strongly correlated with *C. albicans* ($r=0.931$, $p<0.001$). These three oral microbes exhibited strong positive correlations with each other and had various direct or indirect relationships with other species. **Conclusions:** In the OSCC group, *T. denticola*, *L. casei*, and *C. albicans* exhibited strong positive correlations with one another, further emphasizing the need for a deeper understanding of the complex microbial interactions in the OSCC environment.

Keywords: microbiome; oral squamous cell carcinoma; bacteria; fungi; *Candida albicans*; oral cancer

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Introduction

Approximately 38 trillion microorganisms, including bacteria, fungi, viruses, and protozoa, coexist in humans, and their numbers roughly equal those of human cells [1]. These microorganisms play crucial roles in various physiological and pathological processes, including cancer [2]. The concept of intratumoral microbes has emerged to describe microorganisms residing within the tumor microenvironment [3]. These microbes are located within or adjacent to tumor tissues and have been shown to influence various aspects of tumor biology. Their presence can affect tumor development, progression, and therapeutic responses. The roles of these intratumoral microbes in cancer are gaining recognition as they can modulate the tumor's biological behavior and interact with the host's immune system [4]. Understanding these interactions is critical for advancing cancer research and for developing novel therapeutics.

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Oral squamous cell carcinoma (OSCC) affects the oral cavity and oropharynx and is the most common form of head and neck cancer. OSCC accounts for more than 90% of cancer cases in this region [5]. It can occur anywhere in the mouth, including the tongue, upper and lower gums, floor of the mouth, palate, and buccal mucosa [6]. Globally, OSCC is one of the most prevalent human malignant tumors, responsible for 1–4% of all cancers and contributing to 2.4% of all cancer-related deaths, reflecting its high mortality rate [7]. Nonetheless, survival rates for oral cancer have significantly improved, increasing by approximately 27% from the mid-1970s to 2018, according to data from the National Institutes of Health [8]. Currently, the overall 5-year survival rate for individuals with oral cancer is 68%, although this rate varies depending on factors such as sex, race, and cancer stage [9]. While smoking and alcohol consumption are the most common risk factors, other etiological factors, such as genetic predisposition and interactions between the host and microorganisms, remain incompletely understood. Mounting evidence suggests that oral microbes play crucial roles in the initiation and progression of oral cancer [10–12]. Improvements in mortality and treatment outcomes have been supported by advancements in diagnostic tools that allow earlier detection and prevention of disease progression.

Numerous oral microbes have been implicated in OSCC pathogenesis. The oral cavity harbors a highly diverse and complex microbiome, second only to the gut in terms of microbial richness, comprising over 700 bacterial species alongside fungi, viruses, and protozoa [13]. Although the presence of bacteria in human tumors was first documented over a century ago, characterization of the tumor-associated microbiome has proven challenging owing to its low biomass. In 2020, Nejman et al. conducted a comprehensive and rigorous analysis of bacterial communities across various human tumors, including those of the breast, lung, ovary, pancreas, melanoma, bone, and brain, demonstrating that distinct bacterial profiles are associated with specific cancer types [14]. Additionally, the detection of fungi within multiple tumor types highlights the need to further investigate the role of intratumoral fungi in cancer diagnosis and prognosis [15]. However, research has not focused on identifying OSCC-specific oral microbial profiles or elucidating the microbial shifts that occur as a healthy oral cavity progresses to a premalignant state or early-stage OSCC. Such investigations are pivotal for advancing our understanding of OSCC, particularly in the areas of early detection and prevention of disease and development of targeted therapeutic strategies.

Several key factors influence the development and progression of OSCC, including oral and systemic health status, immune responses, and microbial dysbiosis of the oral cavity. This study specifically focused on oral microbes. The primary objective of this study was to present the microbial profiles of patients with OSCC by investigating 15 oral microbes, including 14 predominantly detected oral bacteria and 1 fungus, and to elucidate the correlations among these microbes. The hypothesis of this study was that, instead of a single species being OSCC-specific, certain species may serve as keystone members in forming a pathological microbial network. In addition, we reviewed the significance of OSCC-specific intratumoral microbes.

Materials and methods

Study population

Twelve participants (8 females and 4 males; mean age, 54.33 ± 20.65 years) voluntarily participated in this study at the Kyung Hee University Dental Hospital, recruited through advertising between October 1, 2023, and June 30, 2024. The research protocol for this study was reviewed for compliance with the Declaration of Helsinki and approved by the Institutional Review Board of Kyung Hee University Dental Hospital in Seoul, South Korea (KHD IRB, IRB No-KH-DT20030). Informed consent was obtained from all participants. The participants were divided into three groups: Group 1—healthy controls (3 females and 1 male, 28.25 ± 3.86 years), Group 2—patients with stomatitis (2 females and 2 males, 59.75 ± 5.74 years), and Group 3—patients with OSCC (3 females and 1 male, 75.00 ± 1.82 years). The health status of all participants was assessed by examining oral tissues, including the periodontal tissues and buccal mucosa, as well as general conditions such as oral hygiene and dental calculus deposition.

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1) Inclusion criteria: Participants were required to voluntarily read, understand, and sign the consent form and be capable of participating in the study. Group 1 consisted of medically healthy adults with healthy periodontal and oral mucosal conditions, fewer than two missing teeth in the permanent dentition, and intact oral mucosal integrity. Group 2 comprised patients with stomatitis, characterized by inflammation of the oral mucosa affecting the mouth and lips, with or without oral ulceration. Group 3 comprised patients with OSCC, with inclusion limited to those whose OSCC was confirmed by pathological examination following an incisional biopsy.

2) Exclusion criteria: Individuals with severe xerostomia who were unable to produce 2 mL of saliva, pregnant or lactating women, adults who did not comply with clinical examination or sample collection protocols, and those with insufficient data or who withdrew from the study for any reason were excluded.

Collection of unstimulated whole saliva

For microbial analysis, 2 mL of unstimulated whole saliva was collected from all participants using the spitting method. For patients with stomatitis and OSCC, saliva samples were collected before any treatment. Before the saliva sampling session, the participants were instructed to refrain from consuming caffeine and/or nicotine for at least 4 h and alcohol for at least 24 h. All participants were instructed to abstain from eating, drinking, or brushing their teeth before saliva collection. Unstimulated whole saliva samples were collected between 9:30 and 11:30 a.m. to minimize diurnal variability, with an average time of 3 h between waking up and collection.

Contamination prevention

To minimize contamination, stringent protocols were followed throughout the saliva sample collection and microbial identification processes. The researchers wore masks, sanitized their hands, and used disinfected dental gloves, which were replaced between participants. The experimental table was cleaned with alcohol before each experiment. All equipment and reagents that came into direct contact with the samples, such as pipette tips and tubes, were sterilized before use and discarded after a single use. To avoid aerosol contamination, a centrifuge with a closed lid was used, and the reagent and reaction preparations for PCR were conducted on a clean bench.

Identification and quantification of oral bacterial and fungal species

The bacterial DNA quantity, community composition, and individual abundance of oral bacterial species were determined. In saliva samples, the absolute amount and abundance of 14 bacteria were assessed, namely, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Prevotella nigrescens*, *Eikenella corrodens*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Lactobacillus casei*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Parvimonas micra*, and *Eubacterium nodatum*. Additionally, *Candida albicans* was identified as a representative fungal species. The detailed methods for the identification and quantification of oral bacterial and fungal species are as follows.

1) Oral microbe DNA isolation

The saliva samples were first subjected to vigorous vortexing to ensure thorough mixing. Subsequently, 500 μL from each sample was then combined with 500 μL of lysis buffer (5 mM EDTA, 5 M guanidine hydrochloride, and 0.3 M sodium acetate) in a tube. The mixture was vortexed and incubated at 65°C for 10 min. Thereafter, 20 μL of S2 buffer (0.25 g/mL silicon dioxide; Merck KGaA, Darmstadt, Germany) was added to the sample-lysis buffer mixture, which was then vortexed and incubated at room temperature for 5 min, with periodic inversion using an automatic system. The mixture was centrifuged at 5,000 rpm for 30 s, and the supernatant was carefully removed. Subsequently, 1 mL of PureLink (Invitrogen Corporation, Carlsbad, CA, USA) and PCR purification washing buffer 1 (50 mM 3-(N-morpholino) propanesulfonic acid buffer, pH 7.0, with 1 M sodium chloride) activated with 160 mL of 100% ethanol were added to the tube. The contents were vortexed until the beads were completely resuspended. After another 30 s centrifugation at 5,000 rpm, the supernatant was carefully removed. Next, 1,000 μL of ethanol wash buffer 2 was added, and the beads were resuspended by vortexing. The tube was then centrifuged at 5,000 rpm for 30 s, and the supernatant was removed. To elute the DNA, 100 μL of elution buffer (100 mM Tris-HCl, pH 7.5, and 1 M EDTA) was added to the tube, vortexed, and incubated at 65°C for 10 min. DNA was then

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isolated. For PCR analysis, the samples were centrifuged at 13,000 rpm for 5 min, and the supernatant was transferred to a sterile microcentrifuge tube.

2) Real-time PCR (qPCR) amplification

qPCR amplification was performed to detect 14 salivary bacterial species using species-specific primers. Total bacterial quantities were assessed using 16S ribosomal RNA (rRNA) primers designed to target each bacterium. The primers used were consistent with those used in our previous study [16]. To quantify the total bacterial DNA, a conserved 16S rRNA primer probe was used. To amplify *C. albicans*, primers were selected based on previous studies [17] and verified through in silico analyses. A reaction mixture was prepared by combining 5 μL of DNA template, 2.5 μM of each forward and reverse primer, and 10 μL of 2X master mix (GeNet Bio, Daejeon, Korea). Subsequently, 20 μL of this mixture was used for qPCR. The qPCR thermal cycling protocol included an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The positive controls comprised plasmid DNA from each target bacterium and *C. albicans*, whereas DNase/RNase-free water was used as a negative control.

3) Calculation of DNA copy numbers for oral bacteria and C. albicans

Samples were prepared by mixing 2 mL of saliva with 2 mL of the sample stock solution. For DNA extraction, 500 μL of this preservation solution was used, and 100 μL of the extract was finally eluted. qPCR was conducted using 5 μL of the total 100 μL solution. DNA quantification was performed using the standard curve method, with the analysis based on a 5-μL aliquot. The DNA copy number was calculated for 1 mL of saliva and 20 mL of the preservation solution. The preservation solution consisted of Tris-HCl, urea, sodium acetate, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, sodium ascorbate, and ethanol.

4) α-Diversity based on Shannon's diversity index

The α-diversity of microbial communities was evaluated using Shannon's diversity index, with bacterial richness determined by the total number of bacterial DNA copies. Shannon's diversity index was calculated using the following formula:

$$H = -\sum (pi \times \ln(pi))$$

where pi represents the relative abundance of the ith species, calculated as n/N, where n is the number of individuals of the ith species and N is the total number of individuals across all species in the community. Shannon's diversity index H measures diversity, where a value of zero indicates no diversity, and higher values signify greater diversity [18,19].

Statistical analyses

Descriptive statistics are reported as means ± standard deviations or numbers with percentages, as appropriate. To analyze the distribution of discontinuous data, we used χ^2 tests for equality of proportions, Fisher's exact tests, and Bonferroni tests. Analysis of variance (ANOVA) was used to investigate differences in the mean values related to the oral microbiome among the three groups. Spearman's correlation analysis was used to determine the correlations between the variables, and the correlation coefficient (r) was closer to the absolute value of 1, indicating a stronger correlation. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 26.0; IBM Corp., Armonk, NY, USA) and R Version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at a two-tailed p-value < 0.05.

Results

Demographics and oral examination

Among the three groups in this study, the ages of patients with stomatitis (59.75 ± 5.74 years) and OSCC (75.00 ± 1.82 years) were significantly higher than those of the healthy control group (28.25 ± 3.86 years). Furthermore, the age of the patients with OSCC was significantly higher than that of the patients with stomatitis ($p < 0.001$). There was no statistically significant difference in the female-to-male ratio among the groups, which was 1:1 in the stomatitis group and 3:1 in the OSCC group. Considering the lesion site, visual inspection, X-ray, and CT scans revealed that 100% of the lesions in the stomatitis group were located in the buccal mucosa, while 100% of the lesions in the OSCC

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group were found in the mandible. During oral examinations, the incidence of periodontitis was significantly higher in the OSCC group than in the healthy control group (0.0% vs. 70%, p = 0.018). However, there were no differences between the groups regarding the presence of calculus deposition or poor oral hygiene. In the OSCC group, calculus deposition was observed in 25% of the patients, and poor oral hygiene was noted in 50% of the patients (Table 1).

Table 1. Demographics and oral examination.

Parameters	Healthy control mean ± SD or n (%)	Stomatitis mean ± SD or n (%)	OSCC mean ± SD or n (%)	p-value	post-hoc
Age (years) [a]	28.25 ± 3.86	59.75 ± 5.74	75.00 ± 1.82	**<0.001*****	Healthy control < Stomatitis and OSCC, Stomatitis < OSCC
Sex [b]					
Female	3 (75.0%)	2 (50.0%)	3 (75.0%)	0.687	
Male	1 (25.0%)	2 (50.0%)	1 (25.0%)		
Lesion site					
Buccal mucosa [b]	0 (0.0%)	4 (100.0%)	0 (0.0%)	**<0.001*****	
Mandible [b]	0 (0.0%)	0 (0.0%)	4 (100.0%)	**<0.001*****	
Oral examination					
Periodontitis [b]	0 (0.0%)	2 (50.0%)	3 (75.0%)	**0.018***	
Calculus deposition [b]	0 (0.0%)	1 (25.0%)	1 (25.0%)	0.549	
Poor oral hygiene [b]	0 (0.0%)	0 (0.0%)	2 (50.0%)	0.091	

The results were obtained using: a: Analysis of variance (ANOVA) and post-hoc analysis. b: Chi-square test (two-sided). Statistical significance was set at p < 0.05. Significant differences are indicated in bold. *p < 0.05, **p < 0.01, ***p < 0.001; SD: standard deviation. Healthy control: healthy control participants; Stomatitis: patients with stomatitis; OSCC: patients with oral squamous cell carcinoma.

DNA copy numbers of bacterial and fungal species

The DNA copy numbers of 14 bacteria and 1 fungus were compared across the healthy control, stomatitis, and OSCC groups (Table 2). Among these, *T. denticola, L. casei,* and *C. albicans* exhibited statistically significant differences between groups (all p < 0.05). Specifically, *T. denticola* was found in the highest quantity in the OSCC group (5,358,692.95 ± 3,540,767.33) compared to that in the stomatitis (123,355.54 ± 197,490.86) and healthy control (9,999.21 ± 11,998.40) groups, with a significant increase in DNA copy numbers in the stomatitis group relative to the healthy control group (p = 0.007). *L. casei* was not detected in the healthy control group, but it was significantly more abundant in the stomatitis group (1,653.94 ± 2,981.98) and even higher in the OSCC group (21,336.95 ± 9,258.79) (p = 0.001). A similar pattern was observed for *C. albicans*, with DNA copy numbers increasing from the healthy control (464.29 ± 716.76) to the stomatitis (1,861.30 ± 1,206.15) to the OSCC group (9,347.98 ± 5,128.54) (p = 0.006). *A. actinomycetemcomitans* was not detected in any group. Aside from *T. denticola, L. casei,* and *C. albicans*, no significant quantitative differences were observed among the other 12 microbial species across the groups (Figure 1).

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Table 2. Comparison of DNA copies of bacterial and fungal species, and Shannon's diversity index.

Microbial parameter		Healthy control mean ± SD	Stomatitis mean ± SD	OSCC mean ± SD	p-value	post-hoc
Gram (-)	*Aa*	0 ± 0	0 ± 0	0 ± 0	NA	
	Pi	4721.43 ± 9338.45	97123.55 ± 163965.30	0 ± 0	0.311	
	Pn	313182.60 ± 162170.03	1783400.78 ± 1859234.18	7444085.88 ± 7776100.68	0.124	
	Ec	161638.36 ± 73445.12	497355.67± 376410.96	5455613.30 ± 5929438.11	0.101	
	Cr	17702.63 ± 19738.29	431907.02 ± 725172.58	869577.08 ± 745262.35	0.189	
	Fn	1414677.53 ± 1248019.45	5425576.88 ± 6134874.96	3928871.25 ± 4209210.50	0.453	
	Pg	2076.07 ± 1336.47	7803707.73 ± 10628172.40	2501079.28 ± 1786087.94	0.247	
	Td	**9999.21 ± 11998.40**	**123355.54 ± 197490.86**	**5358692.95 ± 3540767.33**	**0.007****	**Healthy control < Stomatitis and OSCC, Stomatitis< OSCC**
	Tf	35267.53 ± 25096.14	821889.97 ± 910730.89	1150570.3 ± 721737.42	0.106	
Gram (+)	***Lc***	**0 ± 0**	**1653.94 ± 2981.98**	**21336.95 ± 9258.79**	**0.001****	**Healthy control < Stomatitis and OSCC, Stomatitis< OSCC**
	Sm	3258.15 ± 3492.39	18238.71 ± 35986.69	100785.83 ± 181256.31	0.416	
	Ss	0 ± 0	641.81 ± 1283.62	0 ± 0	0.405	
	Pm	88593.26 ± 81242.44	112672.78 ± 85587.27	13204631.90 ± 15038585.05	0.098	
	En	169.37 ± 226.55	22360.99 ± 32330.07	38018.78 ± 37252.07	0.223	
Fugus	***Ca***	**464.29 ± 716.76**	**1861.30 ± 1206.15**	**9347.98 ± 5128.54**	**0.006****	**Healthy control < Stomatitis and OSCC, Stomatitis< OSCC**
Gram (-)		1959265.36 ± 1372737.86	16984317.46 ± 14157382.83	26708490.05 ± 22693303.84	0.128	
Gram (+)		92020.78 ± 82510.78	155568.13 ± 131036.11	13364773.45 ± 14988291.76	0.093	
DNA copies of 15 microbes		2051750.43 ± 1394889.68	17141746.89 ± 14070899.96	40082611.47 ± 37609107.97	0.119	
Total bacteria		246015329.80 ± 250216570.66	671981670.25 ± 645482366.81	827473263.93 ± 781237168.13	0.406	
Shannon's diversity index		1.17 ± 0.35	1.42 ± 0.42	1.85 ± 1.26	0.502	

The results were obtained using analysis of variance (ANOVA) and post-hoc analysis. Differences between groups were considered significant at $p < 0.05$. Significant differences are indicated in bold. **$p < 0.01$; SD: standard deviation. Healthy control: healthy control participants; Stomatitis: patients with stomatitis; OSCC: patients with oral squamous cell carcinoma. *Aa, Aggregatibacter actinomycetemcomitans; Pi, Prevotella intermedia;*

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Pn, Prevotella nigrescens; Ec, Eikenella corrodens; Cr, Campylobacter rectus; Fn, Fusobacterium nucleatum; Pg, Porphyromonas gingivalis; Td, Treponema denticola; Tf, Tannerella forsythia; Lc, Lactobacillus casei; Sm, Streptococcus mutans; Ss, Streptococcus sobrinus; Pm, Parvimonas micra; En, Eubacterium nodatum; Ca, Candida albicans. Gram (−): gram-negative species (*Aa, Pi, Pn, Ec, Cr, Fn, Pg, Td*, and *Tf*); Gram (+): gram-positive species (*Lc, Sm, Ss, Pm*, and *En*).

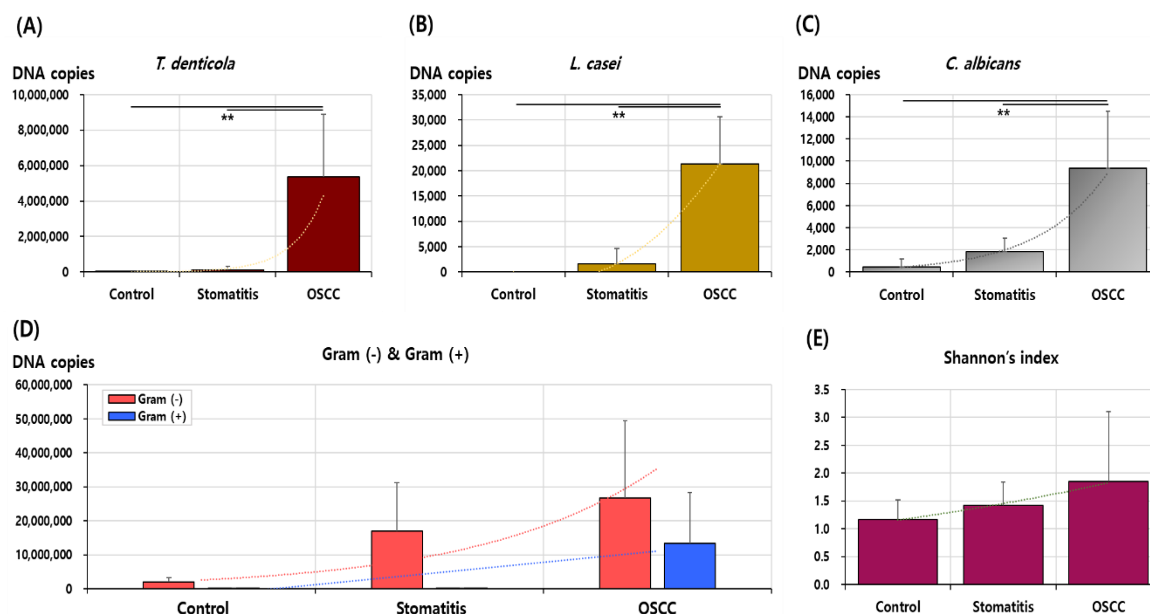

Figure 1. Comparison of DNA copy numbers of oral bacteria and *Candida albicans* between groups.

Total bacterial amount and Shannon's diversity index

The total bacterial DNA copies, measured using a universal probe for oral bacteria, followed the pattern of healthy control (246,015,329.80 ± 250,216,570.66) < stomatitis (671,981,670.25 ± 645,482,366.81) < OSCC (40,082,611.47 ± 37,609,107.97), with no statistically significant differences (p = 0.406). The mean total DNA copies of gram-negative microorganisms followed the trend of healthy control (1,959,265.36 ± 1,372,737.86) < stomatitis (16,984,317.46 ± 14,157,382.83) < OSCC (26,708,490.05 ± 22,693,303.84), although no statistically significant differences were observed between the groups (p = 0.128). Similarly, the total DNA copy numbers of gram-positive microorganisms increased from the healthy control (92,020.78 ± 82,510.78) to the stomatitis (1,861.30 ± 1,206.15) to the OSCC group (9,347.98 ± 5,128.54), yet these differences were not statistically significant (p = 0.119). Shannon's diversity index also increased from the healthy control (1.17 ± 0.35) to the stomatitis (1.42 ± 0.42) to the OSCC group (1.85 ± 1.26), but the difference was not statistically significant (p = 0.502) (Table 2 and Figure 1). In general, Shannon's diversity index falls between 1.5 and 3.5, with values above 4.5 being rare [18].

Prevalence of bacterial and fungal species

The comparison of the prevalence of 15 oral microbes, including 14 oral bacteria and 1 fungus, across the three study groups revealed several significant differences. First, the proportion of *F. nucleatum* relative to the total DNA copies of the 15 oral microorganisms analyzed was significantly higher in the healthy control group (60.47 ± 21.04%) than in the stomatitis (30.82 ± 23.06%) and OSCC groups (7.75 ± 3.62%) (p = 0.008). Second, *P. gingivalis* exhibited a significantly different distribution among the groups, with the highest prevalence in the stomatitis group (43.45 ± 24.93%), followed by the OSCC group (16.39 ± 23.18%) and the healthy control group (0.17 ± 0.17%) (p = 0.035) (Table 3).

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Table 3. Comparison of the prevalence of 14 oral bacterial and 1 fungal species.

Microbial parameters		Healthy control mean ± SD	Stomatitis mean ± SD	OSCC mean ± SD	p-value	post-hoc
Gram (−)	*Aa*	0 ± 0	0 ± 0	0 ± 0	NA	
	Pi	0.47 ± 0.93	1.37 ± 1.86	0 ± 0	0.311	
	Pn	19.88 ± 16.99	11.09 ± 6.83	16.91 ± 7.35	0.561	
	Ec	9.65 ± 6.59	2.78 ± 1.55	8.64 ± 7.23	0.239	
	Cr	1.37 ± 2.07	1.53 ± 1.94	4.17 ± 5.11	0.451	
	Fn	60.47 ± 21.04	30.82 ± 23.06	7.75 ± 3.62	**0.008****	Healthy control > Stomatitis > OSCC
	Pg	0.17 ± 0.17	43.45 ± 24.93	16.39 ± 23.18	**0.035***	Healthy control < Stomatitis, OSCC < Stomatitis
	Td	1.05 ± 1.28	1.32 ± 2.33	20.96 ± 27.89	0.192	
	Tf	1.98 ± 1.72	4.74 ± 2.31	5.28 ± 5.15	0.379	
Gram (+)	*Lc*	0 ± 0	0.05 ± 0.09	0.14 ± 0.15	0.195	
	Sm	0.22 ± 0.27	0.58 ± 1.15	0.77± 1.52	0.783	
	Ss	0 ± 0	0.02 ± 0.04	0 ± 0	0.405	
	Pm	4.68 ± 4.26	1.67 ± 2.04	18.85 ± 20.05	0.148	
	En	0.01 ± 0.01	0.57 ± 1.08	0.07 ± 0.05	0.415	
Fungus	*Ca*	0.04 ± 0.07	0.02 ± 0.02	0.07 ± 0.10	0.658	

The results were obtained using analysis of variance (ANOVA) and post-hoc analysis. Differences between groups were considered significant at $p < 0.05$. Significant differences are indicated in bold. *$p < 0.05$, **$p < 0.01$; SD: standard deviation. Healthy control: healthy control participants; Stomatitis: patients with stomatitis; OSCC: patients with oral squamous cell carcinoma. *Aa, Aggregatibacter actinomycetemcomitans; Pi, Prevotella intermedia; Pn, Prevotella nigrescens; Ec, Eikenella corrodens; Cr, Campylobacter rectus; Fn, Fusobacterium nucleatum; Pg, Porphyromonas gingivalis; Td, Treponema denticola; Tf, Tannerella forsythia; Lc, Lactobacillus casei; Sm, Streptococcus mutans; Ss, Streptococcus sobrinus; Pm, Parvimonas micra; En, Eubacterium nodatum; Ca, Candida albicans.* Gram (−): gram-negative species (*Aa, Pi, Pn, Ec, Cr, Fn, Pg, Td*, and *Tf*); Gram (+): gram-positive species (*Lc, Sm, Ss, Pm*, and *En*).

When ranking the mean prevalence of the analyzed microorganisms within each group from highest to lowest, the following patterns were observed:

(1) Healthy control group: *F. nucleatum* > *P. nigrescens* > *E. corrodens* > *P. micra* > *T. forsythia* > *C. rectus* > *T. denticola* > *P. intermedia* > *S. mutans* > *P. gingivalis* > *C. albicans* > *E. nodatum* > *S. sanguinis* ≈ *L. casei* > *A. actinomycetemcomitans*.

(2) Stomatitis groups were as follows: *P. gingivalis*, *F. nucleatum*, *P. nigrescens*, *T. forsythia* > *E. corrodens*, *C. rectus*, *T. denticola*, *P. micra*, *P. intermedia*, *E. nodatum*, *S. mutans*, *L. casei*, *S. sanguinis*, *A. actinomycetemcomitans*.

(3) OSCC group: *P. micra* > *P. nigrescens* > *E. corrodens* > *T. denticola* > *F. nucleatum* > *P. gingivalis* > *T. forsythia* > *C. rectus* > *S. mutans* > *E. nodatum* > *L. casei* > *C. albicans* > *S. sanguinis* ≈ *P. intermedia* > *A. actinomycetemcomitans*.

Notably, the most prevalent oral bacterial species varied among the groups. *F. nucleatum* was the most prevalent in the healthy control group (60.47%), *P. gingivalis* in the stomatitis group (43.45%), and *P. micra* in the OSCC group (18.85%) (Figure 2A). When the presence of each microbe was analyzed, *T. denticola*, *P. micra*, *L. casei*, *S. mutans*, and *C. albicans* were found to be more prevalent in the OSCC group than in the other groups (Figure 2B).

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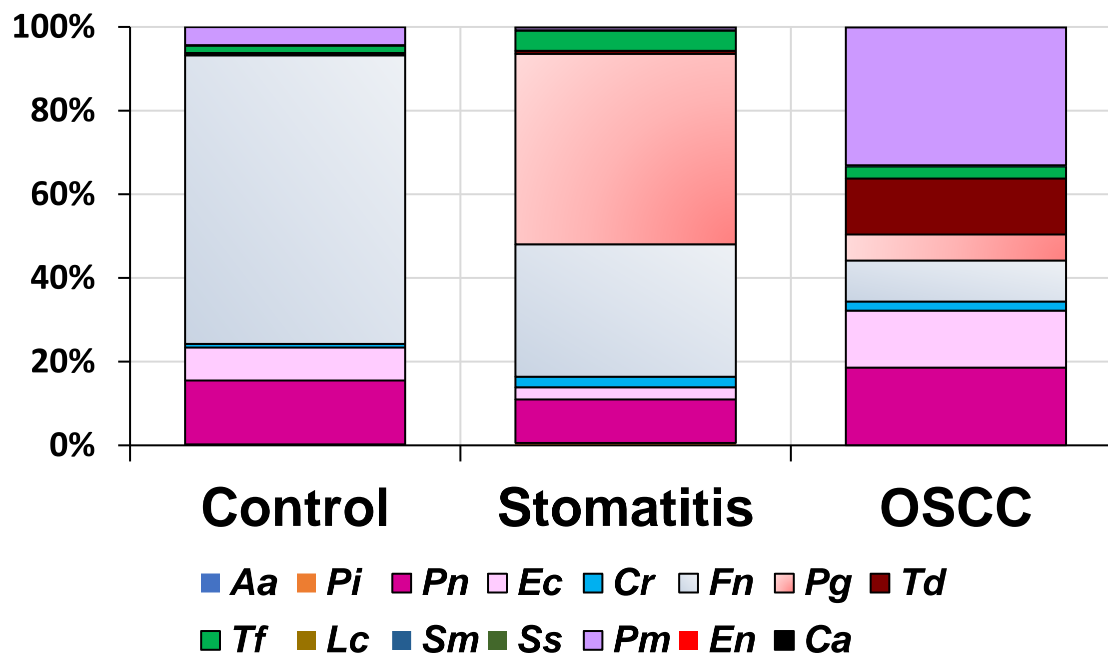
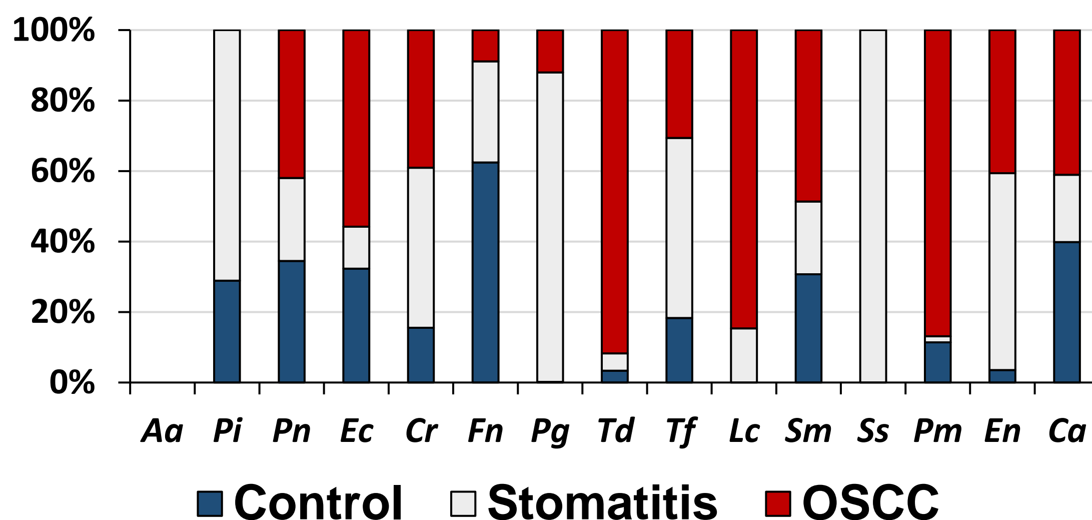


(A) Prevalence of each species by group



(B) Percentage distribution of groups for each microbe

Figure 2. Group-wise comparison of the prevalence of each oral microbe. *Aa, Aggregatibacter actinomycetemcomitans; Pi, Prevotella intermedia; Pn, Prevotella nigrescens; Ec, Eikenella corrodens; Cr, Campylobacter rectus; Fn, Fusobacterium nucleatum; Pg, Porphyromonas gingivalis; Td, Treponema denticola; Tf, Tannerella forsythia; Lc, Lactobacillus casei; Sm, Streptococcus mutans; Ss, Streptococcus sobrinus; Pm, Parvimonas micra; En, Eubacterium nodatum; Ca, Candida albicans.*

Correlations among oral microbes

Spearman's correlation analysis was conducted to examine the relationship between the DNA copies of oral microbes. Importantly, several significant positive correlations were observed between gram-negative and gram-positive bacteria and fungi (Table 4).

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Table 4. Correlation between DNA copies of oral microbes.

Correlation coefficient		Gram (−)							Gram (+)					Fungus
		Pn	*Ec*	*Cr*	*Fn*	*Pg*	*Td*	*Tf*	*Lc*	*Sm*	*Ss*	*Pm*	*En*	*Ca*
Gram (−)	*Pi*	-0.193	-0.160	-0.215	-0.129	-0.006	-0.176	-0.075	-0.233	-0.115	0.045	-0.163	-0.151	-0.112
	Pn		**0.980**	**0.780**	**0.612**	0.097	**0.708**	0.498	**0.849**	-0.130	-0.165	**0.974**	**0.761**	**0.878**
	Ec			**0.790**	0.465	0.087	**0.752**	0.522	**0.884**	-0.091	-0.158	**0.998**	**0.755**	**0.909**
	Cr				0.410	**0.657**	0.515	**0.788**	**0.653**	-0.175	-0.208	**0.761**	0.497	**0.790**
	Fn					0.183	0.198	0.290	0.243	-0.264	-0.220	0.434	0.372	0.277
	Pg						-0.068	**0.698**	-0.058	-0.167	-0.086	0.036	-0.051	0.120
	Td							**0.634**	**0.890**	0.575	-0.180	**0.742**	0.572	**0.724**
	Tf								0.524	0.337	-0.220	0.472	0.296	0.522
Gram (+)	*Lc*									0.265	-0.043	**0.889**	**0.751**	**0.931**
	Sm										0.093	-0.104	0.049	-0.038
	Ss											-0.134	0.500	-0.138
	Pm												**0.766**	**0.913**
	En													**0.674**

The results were obtained through Spearman's correlation analysis. Differences between groups were considered significant at p-value < 0.05. Statistically significant results are highlighted in bold. *Pi, Prevotella intermedia; Pn, Prevotella nigrescens; Ec, Eikenella corrodens; Cr, Campylobacter rectus; Fn, Fusobacterium nucleatum; Pg, Porphyromonas gingivalis; Td, Treponema denticola; Tf, Tannerella forsythia; Lc, Lactobacillus casei; Sm, Streptococcus mutans; Ss, Streptococcus sobrinus; Pm, Parvimonas micra; En, Eubacterium nodatum; Ca, Candida albicans.* Gram (−): gram-negative species (*Pi, Pn, Ec, Cr, Fn, Pg, Td,* and *Tf*); Gram (+): gram-positive species (*Lc, Sm, Ss, Pm,* and *En*). Deeper shades of red indicate correlations closer to +1, while deeper shades of blue signify correlations closer to −1. *Aggregatibacter actinomycetemcomitans* was not detected in the real-time PCR and was therefore excluded from this correlation analysis.

Based on DNA copy numbers, *T. denticola, L. casei,* and *C. albicans* were the oral microbes that exhibited significantly higher levels in the OSCC group; therefore, we focused on the relationships between these species. First, *T. denticola, L. casei,* and *C. albicans* showed strong positive correlations. Considering the strength of the correlations with *T. denticola*, *T. denticola* was positively correlated with *L. casei* ($r = 0.890$, $p < 0.001$), *E. corrodens* ($r = 0.752$, $p = 0.005$), *P. micra* ($r = 0.742$, $p = 0.006$), *C. albicans* ($r = 0.724$, $p = 0.008$), *P. nigrescens* ($r = 0.708$, $p = 0.010$), and *T. forsythia* ($r = 0.634$, $p = 0.027$). Species that were positively correlated with *L. casei* DNA copy numbers presented with the following correlation strengths: *C. albicans* ($r = 0.931$, $p < 0.001$) > *T. denticola* ($r = 0.890$, $p < 0.001$) > *P. nigrescens* ($r = 0.849$, $p < 0.001$) > *P. micra* ($r = 0.889$, $p < 0.001$) > *E. corrodens* ($r = 0.849$, $p < 0.001$), *E. nodatum* ($r = 0.751$, $p = 0.005$) > *C. rectus* ($r = 0.653$, $p = 0.021$). The oral microbes that were positively correlated with *C. albicans* DNA copy numbers were *L. casei* ($r = 0.931$, $p < 0.001$), *P. micra* ($r = 0.913$, $p < 0.001$), *E. corrodens* ($r = 0.909$, $p < 0.001$), *P. nigrescens* ($r = 0.878$, $p < 0.001$), *C. rectus* ($r = 0.790$, $p = 0.002$), *T. denticola* ($r = 0.724$, $p = 0.008$), and *E. nodatum* ($r = 0.674$, $p = 0.016$). Additionally, *P. micra*, which was the most abundant in the OSCC group compared to that in the healthy control and stomatitis groups, was positively correlated with *E. corrodens* ($r = 0.998$, $p < 0.001$), *P. nigrescens* ($r = 0.974$, $p < 0.001$), *C. albicans* ($r = 0.931$, $p < 0.001$), *L. casei* ($r = 0.889$, $p < 0.001$), *C. rectus* ($r = 0.761$, $p = 0.004$), and *T. denticola* ($r = 0.742$, $p = 0.006$). Notably, no significant negative correlations were observed among the microbes, suggesting an inhibition or reduction in the presence of certain species.

In-depth analysis of *T. denticola*, *C. albicans*, and *L. casei*

An in-depth analysis based on the schematic diagram illustrating the direct and indirect relationships between the three prominent microbes in the OSCC group—*T. denticola, C. albicans*, and *L. casei*—revealed the following findings: *T. denticola* was directly correlated with gram-negative species such as *P. nigrescens*, *E. corrodens*, and *P. gingivalis*. *F. nucleatum* exhibited an indirect positive correlation with *T. denticola* via *P. nigrescens*. Although *C. rectus* was not directly correlated with *T. denticola*, it was indirectly correlated with *P. nigrescens*, *E. corrodens*, *T. forsythia*, and *P. gingivalis*. Among the gram-positive species, *T. denticola* showed a direct positive correlation with *L. casei* and

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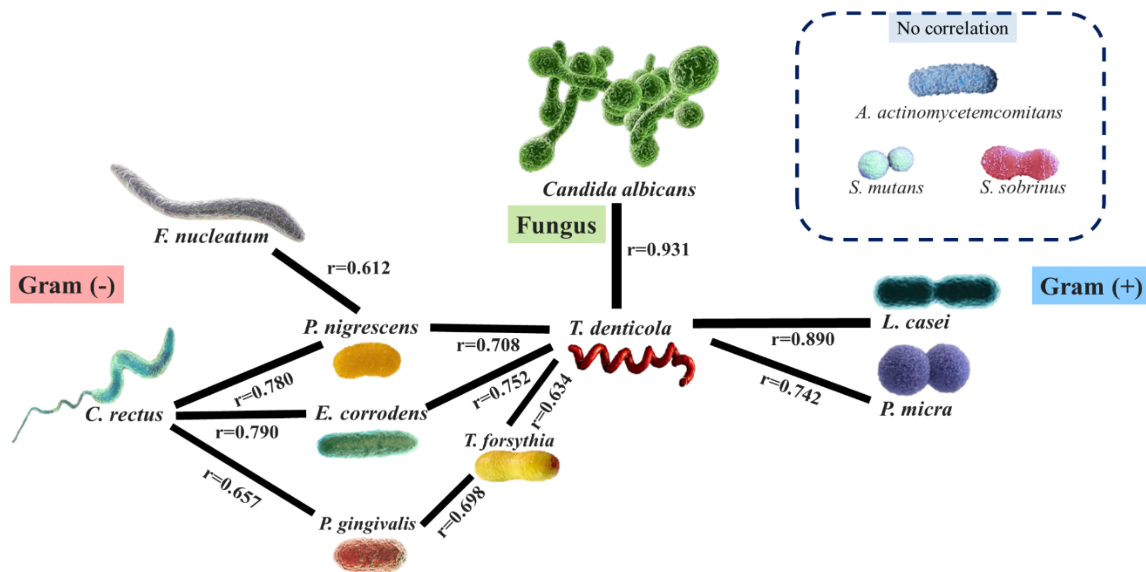
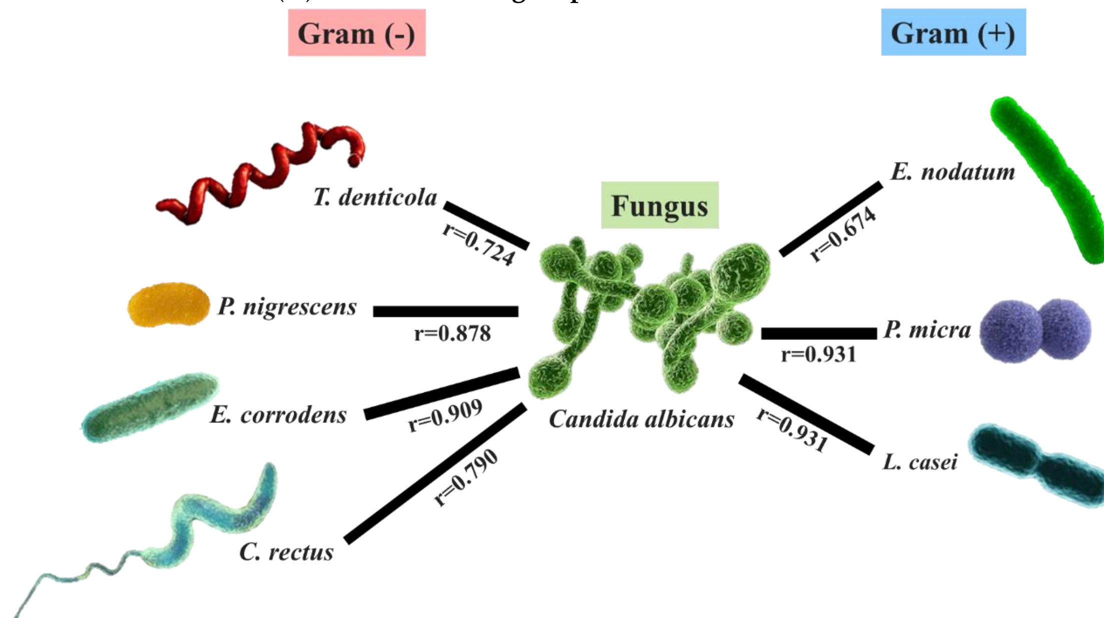
P. micra. A strong, direct positive correlation was observed between *T. denticola* and *C. albicans* (Figure 3A). *C. albicans* was positively correlated with several gram-negative species, including *T. denticola, E. corrodens, P. nigrescens*, and *C. rectus*. Among the gram-positive species, *C. albicans* was significantly and positively correlated with *P. micra*, *E. nodatum*, and *L. casei* (Figure 3B). *L. casei* was positively correlated with several gram-positive species, including *P. nigrescens*, *E. corrodens*, *P. micra*, and *E. nodatum*. Among the gram-negative species, *L. casei* had significant positive correlations with *T. denticola* and *C. rectus* and also showed a strong positive correlation with *C. albicans* (Figure 3C).



(A) Microbial linkages specific to *T. denticola*



(B) Microbial linkages specific to *Candida albicans*

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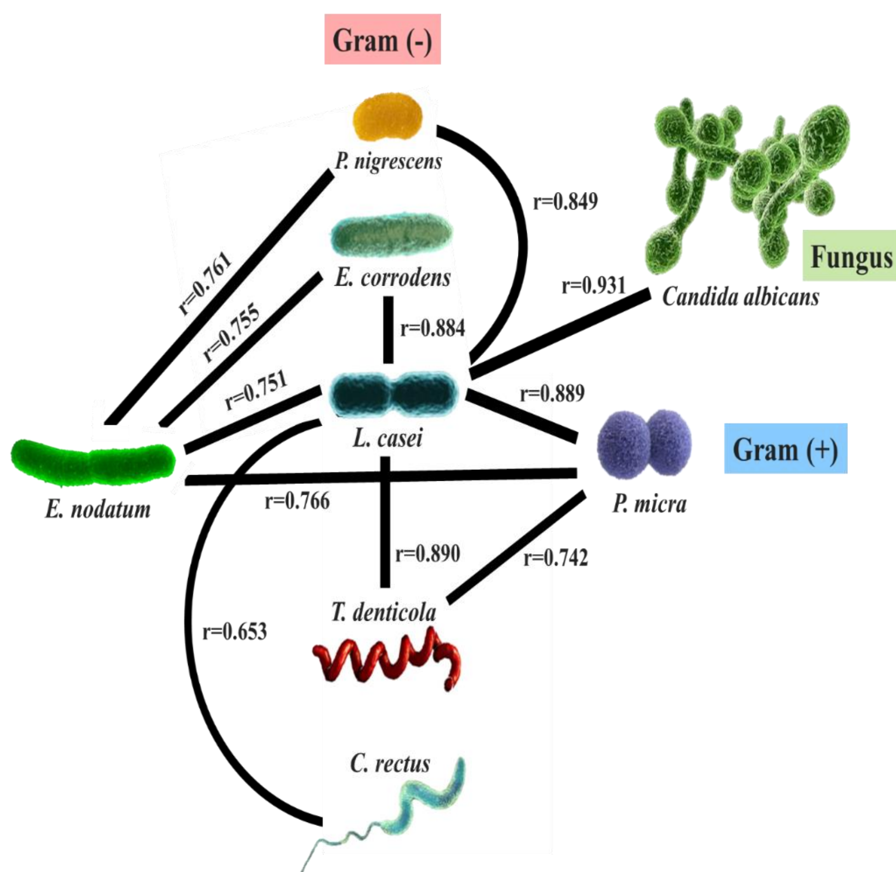

(C) **Microbial linkages specific to *L. casei***

Figure 3. Schematic presentation of the relationships between oral microbes. Based on the results of Spearman's correlation analysis, the diagram illustrates the microbial relationships. The thickness of each bar reflects the strength of the correlation between two oral microbes. *P. intermedia: Prevotella intermedia, P. nigrescens: Prevotella nigrescens, E. corrodens: Eikenella corrodens, C. rectus: Campylobacter rectus, F. nucleatum: Fusobacterium nucleatum, P. gingivalis: Porphyromonas gingivalis, T. denticola: Treponema denticola, T. forsythia: Tannerella forsythia, L. casei: Lactobacillus casei, S. mutans: Streptococcus mutans, S. sobrinus: Streptococcus sobrinus, P. micra: Parvimonas micra, E. nodatum: Eubacterium nodatum. C. albicans: Candida albicans.*

Discussion

In this study, we investigated the distribution of 14 major oral bacteria and *C. albicans* across three groups: healthy controls, patients with stomatitis, and patients with OSCC. Our findings revealed that *T. denticola*, *L. casei*, and *C. albicans* were significantly more abundant in the OSCC group than in the other groups. Specifically, *T. denticola* was present in the highest quantity in the OSCC group, followed by the stomatitis and healthy control groups. *L. casei* was undetectable in healthy controls, but showed a marked increase in both the stomatitis and OSCC groups. Similarly, *C. albicans* exhibited a progressive increase in DNA copy numbers from healthy controls to patients with stomatitis and those with OSCC. Considering the correlations among oral microbes, a wide range of positive associations were observed between gram-negative and gram-negative bacteria, gram-positive and gram-positive bacteria, gram-negative and gram-positive bacteria, *C. albicans* and gram-negative bacteria, and *C. albicans* and gram-positive bacteria. Despite these trends, overall microbial diversity, as measured by the Shannon's diversity index, did not differ significantly between the groups. These results suggest a potential association between these specific microbes and OSCC, warranting further investigation into their roles as OSCC-specific markers.

Among the gram-negative species, *T. denticola* was notably more abundant in the OSCC group than in the healthy control and stomatitis groups. *T. denticola* is a motile, obligate anaerobic bacterium and highly proteolytic spirochete that lives in the oral cavity of humans and is predominantly found

in periodontal lesions associated with adult periodontitis [20]. *T. denticola*, along with *P. gingivalis* and *F. nucleatum*, forms a red complex that plays a crucial role in periodontal disease. Numerous studies have demonstrated an association between periodontitis and OSCC, and periodontal pathogens, such as *T. denticola*, are implicated in the pathogenesis of OSCC [11,21]. In addition, *T. denticola* directly promotes OSCC cell proliferation, and the mechanism has been associated with intracellular TGF-β pathway activation [11]. In the present study, *T. denticola* was directly correlated with gram-negative species, such as *P. gingivalis*, *P. nigrescens*, and *E. corrodens*. *P. nigrescens* plays a bridging role between *T. denticola* and *F. nucleatum*. In a recent study, more abundant *Fusobacteria* (at the phylum level), *Fusobacterium* (at the genus level), and *F. nucleatum* (at the species level) were identified in OSCC patients [22]. *F. nucleatum* is a major pro-tumorigenic bacterium that accumulates at the invasive margins of OSCC tissues and drives tumor-associated macrophage formation [23]. However, it is important to note that the association of these bacteria with OSCC has not been fully established. Further research is required to explore their potential roles in the prognosis and pathogenesis of OSCC.

T. denticola exhibited a direct positive correlation with *L. casei* and *P. micra* and a strong direct positive correlation with *C. albicans*. Additionally, several direct and indirect complex interactions among the oral microbes were observed. Avril et al. proposed a "bacterial driver–passenger" model in colorectal cancer, wherein initial "driver" bacteria induce alterations in the microenvironment, thereby facilitating tumor formation [24]. In the early stages of oral cancer, key pathogens increase, followed by subsequent changes in the associated microbial communities [25]. As the tumor progresses, these driver bacteria are gradually supplanted by "passenger" bacteria, including opportunistic pathogens and commensal or probiotic species. These passenger bacteria may further influence tumor progression through interactions with both the host and existing tumor microenvironment [26]. In patients with OSCC, additional research is required to elucidate driver–passenger microbe relationships and delineate the sequential dynamics of microbial populations within the tumor context.

Among the gram-positive bacteria, *L. casei* has emerged as the most prominent species associated with OSCC. *Lactobacilli* are gram-positive, anaerobic, rod-shaped commensal bacteria typically found in the oral, genitourinary, and gastrointestinal tracts of humans [27]. *Lactobacillus* is one of the most common bacterial genera found in the saliva of individuals with OSCC [28,29]. *L. casei* was positively correlated with several gram-positive species, including *P. nigrescens*, *E. corrodens*, *P. micra*, and *E. nodatum*. Among the gram-negative species, *L. casei* had a significantly strong positive correlation with *T. denticola* and *C. rectus*. Notably, members of the *Lactobacillus* genus exhibit both carcinogenic and anticarcinogenic properties. The production of lactic acid and other organic acids by these bacteria can acidify the tumor microenvironment, potentially promoting OSCC growth [30]. Additionally, variations in the activities of nicotinamide adenine dinucleotide phosphate oxidase and nitric oxide synthase may lead to the accumulation of reactive oxygen and nitrogen species, thereby contributing to chronic inflammation [31]. Some *Lactobacillus* species may exacerbate this process by generating hydrogen peroxide [32]. However, it is crucial to recognize that, while *Lactobacillus* species are prevalent in individuals with OSCC, their presence does not necessarily imply a causative role in the disease [33]. Further research is required to fully elucidate the roles of *Lactobacillus* and other microbes in OSCC development.

C. albicans positively correlated with several gram-negative species, including *T. denticola*, *E. corrodens*, *P. nigrescens*, and *C. rectus*. This study focused on *C. albicans*, which is the fungus most commonly associated with oral diseases. *C. albicans* can cause oral candidiasis, commonly known as thrush [34]. Although extensive research has been conducted on the bacterial components of the OSCC microbiome, fungal components remain relatively underexplored and poorly defined [35]. Nonetheless, *Candida*, a genus of yeast-like fungi, continues to be a prominent subject of study in OSCC. Although *C. albicans* is the most prevalent species, other *Candida* species have also been identified in patients with OSCC [28,36]. Studies have found that 72.2% of these patients have *Candida* species in their saliva [28]. The potential carcinogenic mechanisms of *C. albicans* infection are complex. The initial step in its colonization and invasion involves adhesion to mucosal epithelial cells, which

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are the first line of defense against pathogens [37]. This adhesion can compromise the host's immune defenses, facilitating further infection. Additionally, *C. albicans* produces carcinogens, such as nitrosamines, which can activate proto-oncogenes and induce carcinomatous changes [38]. Chronic inflammation associated with *C. albicans* infection may also contribute to cancer development [39]. Furthermore, *C. albicans* may promote carcinogenesis by stimulating the T helper 17 (Th17) response. Th17 cells play a crucial role in maintaining mucosal barriers and eliminating pathogens from mucosal surfaces [40]. *C. albicans* exhibited significant positive correlations with *L. casei*, *P. micra*, and *E. nodatum* among gram-positive species. However, these complex interactions remain underexplored, and further research employing advanced statistical and analytical methods is required to fully elucidate these relationships.

In this study, although the mean Shannon's index, which is an index of α-diversity, increased from healthy controls to stomatitis patients and then to OSCC patients, the differences were not statistically significant. Consistent with the results of Zhao et al. [41], oral cancer samples exhibited a greater variety of bacterial species than did healthy control samples. This finding is supported by recent reviews that have reported higher α-diversity in OSCC patient samples than in healthy controls [22]. Additionally, patients with OSCC and a history of tobacco use showed an increase in α-diversity [42]. Studies have similarly indicated that cancers at various sites often present with elevated α-diversity in the microbiome compared with that in healthy controls [43]. This increase in microbial diversity may be attributed to several factors, including a chronic inflammatory environment, alterations in local pH and nutrient availability, changes in tumor-associated inflammatory responses, and environmental conditions. In the current study, no specific microbial species were found to be directly correlated with the increase in Shannon's diversity index observed in OSCC. Future research with a larger cohort of OSCC samples is needed to identify the microbes associated with microbial diversity and to further investigate changes in microbial profiles relative to healthy controls.

A limitation of this preliminary study is its small sample size of only 12 participants, including only 4 patients with OSCC. Additionally, we did not utilize advanced analytical techniques such as next-generation sequencing or state-of-the-art AI-driven analyses. Despite these limitations, this study has several strengths. We concurrently assessed both the major bacterial species and fungi using qPCR, which allowed for a comprehensive analysis of the oral microbial profile and the interrelationships within the same saliva samples. This methodology enabled us to investigate the roles of specific microbes and their complex interactions in the pathological mechanisms of OSCC. Previous studies targeting OSCC-specific pathogens have often concentrated on individual microbes, such as *P. gingivalis* or *T. denticola* [10,11,44,45]. Although our preliminary results are encouraging, further studies with larger sample sizes and multicenter designs are essential to validate these findings. Follow-up studies are being planned to address these issues.

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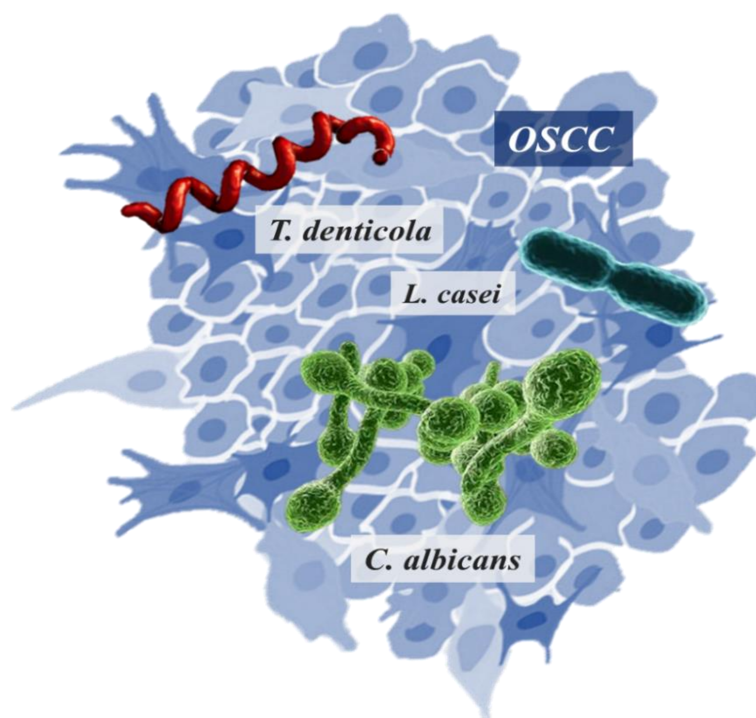

Figure 4. Oral microbes associated with oral squamous cell carcinoma. *T. denticola: Treponema denticola, L. casei: Lactobacillus casei, C. albicans: Candida albicans,* OSCC: oral squamous cell carcinoma.

Author Contributions: Writing and original draft preparation: Y-HL; conceptualization: Y-HL and JJ; methodology: Y-HL, J-YH, and JJ; software: Y-HL; validation and formal analysis: Y-HL; investigation: Y-HL and JJ; resources: Y-HL, J-YH, and JJ; data curation: Y-HL; writing, review, and editing: Y-HL; supervision: Y-HL, J-YH, and JJ; project administration: Y-HL; funding acquisition: Y-HL. All the authors contributed to and approved the submission of the manuscript.

Funding: This work was supported by the Korea Medical Device Development Fund grant funded by the Korean government (Ministry of Science and ICT, Ministry of Trade, Industry and Energy, Ministry of Health & Welfare, Republic of Korea, Ministry of Food and Drug Safety) (Project Number: KMDF_PR_20200901_0023, 9991006696).

Informed consent: Informed consent was obtained from all patients involved in the study.

Data availability statement: The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors extend their gratitude to Sung-Woo Lee and Hee-Kyung Park, Department of Oral Medicine and Oral Diagnosis, Seoul National University.

Conflict of interest: The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as a conflict of interest.

Ethics statement: The research protocol complied with the Declaration of Helsinki and was approved by the Institutional Review Board of Kyung Hee University Dental Hospital, Seoul, South Korea (IRB No-KH-DT20030).

Declaration of generative AI in scientific writing: Not applicable.

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