A Dysbiotic Mycobiome Dominated by *Candida albicans* is Identified within Oral Squamous Cell Carcinomas

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**Running title:** Mycobiome associated with OSCC
Abstract

**Background:** Studies employing next-generation sequencing (NGS) show that the oral fungal community (mycobiome) is far more complex than hitherto thought. However, the role of the oral mycobiome in health and disease, including oral carcinogenesis, has not been explored. **Objective:** To characterize the mycobiome associated with oral squamous cell carcinoma (OSCC). **Methods:** Tissue biopsies [cases: 25 OSCC; controls: 27 intra-oral fibro-epithelial polyp (FEP)] were collected from oral and maxillofacial units in Sri Lanka. Total DNA was extracted and subjected to sequencing of the fungal ITS2 region using Illumina’s 2x300 bp chemistry. High quality, non-chimeric merged reads were classified to species level using a BLASTN-algorithm with UNITE’s named species sequences as reference. Downstream analyses were performed using QIIME and LEfSe. **Results:** 364 species representing 160 genera and 2 phyla (Ascomycota and Basidiomycota) were identified, with *Candida* and *Malassezia* making up 48% and 11% of the average mycobiome, respectively. However, only 5 species and 4 genera were detected in ≥50% of the samples. The species richness and diversity were significantly lower in OSCC. At the genus level, *Candida*, *Hannaella* and *Gibberella* were overrepresented in OSCC while *Alternaria* and *Trametes* were more abundant in FEP. Species-wise, *C. albicans*, *C. etchellsii* and *Hannaella luteola*-like species were enriched in OSCC while *Malassezia restricta*, *Aspergillus tamarii*, *Alternaria alternate*, *Cladosporium halotolerans*, and *Hanseniaspora uvarum*-like species were the most significantly abundant in FEP. **Conclusions:** A dysbiotic mycobiome dominated by *C. albicans* was found in association with OSCC. Whether this dysbiosis plays a role in oral carcinogenesis warrants further investigation.

**Key words:** 18S rRNA; fungi; mycobiome; carcinoma; High-Throughput Nucleotide Sequencing; microbiome; mouth; squamous cell
Introduction

Oral squamous cell carcinoma (OSSC) accounts for more than 90% of cases of oral cancer, a malignancy with poor prognosis representing a public health challenge, particularly in less developed regions where it ranks the 8th most common cancer type [1, 2, 3]. The incidence rates are exceptionally high in certain countries such as Papua New Guinea, India, Sri Lanka, Maldives and Pakistan [1]. There are a number of well-established risk factors for OSSC including smoking, smokeless tobacco usage, areca nut and betel quid chewing, alcohol consumption and HPV infections [4, 5]. However, around 15% of oral cancer cases are not attributable to any of these; the role of other factors such as infection and inflammation is thus emerging [6, 7].

The oral cavity harbors the second most diverse microbiota in our body: the oral microbiome [8]. Oral microorganisms, predominantly bacteria, form homeostatic communities that live in a mutualistic relationship with the host. However, local ecological challenges may disturb the community balance and result in a microbial “dysbiosis” characterized by altered microbiome profile and the potential to cause or contribute to disease [9]. Technological advances, particularly the advent of next-generation sequencing (NGS), have revolutionized the study of oral microbiome in health and disease [10]. Periodontitis and dental caries, the most common oral diseases, are recognized today as sequelae of oral microbial dysbiosis [9, 11]. Recent evidence from NGS studies also suggest a possible role of a dysbiotic microbial community in OSCC [7, 12]. However, all these studies have focused only on the bacterial component of the microbiome (i.e. bacteriome).

Numerous fungi are found in the oral cavity, with the genus Candida being the most commonly isolated and thus studied. Recent studies using NGS have revealed the presence of a complex fungal community (mycobiome) in healthy individuals. In 2010, a study by Ghannoum et al. revealed
presence of a core oral mycobiome consisting of 13 taxa, with *Candida*, Saccharomycetales, *Cladosporium* and *Aspergillus* being the most abundant [13]. In 2014, Dupuy et al. [14] identified an additional 5 core genera, of which *Malassezia* was detected for the first time in the oral cavity and even found to be more abundant than *Candida* in some of the subjects. So far, no attempts have been made to explore the potential role of the oral mycobiome in oral health and disease, including oral cancer. This is surprising given the existing evidence implicating *Candida albicans* in oral carcinogenesis [15].

The objective of this study was to use NGS coupled with a species-level taxonomy assignment algorithm to compare the mycobiome profile within OSSC tissues to benign intra-oral fibro-epithelial polyps (FEP) and identify fungal taxa that may play a role in oral carcinogenesis.

**Methods**

**Study design, setting and subjects**

This was a case-control study. Study subjects were recruited between 17/04/15 and 02/08/15 at nine oral and maxillofacial (OMF) units in six provinces of Sri Lanka. Cases comprised 25 Sinhala, ≥ 40 years old males with histologically confirmed OSCC affecting the buccal mucosa or tongue. The control group consisted of 27 Sinhala males with a clinical diagnosis of FEP also involving the buccal mucosa or tongue. Subjects with history of antibiotic used in the last 2 months were excluded.

Ethical approval of the study was obtained from the Faculty Research committee, Faculty of Dental Sciences, University of Peradeniya, Sri Lanka (FRC/FDS/UOP/E/2014/32) and Griffith University.
Human Research Ethics Committee, Australia (DOH/18/14/HREC). Written informed consent was obtained from each participant.

**History taking and clinical examination**

Data were collected using a pre-tested, interviewer-administered questionnaire which comprised information on socio-demographics and risk habits including betel quid chewing, smoking and alcohol consumption. Clinical oral examinations were conducted by dental public health specialists. The oral mucosa was thoroughly inspected for any growth, ulceration or white patches. Number of missing teeth was recorded. Oral hygiene status was assessed with the simplified oral hygiene index [16] while periodontal status was assessed using bleeding on probing (BOP), periodontal pocket depth (PPD) and clinical attachment loss (CAL) at 4 sites per anterior tooth and 6 sites per posterior tooth.

**Tissue sampling and DNA extraction**

For suspected OSSC cases, tissue samples were obtained from the incisional biopsies taken for diagnosis. The freshly-taken biopsy was laid on a pile of sterile gauze and a small piece of tissue (~3 mm³) was excised from the body of the lesion. A new sterile surgical blade was used for each case. The sample was aseptically transferred into a screw-cap vial and immediately stored at -20°C. Simultaneously, the rest of the biopsy was sent in 10% buffered formalin for histopathological diagnosis. Only samples histopathologically-confirmed as OSSC were included in the study. Control tissue samples were obtained as above from freshly excised, clinically diagnosed FEPs.
Tissue samples (~100 mg each) were finely chopped using a sterile blade. DNA extraction was then performed using Gentra Puregene Tissue kit (Qiagen, Germany) according to the manufacturer’s instructions (solid tissue protocol) with a few modifications: 1) incubation in the lysis buffer was performed overnight; 2) an additional lysis step using 50 units of mutanolysin at 37°C for 1.5 hrs to digest the cell wall of Gram +ve bacteria was included prior to the addition of Gentra Puregene Proteinase K. Total DNA concentration and purity was determined using the NanoDrop 1000 Spectrophotometer (Thermofisher Scientific, USA). The extracts were stored at - 80°C.

**Amplicon Library preparation and sequencing**

Amplicon library was prepared as per Illumina’s protocol except that amplification was performed with the Q5 Hot Start High-Fidelity 2X MasterMix (New England Biolabs, USA). In brief, the fungal ribosomal internal transcribed spacer 2 (ITS2) was amplified using the primers ITS3-F (5’-GCATCGATGAAGAACGCAGC-3’) and ITS4-R (5’-TCCTCCGCTTATTTAGATATGC-3’) [17], linked to Illumina’s specific adapter sequences 803F and 1392wR, in standard PCR conditions. The resultant PCR amplicons (~ 250-590 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter, USA). A second PCR performed to tag the amplicons with unique 8-base barcodes using Nextera XT v2 Index Kit sets A-D (Illumina, USA). The tagged amplicons were then pooled together in equimolar concentrations and sequenced on MiSeq Sequencing System (Illumina, USA) using v3 2x300 bp, paired-end sequencing chemistry in the Australian Centre for Ecogenomics according to the manufacturer’s protocol.
**Processing of sequencing data**

Raw sequencing data were deposited in (and are publicly available from) Sequence Reads Archive (SRA) under project no. PRJNA375780. Reads with primer mismatches were removed and primer sequences were trimmed off. Paired sequences were then merged with PEAR [18] using the following parameters: minimum amplicon length, 213 bp; maximum amplicon length, 552 bp; p-value, 0.001. Preprocessing of the merged reads was performed using the mothur v1.38.1 [19]. Firstly, to stringently minimize sequencing errors, reads with ambiguous bases, with homopolymers > 8 bp or that did not achieve a sliding 50-nucleotide Q-score average of ≥30 were filtered out. Secondly, the high quality reads were cleared of chimeras with Uchime [20] using the self-reference approach [21]. Finally, sequences representing non-fungal lineages, identified by preliminary taxonomy using mothur’s classify.seqs command, were removed.

**Taxonomy assignment algorithm and down-stream analysis**

The high quality, non-chimeric reads were classified to the species level employing a previously described BLASTN-based algorithm, modified to analyze fungal ITS2 region of the 18S rRNA gene instead of bacterial 16S RNA [22]. A set of 23,423 fungal ITS sequences representing all named species (16,595 species) in UNITE’s database version 7.1 (https://unite.ut.ee/repository.php; August 22, 2016 dynamic release; untrimmed sequences) [23] was used as reference; the fasta and taxonomy files of this set can be downloaded at ftp://www.homd.org/publication_data/20170221/

Briefly, the reads were individually BLASTN-searched against the reference set at alignment coverage of ≥99% and percent identity of ≥98.5%. Hits were ranked by percent identity and, when equal, by bit score. Reads were assigned taxonomies of the best hits. Reads with best hits representing more than one species were screened again for chimeras using *de novo* check at 98%
similarity with USEARCH v8.1.1861, and, if not chimeric, were assigned multiple-species taxonomy[24]. Reads with no matches at the specified criteria underwent secondary de novo chimera checking as above, and then de novo, species-level operational taxonomy unit (OTU) calling at 98% using USEARCH. Singleton OTUs were excluded; the rest were considered potentially novel species and a representative read from each was BLASTN-searched against the same reference sequence set again to determine the closest species for taxonomy assignment.

Downstream analysis was performed as previously described [22]. In short, the Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 [25] was employed to perform further analysis including generation of taxonomy plots, rarefaction, calculation of species richness and diversity indices, computing distance matrices, and running principle component analysis (PCoA). Detection of differentially abundant taxa between the cases and controls was done using Linear discriminant analysis Effect Size (LEfSe) [26].

**Results**

Four samples ended up with low read count (< 3000) and one with very high count (an outlier) and were thus excluded. Results are presented hereafter for 22 cases and 25 controls. The clinical characteristics of both groups is presented in Table 1. The cases, albeit older on average than controls, were heavier users of betel quid and alcohol, and smoked more: these being the traditional risk factors for oral cancer. They had significantly poorer oral hygiene, fewer teeth and more severe periodontal disease.
**DNA extracts and sequencing/data processing statistics**

Total DNA extracted averaged 482.08 ng/µL (range: 16.85-1908 ng/µL; <100 ng/µL from 8/47 (17%) samples) with all samples containing DNA of high purity (average 260/280 ratio was 1.93, range: 1.81-2.07). However, the mean 260/230 ratio was 1.54 (range: 0.41-2.22; 260) indicating presence of inhibitors in some of the samples, an issue that was dealt with using dilution before amplification.

Sequencing generated 1,576,427 raw paired reads: 14.3% of these were discarded due to primer mismatches; 96.6% of the remaining reads were successfully stitched with PEAR. Quality filtration and chimera checking removed 7.9% of the merged reads, thus leaving 1,063,430 reads (67.5%), 205-535 bp long. Of these, 1,017,131 reads (95.7%) were successfully classified to the species level; 2.3% did not return BLASTN matches and 2% formed singleton OTUs and were excluded. The number of classified reads per sample averaged 21,641 (range: 3,973 – 54,849).

**Overall mycobione profile**

A total of 364 species belonging to 162 genera and 2 phyla were detected in the samples. The abundances and detection frequencies of these taxa in each of the samples and across the study groups are presented in **Supplementary Tables 1-3**. The number of species per sample ranged from 4 to 64. However, only 125 species and 74 genera were identified in more than one sample; 10 species and 7 genera in ≥ 25% of the samples and 5 species and 4 genera in >50%.

The abundances of the two phyla identified as well as the genera and species detected in ≥ 15% of the samples are shown in **Figure 1**. On average, the phyla Ascomycota and Basidiomycota...
accounted for 78.4% and 21.6% of the mycobiome, respectively. At the genus level, *Candida* was the detected in 100% of the samples and constituted 48% of the average mycobiome. The genera *Malassezia*, *Cladosporium* and *Aspergillus* were identified in ≥75% of the sample with an average abundance of 11%, 6.1% and 3.7%, respectively. Species-wise, *C. albicans* was found in all samples at a mean abundance of 44.4%. *Malassezia restricta*, *Aspergillus penicillioides* and *Malassezia globosa* were identified in 83%, 70.2% and 68.1% of the samples respectively, and accounted for 3.2%, 2.2% and 4.2% of the average mycobiome, respectively. *Cladosporium exasperatum* and a potentially novel species close to *Cladosporium sphaerospermum* were also identified in half of the samples at an average abundance of > 2%.

However, the relative abundance of these taxa varied significantly between the samples. In addition, the mycobiome of some of the samples was dominated by taxa other than the above-mentioned. Examples of species abundant in single samples include *Rhodotorula mucilaginosa*, *Sporidiobolus johnsonii*, *Penicillium toxicarium*, *Toxicocladosporium irritans*, *Gibberella intricans*, *Alternaria infectoria*, *Ophiocordyceps sinensis* and *Aspergillus tamarii* as well as a number of potentially novel taxa.

**OSCC vs. FEP**

The number of species per sample ranged from 4 to 29 for the cases and from 8 to 64 for the controls. The FEP controls had higher species richness and α-diversity than the cases (Table 2). Rarefaction curves show that as few as 1,500 reads per sample represented sufficient sequencing depth (Figure 2A). No separate clusters formed for the cases and controls by PCoA (Figure 2B).
The genera and species found by LEsFe to be differentially abundant between the cases and controls are shown in Figure 3. The genera *Candida*, *Hannaella* and *Gibberella* were significantly more abundant in OSCC. In contrast, *Trametes* and *Alternaria* were strongly associated with FEP. At the species level, *C. albicans*, *Candida etchellii* and a potentially novel species close to *Hannaella luteola* were significantly enriched in OSCC. *C. albicans* was identified in 100% of the samples but the average relative abundance in OSCC was twice that in the controls (61.2% vs. 29.6%). *C. etchellii* was identified in 32% of the cases compared to only 8% of the controls. On the other hand, a potentially novel *Hanseniaspora uvarum*-like species, in addition to *M. restricta*, *A. tamarii*, *Cladosporium halotolerans*, *Alternaria alternata* and *Malassezia furfur* were overrepresented or even exclusively found in FEP (See Supplementary Table 4 for a list of taxa exclusively found in either group at prevalence ≥ 10%)

**Discussion**

To our knowledge, this is the first study to explore the mycobiome associated with oral cancer. The study subjects were recruited from Sri Lanka, a developing lower-middle income country where oral cancer is the most common malignancy among men [1]. Tissue biopsies rather than surface swabs were used to ensure that any association identified is more relevant to carcinogenesis. Fibro-epithelial polyps (FEP) were chosen as controls with the assumption they would offer a more valid comparison (benign vs. malignant growths) than that with buccal epithelium or even adjacent normal tissue. However, some study limitations should be noted. Firstly, contamination of samples by saliva cannot be entirely excluded although great care was taken during biopsy to avoid this, and all biopsies were blotted with fresh gauze immediately after
excision. Secondly, some DNA samples had low 260/230 ratios, suggestive of the presence of inhibitors. Nevertheless, these samples amplified successfully after dilution. Finally, a few reads were detected in the control samples which, while having negligible effect on the results, suggests some sort of contamination at some stage of the laboratory work. The latter could have been due to contamination of the DNA extraction or sequencing reagents by extraneous organisms, now known to be a problem around the world [27].

Coupling Illumina’s 2x300 bp sequencing chemistry and merging of paired reads ensured full ITS2 amplicons were obtained and maximizing taxonomic resolution. This, in addition to limiting the reference set to sequences from named species only, enabled us to classify the reads to the species level. A percent identity threshold of 98.5% (i.e. like that used to generate species hypothesis in UNITE’s dynamic release) and high query alignment coverage (≥98%) cutoff increased the reliability of taxonomic assignment and eliminated the possibility of forced classifications by BLASTN. However, this approach carries the risk of assigning reads belonging to species not represented in the reference set to another closely related species.

Of the species identified 239/364 (65.6%) were from single samples indicating that they represent transient environmental fungi. Although the scope of the current study is different from that of the previous two oral mycobiome studies in which salivary samples from healthy subjects were analyzed, some comparisons can be made. Ghannoum et al., using a relative abundance cutoff of 1%, identified 101 species in their samples [13]. Applying the same cutoff to our samples left 137 species, which is comparable. They also described a basal mycobiome comprising 13 taxa that was present in ≥20% of the subjects. Consistently, 7 of these genera, namely Candida,
Aspergillus, Cladosporium, Alternaria, Cryptococcus, Gibberella and Saccharomyces were detected at the same frequency in at least one of our two groups. Interestingly, Malassezia, which was not detected by Ghannoum et al., was identified in this study as the second most common and abundant genus after Candida. This substantiates the more recent findings by Dupuy et al., who, for the first time, described this genus in the oral cavity and even found it to be more abundant than Candida [14]. The same study described a core mycobiome comprising 14 core genera, detected in ≥ 50% of the subjects. Four of these (Candida, Malassezia, Aspergillus, Cladosporium) were also found in more than half of our samples. Put together, these findings provide evidence for the existence of a resident, core oral fungal community.

Candida, specifically C. albicans and etchellsii, showed the strongest association with OSCC in the current study. In fact, candidiasis has for long been proposed as a risk factor for malignant transformation of oral potentially malignant disorders, including leukoplakia [28]. Some strains of C. albicans have high nitrosation potential and have been experimentally shown to induce dysplasia [29]. Recently, there is increasing evidence of the association between candida infection and OSCC [15]. Nagy et al. recovered C. albicans from the surface of 8/21 (38%) neoplasms but from none of the control tissues [30]. Similarly, Čanković et al. isolated Candida from 9/30 (4 C. albicans and 5 non–albicans) cancer surfaces (30%) but from only 2/30 (6.6%) benign control samples [31]. Gall et al. identified Candida spp in 31/48 (65%) oral cancer cases, but the study did not include healthy tissue samples for comparison [32]. More recently, Berkovits et al. isolated yeasts, predominantly Candida spp., from 18/20 (90%) OSCC samples compared to only 12/40 (30%) control tissues; in addition, they found the “fungal burden” to be higher in OSCC [33]. In our study, Candida albicans was detected in 100% of the samples, which is probably a reflection of the much higher sensitivity
of NGS compared to cultural techniques used in previous studies. The difference between the cases and controls in our material was rather in terms of relative abundance (average of 61.2% in the cases vs. 29.6% in the controls). This is somewhat consistent with the high “fungal burden” described by Berkovits et al. and indicates that a fungal community dysbiosis characterized, by overall simplification, with increased abundance of *C. albicans* is associated with OSCC.

*C. etchellsii* was identified in 32% of the cases vs. 8% of the controls: this is the first time a specific *Candida* species other than *C. albicans* is implicated in oral cancer, a finding worth further investigation. *Hannaella* and *Gibberella* spp were also overrepresented in OSCC tissues. These are typically found on plants as commensals and pathogens, respectively [34, 35]. Therefore, they probably represent contaminants of the OSCC rather than members of the oral mycobiome community, especially that they were found in only one control sample. However, their contribution to the carcinogenic process cannot be excluded; in fact one *Gibberella* species (*G. moniliformis*) produces a mycotoxin that has been shown to induce liver cancer in rodents [36].

Species belonging to *Malassezia, Aspergillus, Alternaria, Cladosporium* and *Hanseniaspora* were significantly enriched or exclusively found in the controls. *Malassezia* spp are normal colonizers of healthy skin [37] and have been recently found to be dominant members of the salivary mycobiome [14]. *Aspergillus, Alternaria*, and *Cladosporium* have also been described as core oral fungal taxa [13, 14]. Interestingly, some species of these genera, including *A. tamarii* and *A. alternata*, which were identified in our samples, are known to produce compounds with anticancer activity [38, 39, 40, 41]. In addition, statins produced by *A. tamarii* have been shown to inhibit growth of *C. albicans* [40], which possibly explains why all samples with high abundance of this
species had very low levels of *C. albicans* (Supplementary Table 3). Therefore, while these species may simply represent transient environmental fungi or passenger oral fungal taxa, it is also possible that carriage of some of these species confers some protection against development of oral cancer. Further research to explore these scenarios is needed to harness their potential for novel prevention and control strategies.

In conclusion, the current study uncovered a dysbiotic mycobiome characterized by lower species diversity and increased *C. albicans* abundance in association with OSCC. It also identified a number of fungal taxa capable of producing anti-cancer compounds exclusively in non-cancerous tissues. The relevance of these findings for development of risk markers and for prevention and control of OSCC warrants further investigation.

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**Competing interests**

None to declare.


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Supplementary Information

Supplementary Table 1. Abundances (Sheet 1) and detection frequencies (Sheet 2) of phyla detected in each of the samples and across the study groups.

Supplementary Table 2. Abundances (Sheet 1) and detection frequencies (Sheet 2) of genera detected in each of the samples and across the study groups.

Supplementary Table 3. Abundances (Sheet 1) and detection frequencies (Sheet 2) of species detected in each of the samples and across the study groups.

Supplementary Table 4. List of taxa exclusively identified in either group at prevalence $\geq 10\%$. 
Figure Legends

**Figure 1. Mycobiome profile.** Average abundances of the two phyla identified (A) as well as the genera (B) and species (C) detected in ≥ 15% of the samples.
Figure 2. Rarefaction and β-diversity. A. Rarefaction curves showing the number of observed species as a function of sequencing depth. B. Non-clustering of the study subjects by PCoA (weighted Unifrac).

Figure 3. Differentially abundant taxa. Linear Discriminant Analysis Effect Size (LEfSe) analysis showing genera (A) and species (B) that were significantly differentially abundant between the cases and controls (LDA score ≥ 3).
### Table 1. Clinical characteristics of the study subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=22)</th>
<th>Controls (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> (mean±SD) *</td>
<td>61.00±9.5</td>
<td>50.58±13.5</td>
</tr>
<tr>
<td>% males</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Betel quid:</strong> No. (%) ¶</td>
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<td></td>
</tr>
<tr>
<td>Never</td>
<td>0 (0.0)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Past</td>
<td>3 (13.6)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Sometimes</td>
<td>1 (4.6)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Daily</td>
<td>18 (81.9)</td>
<td>12 (48)</td>
</tr>
<tr>
<td><strong>Smoking:</strong> No. (%) ¶</td>
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<td></td>
</tr>
<tr>
<td>Never</td>
<td>4 (18.2)</td>
<td>8 (32)</td>
</tr>
<tr>
<td>Past</td>
<td>5 (22.7)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Sometimes</td>
<td>4 (18.2)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Daily</td>
<td>9 (40.9)</td>
<td>6 (24)</td>
</tr>
<tr>
<td><strong>Alcohol use:</strong> No. (%) ¶</td>
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<td></td>
</tr>
<tr>
<td>Never</td>
<td>0 (0.0)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Past</td>
<td>5 (22.7)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Sometimes</td>
<td>4 (18.2)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Daily</td>
<td>13 (59.1)</td>
<td>4 (16)</td>
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<tr>
<td><strong>Oral hygiene:</strong> No. (%) ¶</td>
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<tr>
<td>Bad</td>
<td>6 (27.3)</td>
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<tr>
<td>Fair</td>
<td>14 (63.6)</td>
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<td>Good</td>
<td>2 (9.1)</td>
<td>12 (48)</td>
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<td><strong>Missing teeth</strong> (mean±SD) *</td>
<td>14.73±10.01</td>
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<td><strong>Periodontal status:</strong> No. (%) ¶§</td>
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<tr>
<td>Severe Periodontitis</td>
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<td>Moderate Periodontitis</td>
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<td>6 (24)</td>
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<td>No or Mild Periodontitis</td>
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<td><strong>Site affected:</strong> No. (%)</td>
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<tr>
<td>Tongue</td>
<td>14 (63.6)</td>
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<tr>
<td>Buccal mucosa</td>
<td>8 (36.4)</td>
<td>6 (24)</td>
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<td><strong>Histopathology:</strong> No. (%)</td>
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<tr>
<td>Well differentiated SCC</td>
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<td>19 (76)</td>
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<tr>
<td>Moderately differentiated SCC</td>
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<td>6 (24)</td>
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<tr>
<td>Fibro-epithelial polyp</td>
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<td>25 (100)</td>
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</table>

* Statistically significant: $P \leq 0.05$; t-test

¶ Statistically significant: $P \leq 0.05$; Chi-squared test

§ Classification made according to Page and Eke [42]
Table 2. Species richness, α-diversity and coverage (mean±SE) calculated from the rarefied biom.

<table>
<thead>
<tr>
<th>Group</th>
<th>Observed richness</th>
<th>Chao1</th>
<th>Shannon index</th>
<th>Good’s coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSCC</td>
<td>11.7±6.4</td>
<td>13.6±7.0</td>
<td>1.5±0.9</td>
<td>0.999±0.000</td>
</tr>
<tr>
<td>FEP</td>
<td>17.7±9.8</td>
<td>19.9±12.2</td>
<td>2.1±1.2</td>
<td>0.999±0.001</td>
</tr>
</tbody>
</table>

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