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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Microbiome and oral squamous cell carcinoma: a possible interplay on iron metabolism and its impact on tumor microenvironment

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Abstract

There is increasing evidence showing positive association between changes in oral microbiome and the occurrence of oral squamous cell carcinoma (OSCC). Alcohol- and nicotine-related products can induce microbial changes but are still unknown if these changes are related to cancerous lesion sites. In an attempt to understand how these changes can influence the OSCC development and maintenance, the aim of this study was to investigate the oral microbiome linked with OSCC as well as to identify functional signatures and associate them with healthy or precancerous and cancerous sites. Our group used data of oral microbiomes available in public repositories. The analysis included data of oral microbiomes from electronic cigarette users, alcohol consumers, and precancerous and OSCC samples. An R-based pipeline was used for taxonomic and functional prediction analysis. The *Streptococcus* spp. genus was the main class identified in the healthy group. *Haemophilus* spp. predominated in precancerous lesions. OSCC samples revealed a higher relative abundance compared with the other groups, represented by an increased proportion of *Fusobacterium* spp., *Prevotella* spp., *Haemophilus* spp., and *Campylobacter* spp. Venn diagram analysis showed 52 genera exclusive of OSCC samples. Both precancerous and OSCC samples seemed to present a specific associated functional pattern. They were menaquinone-dependent protoporphyrinogen oxidase pattern enhanced in the former and both 3′,5′-cyclic-nucleotide phosphodiesterase (purine metabolism) and iron(III) transport system ATP-binding protein enhanced in the latter. We conclude that although precancerous and OSCC samples present some differences on microbial profile, both microbiomes act as "iron chelators-like" potentially contributing to tumor growth.

Keywords Microbiome · Oral squamous cell carcinoma · Alcohol · Cigarette · Functional pathways prediction · Iron(III) transport system

Introduction

Cancer has been considered one of the main causes of premature deaths in adults worldwide affecting more than 4.5 million persons [1], being head and neck cancer the seventh most common one [2]. Approximately half of the head and neck cancerous lesions diagnosed in 2018 belonged to oral cavity, with about 350,000 affected individuals [2].

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Consumption of alcoholic beverages, smokeless tobacco, and tobacco smoking are considered the main agents associated with the occurrence and development of oral cancer [3]. In this sense, alcohol consumption increases up to 5 times the risk for oral cavity cancer development [4], and the simultaneous consumption of alcohol and smokeless tobacco or tobacco smoking increases up to 16 times the chance for oral cancer development [5]. Around 2.4 million tobacco-related cancer deaths per year have been reported [6] being smoking-attributable lip and oral cancer responsible for about one-third of worldwide cancer deaths during the last 30 years [7]. However, although the carcinogenicity of tobacco has been well-established, it is still unclear how and whether electronic cigarettes and/or electronic nicotine delivery systems actually increase the risk of users developing cancer, which raises the need for further investigation on this matter [8].

In addition to the abovementioned risk factors, it is well understood that shifts in the commensal microbiota at specific sites might be associated with cancer development [9–12]. Studies have suggested that *Helicobacter pylori* have a direct effect on stomach cancer [13, 14] and, therefore, this is the only well-established bacteria considered as carcinogenic to humans [15]. On the other hand, there is increasing evidence showing a positive association between the presence of *Fusobacterium* spp., *Pseudomonas* spp., *Prevotella* spp., *Campylobacter* spp., *Rothia* spp., *Leptotrichia* spp., and others and the occurrence of oral squamous cell carcinoma (OSCC), suggesting that a microbial signature might also be associated with oral cancerous lesions, although the causal effect has not been shown so far [16–19]. Yet, both alcohol consumption and electronic cigarette use can induce microbial changes in the oral cavity [20, 21], but it is unknown whether the microbial profile found under those conditions resembles the one related to cancerous lesion sites.

Several mechanisms have been proposed to explain the interplay between host cells and the associated microbiome and cancerous lesion development. An overrepresentation of peptidases and the enrichment of the lipopolysaccharide biosynthesis pathway have been reported in the microbiome associated with oral cancerous lesions [17, 22], revealing a microbiome-derived pro-inflammatory environment correlated with carcinogenesis. Dysregulation of the host immune system has also been warranted as a possible direct and end-effect of tissue-associated microbiota [9]. Moreover, alterations in epithelial barriers and epigenetic modulation have also been considered as effects of the microbiome over host cells [12]. Whether microbial changes and the associated functional patterns are consequences of cancer development or whether they both act modifying the lesion behavior and progression is still under debate. This way, the identification of potential microbial biomarkers and specific functional patterns related to precancerous or cancerous lesions has been encouraged as an aid to the development of further clinical strategies attempting to prevent cancer development and progression [23, 24]. Hence, the present study aims to investigate the oral microbiome linked with precancerous lesions and with oral squamous cell carcinoma. We also intend to identify functional signatures of oral dysbiosis and associate them with healthy or cancerous and precancerous sites. The tested hypotheses were that oral microbial dysbiosis is associated with cancer sites whose microbiomes present specific functional patterns.

Materials and methods

Sample description

To assess the differences on the oral microbiome among 4 different conditions (2 related to lifestyle and 2 related to pathogenic processes), we used public data available in different biological databases. First, we performed a search in the literature and in specialized databases like MGnify [25] and GenBank [26] to generate concise data. The options selected in MGnify database was human biome; oral (16S). For the purpose of this study, only data generated on Illumina platform were selected. In this way, we gathered 41 samples with a total of 204,380 sequences from 2 different studies divided into "alcohol consumers" and "electronic cigarette users (PRJNA413706)" and a group with precancerous and with OSCC (PRJEB4953) [27, 28]; the number of raw sequence reads varied by >10-fold across samples, ranging from 1231 to a maximum of 17,682 raw reads.

Bioinformatics pipeline

The FastQC version 0.11.9 [29] was used to verify the quality of the sequences as well as the number of duplicates, ambiguous bases, and bases to be truncated. Subsequently the sequences were processed in R script, using the pipeline recommended by the DADA2 package [30]. First, we filtered low-quality sequences with the following parameters: maximum number of *N* allowed was 0 (maxN = 0), the sequences were truncated at 240 in reads forwards and 160 in reads reverse (truncLen = 240,160), and maximum number of expected errors allowed was 2 and 5 forwards and reverse reads, respectively (maxEE = 2,5). After we used a machine learning model to correct error rate, within the use of a parametric error model, the implemented method learns this error model from the data, by alternating estimation of the error rates and inference of sample composition until they converge on a jointly consistent solution. After this stage, the same/similar sequences were clustered for later creation of the amplicon sequence variant table (ASV); after that, the chimera was removed in order to minimize possible PCR bias. Taxonomic analysis was performed considering SILVA database version 132 [31]

available online, and the composition table was generated from this process. Data were also processed by Piphillin [32] to predict functional analysis of microbiome to a better elucidation of mechanisms by which microbiome perturbation can cause any change in the patient. To proceed with the Piphillin output analysis, we used "in-house" R scripts. All diversity graphics, abundance, and heatmaps were generated in MicrobiomeAnalyst [33] web server. The statistical methods used to calculate the alpha diversity were analysis of variance (ANOVA) and for the beta diversity the analysis of similarities (ANOSIM). Abundance of phyla and genera between T1/T2 and T3/T4 staging lesions and abundance of the main bacteria that are involved in the process of regulating molecules between precancerous and cancerous lesions were compared by the Mann-Whitney test; *p*-values less than 0.05 were considered statistically significant. The two studies used to conduct this work are available under the following accession number: PRJNA413706 (alcohol drinkers and electronic cigarette smokers) and PRJEB4953 (precancerous and OSCC) in MGnify and GenBank databases.

Results

Taxonomic profiles at the phyla and genus level

The analysis of the taxonomic oral microbiota composition of the four studied groups was explored by comparing the relative abundance at phyla (Figure S1) and the genera levels (Fig. 1). *Firmicutes* and *Proteobacteria* represent around 80% of identified phyla in cigarette users, alcohol consumers, and precancerous samples. Furthermore, in precancerous samples, the proportion of *Proteobacteria* increased and the proportion of *Firmicutes* decreased compared with cigarette users and alcohol consumers. Interestingly, both phyla accounted for less than 50% of those associated with OSCC lesions, being *Bacteroidetes* the most prevalent one (30% of the total abundance) in cancerous lesions. In contrast, this phylum ranged from 6 to 8% of total abundance in the microbiome of the other conditions. The prevalence of *Fusobacteria* on OSCC samples was about four times greater than in cigarette users and alcohol consumers (Figure S1) and two times greater than in precancerous samples. Taxa belonging to the genus *Streptococcus* and *Haemophilus* occurred at higher relative abundances in electronic cigarette users (group 1), alcohol consumers (group 2), and individuals diagnosed with precancerous lesions (group 3), being the relative abundance of *Haemophilus* greater in precancerous lesions. Moreover, an increase in taxonomic diversity, in terms of *Fusobacterium*, *Prevotella*, *Haemophilus*, *Campylobacter*, *Alloprevotella*, and *Corynebacterium*, was found associated with OSCC (group 4) (Fig. 1).

Alpha- and beta-diversity analyses

In terms of alpha-diversity (number of genera, richness, and abundance), although slight differences have been found among the groups, the general diversity pattern was similar among them (Fig. 2a, b, c, d). In terms of beta-diversity (community level), a similar pattern is likely to be found on electronic cigarette users (group 1), alcohol consumers (group 2), and precancerous samples (group 3). In contrast, a specific beta-diversity pattern was found on OSCC samples (purple circle) that was different compared to the other conditions (Fig. 2e). Moreover, the beta-diversity in OSCC samples is gender dependent (Figure S2). Overall, the Venn diagram shows that the greatest genera diversity was found in OSCC samples (140 identified genera), followed by electronic cigarettes (94 genera), alcohol consumers (78 genera), and precancerous lesions (71 genera) (Fig. 2f). Furthermore, the co-occurrence of 52 genera was found among the groups, while OSCC samples harbored 52 exclusive genera not found on the other conditions (Fig. 2f). Within those OSCC-exclusive genera, 36% and 30% belonged to *Firmicutes* and *Proteobacteria* phyla, while 13.5% and 7.5% belonged to *Actinobacteria* and *Bacteroidetes*, respectively. The reads counts of *Bacteroidetes* in OSCC samples were greater than in the electronic cigarette users and alcohol consumers. Table S1 showed a description of shared and exclusively found genera among electronic cigarette users, alcohol consumers, and precancerous and OSCC samples.

Staging analysis in OSCC

In the comparison between T1/T2 and T3/T4 staging lesions, the abundance of *Bacteroidetes* and *Firmicutes* was statistically higher (two times greater; $p = 0.00423$ and 0.00436, respectively) on T3/T4 lesions, whereas statistically lower abundance of *Proteobacteria* and *Fusobacteria* (half-fold and four-fold, respectively; $p = 0.00032$ and 0.03237) was found on more advanced lesions (Figure S3). In terms of abundance at the genus level, *Fusobacterium*, *Prevotella*, *Akkermansia*, *Actinobacillus*, and *Corynebacterium* comprised about 50% of genera found in T1/T2 lesions, being the abundance of *Akkermansia* ($p = 0.02147$), *Corynebacterium* ($p = 0.01443$), and *Fusobacterium* ($p = 0.00388$) statistically greater in T1/T2 (since they were virtually absent on T3/T4 lesions). *Prevotella* and *Haemophilus* besides comprising up to 50% of the identified genera in T3/T4 were statistically more abundant in more advanced lesions (four-fold and three-fold, respectively; $p = 0.003112$ and 0.01253) (Fig. 3). The abundance at the genus level on OSCC samples seems to be affected by tumor staging, gender, and use/consumption or not of electronic cigarettes or alcohol (Fig. 4). Overall, it seems that the abundance is greater in men than that in women and in the most advanced stages of the disease, being the abundance also dependent on

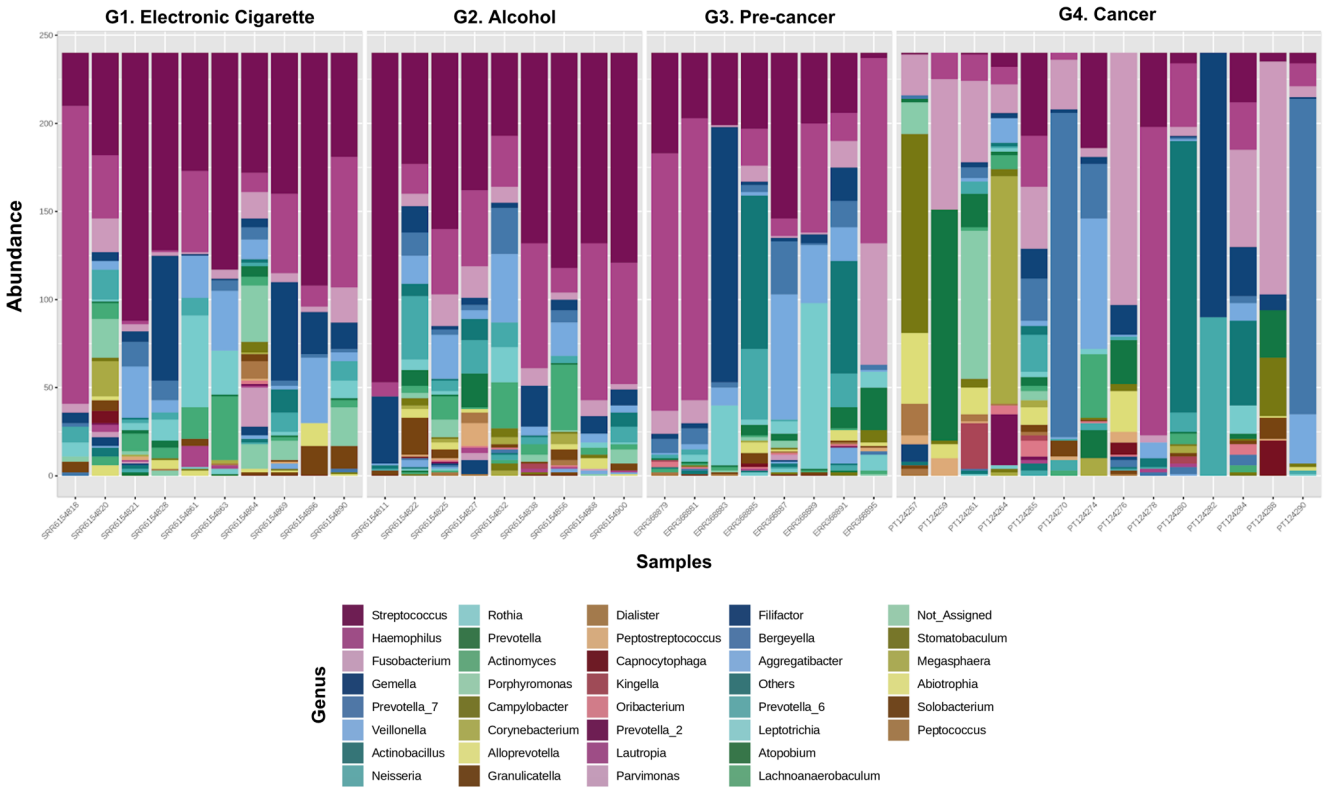

Fig. 1 Stacked bar chart showing the relative abundance of the bacterial taxonomic hits at the genus level in buccal mucosa samples in the different studied groups: G1, electronic cigarette users; G2, alcohol consumers; G3, individuals diagnosed with precancerous lesions; and G4, individuals diagnosed with OSCC obtained by the MiSeq sequencing pipeline

use/consumption or not of electronic cigarettes or alcohol. Some genera, such as *Streptococcus*, *Rothia*, *Actinomyces*, *Veillonella*, *Prevotella*, *Bifidobacterium*, *Atopobium*, *Megasphaera*, *Haemophilus*, *Peptostreptococcus*,

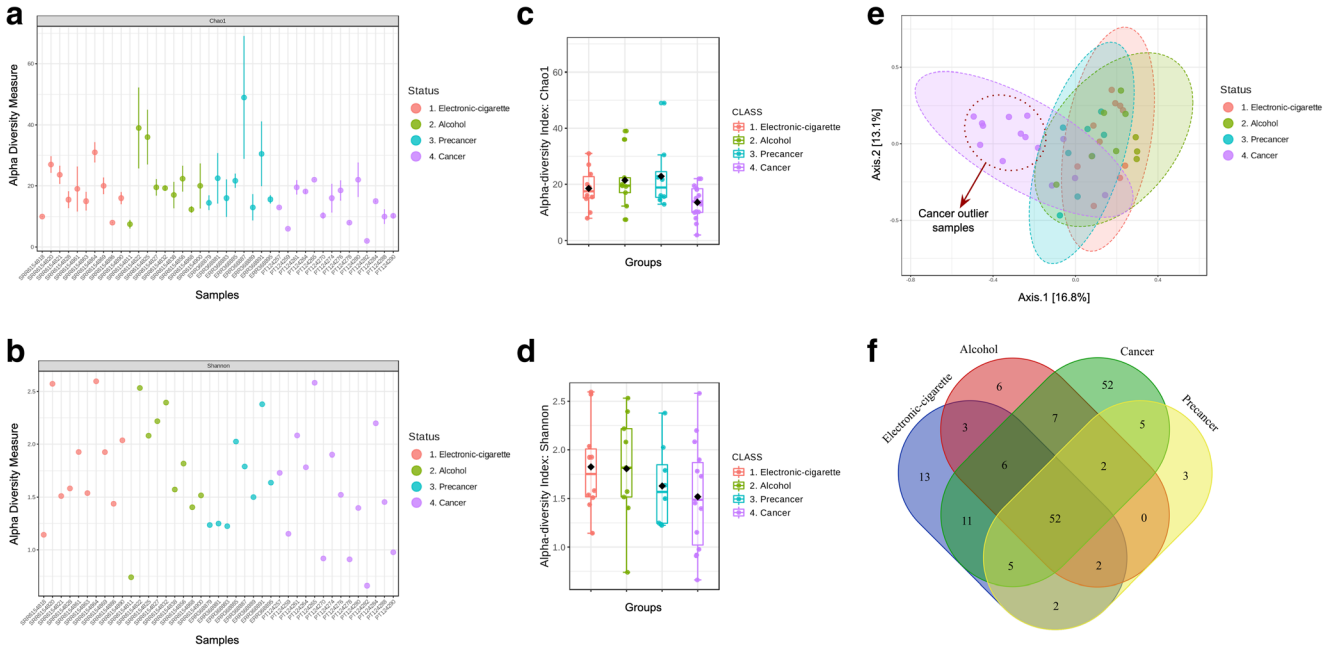

Fig. 2 Representation of alpha- and beta-diversity on samples and community levels. **a** and **b** Alpha diversity analysis with Chao and Shannon metrics (Chao1 p=0.15125; Shannon p=0.476094), and boxplot graph analyzed by the same metrics (**c** and **d**); **e** principal components analysis (PCA; Bray-Curtis Index) with beta-diversity metric (PCA p<0.001), revealing an outlier group counting only cancer samples; **f** Venn diagram showing the occurrence and co-occurrence of the genera between the four groups of samples

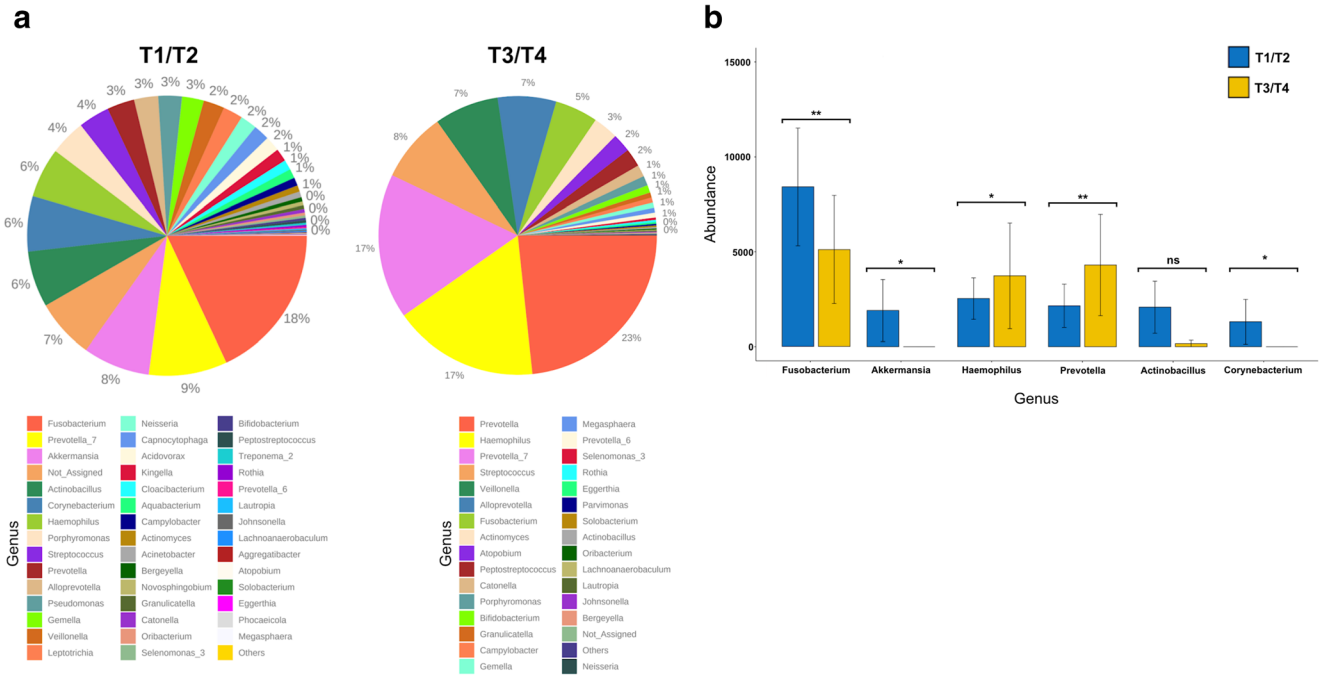

Fig. 3 Pie chart and statistical analysis at genus level in OSCC samples according to tumor staging. **a** Pie chart showing the relative abundance at genus level in OSCC samples according to tumor staging (Union for International Cancer Control-American Joint Committee on Cancer—UICC-AJCC). **b** Statistical analysis at genus level in OSCC samples according to tumor staging. **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.0001 by Mann-Whitney test

Granulicatella, and *Solobacterium*, were mostly found in electronic cigarette users who were also alcohol consumers and presented advanced OSCC lesions, showing a synergistic effect of such habits on the microbial composition. Abundance of *Streptococcus* seems to be greater in men than that in women.

Functional prediction analysis

The predictive enrichment analysis showed that there is no difference among the groups in the top ten predicted functional patterns (Figure S4). Considering the functional prediction "metabolism," we then decided to investigate the seven most representative molecules regulated by bacteria in four different conditions: electronic cigarette users, alcohol consumers, precancerous lesions, and OSCC samples (Fig. 5). The results revealed that poly(A) polymerase pathway (involved in RNA degradation) is likely to be more found in cigarette users and alcohol consumer individuals (Fig. 5(3)) and succinate dehydrogenase/fumarate reductase (involved in many pathways, such as citrate cycle, oxidative phosphorylation, biosynthesis of secondary metabolites, and others) (Fig. 5(2)) and sedoheptulose-bisphosphatase (a hydrolase) (Fig. 5(4)) are prone to be found in both precancerous and OSCC samples. Interestingly, menaquinone-dependent protoporphyrinogen oxidase (involved in the biosynthesis of secondary metabolites) seems to be an important pathway on precancerous samples (Fig. 5(1)), whereas 3′,5′-cyclic-nucleotide phosphodiesterase (purine metabolism) and iron(III) transport system ATP-binding protein (an ABC transporter) (Fig. 5(5) and 5(7)) are likely to be more found in OSCC samples . The results also revealed that excluding the precancerous group, which included pullulanase enzyme (C6. PulnA; pullulanase [EC: 3.2.1.41]), and cancerous group which included arylsulfatase enzyme (aslA; arylsulfatase [EC:3.1.6.1]), all other groups had the same molecules (Fig. 6).

A heatmap was analyzed considering the four groups together and individually (Fig. 6). Figure 6a indicates that *Actinobacillus pleuropneumoniae* and *Actinobacillus equuli* expresses the most significant number of molecules (Fig. 6 and Table 1), followed by bacteria *Actinomyces oris* with four molecules and *Prevotella melaninogenica* and *Prevotella jejuni* with three molecules. The majority of the bacteria express between two and one molecules (Fig. 6 and Table 1). The bacteria, *Leptotrichia* sp., *Parvimonas micra*, *Fusobacterium nucleatum*, and *Fusobacterium hwasookii*, expressed only the molecule 3′,5′-cyclic-nucleotide phosphodiesterase. Interestingly and considering all groups, *Fusobacterium hwasookii* presented the higher level of expression of that molecule. Analyzing the precancerous samples, the molecules menaquinone-dependent protoporphyrinogen oxidase, poly(A) polymerase, 3′,5′-cyclic-nucleotide phosphodiesterase, and arylsulfatase were expressed by *Actinobacillus pleuropneumoniae* and *Actinobacillus equuli*, whereas *Haemophilus influenzae*,

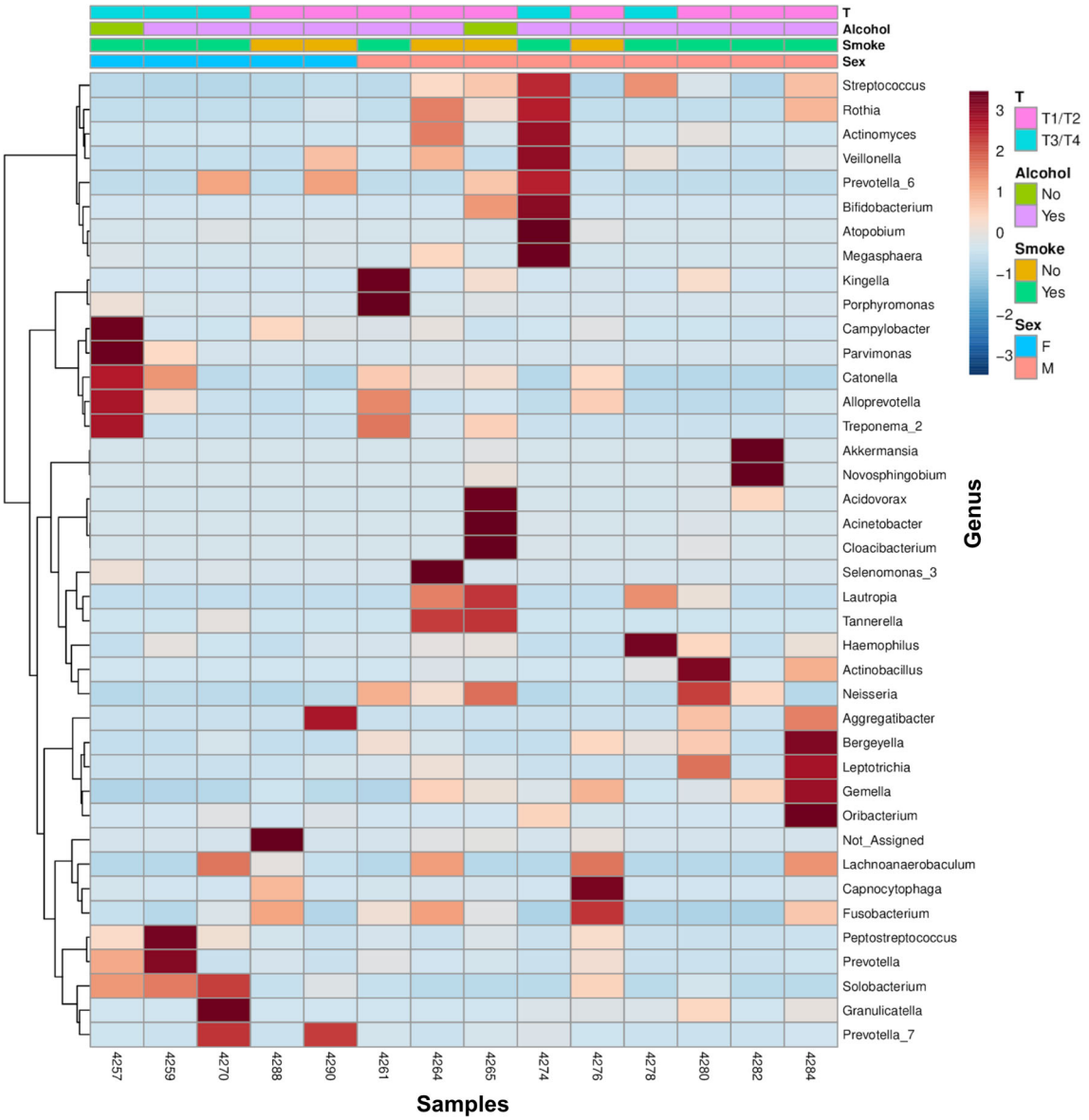

Fig. 4 Heatmap clustering taxonomy abundance at genus level in OSCC samples according to gender, use or not of electronic cigarettes, consumption of alcohol, and cancer staging (T, tumor size according to the Union for International Cancer Control-American Joint Committee on Cancer—UICC-AJCC)

Rothia mucilaginosa, *Prevotella melaninogenica*, and *Prevotella jejuni* by two molecules each. Twelve bacteria expressed only one molecule (Fig. 6b(3)). In OSCC samples, *Prevotella melaninogenica* and *Aggregatibacter aphrophilus* [NJ8700] accounted for three molecules, whereas *Neisseria weaveri*, *Prevotella jejuni*, *Aggregatibacter aphrophilus* [W10433], *Haemophilus influenzae*, and *Limnohabitans* sp. with two molecules. Twelve bacteria expressed only one molecule (Fig. 6b(4)). For a better understanding of the interactions of bacteria between each other, we perform a network analysis, thus being able to see how these organisms can regulate the environment in which they are present, in this case the oral cavity, in a negative or positive way (Fig. 7a). The main bacteria that are involved in the process of regulating molecules are shown in a barplot (Fig. 7b), where we can see a low difference in abundance for most of the genera found there. The contributions of *Akkermansia*, *Bacteroides*, *Neisseria*, and *Prevotella* were greater on OSCC samples. The contribution of *Veillonella* and *Rothia* was higher on precancerous samples. *Aggregatibacter* and *Actinobacillus* were similar between OSCC and precancerous samples.

Discussion

The resident oral microbiome is diverse and distinct from any other part of the human body. The composition of the microbiome tends to remain stable over time unless external

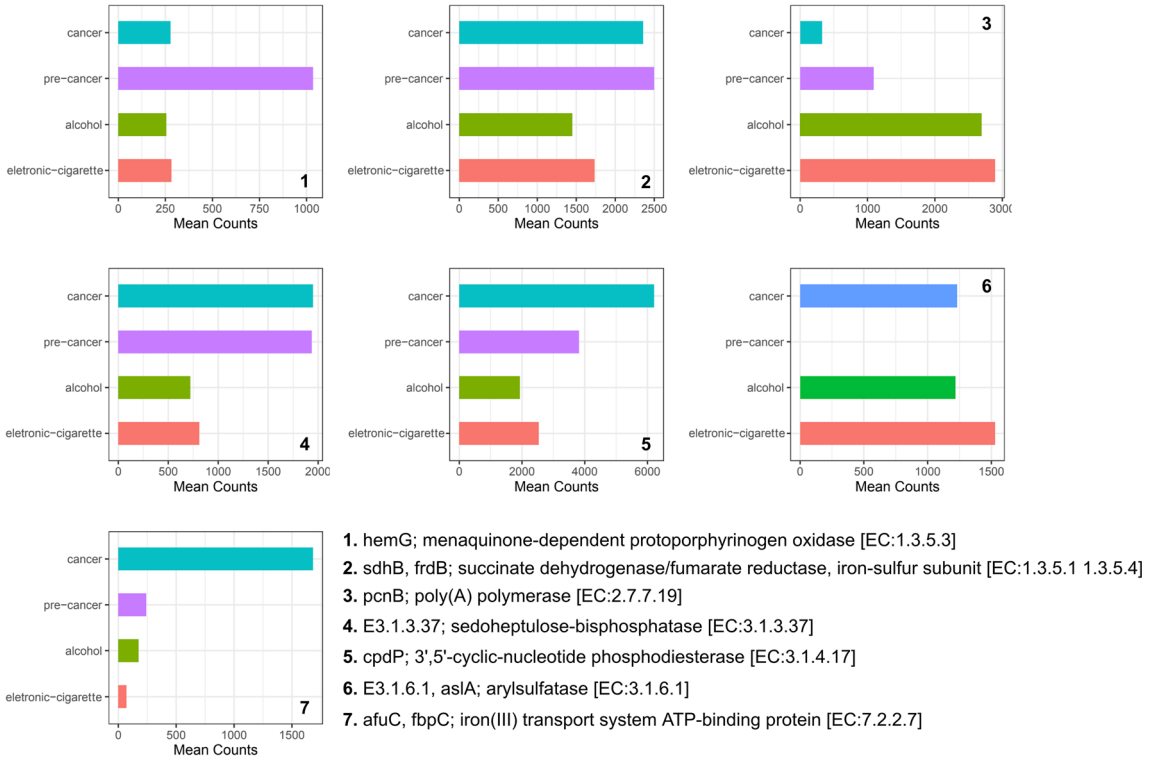

Fig. 5 Metabolic pathways predicted by the genera counts. The different pathways are regulated by bacteria in four different conditions, all the four groups are represented in the graphics in the following order: Cancer, precancer, alcohol and electronic cigarette users. The different molecules expressed are represented by the numbers: **1** hemG; menaquinone-dependent protoporphyrinogen oxidase [EC:1.3.5.3]; **2** sdhB, frdB; succinate dehydrogenase/fumarate reductase, iron-sulfur subunit [EC:1.3.5.1 1.3.5.4]; **3** pcnB; poly(A) polymerase [EC:2.7.7.19]; **4** E3.1.3.37; sedoheptulose-bisphosphatase [EC:3.1.3.37]; **5** cpdP; 3′,5′-cyclic-nucleotide phosphodiesterase [EC:3.1.4.17]; **6** E3.1.6.1, aslA; arylsulfatase [EC:3.1.6.1]; **7** afuC, fbpC; iron(III) transport system ATP-binding protein [EC:7.2.2.7]

or internal stressors disrupt the microbial homeostasis. In this context, studies have shown shifts in the oral microbiome of healthy individuals in comparison to those with chronic periodontitis or dental caries [34–37]. While smoking is considered as one of the main risk factors for periodontitis development [38, 39], carbohydrate intake, specifically through sucrose ingestion, is considered as the main factor inducing microbiota dysbiosis [40]. In line with these findings, the role of oral microbiota on both precancerous and OSCC lesion development has also been investigated in an attempt to identify potential microbial diagnostic markers or targets for cancer treatment [23, 41].

Our data show some differences among the studied groups at both phylum and at genus abundance levels. *Firmicutes* were the main phylum identified in electronic cigarette users and in alcohol consumers, which was mostly represented by the *Streptococcus* spp. genus. On the other hand, *Proteobacteria* and *Haemophilus* spp. predominated in precancerous lesions (Figure S1 and Fig. 1). Greater relative abundance at the genus level was found in OSCC samples in comparison to the other groups (Fig. 1), which was represented by an increased proportion of *Fusobacterium* spp., *Prevotella* spp., *Haemophilus* spp., *Campylobacter* spp., and others. Moreover, the cancer-associated microbiome possesses 140 different genera in total (Fig. 2f). Abundance at the community level also tended to be different in OSCC samples (Fig. 2e). *Bacteroidetes* predominated in the cancer-associated microbiome (Figure S1).

Previous studies also found that *Streptococcus* and *Haemophilus* tended to be more abundant in healthy individuals [42, 43]. Moreover, greater abundance of *Bacteroidetes* has also been shown to be more associated with cancerous lesions than to precancerous ones [42, 44], which agrees with our data (Fig. 1 and Figure S1). It has been reported that the risk for OSCC development is increased up to two times in the presence of some periodontopathogens, such as *Prevotella tannerae*, *Prevotella intermedia*, and *Fusobacterium nucleatum*, on saliva [19]. This way, it seems that poor oral hygiene may lead to increased salivary levels of such periodontopathogens, which in turn may affect the prognosis of individuals presenting head and neck cancer [19, 45]. Although we cannot make any inference about the oral hygiene of our studied individuals, it is important to highlight that a greater abundance of both *Fusobacterium* and *Prevotella* was also found in our OSCC samples. In line with this finding, *F. nucleatum* might be also used as biomarkers for oral cancer [23].

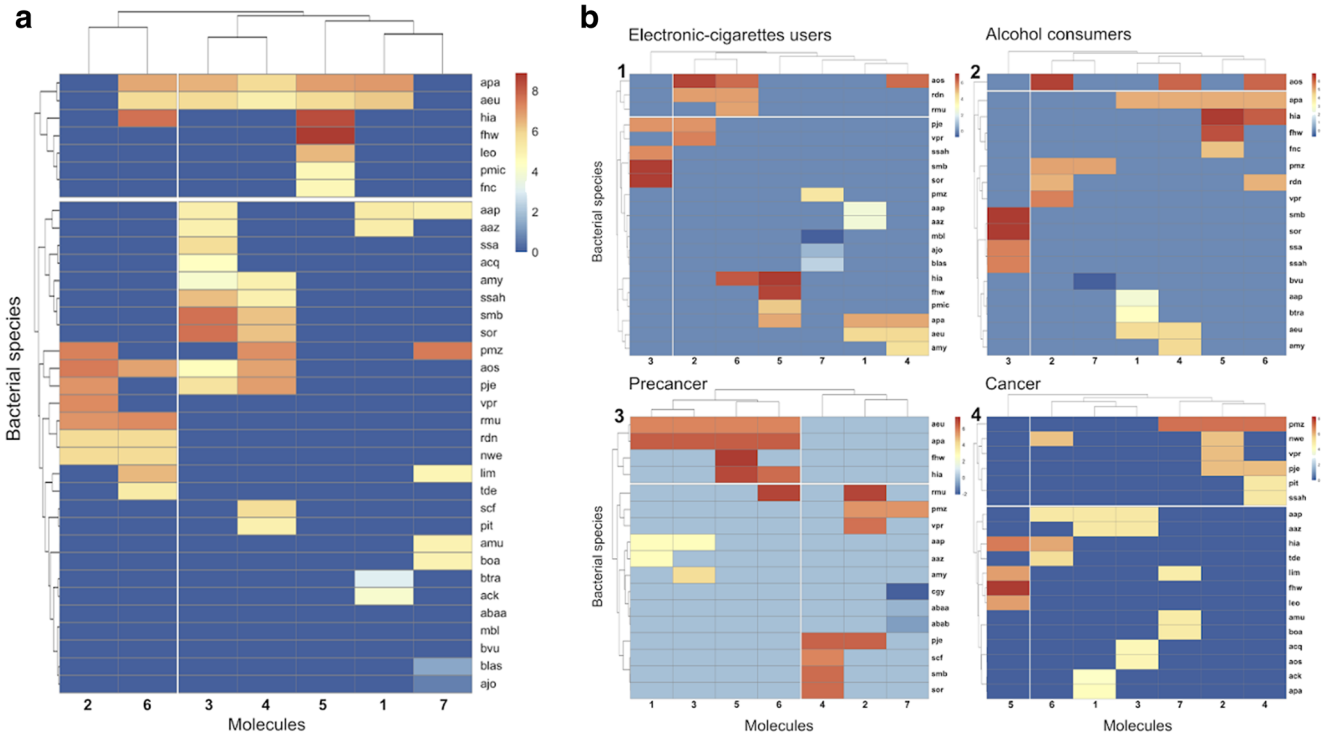

Fig. 6 Heatmap indicating the number of molecules expressed per bacteria on two scenarios. **a** Shows the number of molecules expressed per species considering the four groups of samples; **b**(1) number of molecules expressed in electronic cigarette users; **b**(2) number of molecules expressed in alcohol consumers group; **b**(3) number of molecules expressed in precancerous group; **b**(4) number of molecules expressed in cancer. The top seven molecules expressed in the functional prediction more enriched pathways (Figure S4): 1 hemG; menaquinone-dependent protoporphyrinogen oxidase [EC:1.3.5.3]; 2 sdhB, frdB; succinate dehydrogenase/fumarate reductase, iron-sulfur subunit [EC:1.3.5.1 1.3.5.4]; 3 pcnB; poly(A) polymerase [EC:2.7.7.19]; 4 E3.1.3.37; sedoheptulose-bisphosphatase [EC:3.1.3.37]; 5 cpdP; 3′,5′-cyclic-nucleotide phosphodiesterase [EC:3.1.4.17]; 6 E3.1.6.1, aslA; arylsulfatase [EC:3.1.6.1]; 7 afuC, fbpC; iron(III) transport system ATP-binding protein [EC:7.2.2.7]. Molecules expressed exclusively in the precancerous group: **b**(3) 6 pulnA; pullulanase [EC:3.2.1.41], and cancerous group: **b**(4) 6 E3.1.6.1, aslA; arylsulfatase [EC:3.1.6.1]. Full species name: *aap* (*Aggregatibacter aphrophilus*), *aaz* (*Aggregatibacter aphrophilus*), *abaa* (*Acinetobacter baumannii*), *ack* (*Acidovorax* sp.), *acq* (*Actinomyces* sp.), *aeu* (*Actinobacillus equuli*), *ajo* (*Acinetobacter johnsonii*), *amu* (*Akkermansia muciniphila*), *amy* (*Schaalia meyeri*), *aos* (*Actinomyces oris*), *apa* (*Actinobacillus pleuropneumoniae*), *blas* (*Blastomonas* sp.), *boa* (*Bacteroides ovatus*), *btra* (*Bibersteinia trehalosi*), *bvu* (*Bacteroides vulgatus*), *fhw* (*Fusobacterium hwasookii*), *fnc* (*Fusobacterium nucleatum*), *hia* (*Haemophilus influenzae*), *leo* (*Leptotrichia* sp.), *lim* (*Limnohabitans* sp.), *mbl* (*Moraxella bovoculi*), *nwe* (*Neisseria weaveri*), *pit* (*Prevotella intermedia*), *pje* (*Prevotella jejuni*), *pmic* (*Parvimonas micra*), *pmz* (*Prevotella melaninogenica*), *rdn* (*Rothia dentocariosa*), *rmu* (*Rothia mucilaginosa*), *scf* (*Streptococcus parasanguinis*), *smb* (*Streptococcus mitis*), *sor* (*Streptococcus oralis*), *ssa* (*Streptococcus sanguinis*), *ssah* (*Streptococcus salivarius*), *tde* (*Treponema denticola*), *vpr* (*Veillonella parvula*)

By comparing genera abundance among the groups, we found a commonly shared core comprising 52 genera. At the community level, it seemed that electronic cigarette users, alcohol consumers, and precancerous samples presented an overall pattern that does not resemble the one found in OSCC samples (Fig. 2e). Other 52 genera were exclusively found on OSCC samples (Fig. 2f, Table S1). It is important to highlight those OSCC-related genera do not belong to the group of microorganisms commonly associated with healthy oral cavity [46], suggesting that either a microbial dysbiosis might have led to malignant lesion development or that the tumor environment induced such changes on microbial composition. The cause-effect mechanism is still unclear. Yet, the role of those exclusively found and OSCC-related genera on cancer development is inconclusive or even unknown, and it deserves further investigation. Specifically, *Solobacterium* has been considered as a malignant lesion-associated genus [44]. However, in the present study, this genus was co-shared among all the studied groups. Although we understand that oral microbiota is highly diverse which limits the comparison among different studies, we acknowledge that differences between our data and those from Hashimoto et al. might be also related to sampling techniques, since saliva was used as a biological sample in the former and mucosal swabs were used to create the microbiome database used in the present study [27]. Besides, it is not possible to infer from the data from Schmidt et al. which type of malignant/precancerous lesions were assessed.

Gender-specific differences in microbiome have been reported. Salivary microbiome is different between male and female children which could pose girls to an increased risk for dental caries development [47]. Gut microbiome gender-

Table 1 Number of molecules expressed by bacteria

Bacteria*	Molecules						
	1	2	3	4	5	6	7
Actinobacillus pleuropneumoniae (apa), *Actinobacillus equuli* (aeu)	■		■	■	■	■	
Actinomyces oris (aos)		■	■	■		■	
Aggregatibacter aphrophilus [NJ8700] (aap)	■		■				■
Prevotella melaninogenica (pmz)		■		■			■
Prevotella jejuni (pje)		■	■	■			
Haemophilus influenzae (hia)					■	■	
Aggregatibacter aphrophilus [W10433] (aaz)	■		■				
Schaalia meyeri (amy), *Streptococcus salivarius* (ssah), *Streptococcus mitis* (smb), *Streptococcus oralis* (sor)			■	■			
Rothia mucilagenosa (rmu), *Rothia dentocariosa* (rdn), *Neisseria weaveri* (nwe)		■				■	
Limnohabitans sp. (lim)						■	■
Fusobacterium hwasokii (fhw), *Leptotrichia sp.* [oral taxon 212] (leo), *Parvimonas micra* (pmic), *Fusobacterium nucleatum* (fnc)					■		
Streptococcus sanguinis (ssa), *Actinomyces sp.* oral [taxon 414] (acq)			■				
Veillonella parvula (vpr)		■					
Treponema denticola (tde)						■	
Streptococcus parasanguinis (scf), *Prevotella intermedia* (pit)				■			
Akkermansia muciniphila (amu), *Bacteroides ovatus* (boa)							■
Bibersteinia trehalosi (btra), *Acidovorax sp.* [KKS102] (ack)	■						
Acinetobacter baumannii (abaa)							■
Moraxella bovoculi (mbl)							■
Bacteroides vulgatus (bvu)							■
Blastomonas sp. (blas)							■
Acinetobacter johnsonii (ajo)							■

*KEEG bacteria full name. 1 hemG | menaquinone-dependent protoporphyrinogen oxidase [EC:1.3.5.3]; 2 sdhB, frdB | succinate dehydrogenase/fumarate reductase, iron-sulfur subunit [EC:1.3.5.1 1.3.5.4]; 3 pcnB | poly(A) polymerase [EC:2.7.7.19]; 4 E3.1.3.37 | sedoheptulose-bisphosphatase [EC:3.1.3.37]; 5 cpdP | 3′,5′-cyclic-nucleotide phosphodiesterase [EC:3.1.4.17]; 6 E3.1.6.1, aslA | arylsulfatase [EC:3.1.6.1; **7**) afuC, fbpC | iron(III) transport system ATP-binding protein [EC:7.2.2.7]

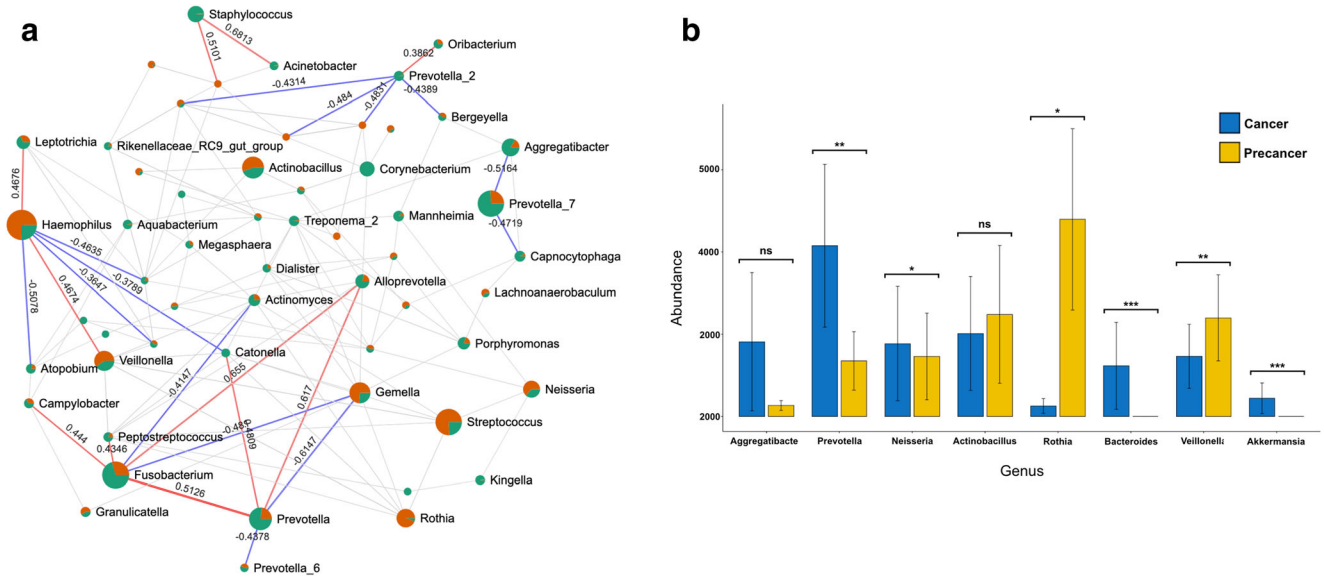

Fig. 7 Correlation analysis between the bacterial community and the main bacteria that regulate the predicted molecules. **a** Network demonstrating positive and negative regulations between the different bacteria that make up the oral microbiome, **b** barplot showing different abundances taxa between the main bacteria that regulate molecules in cancerous and precancerous samples. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.0001 by Mann-Whitney test

related differences might also be associated with distinct susceptibility to neurologic diseases, to arterial hypertension, to inflammatory bowel disease, and to major depressive disorder [48–51]. A few studies, though, report gender differences on microbiome and their role on carcinogenesis. Urinary-associated microbiome seems also to be gender-specific and the role of the gender-distinct microbial diversity on bladder cancer has been discussed [52]. Distinct gut microbiota between male and female animals also suggest a possible link to an increased risk for liver cancer [53]. However, it seems this association has not been reported elsewhere in relation to OSCC. We found the abundance at both genus and community levels is gender dependent (Fig. 4 and Figure S2). Whether those differences possess any clinical significance is unclear at the moment. Nevertheless, it is important to emphasize that most of T3/T4 staged lesions were found in men (Fig. 4).

Microbiome and metabolome shifts have been found in more advanced and aggressive malignant lesions, such as in colorectal and in breast cancerous lesions [54, 55], being the abundance of *F. nucleatum* increased in advanced lesions [55]. Higher abundance of *Streptococcus* and *Prevotella* in T3/T4 staged esophageal squamous cell carcinoma was associated with an increased hazard ratio and with a worse prognosis [56]. Concerning to OSCC and by assessing samples from individual's oral rinse, the abundance of *Fusobacteria* increased from T1 to T4 staged lesions, but *Streptococcus*, *Haemophilus*, *Porphyromonas*, and *Actinomyces* decreased in more advanced lesions [57]. *Acinetobacter* and *Fusobacterium* abundance increased in mucosal samples of more advanced OSCC lesions [41]. Our results show an increase in *Bacteroidetes* and *Firmicutes* abundance and a decrease in the abundance of *Proteobacteria* and *Fusobacteria* in T3/T4 staged lesions. Moreover, we observed an increase in the abundance of *Prevotella* and *Haemophilus* in more advanced OSCC lesions (Fig. 3 and Figure S3). Indeed, microbiome composition is different among saliva, oral rinse, and mucosal sites samples, being mucosal sites showing less diverse microbial communities [41]. Nevertheless, there is a consistency among different studies that a microbial signature might be associated with malignant mucosal lesions. The bulk of evidence also suggests the importance of microbial dysbiosis in malignant lesion development, which is in agreement with the data shown in the present study (Fig. 1, Figure S1, Fig. 2e and f).

Many hypotheses have encouraged discussions to understand the relationship between bacterial dysbiosis and tumorigenesis. It has been discussed that oral microbiota may either act inducing a chronic tissue inflammation or producing deleterious metabolites, interfering on cell signaling triggering intracellular pathways potentially harmful for the cell functionality [58–60]. Alternatively, some other evidence might support the concept that the development of malignant lesions may create a different environment that, in turn, affects the microbial composition of its surrounding [11]. It was also suggested that mucosal cell surface receptors' expression changes during the development of malignant lesions [61], which could affect the microbiome by a direct effect on microorganism adherence [43]. The exact causality, however, remains inconclusive at the moment. It is essential to elucidate the shifts on the microbiome associated with precancerous and malignant lesions as potential biological indicators of worse

prognosis. Nevertheless, those data may contribute to the discussion of preventive strategies for oral cancer therapies, as well as to facilitate cancerous lesion diagnosis at early stages. Ultimately, the knowledge about cancer-specific–associated microbiota might also be used as potential indicators for predicting prognosis.

An enrichment of metabolic pathways associated with bacterial motility, flagellar assembly, bacterial chemotaxis, and LPS synthesis has been found in the microbiome associated with malignant lesions [17, 41, 62]. Other studies have discussed that acetaldehyde might induce damages to cell DNA, and, therefore, an enrichment of metabolic pathways related to acetaldehyde production might be associated to carcinogenesis [63], being this production enhanced by smoking and alcohol consumption [64, 65]. Interestingly, *Rothia* and *Prevotella* are microorganisms able to produce acetaldehyde [66]. Moreover, *Rothia* has also been found in the microbiome of precancerous lesions [18, 43] which could also indicate a potential acetaldehyde-driven malignant lesion development.

Our data suggest that a distinct microbial profile is associated with OSCC lesions. A greater abundances of *Bacteroidetes*, *Fusobacterium*, *Prevotella*, and others are likely to be found in cancer-related microbiomes. Moreover, genera abundance varies according to cancerous lesion staging. Furthermore, vitamin K2 and iron-related metabolic pathways seem to be enhanced in OSCC lesions. Microbial abundance was similar among electronic cigarette users, alcohol consumers, and precancerous lesions, being the latter presenting some distinct functional patterns in comparison to the former ones. In addition to the qualitative and quantitative changes on microbial abundance among electronic cigarette users, alcohol consumers, and precancerous and OSCC samples, the predictive metabolic analysis of such microbiomes also suggests differences in metabolic patterns (Fig. 5). It is important to emphasize that both precancerous and OSCC samples seemed to present a specific associated functional pattern, being menaquinone-dependent protoporphyrinogen oxidase pattern enhanced in the former and both 3′,5′-cyclic-nucleotide phosphodiesterase (purine metabolism) and iron(III) transport system ATP-binding protein enhanced in the latter. *Actinobacillus* spp. was the main one responsible for precancerous functional patterns, while in OSCC lesion was the *Prevotella* spp. This demonstrates a change in the patterns of both diversity and the phylum that regulate these pathways. So we can also observe that even high levels of a phylum (in the case of *Fusobacterium* highly found in OSCC) may not be the main component of molecule regulation. Besides, as reported above, in addition to the distinct predictive metabolic pathways found among electronic cigarette users, alcohol consumers, and precancerous lesion and malignant lesion individuals, the microbial contribution to each of the identified pathways was also different (Fig. 6).

Within the specific enriched molecules, the presence of pullulanase restricted to the precancerous group may indicate an advanced stage of the malignant process of tumor cells. Zhang et al. demonstrated that the combined use of pullulan with ovalbumin inhibited tumor growth and liver metastasis [67]. Pullulanase is an enzyme that degrades pullulan, a purified polysaccharide from *Aureobasidium pullulans* that has a pro-inflammatory effect on tumor cells and can be an excellent adjunct to anticancer therapies. We envisage the presence of pullulanase in the context of the tumor microenvironment might promote the activation of biological processes involved in tumorigenesis of precancer to cancer status

Iron is a nutrient needed for many metabolic processes of both eukaryotic and prokaryotic cells. Specifically, a perfect equilibrium is needed for iron metabolism in terms of its absorption, transportation, uptake, and storage by eukaryotic cells [68]. Any dysregulation of those pathways may increase the risk for cancer development, being the growth of some tumors directly dependent on iron availability [68]. However, elevated levels of iron may induce cell death by ferroptosis that is an iron-triggered programmed cell death. The mechanisms involved in ferroptosis are still not very clear, but there is evidence to show that ferroptosis occurs with an adaptive response important for the removal of cancerous cells, and it can also act as an important factor in excessive levels of free iron [69]. It is clear then a balanced iron metabolism is essential for tumor growth. Cancer cells have a strong capacity to proliferate and metastasize since proliferation is closely associated with the vast biosynthesis of nucleic acids and proteins; the acquisition of energy is vital. Mitochondria generate energy and contain diverse enzymes involved in the synthesis. Iron is a crucial element of biosynthesis of these enzymes, and one of the most important pathways of mitochondrial iron is the iron-sulfur cluster (Fe-S cluster) biogenesis [70, 71]. The Fe-S cluster contains multiple enzymes, including NADH-ubiquinone oxidoreductase, one of the most substantial membrane-bound enzymes in the cell and is the largest complex of the mitochondrial respiratory chain. This enzyme's primary function is ATP production; in total, NADH-ubiquinone oxidoreductase is responsible for ~40% of ATP synthesis [72, 73]. In cancer cells, a high concentration of iron and these enzymes, such as NADH-ubiquinone oxidoreductase, which promote cellular growth, both labor a critical role in proliferation [74]. Small molecular NADH-ubiquinone oxidoreductase inhibitors have been identified as anticancer agents. For example, rotenoids, polyphenols AG311, metformin, BAY 87-2243, fenofibrate, canagliflozin, and kalkitoxin offer potential anticancer treatment [75, 76].

In both microbiomes associated with precancerous and OSCC samples, an enhanced predictive functional pattern related to uptake of iron was found (Fig. 5). Menaquinone-dependent protoporphyrinogen oxidase (hemG) predictive pattern is enhanced in microbiomes associated with precancerous

lesions. This pattern is related to heme synthesis in both aerobic and anaerobic conditions. Heme is an iron-rich prosthetic group considered an iron source for bacterial metabolism [77]. Moreover, ABC-type iron(III) transporter is a predictive pattern enhanced in microbiomes associated with OSCC samples. This transporter is responsible for interacting with a periplasmic iron-binding protein to import iron(III) ions into the cytoplasm. This finding agrees with a recent study that showed an enhanced pattern for iron transport in microbiomes associated with OSCC [78]. It is interesting to observe that, in addition to the protoporphyrinogen oxidase (hemG) mentioned above, the succinate dehydrogenase/fumarate reductase (sdhB/frdB) patterns were also enhanced in both precancerous and OSCC associated microbiome. Those pathways are menaquinone (vitamin K2) dependent. It has been discussed that menaquinone inhibits proliferation and invasiveness of cancer cells and also induces apoptosis and autophagy [20]. Some evidence also suggests vitamin K2 may exert an anticancer activity over the liver, bladder, and prostate tumor cells [79–83]. Altogether, our results indicate that microbiomes of precancerous and OSCC samples are acquiring extracellular iron and vitamin K2, reducing their availability to tumor cells. Considering the excess of iron is toxic to tumor cells [69] and that vitamin K2 may exert an anticancer effect, we hypothesized the metabolism of the precancerous and OSCC associated microbiomes is somehow creating ideal iron/vitamin K2 concentrations for both precancerous and cancer cell growth, which means the microbiome might be contributing to tumor growth.

Some studies have already demonstrated the importance of iron for both bacteria and for the growth of tumor cells, but this correlation between microbiome and tumor environment and how iron regulates them is an original observation. Therefore, we conclude that although precancerous and OSCC samples present some differences in microbial profile, both microbiomes present a common microbial functional signature that is potentially contributing to tumor growth. Further studies are necessary to further infer how these mechanisms work more clearly, which may arouse interest in investigating whether this pattern is reproducible in other types of cancer and even if it can be inferred in its aggressiveness or treatment.

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