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Research

A study on the microbiome within oropharyngeal cancer tissues

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Abstract
Oropharyngeal cancer (OPC), a prevalent head and neck malignancy, is witnessing a rise in incidence and mortality rates annually. Our study aimed to understand the microbial composition within OPC tissue, utilizing the 2bRAD-M technique to analyze microbiome characteristics of tissue samples from 46 OPC patients and 31 with tonsillitis, followed by bioinformatics analysis. We identified higher relative abundances of Selenomonas sputigena, Nocardia farcinica, and other species in the OPC group compared to the tonsillitis group. KEGG functional prediction revealed enrichment in bile secretion, type II polyketide backbone biosynthesis, staurosporine biosynthesis, and cGMP-PKG signaling pathways. HPV-positive OPC patients showed greater abundances of Pseudomonas and other species, with differential gene enrichment in "ATP-binding cassette" and "ACSL" processes. These microbial disparities may offer potential biomarkers for OPC prediction and insight into its progression, informing treatment strategies for HPV-positive and HPV-negative patients.

Keywords Microbiota · Oropharyngeal cancer · HPV

1 Introduction

Oropharyngeal cancer (OPC) is a common malignant tumour of the head and neck. Epidemiologically, there has been a consistent annual increase in both the incidence rate and mortality rate of this disease. Notably, approximately 90% of OPC cases are classified as squamous cell carcinomas [1]. The palatine tonsils and the base of the tongue are the predominant anatomical sites susceptible to carcinogenesis in the oropharyngeal region [2]. Currently, smoking and alcohol consumption are recognized as the primary risk factors [3]. Additionally, infections caused by human papillomavirus (HPV) and imbalances in the oral microbiota are implicated as contributing risk factors.

Ran Sun and Lu Xing have contributed equally to this work.

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The human microbiota, which comprises trillions of microorganisms, plays a crucial role in our health, with diverse functionalities closely intertwined with our well-being. Dysbiosis, an ecological imbalance within these symbiotic microbial populations, exerts significant and enduring effects on individuals with cancers of the gastrointestinal tract, hepatobiliary system, pancreas, and colorectum. Such imbalances are frequently induced by nutritional deficiencies or a range of other contributing external factors [4, 5]. The influence of microbes, particularly *Helicobacter pylori*, on the aetiology and progression of gastric cancer is well established in the scientific community [6]. Recent high-throughput sequencing analyses have revealed associations between colorectal cancer and infections caused by Fusobacterium [7] and Porphyromonas [8] species, suggesting that the microbiome plays a broader role in the process of carcinogenesis.

Research in the field of head and neck malignancies has focused largely on characterizing the distinct microbial communities related to laryngeal carcinoma. The association between oral microbiome dysbiosis and the emergence and progression of oral cancer is now widely accepted in the scientific community. Moreover, suboptimal oral hygiene and periodontal disease, factors that can alter bacterial ecosystems, have been found to be independently correlated with oropharyngeal cancer risk in epidemiological studies. However, research data on the intratumoural microbiome of OPC are exceedingly limited. This study aimed to investigate the differences in the microbial communities between OPC tissue samples and tonsillitis samples, the microbial composition of tumour samples from the HPV-positive and HPV-negative groups was examined.

2 Materials and methods

2.1 Sample collection

Patients were enrolled at the Affiliated Provincial Hospital of Shandong First Medical University from December 2022 to June 2023. All participants were divided into two groups: (1) the tumour group (T group): 48 patients with pathologically confirmed oropharyngeal cancer who underwent curative resection surgery; and (2) the control group (T_C group): 31 patients with pathologically confirmed tonsillitis. In the tumour group, 8 patients with HPV positivity were selected to form Group A, and 9 patients with HPV negativity were selected to form Group B. The present study has obtained formal approval from the Medical Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. All participants were thoroughly informed of the research content and provided written informed consent prior to their involvement. This ensured the ethical compliance of the research process and safeguarded the rights and interests of the participants.

Exclusion Criteria:

1) Infection with hepatitis B virus (HBV), hepatitis C virus (HCV), Treponema pallidum (syphilis), or human immunodeficiency virus (HIV)
2) History of malignancies, chemotherapy, or radiotherapy.

Clinical information, such as age, sex, and smoking and alcohol consumption habits, was obtained from the participants' medical histories. Tumour tissue and tonsil tissue were harvested from the central region of the lesion during surgery, embedded in paraffin blocks for preservation, and processed for further analysis.

The paraffin blocks are sealed and stored in a dry environment at 4℃, avoiding direct sunlight to prevent the degradation of biomolecules. The paraffin blocks are fixed in paraffin embedding cassettes and sealed, which can be further ensured by placing them in a desiccator.Formalin-fixed paraffin-embedded (FFPE) specimens, characterized by their capacity for long-term preservation at ambient temperatures and the maintenance of intact tissue architecture, have emerged as a vital resource in the realms of pathology and molecular biology [9]. Owing to technological innovations, the utility of FFPE specimens in microbiome research has been progressively expanding. Empirical studies have demonstrated that, despite a potential diminution in microbiome α-diversity, FFPE specimens remain amenable to microbiome analysis and are capable of yielding microbiome data of significant value [10].

2.2 2B-RAD-M gene sequencing and analysis

DNA isolation: DNA extraction was performed using the Upure FFPE Tissue DNA Kit (Cat. No. M2015-A96) from Biokeystone. Specifically, RNase-free and DNase-free reagents and consumables were utilized throughout the extraction process to minimize the risk of contamination. The integrity of the extracted DNA was assessed via agarose gel electrophoresis, while its purity was evaluated using a NanoDrop spectrophotometer.

Library preparation: The 2bRAD-M library preparation basically followed the original protocol developed by Wang et al.with minor modifications [11]. DNA was digested with the BcgI enzyme, followed by ligation of adaptors via T4 DNA ligase. The ligation products were amplified and purified; the bands of interest were those under 100 bp on the polyacrylamide gel.

Sequencing: PCR products were sequenced via the Illumina Nova PE150 platform at Qingdao OE Biotech Co., Ltd.

Data analysis and marker identification: From 173,165 microbial genomes, 2bRAD tags were sampled via Perl scripts and compared to form a database of species-specific markers.

Relative abundance calculation: Sequenced 2bRAD tags were mapped against the marker database. The G score was calculated to control false-positives, considering read coverage and the number of markers. The average read coverage was used to estimate the number of individual reads per species, and the relative abundance was calculated as the ratio of individual reads of a species to the total number of detectable individual reads [12].

2.3 Statistical analysis

Statistical analysis was conducted via R software. The alpha diversity between the tumour group (T) and the tonsil group (T_C) was compared via paired t tests and Wilcoxon tests for the Chao1 index (species richness and abundance) and Simpson index (species diversity). Beta diversity was assessed via principal coordinate analysis (PCoA) based on binary Jaccard distances to compare the differences between the T and T_C groups.

2.4 PICRUSt2 analysis

PICRUSt2 software (version 2.3.0b0) was used to infer the functional profiles of known microbial genes, facilitating a statistical analysis to discern functional disparities between the tumour and tonsil groups. We performed KEGG pathway enrichment analysis on the basis of 16S rRNA data and assessed the KEGG results at three different levels (via Wilcoxon and Kruskal–Wallis analyses). A false discovery rate (FDR) threshold of less than 0.05 was set as the criterion for statistical significance. On the basis of the COG functional prediction derived from 16S rRNA, we performed differential statistical analysis on the results, considering a false discovery rate (FDR) threshold of less than 0.05 as the criterion for statistical significance.

2.5 Ethics approval and consent participate.

The study was performed in accordance with the ethical standards of the Declaration of Helsinki (1964) and its subsequent amendments. All experimental protocols were approved by the Medical Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. All participants provided written informed consent before participating.

3 Results

3.1 Analysis of dysbiosis of microbial diversity in oropharyngeal cancer and tonsil groups

We collected a total of 77 samples from 77 patients, comprising 46 oropharyngeal cancer patients and 31 tonsillitis patients. The majority of patients were within the age range of 5070 years, with an average age of 61 years, and the sex ratio was 37:9. In ac-cordance with cancer statistics, the sex ratio and age range of the tumour group patients in this study were generally consistent with the epidemiological features of oropharyngeal cancer. The characteristics of the entire cohort are presented in Table 1. As depicted in the table, smoking and alcohol consumption were more common in the tumour group.Bulleted lists look like this:

Table 1 Clinical features of patients with OPC and tonsillitis include

Characteristics	OPC (n = 46)	tonsillitis (n = 31)	P
Age (years)			< 0.001
> 60	25	6	
50–60	14	10	
≤ 50	7	15	
Sex			0.064
Male	37	19	
Female	9	12	
Smoking status			0.660
Never	16	13	
Current	30	18	
Alcohol consumption			0.725
< 1 standard drink/day	15	12	
Current	31	19	

3.2 Microbial tissue composition: marked inter-group variations in oropharyngeal cancer vs. Ton-sillitis

The statistics of data volume changes during sequencing quality control are shown in Supplementary Table S1, including raw reads, enzyme reads, and clean reads. We subsequently analysed microbial alpha diversity, as depicted in Fig. 1a and Fig. 1b. The differences in the Chao1 ($p = 0.61$) and Simpson ($p = 0.33$) indices between the tumour group (T) and the tonsil group (T_C) were not statistically significant. However, both species richness and species diversity tended to be greater in the T group than in the T_C group.

Principal coordinate analysis (PCoA) was utilized to assess beta diversity. As illustrated in Fig. 1 c, the samples from the tumour group (T) were closely associated and formed tight clusters, which were distinct from those of the tonsil group (T_C) samples ($P = 0.003$). This finding indicates a significant difference in the microbial composition within the tissues between the T group and the T_C group.

3.3 Differential genera and species: distinct patterns in tumor and tonsillitis groups

We compared the relative abundance of the top 10 differential genera between the tumour group and the tonsil group. As anticipated, significant differences in microbial community composition at the genus level were observed across the different groups. According to the boxplot of the relative abundances of the genera (Fig. 2a), the relative abundances of Selenomonas and Nocardia species in tumour tissues were significantly greater in the tumour group than in the control group. In contrast, the relative abundance of Fusobacterium was significantly lower in the tumour tissue group than in the tonsil group.

An analysis of the differences in relative abundance at the species level between the tumour group and the tonsil group revealed that the relative abundances of Sele-nomonas sputigena, Nocardia farcinica, Capnocytophaga ochrecea, Cutibacterium acnes, and Capnocytophaga periodontitidis were greater in the tumour group than in the tonsil group, as shown in the boxplot. (Fig. 2b).

The composition of the differentially abundant species between the two groups was analysed via linear discriminant analysis (LDA) effect size (LEfSe). LEfSe identified 34 discriminative features with an LDA score ≥ 2.0, demonstrating significant differences in relative abundance between the tumour group T and the tonsil group T_C (Fig. 3).

3.4 Unveiling the distinct pathways in tumor and tonsillitis groups

KEGG prediction analysis revealed significantly upregulated pathways that were enriched in the tumour group (T) compared with the tonsil group (T_C). The results revealed enrichment in the following four pathways: 'Bile secretion', 'biosynthesis of type II polyketide backbone', 'staurosporine biosynthesis', and the 'cGMP-PKG signaling

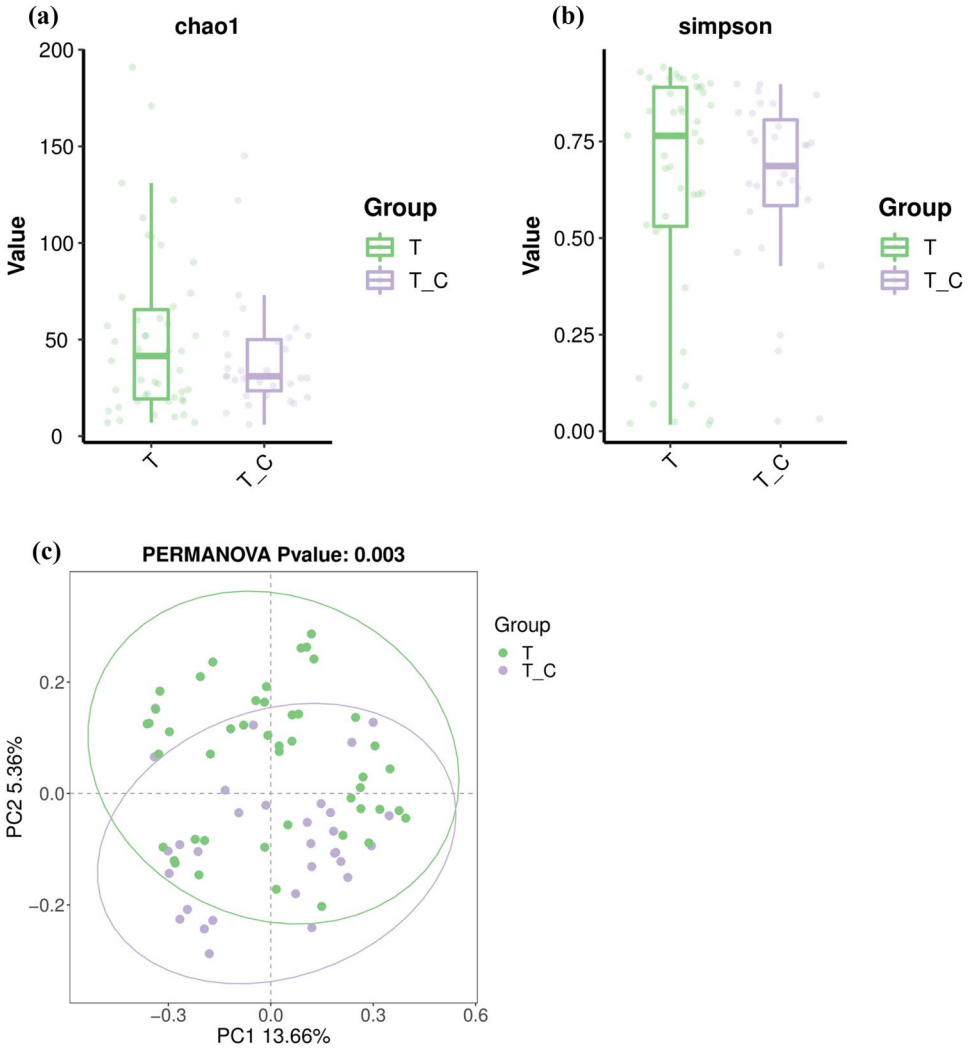

Fig. 1 The alpha diversity analysis results of Group T and Group T-C. **a** Comparison of alpha diversity between the tumour group (T) and the ttonsil group (T_C): The relative abundance in the T group was greater than that in the T_C group. **c** Comparison of beta diversity between the tumour group (T) and the tonsil group (T_C) on the basis of two-dimensional principal coordinate analysis (2D-PCoA). The T and T_C groups exhibit tight intragroup clustering, with significant intergroup distances

pathway'.COG functional prediction based on 16S rRNA data: Statistical analysis of the predictive results revealed significant differences, leading to the selection of the ABC-type glycerol-3-phosphate transport system, specifically the periplasmic component pathway, which was markedly greater in the tumour group (T) than in the tonsil group (T_C).

3.5 The microbial community composition differed between the HPV-positive and -negative groups

The observed Simpson index suggested that our sequencing depth was adequate (Fig. 4a). On the basis of the species abundance of samples from the HPV-positive and HPV-negative groups, a principal coordinate analysis (PCoA) was conducted via the Bray—Curtis distance matrix. This analysis revealed that samples within the HPV-positive group were closely clustered and distinctly separated from those in the HPV-negative group (Fig. 4b).

The T test and Wilcoxon test algorithms revealed significant differences fo Pseudomonas viridiflava, Stenotrophomonas_maltophilia, Bacillus bombysepticus, and Prevotella sp905372445, with species exhibiting a greater relative abundance in Group A than in Group B. The Kyoto Encyclopedia of Genes and Genomes (KEGG) functional prediction results indicated an increase in gene expression within Group A, particularly in processes such as 'ATP-binding cassette' and 'acyl-CoA synthase long-chain' (ACSL).

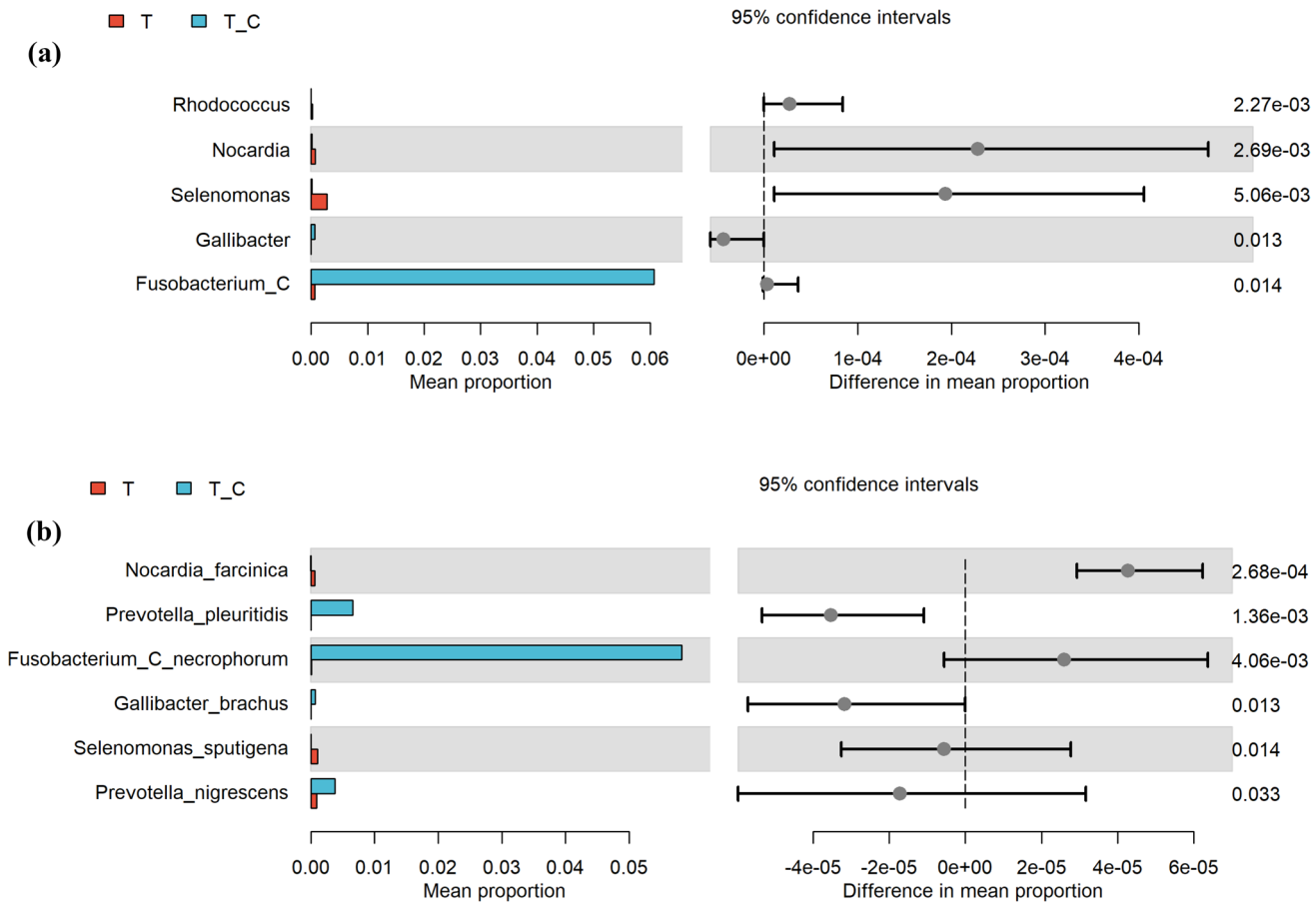

Fig. 2 Different genera and species between the tumor group and the tonsillitis group. The y-axis represents the relative abundance values of the species, with the species labels indicated on the upper section of the facet. **a**The relative abundances of Selenomonas and Nocardia species in tumour tissues were significantly greater in the tumour group than in the tonsillitis group; the relative abundance of Fusobacterium_species was significantly lower in the tumour tissue group than in the tonsil group; **b** The relative abundances of Selenomonas sputigena and Nocardia farcinica species in tumour tissues were significantly greater in the tumour group than in the tonsillitis group

4 Discussion

In this study, to ensure the accuracy and reliability of the results, we have implemented a series of measures to minimize potential biases introduced during the experimental process. We optimized the DNA extraction method by selecting a kit specifically designed for formalin-fixed paraffin-embedded (FFPE) samples, which enhances the extraction efficiency of microbial DNA and ensures its integrity. Additionally, we standardized operating procedures, strictly controlled experimental conditions, and judiciously employed automated technologies to reduce human-induced biases, thereby enhancing the reproducibility and accuracy of our experiments. Furthermore, the application of 2bRAD-M technology ensures consistent length of enzyme-cut short tags (32 bp) without amplification bias during PCR, which further reduces biases in library preparation.

Oropharyngeal cancer (OPC) is a highly complex multifactorial disease, with human papillomavirus (HPV) infection recognized as one of the primary aetiological factors. Hehes in the oral microbiome are also believed to be associated with the development and progression of oropharyngeal cancer. Specific microbial communities, such as Porphyromonas gingivalis and Fusobacterium nucleatum, have been linked to an increased risk of oropharyngeal cancer. As the incidence of oropharyngeal cancer continues to rise an-nually, a comprehensive study of its microbiome is indispensable. In this section, we investigated the differences in the microbial composition between oropharyngeal cancer tissues and tonsillitis tissues. Oropharyngeal cancer is commonly found in the elderly population, while

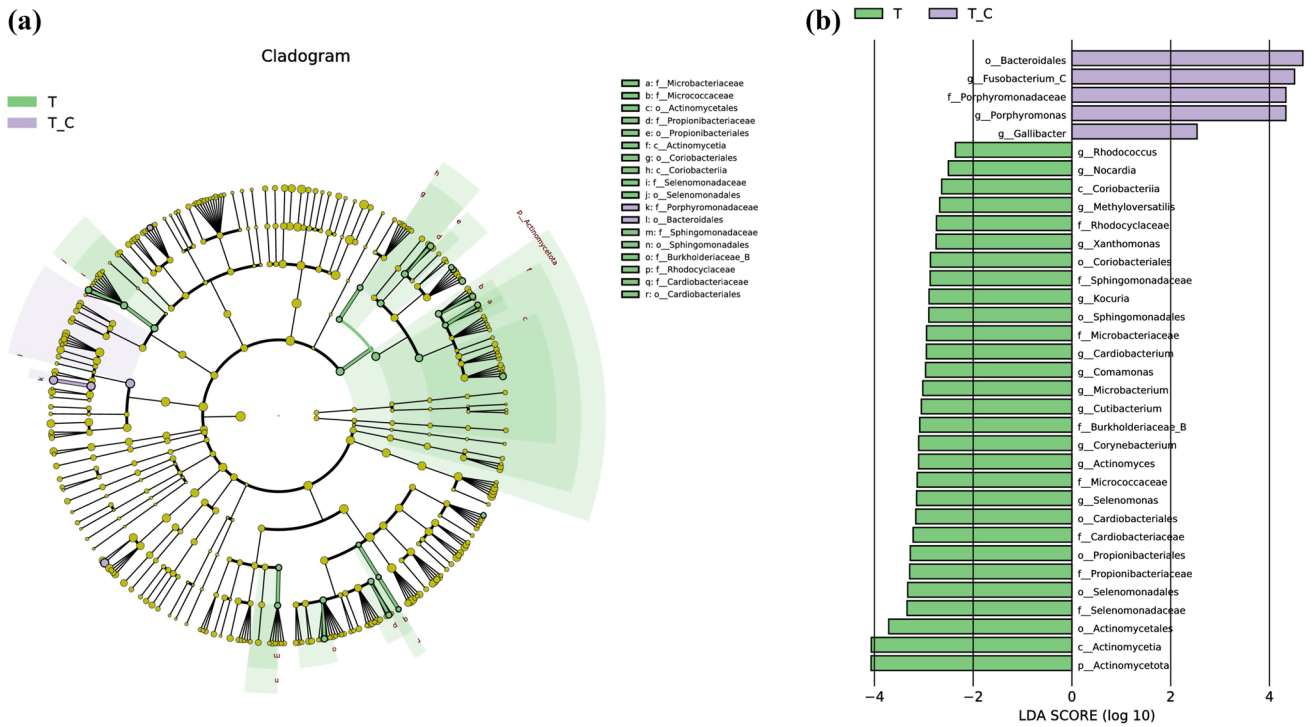

Fig. 3 LEfSe was performed to identify differentially abundant bacterial taxa be-tween the two groups. **a** The Cladogram shows the taxonomic hierarchical structure biomarkers identified by LEfSe. **b** The histogram of the LDA score shows significantly different biomarkers between the two groups

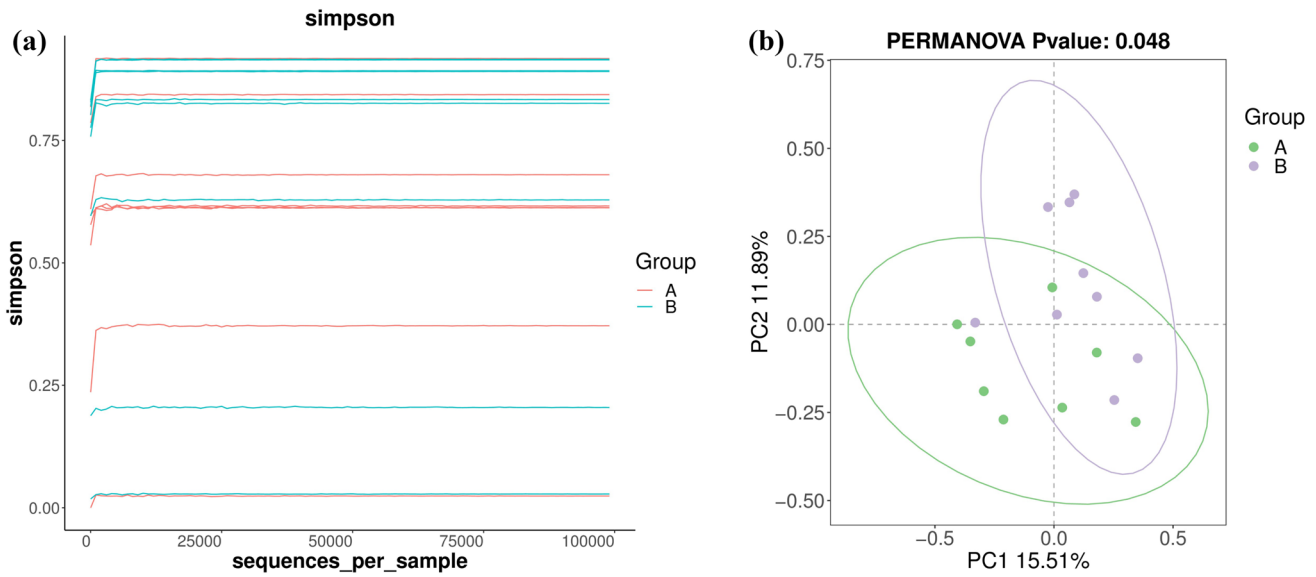

Fig. 4 Alpha diversity analysis results of HPV-positive group and HPV-negative group. **a** In the figure, each curve corresponds to an individual sample, with the x-axis representing the depth of random sampling (i.e., the number of sequences sampled) and the y-axis representing the exponential values. **b** The x-axis (PC1) and y-axis (PC2) rep-resent the two principal coordinates that account for the greatest degree of variation be-tween samples

the control group of tonsillitis patients is primarily made up of mid-dle-aged individuals. Consequently, there is a statistically significant age difference at baseline between the two groups ($P < 0.05$).

In the present study, alpha diversity analysis revealed slightly greater microbial species richness in oropharyngeal cancer tissues than in tonsillitis tissues. However, there were no significant differences in microbial abundance or diversity

between the tumour group (T) and the tonsillitis group (T_C). We hypothesize that the limited number of oropharyngeal cancer tissue and tonsillar group samples collected and the unequal numbers between the two groups may be the reasons for the nonsignificant differences observed. With respect to beta diversity, we observed certain differences in the microbial composition between tonsillitis tissues and oropharyngeal cancer tissues, particularly as evidenced by a notable difference in binary Jaccard distances between the two groups. Consequently, we postulate that the microbiota within oropharyngeal cancer tissues may serve as a potential biomarker for the diagnosis of oropharyngeal cancer.

Recent studies have established a significant link between Fusobacterium species and the development of multiple cancers. Notably, Fusobacterium has been observed to be particularly prevalent in head and neck, as well as oral cancer patients, with Fusobacterium nucleatum being especially notable. Its abundance appears to be correlated with tumor progression [13, 14]. The existing body of research predominantly centers on investigating the microbial communities residing within the oral cavity, acknowledging the potential for variations between the oral microbiome and the microbiota present in deeper tissues. The inconsistencies observed between our results and those reported in prior studies may be attributable to several factors, including the limited sample size and the unequal distribution of samples across groups. Moreover, a critical consideration is the divergence in sample types; our study specifically targeted oropharyngeal tissues, whereas the majority of previous investigations have focused on oral cancer tissues. This discrepancy in sample types likely constitutes a significant factor underlying the divergent microbial profiles reported. To achieve a more precise delineation of differential microbes, further research endeavors, encompassing larger and more balanced sample cohorts, are essential to conduct a comprehensive comparison of microbial profiles between tumor and tonsillitis groups.

Notably, among the differentially abundant bacteria identified, both *Capnocytophaga periodontitidis* and *Selenomonas sputigena* are likely to play roles in the onset and progression of periodontal disease, albeit potentially through different mechanisms. *Capnocytophaga periodontitidis* may be directly involved in the pathogenic process of periodontal disease [15], whereas Selenomonas sputigena may impact periodontal health through interactions with primary cariogenic bacteria [16]. Research has shown that individuals with periodontal disease are at increased risk of developing oral cancer, and the severity of periodontal inflammation has been correlated with an increased risk of oral cancer [17]. Additionally, studies have demonstrated that the association between periodontal disease and cancer is not limited to the oral cavity [18]. Studies have confirmed that chronic inflammation promotes the occurrence and development of cancer [19]. *Capnocytophaga periodontitidis* and *Selenomonas sputigena* can lead to the occurrence and development of chronic inflammation, which may be associated with the progression of tumours. The abundances of *Capnocytophaga periodontitidis* and *Selenomonas sputigena* were greater in the oropharyngeal cancer group than in the control group, so these species may influence the progression of oropharyngeal cancer through their impact on chronic inflammation. This study did not involve elucidation of the pathogenic mechanisms of the differentially abundant species, and further in-depth studies are needed to confirm this.

Through Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis aimed at determining the impact of gene expression levels in oropharyngeal cancer, we analysed the biological processes involved in the enriched pathways, the affected cellular components, and the executed molecular functions. The results revealed that the cGMP−PKG signalling pathway was enriched in the tumour group. Research has indicated that this pathway is closely associated with the occurrence and progression of breast and gastric cancers. Furthermore, an increase in de novo nucleotide synthesis in metastatic breast cancer cells is a critical metabolic signature that is related to the stemness and metastasis of tumour cells [20]. Given the biological processes closely associated with this pathway, we speculate that the enrichment of the cGMP-PKG signalling pathway in the tumour group may be related to the stemness and metastasis of oropharyngeal cancer tumour cells. The novel protein PRTG, which is induced by *Helicobacter pylori* infection, promotes gastric cancer development through the cGMP/PKG signalling pathway [21]. *Helicobacter pylori* has been recognized as a contributing factor in the development and progression of gastric cancer, and research into its carcinogenic pathways is more extensive than that of other microorganisms. Therefore, its carcinogenic pathways may extend beyond the occurrence and development of gastric cancer; however, our research on these pathways is limited, and we are not yet able to fully elucidate all the biological processes involved in this pathway. The bile secretion pathway has been identified as a significant factor in the pathogenesis of colorectal cancer, which shares some similar pathological features with oropharyngeal squamous cell carcinoma (OPSCC) [22]. Colorectal cancer is intricately linked to diets high in animal protein and saturated fats, which in turn trigger elevated bile secretion within the intestine. This heightened bile secretion subsequently alters the composition of the gut microbiota, fostering the production of secondary bile acids that are implicated in tumorigenesis. Moreover, bile secretion has been identified as a significant factor contributing to malignant transformation in various tumor tissues, including gastric

cancer and Barrett's adenocarcinoma [22, 23]. The link between oropharyngeal cancer and the gastrointestinal tract primarily manifests in the interplay of their respective microbiota. In the study of this pathway, we hypothesize that enrichment of the bile secretion pathway is associated with the malignant transformation of oropharyngeal tissue. Whether the increased intratumoural abundance of microbes in our study promotes tumorigenesis through these pathways remains to be elucidated.

Human papillomavirus (HPV) is closely associated with oropharyngeal cancer. Through comparative microbiome analysis between the HPV-positive and HPV-negative groups, we identified differential species, including Pseudomonas viridiflava, Stenotrophomonas maltophilia, Bacillus bombysepticus, and Prevotella sp905372445. Owing to the limited research on these microorganisms, it is currently difficult to ascertain their relationship with oropharyngeal cancer and HPV and whether they play a role in the development and progression of oropharyngeal cancer. The limited number of samples we selected may also have contributed to the difficulty in identifying differential species. The limited sample size of the present study, particularly within the HPV-positive and HPV-negative subgroups, restricts the statistical power and may introduce increased variability in the results. While the sequencing depth was adequate for both the HPV-positive and HPV-negative cohorts in our investigation, the robustness and generalizability of our findings would benefit from validation through larger sample sizes in future studies. Additionally, future research endeavors should incorporate clinical data to elucidate the mechanisms underlying the impact of the microbiome on oropharyngeal cancer and to explore its clinical significance.

With a similar approach, KEGG pathway enrichment analysis was conducted on tumour samples from the HPV-positive and HPV-negative groups. The findings revealed an increase in the expression of genes associated with pathways such as the 'ATP-binding cassette" and 'ACSL' pathways within the HPV-positive cohort. Studies have indicated that ATP-binding cassette (ABC) transporters are associated with the maintenance of intracellular cholesterol homeostasis [24]. Thus, the increased activity of ABC pathways in the HPV-positive group may suggest that cholesterol homeostasis in HPV-positive oropharyngeal cancer patients is compromised, potentially associated with the progression of their cancer. Furthermore, Quan J et al. [25] Members of the acyl-CoA synthetase long-chain (ACSL) family are responsible for the activation of FAs and play diverse roles in the development of cancer. HPV-positive oropharyngeal cancer may activate FAs through the ACSL, thereby regulating the onset and progression of cancer. However, our knowledge of the ATP-binding cassette and ACSL is currently limited, and we cannot conclusively determine their specific impact on HPV-positive oropharyngeal cancer; further in-depth research is warranted.

Concurrently, bioinformatics data confirmed that certain intratumoural microbial communities differed between the two groups of samples. However, more experimental data are currently needed. Although the potential role of the microbiota in oropharyngeal cancer has garnered attention, there are still some research challenges, including the accurate identification of microbial compositions, the specific mechanisms of interaction between microbes and tumours, and the translation of these findings into clinical applications. Future studies will need to further explore the complex relationship between the microbiota and oropharyngeal cancer and develop diagnostic and therapeutic strategies based on the microbiota.

5 Conclusion

In summary, comparative analysis revealed differences in the microbial communities between tumour and tonsillar samples; this analysis revealed microbes at the genus and species taxonomic levels with significantly greater relative abundances in the tumour group and revealed the significantly upregulated pathways. Additionally, the analysed the differences in microbial communities between HPV-positive and HPV-negative samples from oropharyngeal cancer patients were analysed. These newly discovered differentially abundant microbes or significantly upregulated pathways in the tumour microenvironment may serve as potential biomarkers for predicting oropharyngeal cancer (OPC) and provide guidance for the advancement of oropharyngeal cancer treatment.

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Data Availability The datasets generated and/or analysed during the current study are available in the Sequence Read Archive (SRA) repository, accession number PRJNA1198294.

Declarations

Competing Interests The authors declare no competing interests.

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