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REVIEW ARTICLE

Microbiome and Cancer in the Oral Cavity: A Bioinformatics Perspective

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ABSTRACT

The relationship between microbiome and cancer in the oral cavity has been under intense scrutiny over the past decade due to the advancement in next-generation sequencing. However, rapid accumulation of sequencing data may yield more questions than answers. Although such inconclusiveness can be attributed to the heterogeneous nature of biological phenomena, inconsistency in complex bioinformatics analysis may also play a role. In this review, we aim to provide a comprehensive and concise overview of common bioinformatics analysis processes used in microbiome studies focusing on 16S rDNA sequencing. By taking this bioinformatics perspective as a conceptual framework, we further discussed the consistency and discrepancies among numerous studies on the relationship between oral microbiome and oral cancer. This review aims to elucidate the bioinformatics methodologies and their impact on the current understanding of the oral microbiome's role in cancer development.

Keywords: Oral cavity; Bioinformatics; Microbiome

1 INTRODUCTION

The oral microbiome, the second largest microbiome in the human body, plays a critical role in maintaining both oral and systemic health[1,2]. Dysbiosis within this microbiome is a primary factor in dental diseases such as caries[3,4] and periodontal disease[5]. Moreover, the oral microbiome has been linked either directly or indirectly to systemic conditions, including diabetes[6], infective endocarditis[7], stroke[8], and Alzheimer's disease[9]. Thus, the oral microbiome is considered an integral part of the human body, co-evolving with the host's physiological and pathological states.

The relationship between the oral microbiome and cancer development has gained significant attention, particularly concerning oral squamous cell carcinoma (OSCC), which accounts for 90% of all oral cancer incidences[10]. Motivated by the well-documented association between Helicobacter pylori and gastric cancer[11], as well as the more recently recognized link between Fusobacterium nucleatum and colorectal cancer[12], researchers are increasingly concerned about the role of the oral microbiome in the development and progression of OSCC[13]. This concern has prompted numerous studies over the past decade, primarily using 16S rDNA sequencing, to explore the intricate pathophysiological mechanisms involving bacteria, tumor microenvironment, and cancer cells[14,15]. Given the multitude of studies on the oral microbiome and cancer, a deep understanding and robust methodology in bioinformatics analysis is crucial, yet often underestimated. The inconsistency in analysis processes across studies highlights the need for standardization and precision. In this review, we aim to present a comprehensive and concise overview of the bioinformatics analysis methods used in 16S rDNA amplicon sequencing. Additionally, we will outline the association between the microbiome and oral cancer, address microbiome-associated clinical factors and their influences on treatment and prognosis and discuss potential future research directions in this rapidly growing field.

1.1 *Overview of bioinformatics analysis process*

The Qiime2 computational ecosystem, a suite of analysis toolkits integrating methods from various sources, is commonly used to process and analyze microbiome sequencing data[16]. Starting from raw sequencing reads produced by sequencing machines (either Illumina or PacBio), the first step of the computational pipeline is to group reads with similar sequences together. There are two algorithms to achieve this: denoising and clustering, which generate amplicon sequence variants (ASVs) and operational taxonomic units (OTUs), respectively. Denoising algorithms, such as DADA2[17], monitor the error rate introduced during the sequencing process and recognize reads originating from the exact same sequence, making them highly reproducible and precise. However, in some cases, ASVs may be too granular for comparing sequences across different samples. Therefore, studies have used clustering algorithms like Vsearch[18] to group similar ASVs into OTUs at an arbitrary cutoff, such as 97% for species-level resolution. For each OTU, one representative sequence is chosen and compared against known databases such as SILVA[19] and Greengenes[20], to assign taxonomy (i.e., species identification) to the corresponding OTU. The number of reads is then counted for each OTU in each sample to construct a feature table, which is the core data structure for subsequent bioinformatics analyses.

The general purpose of microbiome studies is to comprehensively describe collections of diverse microbes. Therefore, methodologies in ecological studies, which considers complex ecosystems of diverse organisms, are widely used[21]. Using the feature table (produced by denoising or clustering algorithms) as a foundation, analyses of microbiome commonly fall into four categories of numerical methods: alpha diversity, beta diversity, differential abundance, and co-occurrence analysis.

1.2 *Alpha and beta diversity*

Alpha diversity measures how diverse the microbes are contained in each sample, which can be viewed as "intra-sample" diversity. From each sample, quantitative values for richness (e.g. observed features), evenness (e.g. Simpson index[22]) or both (e.g. Shannon entropy[23]) can be generated. These values are then aggregated by groups to be tested statistically. For pairwise comparison between groups, Mann-Whitney U test is advised, as alpha diversity values are not normally distributed (hence not using t-test)[24]. A healthy state of microbiome is commonly characterized by sufficient alpha diversity, composed of symbiotic, commensal microbes that outcompetes incoming pathogens[25]. On the contrary, a disease state of the microbiome - i.e. dysbiosis-harbors more pathogenic bacteria and an imbalanced microbial community[26]. Furthermore, under certain suboptimal health conditions, e.g. inflammatory bowel disease, the intrinsic alpha diversity could decrease, exhibiting a more fragile ecological system of microbial community[27].

Beta diversity measures the difference between a given pair of samples, i.e. "inter-sample" diversity. Various metrics are calculated to quantify these differences, including Euclidean distance, which calculates the straight-line distance between points in a multi-dimensional space; Bray-Curtis dissimilarity, which quantifies compositional dissimilarity based on relative abundances[28]; and UniFrac, a phylogenetic metric that incorporates evolutionary relationships between observed organisms[29]. The resulting distance matrix can be visualized on a 2D plane (i.e. plotted on a paper) via dimensionality reduction techniques, such as principal coordinates analysis (PCoA)[30], non-metric multidimensional scaling (NMDS)[31], t-distributed stochastic neighbor embedding (t-SNE)[32], or uniform manifold approximation and projection (UMAP)[33]. To date, PCoA is still the most widely used visualization technique due to the fact that it's simple, linear, and non-stochastic, hence suitable for moderate sample sizes. Nonetheless, the rapid increase in sequencing capability begins to result in large numbers of samples (possibly up to tens of thousands) in a single study[34]. In such cases, mathematically sophisticated techniques like t-SNE and UMAP are required to resolve subtle patterns happening at different levels of biological phenomena. Together, these methods enable researchers to visualize and interpret complex microbial community relationships across different samples in an intuitive manner.

Both alpha and beta diversity consider the collective state of microbial communities, providing a holistic, non-granular overview of the diversity and differences of samples without focusing on specific taxa of microbes. Nonetheless, the precision and depth in high-throughput sequencing allows researchers to inspect the abundance of specific microbes with regard to disease conditions. By referencing sequence databases of known microbes[19,20], enrichment and depletion analysis can be performed on a per-taxon basis, to identify key pathogens related to disease states.

1.3 *Differential abundance and co-occurrence analysis*

One of the most widely used methods to reveal microbes that are differentially abundant across different patient groups is Linear discriminant analysis Effect Size (LEfSe) analysis[35]. LEfSe combines linear discriminant analysis (LDA) with non-parametric statistical testing to identify features (e.g., microbial species) that are both statistically significant and biologically relevant by estimating the effect size (LDA score), which represents the magnitude of the difference in abundance between groups. This approach allows researchers to pinpoint specific microbial features that may play crucial roles in disease or health states. Microbial features reported by LEfSe can be further

validated statistically using methods like the Mann-Whitney U test[24] for pairwise comparisons and the Kruskal-Wallis test[36] for multi-class (more than two classes) comparisons, respectively.

It is noted, however, that there are hundreds of oral microbial species to be tested. This results in increased false-positive results, a problem known as the multiple testing problem[37]. To address this, corrections for multiple comparisons are essential to control the false discovery rate (FDR). One of the earliest methods developed for this purpose is the Bonferroni correction[38], which adjusts the significance threshold by dividing it by the number of tests performed. While this method is straightforward, it is often overly stringent, limiting the statistical power to reveal any biologically relevant targets. In contrast, the Benjamini-Hochberg procedure is a more modern and widely used approach that controls the FDR, providing a balance between identifying true positives and controlling false positives[39]. The Benjamini-Hochberg method is generally preferred in microbiome research for its ability to maintain statistical power while appropriately managing the risk of false discoveries. Through these methods, individual microbial taxa that are significantly associated with different conditions can be identified.

In a complex microbial community like the oral microbiome, various bacteria coexist either in a symbiotic relationship or exhibit antagonistic competition. These interactions can be reflected by the abundance of sequencing reads across multiple samples under different host physiological conditions. For instance, Streptococcus and Veillonella were shown to co-occur in the tongue microbiome[40], consistent with their metabolic interdependence demonstrated in vitro[41]. Such co-occurrence analysis is generally based on Spearman's correlation[42], as the relationships between microbes usually do not follow a linear pattern. Co-occurrence analysis produces a correlation matrix representing pairwise correlations between any given pair of microbes. Positive and negative values indicate co-occurring and mutually exclusive relationships, respectively. This correlation matrix can be further visualized in a microbial network to highlight clusters of co-occurring microbes potentially exhibiting symbiosis.

1.4 Patterns of microbiome associated cancer in the oral cavity

The relationship between oral microbiome and oral cancer has long been a subject of research, which has been addressed in great depth in the context of common oral diseases such periodontitis[43–47]. Some studies used oral mucosal swabs which allow the collection of paired contralateral normal samples, while others use saliva for a more consistent sampling process.

The first 16S rDNA study on oral microbiome and oral cancer was by Schmidt et al., which found that oral cancer and precancerous samples had a significantly decreased abundance of phyla Firmicutes (genus *Streptococcus*) and *Actinobacteria* (genus *Rothia*)[48]. By using the UniFrac distances based on only 12 taxa, the authors were able to separate oral cancer samples from the normal ones[48]. A subsequent study on saliva microbiome showed that HNSCC patients exhibited significantly reduced alpha diversity, and higher levels of *Streptococcus*[49]. These saliva-derived findings were opposite to many other studies based on mucosal swabs, which all indicated increased levels of alpha diversity, and higher prevalence of *Fusobacterium, Prevotella, Porphyromonas*, and *Peptostreptococcus* on OSCC sites compared to normal mucosa[50–52]. On the contrary, *Streptococcus* was consistently shown to be depleted on OSCC lesion sites[50–52]. Of note, most of the OSCC-associated taxa were periodontal pathogens, which were further demonstrated in a study using metagenomic shotgun sequencing[53]. An additional saliva-based study also showed increased *Fusobacterium* and decreased *Streptococcus* in OSCC patients compared to oral leukoplakia and post-operative patients[54]. Furthermore, a saliva study involving 248 subjects of all OSCC stages (I to IV) clearly showed increasing *Fusobacterium* and decreasing *Streptococcus* with cancer progression[55]. Finally, a meta-analysis by Yu et al. integrating 18 studies involving 1056 participants concluded that OSCC patients are enriched in *Fusobacteria* (genus *Fusobacterium*) but depleted in *Actinobacteria* and Firmicutes (genus *Streptococcus)*[56].

1.5 Treatment and prognostic factors

To date, various oral cancer-related clinical factors have been examined with regard to the oral microbiome. Similarly, information in the oral microbiome was utilized as a prognostic biomarker during the treatment process of OSCC. An intriguing study related to HPV status indicated that the presence of *Fusobacterium* is linked to better survival outcomes in OSCC patients, particularly those without traditional risk factors[57]. In addition, two studies have examined the effect of conventional treatment on the saliva microbiome, revealing a prolonged decrease in alpha diversity for months to years following surgery and chemotherapy[58,59]. Compositional differences in microbiome were reported between responders and non-responders of chemotherapy, suggesting the use of microbial signatures as prognostic indicators[59]. Given the robust associations between microbiome composition and cancer prognosis, as well as the observed impact of treatment on microbial diversity, a non-invasive early detection test (The CancerDetect for Oral & Throat cancer™, or CDOT) utilizing salivary host and microbiome RNA signature was developed to offer high specificity (94%) and sensitivity (90%) for OSCC detection[60].

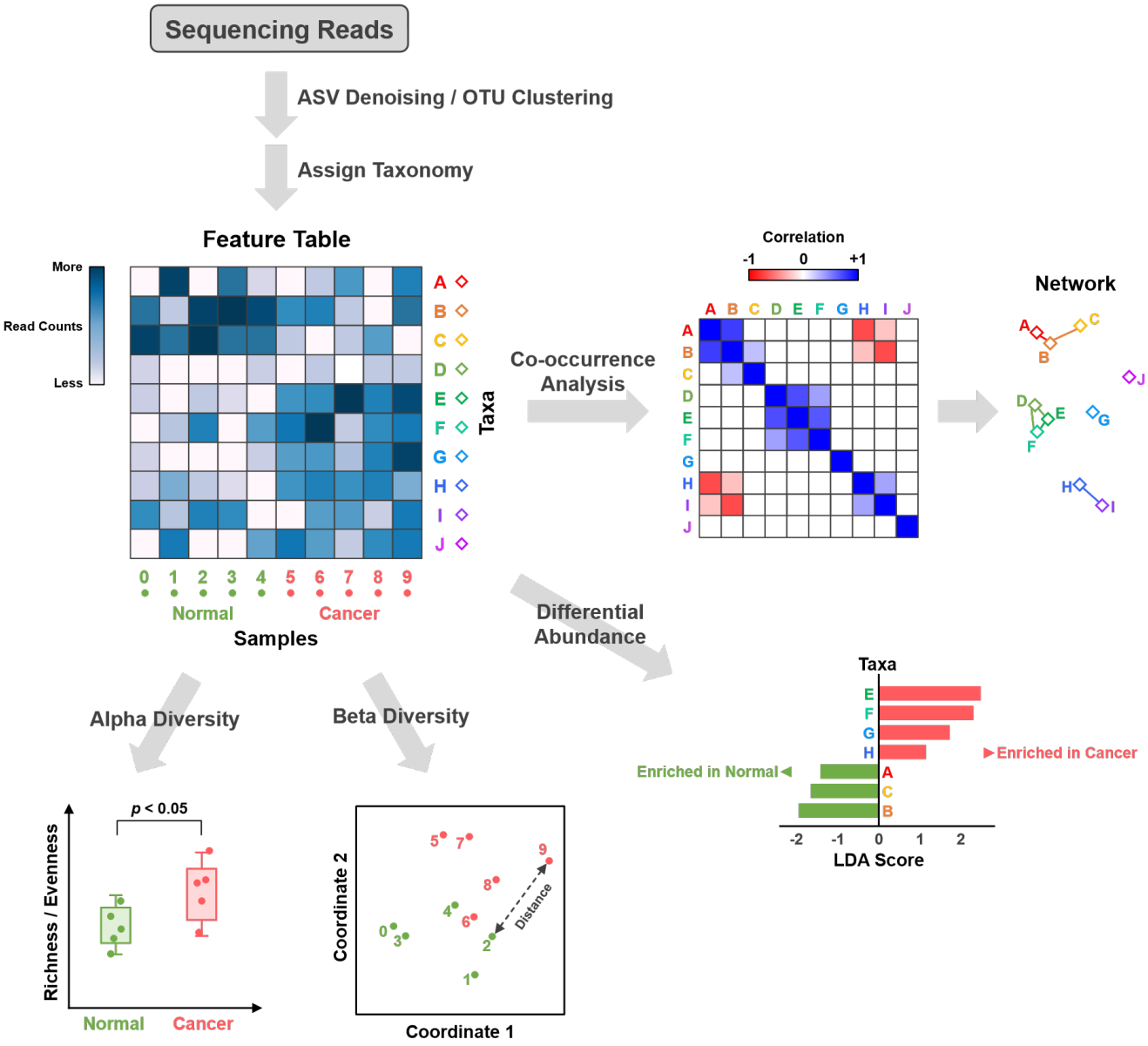

Fig. 1: Computational workflow for microbiome analysis (The analysis starts with sequencing reads, followed by ASV denoising/OTU clustering and taxonomy assignment to create a feature table. Downstream analyses include alpha and beta diversity, co-occurrence analysis, and differential abundance, highlighting taxa enriched in normal versus cancer samples)

2 CONCLUDING REMARKS

This review highlights the critical role of the oral microbiome in OSCC development and progression, in the context of the bioinformatics analysis for 16S rDNA sequencing. Standardizing bioinformatics methodologies and facilitating collaborative data sharing will be essential to ensure consistency and reproducibility in research. Future directions include the use of mouse models to elucidate mechanistic insights and validate the causative roles of specific microbes in cancer[61,62]. Additionally, integrating multi-omics data-including metagenomics, metatranscriptomics, metabolomics, and host genomics-will provide a comprehensive view of the complex host-microbiome interactions and uncover hidden mechanisms driving OSCC[63]. Advancements in oral microbiome research may pave the way for developing precise, non-invasive diagnostic tools and personalized therapies, ultimately improving management of oral cancer.

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