THE ORAL MICROBIOME IS A KEY FACTOR IN ORAL AND SYSTEMIC HEALTH

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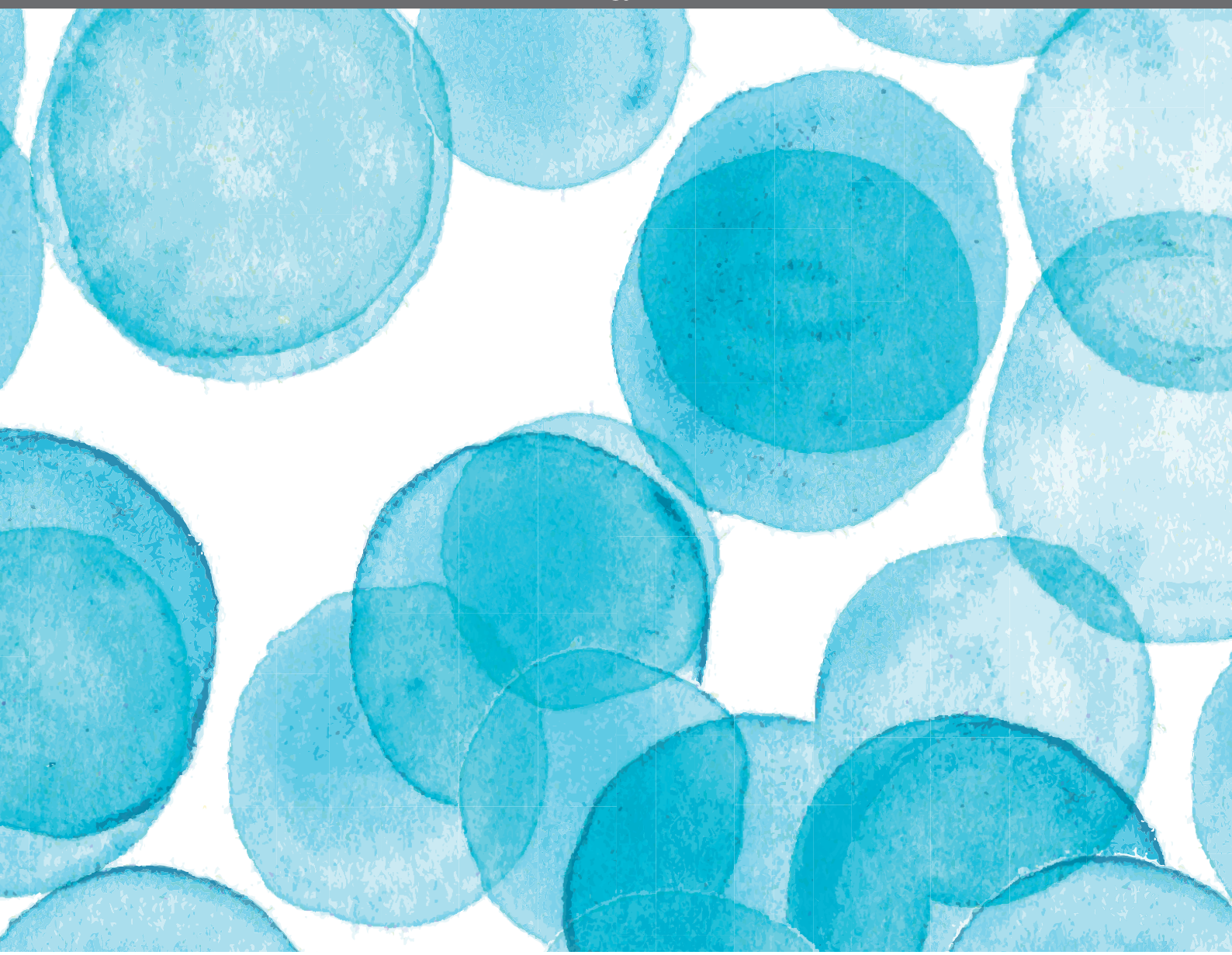

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THE ORAL MICROBIOME IS A KEY FACTOR IN ORAL AND SYSTEMIC HEALTH

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Table of Contents

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EDITORIAL
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Editorial: The Oral Microbiome Is a Key Factor in Oral and Systemic Health

Denis Bourgeois[1], *Lucio Souza Gonçalves*[2], *Josué da Costa Lima-Junior*[2,3] *and Florence Carrouel*[1]*

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Editorial on the Research Topic

The Oral Microbiome Is a Key Factor in Oral and Systemic Health

The oral cavity is a dynamic ecosystem comprising an assemblage of microbial communities, including many pathogenic or opportunistic species (Proctor and Relman, 2017). After the gut microbiome, the human oral microbiome (HOM) is the largest microbial community in the human body. The HOM plays a role in the onset and progression of several localized and systemic diseases including those of bacterial, viral and fungal origin (Soffritti et al.). In this Special Issue, there is a consensus: the oral microbiome is a key factor in oral and systemic health. Also, considering HOM as biomarkers for diseases is a significant emerging orientation. Saliva, the cornerstone of the HOM and systemic health relationship, has gained popularity as a readily available source of biomarkers useful for diagnosing specific oral and systemic conditions (Ferrari et al., 2021).

In oral health, biomarkers in saliva (e.g., enzymes, antibodies, protein markers, or oxidative stress markers) can be used for activity determination and for periodontal disease prognosis (Podzimek et al., 2016). The presence of key pathobionts and ongoing gingival inflammation are critical to the progression of periodontal disease. Mediators of periodontal disease initiation, progression, and recurrence are related to dysbiosis within the subgingival biofilm microbial community and the host immune response generated (Martínez et al., 2021). Abnormal changes in bacterial correlations, community structures, and local stability are linked to the dysbiosis observed in periodontal or peri-implant disease (Zhang et al.). Current findings indicate that salivary markers of oxidative stress are indicative of other clinical disease indices such as the papillary bleeding index and the caries index. Fungal species *Candida dubliniensis* and *Candida tropicalis* more abundant in the saliva of children with severe early childhood caries should play a role as caries risk markers (de Jesus et al.).

Recent studies have also demonstrated the role of periodontal disease as a risk factor or potentiator of distant systemic pathologies such as diabetes, inflammatory bowel diseases, Alzheimer's disease and oral cancer, further highlighting the importance of the oral cavity in systemic health (Kapila, 2021). This allowed for further characterization of oral microbial dysbiosis, especially putative bacterial periodontopathogens and changes in the composition of the oral virome during disease. New microorganisms (viruses, phages and bacteria) have recently been identified for their role in disease progression (Sedghi et al., 2021).

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Thus, oral bacteria could be biomarkers for diseases such as specific cancer types. Some specific bacteria such as *Capnocytophaga gingivalis*, *Peptostreptococcus sp.*, *Porphyromonas gingivalis*, *Prevotella sp.* and *Streptococcus sp.* are strongly associated with oral cancer (Karpiński, 2019). Bacteria belonging to genera *Actinomyces*, *Clostridium*, *Enterobacteriaceae, Fusobacterium*, *Haemophilus*, and *Veillonella* are linked to epithelial precursor lesions and oral cancer (La Rosa et al., 2020). Oral bacteria are detected in tumors outside the oral cavity. Every day, about 10^{11} bacteria from the oral cavity migrate into the lower gastrointestinal tract (DeClercq et al., 2021). Microorganisms act as chemical converters and metabolize nutrients from the host and from the diet (Anand et al., 2016). HOM can generate an ectopic colonization and produce numerous microbial metabolites capable of promoting tumorigenesis through the modulation of pathways related to energy homeostasis, immunological balance and nutritional intake (Zhang et al., 2016).

Oral pathobionts are essential in the development of colorectal and pancreatic cancer with current evidence showing differences in oral microbiota composition between patients with and without digestive cancers (Reitano et al., 2021). In cases of colorectal cancers, two periopathogenic species in particular have been frequently mentioned: Fusobacterium nucleatum and *Porphyromonas gingivalis*. In pancreatic cancers, in addition to previously mentioned bacteria, strains of *Aggregatibacter actinomycetemcomitans*, *Neisseria elongata*, and *Streptococcus mitis* have been described (Fan et al., 2018). Oral *Prevotella* species play an important role as commensals in health but can also be involved in diseases of the lower airways and upper gastrointestinal tract (Könönen and Gursoy). Oral bacteria from genera *Capnocytophaga* and *Veillonella* are apparently present in increased amounts in lung cancer patients (Yan et al., 2015). The study of complex interaction between the oral and gut microbiome in the pathogenies of type 1 diabetes is advanced, and suggests the use of saliva microbiome composition for early diagnosis (Moskovitz et al.). Also, bacterial dysbiosis plays an important role in the esophageal carcinogenesis process through microbial metabolism, inflammation and genotoxicity (Dan et al.) and *Capnocytophaga* and *Veillonella* are reportedly present in increased amounts in lung cancer patients (Najafi et al., 2021). At least, a predictive salivary microbiome signature is associated with a high risk of developing cardiovascular diseases (Murugesan et al.).

It is important for prevention of viral infection to draw a perspective on the role of the oral cavity in the virus infection (Tada and Senpuku, 2021). Salivary markers for viral infections involve direct detection of specific viral antigens, such as proteins and nucleic acids or host antibodies to viral infections and may provide a high accuracy point-of-care platform for detection of viral infections. HOM dysbiosis may facilitate inflammation and virus replication, limiting the development of a protective IgA response (Soffritti et al.). Immunity in saliva is, in particular, thought to have considerable impacts on the incidence and progression of respiratory viral infection. Parts of antiviral mechanisms against influenza virus and SARS-CoV-2 by immunity in saliva are similar.

Saliva based biomarkers are useful in diagnosis of several viral infections such as hepatitis A virus, hepatitis B virus, hepatitis C virus, Human immunodeficiency virus (HIV) 1, etc. (Zhang et al., 2016). Of course, several viruses have previously been isolated from saliva such as cytomegalovirus, Ebola virus, human herpes virus, herpes simplex virus, Influenza virus A, etc. (Corstjens et al., 2016). More recently, Zika and SARS-CoV-2 have been identified (To et al., 2020). In people living with HIV saliva might be used as a diagnostic tool for antioxidant changes in the future (Amjad et al., 2019). Oral bacterial species (e.g., *Leptotrichia spp.*), possessing unique niches and invasive properties, coexist with Human Papilloma Virus (HPV) within HPV-induced oral lesions in head and neck cancer patients (Mougeot et al.).

The fact that oral cavity is an important site for SARS-CoV-2 infection implicates saliva as a potential route of SARS-CoV-2 transmission (Carrouel et al.). The susceptibility of each individual to SARS-CoV-2 infection could therefore be characterized by HOM profile, which could facilitate virus replication and inflammation or conversely induce a protective IgA response (Soffritti et al.). Several routes of SARS-CoV-2 viral entry into the saliva have been suggested. There is direct entry to the oral cavity from upper and lower respiratory tract secretions, while circulatory viruses in the blood enter the gingival crevicular fluid. Studies reported a high yield of virus particles in the gingival sulcus and crevicular fluid, which are suspected to provide favorable conditions for virus replication and maintenance (Sri Santosh et al., 2020). Informations from the 2019 coronavirus pandemic highlight the link between oral and systemic health in a setting of viremias/bacteremias/microbemias, systemic inflammation, and/or immune system disruption in a susceptible host (Martínez et al., 2021).

Overall, the 12 contributions that make up this Special Issue highlight the fundamental relationship between HOM and systemic health providing potential microbiome-based clinical applications improving prevention, diagnosis, or drug response, which is of great significance. Characterization of microbial biomarkers is of great interest for precision medicine and represents a simple method to transfer microbiome research into clinical practice (Gilbert et al., 2018). If during the last years, an extraordinary effort has been made to identify biomarkers, today, HOM investigations have reached a critical inflection point. With the deepening understanding of the association between the HOM and other human microbiomes certain pathogens may be utilized as potential diagnostic biomarkers, screening tools, and prognostic indicators and interventions related to an altered human microbial composition may become the new adjuvant treatment in oral and systemic health.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Oral Microbiome Dysbiosis Is Associated With Symptoms Severity and Local Immune/Inflammatory Response in COVID-19 Patients: A Cross-Sectional Study

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The human oral microbiome (HOM) is the second largest microbial community after the gut and can impact the onset and progression of several localized and systemic diseases, including those of viral origin, especially for viruses entering the body via the oropharynx. However, this important aspect has not been clarified for the new pandemic human coronavirus SARS-CoV-2, causing COVID-19 disease, despite it being one of the many respiratory viruses having the oropharynx as the primary site of replication. In particular, no data are available about the non-bacterial components of the HOM (fungi, viruses), which instead has been shown to be crucial for other diseases. Consistent with this, this study aimed to define the HOM in COVID-19 patients, to evidence any association between its profile and the clinical disease. Seventy-five oral rinse samples were analyzed by Whole Genome Sequencing (WGS) to simultaneously identify oral bacteria, fungi, and viruses. To correlate the HOM profile with local virus replication, the SARS-CoV-2 amount in the oral cavity was quantified by digital droplet PCR. Moreover, local inflammation and secretory immune response were also assessed, respectively by measuring the local release of pro-inflammatory cytokines (L-6, IL-17, TNFα, and GM-CSF) and the production of secretory immunoglobulins A (sIgA). The results showed the presence of oral dysbiosis in COVID-19 patients compared to matched controls, with significantly decreased alpha-diversity value and lower species richness in COVID-19 subjects. Notably, oral dysbiosis correlated with symptom severity ($p = 0.006$), and increased local inflammation ($p < 0.01$). In parallel, a decreased mucosal sIgA response was observed in more severely symptomatic patients ($p = 0.02$), suggesting that local immune response is important in the early control of virus infection and that its correct development is influenced by the HOM profile. In conclusion, the data

presented here suggest that the HOM profile may be important in defining the individual susceptibility to SARS-CoV-2 infection, facilitating inflammation and virus replication, or rather, inducing a protective IgA response. Although it is not possible to determine whether the alteration in the microbial community is the cause or effect of the SARS-CoV-2 replication, these parameters may be considered as markers for personalized therapy and vaccine development.

Keywords: oral microbiome, COVID-19, symptom severity, inflammatory cytokines, secretory IgA

INTRODUCTION

The human oral microbiome (HOM) is the second largest and complex microbial community after that of the gut in the human body (Wade, 2013; Caselli et al., 2020a). HOM dysbiosis is often associated with periodontal inflammation and has been reportedly associated with several local and systemic disease conditions (Baghbani et al., 2020; Caselli et al., 2020a), including those sustained by viral infections (Cagna et al., 2019; Baghbani et al., 2020). Indeed, the role of HOM in the establishment of the infection of many viruses entering the body via the oropharynx has been reportedly recognized (Baghbani et al., 2020). The microbial component of a eubiotic HOM can inhibit pathogen colonization by competitive exclusion and/or by empowering the immune response (Wilks et al., 2013). There is evidence that crucial mutual interactions occur between viruses and the microbiome (Wilks and Golovkina, 2012) and that the microbiome can regulate and is in turn regulated by viruses via different mechanisms (Li et al., 2019). Respiratory viruses spread by aerosol transmission encounter oral and upper respiratory microbiota and are modulated in their ability to establish infection and able to induce changes in the resident microbiota (Li et al., 2019). The microbiota can produce antiviral compounds (defensins) against several viruses, including respiratory or oral viruses such as adenoviruses, herpesviruses, papillomaviruses, orthomyxoviruses, and coronaviruses (Pfeiffer and Sonnenburg, 2011). On the other hand, viruses can alter the microbiota, favoring dysbiosis and disease progression (Lynch, 2014).

The new pandemic human coronavirus SARS-CoV-2, causing COVID-19 disease, is a respiratory virus that uses the oropharynx as the primary site of replication, but the potential impact of HOM in the development of infection is still not elucidated. In particular, no data are available about the non-bacterial components of the HOM (fungi, viruses), which have been shown crucial for other diseases. Concerning the current pandemics by SARS-CoV-2, the presence of gingival inflammation/periodontitis has been associated with a 3.5-fold increased risk of admission to intensive care units (ICU), a 4.5-fold greater risk of assisted ventilation, and a consistent impressive 8.81-fold higher risk of death in COVID-19 patients, independently from other concomitant risk factors (Marouf et al., 2021).

The novel human Severe Acute Respiratory Syndrome Coronavirus type (SARS-CoV-2) is a single strand RNA virus belonging to the *Coronaviridae* family, β-coronavirus genus (Contini et al., 2020), which has spread worldwide. The associated disease, Corona Virus Disease 2019 (COVID-19), is currently reported by the World Health Organization (WHO) to have caused about 120 million cases with >2.6 million deaths (World Health Organization [WHO], 2021). In Italy, to date over 3.2 million cases have been reported, with other 102,000 deaths. The disease is characterized by the involvement of the lower respiratory tract, often accompanied by elevated blood levels of inflammatory cytokines/chemokines, the so-called "cytokine storm" (de la Rica et al., 2020; Jose and Manuel, 2020), by ageusia and/or hyposmia (Contini et al., 2020; Li et al., 2020; Prasad et al., 2020), and neurological and enteric symptoms in severely symptomatic patients (Contini et al., 2020; Gupta et al., 2020).

An extraordinarily high number of studies were published the last year, yet the mechanisms underlying virus proliferation in the primary site of infection and understanding of how the virus can become more invasive at the site of entry is still unclear, even though this could shed important light on the very first phases of the infection. It is recognized that SARS-CoV-2 enters the body mainly via the oropharynx, where it finds epithelial cells expressing the ACE2 and TMPRSS2 virus receptors (Herrera et al., 2020), and the virus has been detected in saliva (Henrique Braz-Silva et al., 2020; To et al., 2020). Thus, the resident oral microbiome may influence the ability of SARS-CoV-2 to take root and establish the infection. Similar to what is reported for other viruses affecting the oral and respiratory tract, the virus-host interplay in this site may define the vulnerability of the infected subject and the subsequent development of the disease or rather the early control of virus infection and prevention of severe disease. Like other microbial communities in the body, the oral microbiome can represent a protective barrier against exogenous pathogens (Zaura et al., 2009; Wade, 2013; He et al., 2015; Deo and Deshmukh, 2019) and it contributes to the lung microbiome, thus potentially affecting also the microbial environment in the lungs (Bassis et al., 2015). The oral microbiome can contribute to regulating mucosal immunity and inflammation, which might affect pathogenic potential directly or indirectly (Belkaid and Hand, 2014; Lamont et al., 2018).

Although there is potential interest in understanding these networks in SARS-CoV-2 infection, no information is yet available about the microbiome profile in COVID-19 patients, except for a two as yet unpublished reports describing the bacterial component of the oral microbiome by NGS (Iebba et al., 2020; Ward et al., 2021). However, several reports have evidenced that the non-bacterial components of the microbiome can be very important in defining individual susceptibility to diseases besides bacteria (the mycome and virome), thus the use of Whole

Genome Sequencing (WGS) technology may be more useful in elucidating the microbial environment potentially impacting on SARS-CoV-2 infecting ability.

The present work aimed to characterize, for the first time, the oral microbiome of COVID-19 patients by WGS, comparing its profile to controls, and simultaneously evaluating the presence of inflammatory cytokines and local IgA immune response, to better understand the features of the oral environment that could potentially support SARS-CoV-2 infection and related disease, and to identify eventual markers for the risk of developing a severe infection.

MATERIALS AND METHODS

Ethics Statement

Recruitment of study participants was performed according to the protocol approved by the Ethics Committee Area Vasta Emilia Centro della Regione Emilia-Romagna (CE-AVEC): approval document no. 408/2020/Oss/UniFe, approved on April 21, 2020.

Design of the Study

A cross-sectional observational study was performed to characterize the oral microbiome and local response in COVID-19 patients compared to non-COVID-19 subjects. All participants were recruited from the University Hospital of Ferrara, in the COVID-19 and the non-COVID Infectious ward, respectively. Study participants were recruited in the period April to July 2020. Each study participant was recruited after signing informed consent. Clinical and epidemiological data were collected from the clinicians of the enrolled ward. The study was registered and published prospectively in the ISRCTN International Registry (study n° ISRCTN87832712; doi: 10.1186/ISRCTN87832712).

Study Participants

All study participants were recruited among the hospitalized patients of the University Hospital of Ferrara. Inclusion criteria were: age >18 years, written consent to participate in the study, and molecular diagnosis of SARS-CoV-2 infection (for COVID-19 group only). Exclusion criteria included: pregnancy, breastfeeding, uncooperative patient (inability to perform oral rinse to collect samples), lack of written agreement. COVID-19 patients were stratified into four categories based on symptoms: asymptomatic (1, no symptoms), paucisymptomatic (2, aspecific flu-like symptoms), symptomatic (3, including specific respiratory symptoms), severely symptomatic (4, needing ventilation). The control group consisted of SARS-CoV-2-negative subjects affected by non-respiratory diseases. The number of study participants was decided based on the subjects hosted at the University Hospital of Ferrara in the study period.

Clinical Specimens

Oral rinse samples were collected in 5 mL of sterile phosphate-buffered saline (PBS), as previously described (Caselli et al., 2020a). The specimens were immediately inactivated with 0.1% SDS, refrigerated (2–8°C), and processed within 4 h. Briefly, all samples were vortexed and centrifuged at 15,000 × *g* for 10 min at 4°C to divide the corpuscular part from the supernatant, which were immediately frozen in liquid nitrogen and kept at −80°C until use.

Nucleic Acid Extraction From Clinical Specimens

Total nucleic acids (DNA and RNA) were extracted from the pellets by using the Maxwell CSC platform equipped with the HT Viral TNA Kit (Promega, Milan, Italy), following the manufacturer's instructions (Comar et al., 2019). Extracted total nucleic acids (TNAs) were checked and quantified by nanodrop spectrophotometric (Thermo Fisher Scientific, Milan, Italy) reading at 260/280 nm. The amplificability of extracted DNA was checked by PCR amplification of human, bacterial, and fungal genes. Namely, human β-actin, bacterial 16S rRNA gene (pan bacterial PCR, *panB*), and mycetes ITS gene (pan fungal PCR, *panF*) were respectively, analyzed, as previously described (Borghi et al., 2016; Caselli et al., 2016, 2018).

Library Preparation and Sequencing

Extracted TNA (100 ng) were retrotranscribed and analyzed by WGS by the NGS Service of the University of Ferrara (Department of Morphology, Surgery and Experimental Medicine, University of Ferrara), who carried out library preparation, sequencing, and taxonomic analysis. Briefly, WGS libraries were prepared using NEBNext® Fast DNA Fragmentation and Library Prep Kit for Ion Torrent TM (Thermo Fisher Scientific, Milan, Italy), following the manufacturer's protocol. Samples were then sequenced by using the Ion Gene Studio S5 System (Thermo Fisher Scientific, Milan, Italy). Low-quality sequence data removal was performed directly on the Ion S5 GeneStudio sequencer, as part of in-built processing. Briefly, the Torrent Suite software (Thermo Fisher Scientific, Milan, Italy), installed in the sequencer, automatically clips adapter sequences and trims low-quality bases from the 3′ end of each read. Reads with quality less than Q20 were also discarded. Additionally, PRINSEQ open source application (Schmieder and Edwards, 2011) was used to remove reads with lengths of less than 100 nucleotides. The taxonomic assignment has been performed using Kraken2 (Pubmed ID: 24580807) and a database consisting of archaea, bacteria, fungi, protozoa, and viruses. Raw sequencing data and bioinformatics analyses have been deposited in the European Nucleotide Archive (ENA) website (accession number PRJEB42999).

SARS-CoV-2 Detection and Quantification

Extracted TNA (100 ng) was used for SARS-CoV-2 detection and quantification by droplet digital PCR (ddPCR), by using the SARS-CoV-2 ddPCR Kit (Bio-Rad Laboratories, Milan, Italy). Briefly, three targets are analyzed in each sample by FAM and HEX labeled probes, targeting SARS-CoV-2 N1 and N2 genes, and human RPP30 gene, this last was used as a control and to normalize the virus counts. The assay sensitivity was between

0.260 copies/μl to 0.351 copies/μl, respectively, for the genetic markers N1 and N2.

IgA Analysis

The presence of anti-SARS-CoV-2 secretory IgA (sIgA) in the oral samples was evaluated by a CE-IVD ELISA assay designed to detect IgA directed against the virus S1 protein (Euroimmun, Lubeck, Germany). The test was previously reported to have high specificity/sensitivity for IgA detection in serum/plasma samples (>95%) and ocular fluids (Caselli et al., 2020b). For oral rinse analysis, the samples were diluted 1:5 in saline, allowing optimal detection of IgA and differentiation between positive samples and controls, as detected in preliminary assays. Each sample was assessed in triplicate. Sample positivity was expressed following the manufacturer's instruction, as the ratio (R) between the absorbance ($OD_{450\ nm}$) value detected in samples and that detected in the calibrator sample provided by the manufacturer. Samples were considered negative if R values were < 0.8, weakly positive with R values comprised between 0.8 and 1.1, and strongly positive with $R \geq 1.1$.

Cytokines Analysis

Oral specimens were analyzed for the presence of pro-inflammatory cytokines, by using ELISA assays specifically detecting and quantitating the following cytokines: IL-6, IL-17, TNFα, and GM-CSF (Thermo Fisher Scientific, Life-Technologies, Milan, Italy).

Statistical Analyses

Statistical analyses were performed with Agilent GeneSpring GX v11.5 software (Agilent Technologies, Santa Clara, CA, United States) and R (R 2019, R Core Team, available as free software at https://www.r-project.org/). Microbiome data were expressed as the relative abundance of each taxonomic unit at the genus or species level. The null hypothesis was tested by the Kruskal–Wallis test. Pairwise *post hoc* analysis was performed by the non-parametric Dunn test which includes correction for multiple comparisons. A Chi-square test was used to assess gender distribution significance. Alpha-diversity obtained by measuring the Shannon H' diversity index was used to describe the microbiome diversity between clinical samples. ELISA results were analyzed by Student's *t*-test. Linear regression and correlation analyses (Spearman r correlation coefficient) were conducted to evaluate the correlation between patients' clinical parameters (a non-continuous discrete variable), and continuous variables including microbiome profile, immune and inflammation responses. A p-value ≤ 0.05 was considered significant.

RESULTS

Patients' Characteristics

Seventy-five eligible subjects, including 39 COVID-19 patients and 36 controls, were enrolled in the study. COVID-19 patients included 20 males (51.3%) and 19 females (48.7%), with a mean age of 71.1 ± 18.4 years (range 25–99). Oral rinses were collected from COVID-19 patients at 0–43 days since the first SARS-CoV-2-positive nasopharyngeal swab. At the time of sample collection, 11/39 (28.2%) COVID-19 patients were asymptomatic, 7/39 (17.9%) presented mild symptoms, 21/39 (55.3%) were symptomatic, with 2 of them (2/39, 5.1%) showing severe respiratory symptoms requiring ventilation. All recruited COVID-19 patients received hydroxychloroquine and azithromycin on hospitalization (Gautret et al., 2020). The control group consisted of SARS-CoV-2-negative subjects admitted for non-respiratory diseases at the non-COVID Infectious Disease ward, and included 22 males and 14 females (respectively, 61% and 39% of the group), with a mean age of 66.5 ± 18.8 years (range 20–94). The characteristics of study participants are reported in **Table 1**. No statistical differences were evidenced between COVID-19 and control group with regard to age (Kruskal–Wallis test; $p = 0.27$, n.s.) and gender (Chi-square test; $\chi^2 = 0.734$, $p = 0.39$, n.s.). Similarly, no statistically significant differences were evidenced between COVID-19 disease sub-groups (asymptomatic, pauci-symptomatic, and symptomatic) regarding age (Kruskal–Wallis test; $p = 0.21$, n.s.) or gender distribution (Chi-square test; $\chi^2 = 0.256$, $p = 0.88$, n.s.).

SARS-CoV-2 Load in COVID-19 Patients

Although all the enrolled COVID-19 patients were confirmed to be SARS-CoV-2 positive at hospital admission by the routine molecular test performed on nasopharyngeal swab by the Hospital microbiology laboratory, we wanted to assess the presence of SARS-CoV-2 in the oral cavity of all the enrolled subjects at the time of oral rinse withdrawal. The oral rinse samples were analyzed by digital droplet PCR (ddPCR), able to detect and quantify the virus genomes, contrarily to the routinely used diagnostic assays (Falzone et al., 2020; Suo et al., 2020). While the results confirmed the absence of positivity in the control group, in the COVID-19 group both positive and negative oral rinse specimens were observed, as summarized in **Figure 1**. Quantitative analysis showed that 16/39 subjects harbored a high load of SARS-CoV-2 (from 101 to 3,963 genome copies in 20 μl of the amplified sample), 17/39 had lower but detectable amounts of virus (from 3 to 100 genome copies in 20 μl), whereas 6/39 patients did not display any detectable virus copy in the oral cavity at the time of oral withdrawal (<3 copies in 20 μl). It is noteworthy that the virus load detected in the oral cavity correlated with symptom severity (Spearman $r = 0.774$; 95% CI 0.608–0.875) ($p < 0.0001$), defining specific subpopulations of COVID-19 patients.

Oral Microbiome in COVID-19 Patients

Whole Genome Sequencing analysis of the oral microbiome evidenced significant differences in the profiles of the COVID-19 compared to controls. Alpha-diversity values were lower in COVID-19 patients vs. controls ($p = 0.01$) (**Figure 2A**). Interestingly, the comparison between severely symptomatic COVID-19 subgroups and controls revealed the most significant differences (**Figure 2B**), with an inverse correlation between alpha-diversity value and symptoms (Spearman $r = -0.431$, 95% CI −0.666/−0.120,

TABLE 1 | Characteristics of COVID-19 and control study participants.

Subject n°	Control group		COVID-19 group				Age/gender distribution
	Gender	Age	Gender	Age	Days after NPS	COVID-19 symptoms (*)	
1	F	74	M	76	13	3	***Age:***
2	M	72	F	72	4	3	**CTR:** 66.5 ± 18.8 years
3	M	73	F	56	0	1	**COVID-19:** 71.1 ± 18.4 years
4	F	86	M	49	3	1	**CTR vs. COVID-19:** $p = 0.27$, n.s.
5	F	38	F	49	6	2	
6	F	66	M	99	6	2	
7	F	67	F	80	18	4	
8	F	40	F	73	16	3	
9	M	53	F	68	2	1	
10	M	42	F	33	18	2	
11	M	75	F	51	2	2	
12	F	86	M	76	4	3	
13	M	60	M	82	8	3	
14	M	59	F	87	29	1	
15	M	83	M	47	6	1	
16	M	86	F	91	18	3	
17	F	86	M	89	5	3	
18	M	71	F	94	16	3	***Gender:***
19	M	88	M	94	20	2	**CTR:** 22/36 males (61%)
20	M	84	M	80	15	3	**COVID-19:** 20/39 males (51.3%)
21	F	88	M	85	18	3	**CTR vs. COVID-19:** $p = 0.39$, n.s.
22	F	86	F	83	7	3	
23	M	20	M	25	10	3	
24	F	94	F	78	18	4	
25	F	46	F	83	49	3	
26	F	76	F	45	17	3	
27	M	50	M	82	1	1	
29	M	51	F	82	2	1	
30	M	53	M	59	0	2	
31	M	45	M	45	43	1	
32	M	70	M	57	5	3	
33	F	85	F	86	4	3	
34	M	49	F	48	3	2	
35	M	67	M	90	11	3	
36	M	49	F	70	0	1	
37	M	76	M	81	51	1	
38	–	–	M	87	23	3	
39	–	–	M	78	11	1	
	–	–	M	63	18	3	

(*) Symptom score was: 1, asymptomatic; 2, paucisymptomatic; 3, symptomatic; 4, severely symptomatic.
NPS, nasopharyngeal swab.
Age and gender distribution significance were assessed, respectively, by Kruskal–Wallis and Chi-square tests.

$p = 0.006$). The decrease of alpha diversity was higher in male compared to female patients (**Figure 2C**), which paralleled symptoms severity.

The microbiome profile appeared profoundly altered in COVID-19 patients compared to controls (**Figure 3**). In particular, the relative abundance of the bacterial genera *Streptococcus*, *Veillonella*, *Prevotella*, *Lactobacillus*, *Capnocytophaga, Porphyromonas, Abiotrophia, Aggregatibacter, Atopobium* was increased in COVID-19 compared to controls, whereas *Rothia*, *Haemophilus*, *Parvimonas*, *Fusobacterium*, and *Gemella* spp. were decreased (**Figure 3A**). Notably, *Enterococcus* and *Enterobacter* genera were exclusively present in COVID-19 patients, and not detectable in control subjects. At the species level (**Figure 3B**), COVID-19 patients had decreased amounts of *Haemophilus parainfluenzae* and *parahaemolyticus*, *Gemella morbillorum* and *sanguinis*, *Parvimonas micra*, and

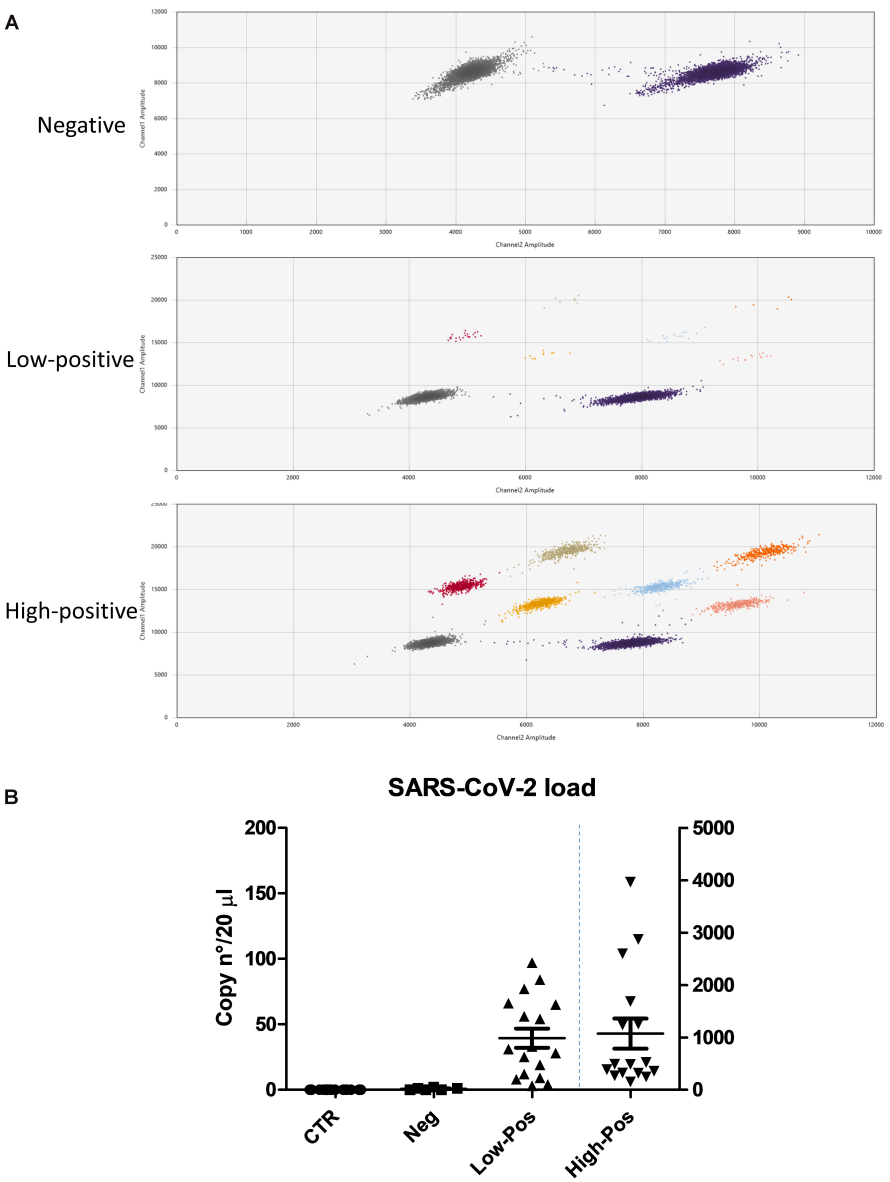


FIGURE 1 | SARS-CoV-2 load in control and COVID-19 subjects, as measured by ddPCR. **(A)** Graphical representation of the values detected by the use of three different molecular probes: negative samples, only the clouds corresponding to the housekeeping control genes are detectable (gray and purple clouds); low- and high-positive samples, the clouds corresponding to the virus genes are detectable and counted (positives to individual FAM probes: gray, red, and yellow; positives to individual HEX probes: purple, blue, and pink; double positives to FAM/HEX probes: beige and orange). **(B)** Virus load, expressed as genome copy number per analyzed sample (20 µl of extracted nucleic acid); left *y* axis refers to control, negative and low-positive values, whereas right *y* axis refers to high-positive COVID-19 subjects. Mean value ± SEM is also reported.

Neisseria subflava, whereas *Neisseria mucosa*, *Veillonella parvula*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Atopobium parvulum*, *Acinetobacter baumannii* were increased. Notably, many species of periodontopathogenic bacteria (*Prevotella melaninogenica*, *jejuni*, *denticola*, and *oris*; *Eikenella corrodens*; *Capnocytophaga sputigena* and *gingivalis*; and *Aggregatibacter aphrophilus*) were significantly increased in COVID-19 compared to control subjects. **Figure 4** summarizes the taxa significantly altered in COVID-19 patients compared to controls, with significance values.

It is of note that high differences were observed relative to the fungal component of the oral microbiome (**Figure 5**). Contrary to the decreased richness of the bacterial component, the fungal fraction of the oral microbiome was increased in COVID-19 patients compared to controls, both as total normalized counts and as species richness. In detail, while the oral mycobiome of controls was essentially constituted by *Candida* and *Saccharomyces* spp. (47% and 52% of relative abundance, respectively), in COVID-19 patients *Aspergillus*, *Nakaseomyces*, and *Malassezia* spp. were detectable at a fair

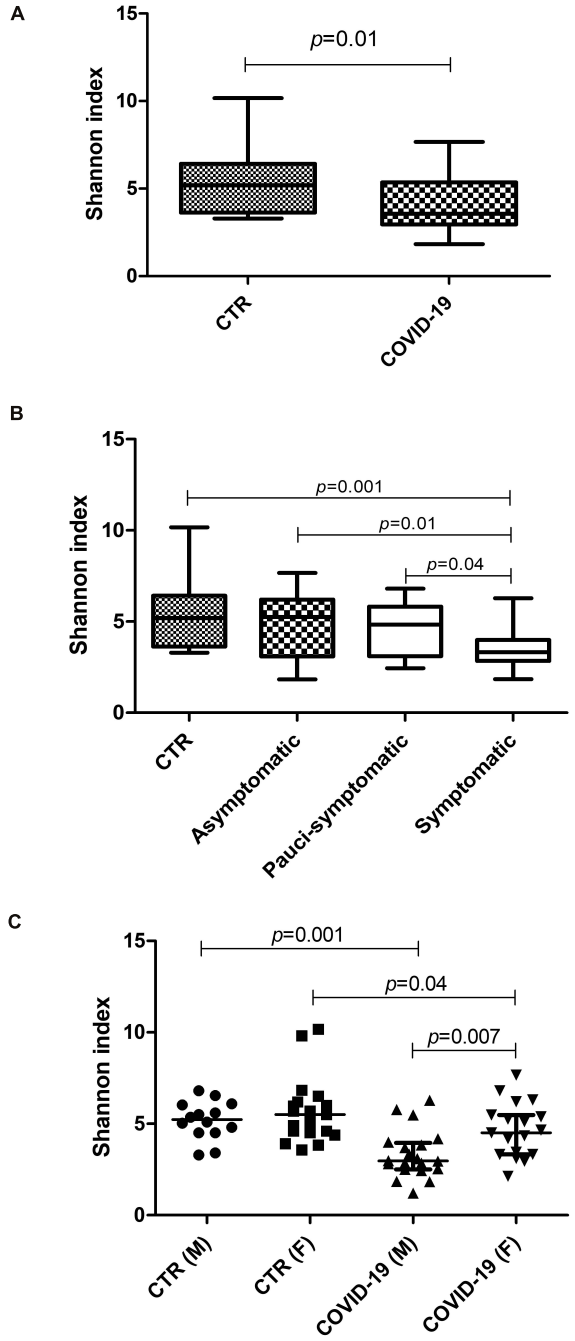


FIGURE 2 | Alpha-diversity values in the oral microbiomes of control and COVID-19 subjects. **(A)** Comparison between control and COVID-19 patients, expressed as median and range values. **(B)** Comparison between controls and COVID-19 asymptomatic, pauci-symptomatic, and symptomatic subjects; median and range values are shown. **(C)** Comparison between genders (M, male; F, female) in the control and COVID-19 groups; median values with interquartile range are shown for each group.

by *Candida* and *Saccharomyces* spp. (47 and 52% of relative abundance, respectively), in COVID-19 patients *Aspergillus*, *Nakaseomyces*, and *Malassezia* spp. were detectable at a fair level, with respective relative abundance values of 4%, 3%, and <1%. The species *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, and *Malassezia restricta* were identified.

Interestingly, the oral virome also appeared more abundant in COVID-19 patients compared to controls (**Figure 6**). While viruses represented 0.07% of the microbial community in controls, their relative abundance increased to 1.12% in COVID-19 patients. *Lymphocryptovirus* and *Simplexvirus* genera of the Herpesviridae family were detected both in COVID-19 and control subjects. However, Epstein Barr virus (EBV) resulted reactivated in 11/39 COVID-19 patients and in only 2/36 controls. Moreover, Herpes simplex virus type 1 (HSV-1) and four bacteriophages targeted, respectively, toward *Staphylococcus* (Staphylococcus phage ROSA), *Streptococcus* (Streptococcus phage EJ-1 and phage PH10), and *Lactobacillus* (Lactobacillus phage phiadh), were also increased in COVID-19 patients compared to controls.

Oral IgA Response in COVID-19 Patients

To assess the development of a mucosal immune response against SARS-CoV-2 in the oral cavity, oral secretory IgA was searched and quantified by specific ELISA in the oral rinse samples of COVID-19 patients and controls. A mucosal IgA response was detected in 25/39 (64.1%) COVID-19 patients and no controls (p = 0.0008). Interestingly, the extent of mucosal response was different among the symptom subgroups of patients (**Figure 7**). In fact, 10/39 patients (25.6%) exhibited a very high concentration of sIgA (R > 2.0), whereas 15/39 patients (38.5%) had intermediate values (0.8 < R < 2.0), and 14/39 (35.9%) showed the presence of a barely detectable (R∼0.8 threshold value) or no sIgA response. Of note, 6/10 COVID-19 patients displaying high oral sIgA titer were asymptomatic/paucisymptomatic, evidencing a trend toward an inverse correlation between the salivary sIgA concentration and symptom severity (Spearman r−0.355; 95% CI −0.600 to 0.047; p = 0.02).

Oral Cytokines in COVID-19 Patients

Since the so-called "cytokine storm" is a hallmark of severe COVID-19 disease, we investigated the release of pro-inflammatory cytokines in the oral cavity. Namely, the four main cytokines/chemokines detected in the blood of COVID-19 patients were analyzed: IL-6, IL-17, TNFα, and GM-CSF. The results showed that both IL-6 (p = 0.005) and IL-17 (p = 0.02) were significantly higher in COVID-19 oral samples than in controls (**Figure 8**). TNFα and GM-CSF were also more concentrated in COVID-19 patients compared to controls, but the differences were not statistically significant. However, the differences became significant by comparing COVID-19 symptomatic subgroup with controls (TNFα p = 0.005; GM-CSF p = 0.002), highlighting that more inflammation was detectable in the subjects undergoing a more severe course of the disease.

Inflammation also correlated with the oral microbiome dysbiosis, being more pronounced in subjects with a more evident decrease of alpha-diversity and species richness (p < 0.01).

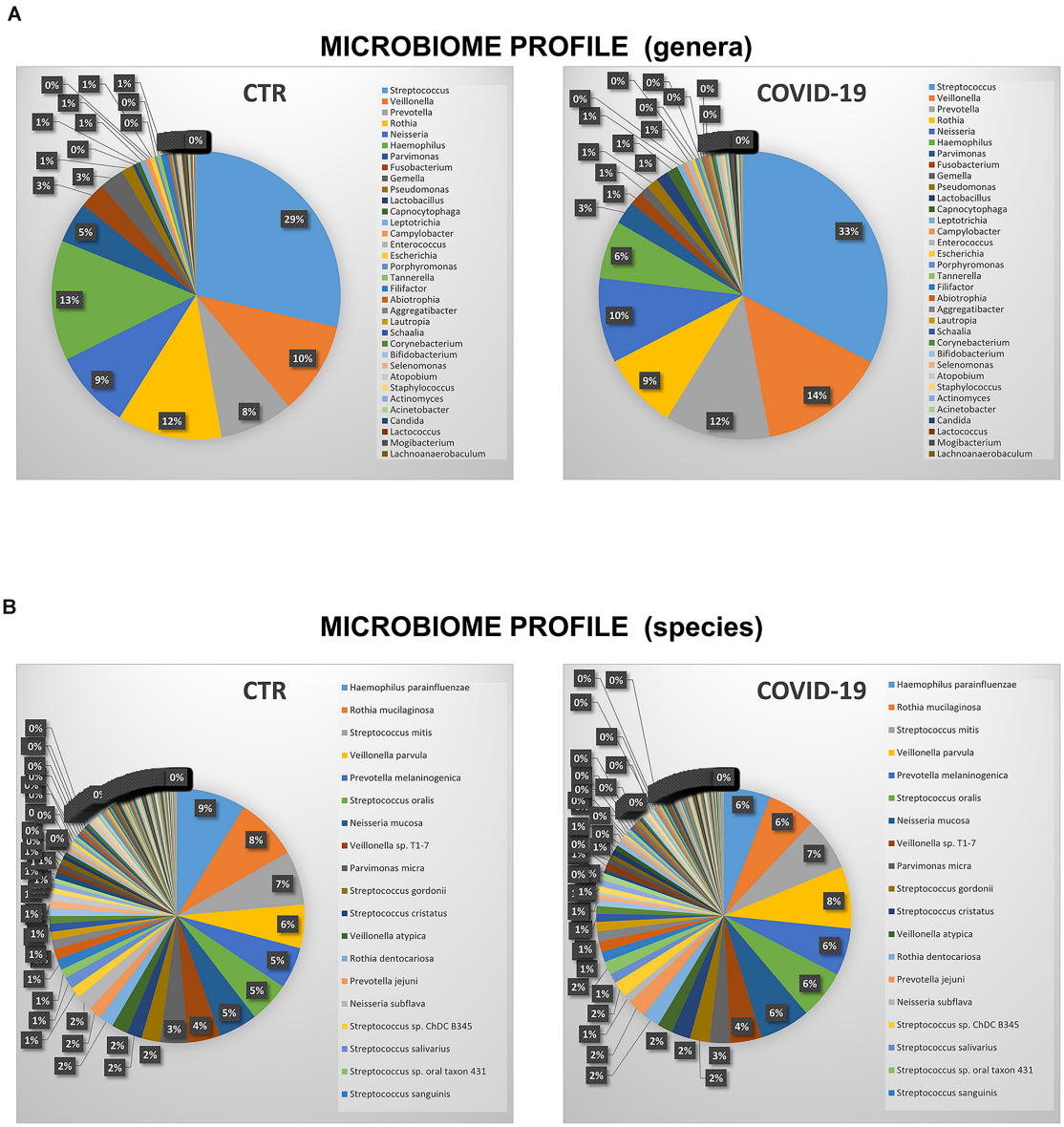


FIGURE 3 | Relative abundance and distribution of microorganisms in the oral cavity of control (CTR) and COVID-19 subjects. **(A)** Percentage distribution of most detected microbial genera. **(B)** Percentage distribution of most detected microbial species.

DISCUSSION

Recent reports have shed light on the role of the microbiome in several diseases, including those of viral origin, suggesting that the commensal microbiota may potentially favor or hamper viral infections. However, most studies consider the gut microbiome, neglecting the role of an oral one. In addition, most if not all studies discuss only bacterial microbiota, whereas fungi and viruses are also important components of the commensal microbiota. Concerning SARS-CoV-2 infection, COVID-19 patients have been reported to harbor oral pathogenic bacteria (such as cariogenic or periodontopathic pathogens) (Bao et al., 2020; Patel and Sampson, 2020; Xiang et al., 2020). Oral dysbiosis might favor the establishment of SARS-CoV-2 infection through different mechanisms, as known for other respiratory viruses, including alteration of the respiratory epithelium, promotion of adhesion of respiratory pathogens, and increase of local inflammation (Baghbani et al., 2020). Despite such suggestions, the profile of the HOM is currently still not clarified, especially in the non-bacterial components, rendering it difficult to understand whether the HOM dysbiosis may be considered a risk factor for COVID-19 development (Patel and Sampson, 2020). Two recent preprints reported on the bacterial profile of HOM in COVID-19 patients, suggesting relationships between some bacteria and SARS-CoV-2 infection (Iebba et al., 2020; Ward et al., 2021). However, to date, no studies have

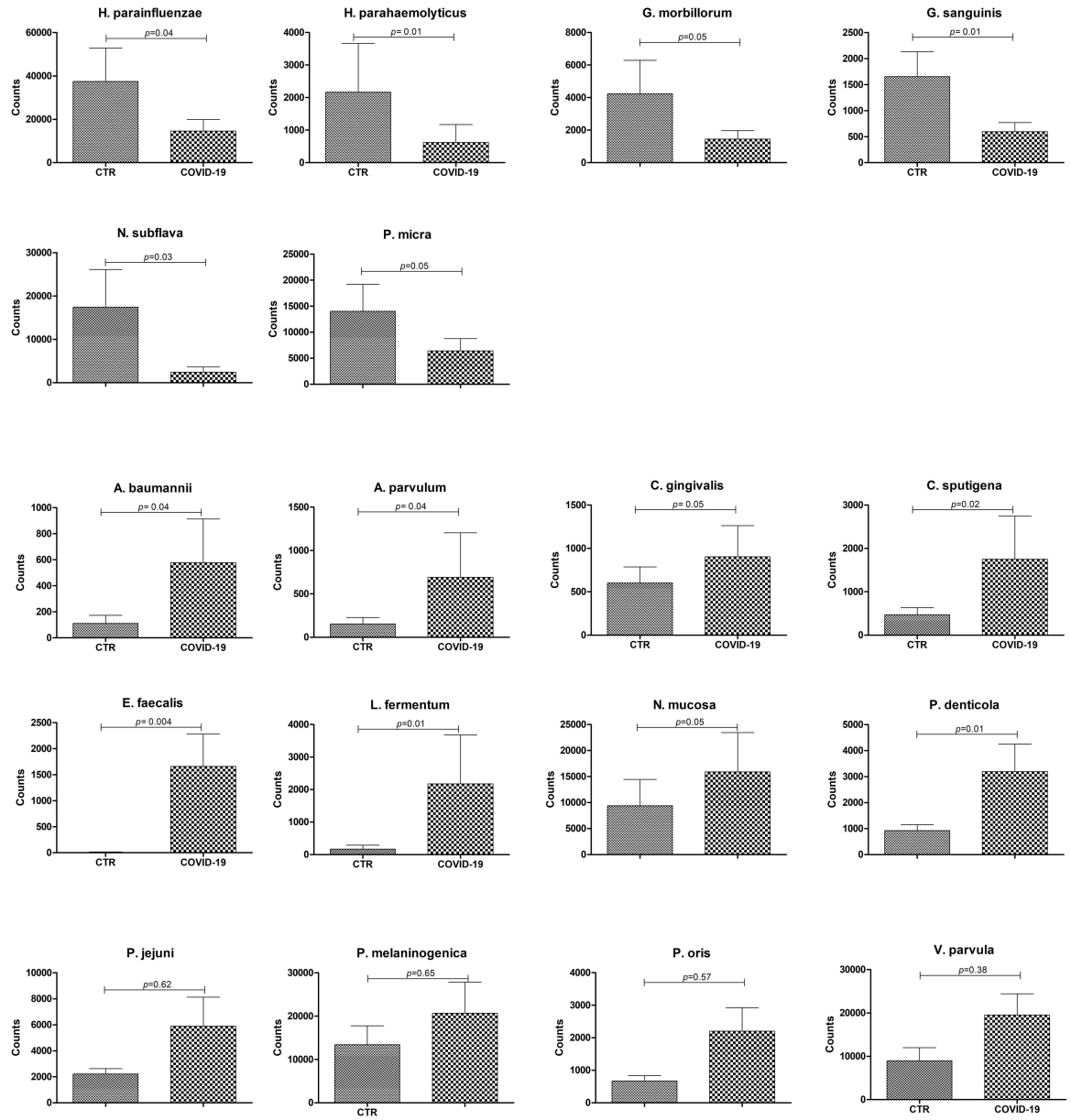


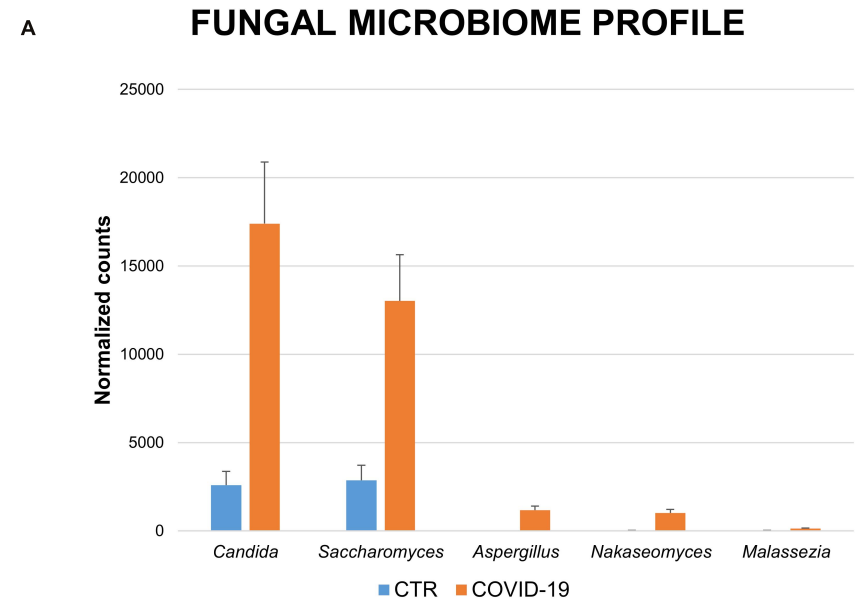
FIGURE 4 | Significantly altered taxa in control (CTR) and COVID-19 subjects. The results are expressed as normalized counts ± SEM values. Significance *p*-values for each comparison are also displayed.

completely addressed HOM profiling, including fungal and viral components.

Thus, our study aimed to characterize by metagenomics (WGS deep sequencing) the oral microbiome of COVID-19, to get a comprehensive view of its bacterial, fungal, and viral components.

The results showed very significant differences in the HOM composition between COVID-19 and control subjects, highlighting a decrease in the alpha-diversity and bacterial species richness in COVID-19 patients compared to controls, and a significant correlation between such decrease and symptom severity (p = 0.006). These data are in line with previous observations highlighting a decrease in the alpha variety and species richness upon HCV, HIV, and influenza infection (Sun et al., 2016; Inoue et al., 2018), with a parallel increase of pro-inflammatory cytokines like IL-6, TNFα, and IL-1β (Yildiz et al., 2018; Ramos-Sevillano et al., 2019).

Our results also showed an increase in the relative abundance of genera associated with poor oral hygiene and periodontitis in COVID-19 patients (*Prevotella*, *Lactobacillus*, *Capnocytophaga, Porphyromonas, Abiotrophia, Aggregatibacter,* and *Atopobium*), suggesting an association between those bacteria and SARS-CoV-2 infection, similar to that reported for other respiratory viruses (Andrews et al., 2012; Wang et al., 2016). The exclusive



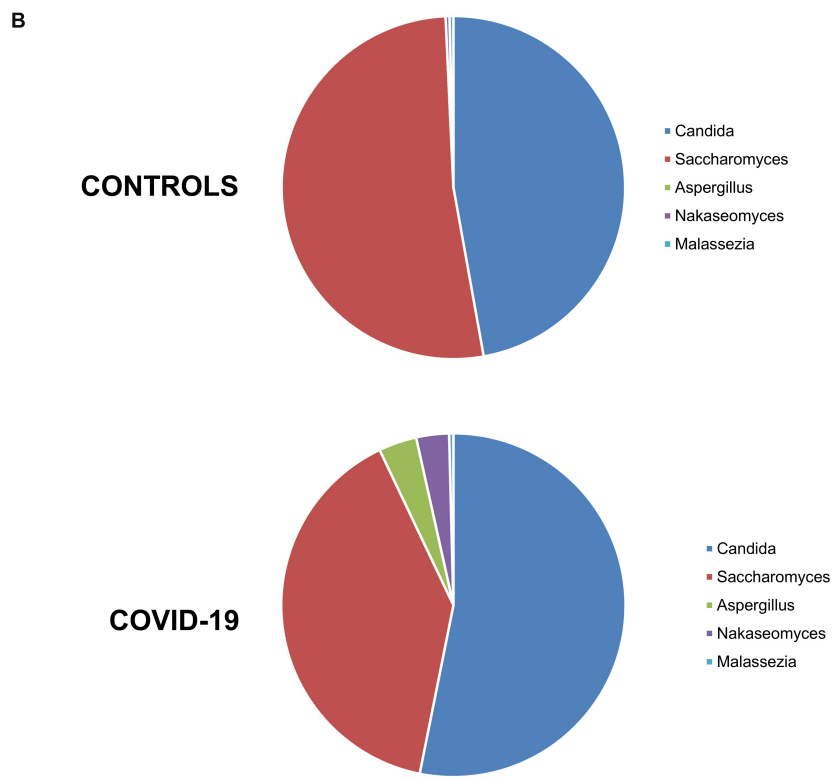


FIGURE 5 | Mycome profile in the oral cavity of control and COVID-19 subjects. **(A)** Abundance of fungi expressed as total normalized counts for each individually detected mycetes. **(B)** Percentage distribution of the fungal genera in controls and COVID-19 patients.

presence of *Enterococcus* and *Enterobacter* genera in COVID-19 patients suggests that they might be a microbial marker of susceptibility for SARS-CoV-2 infection. Even more interesting, fungi were instead more abundant in COVID-19 patients than in controls, with some genera (*Aspergillus*, *Nakaseomyces*, and *Malassezia*) only detectable in COVID-19 subjects, besides the

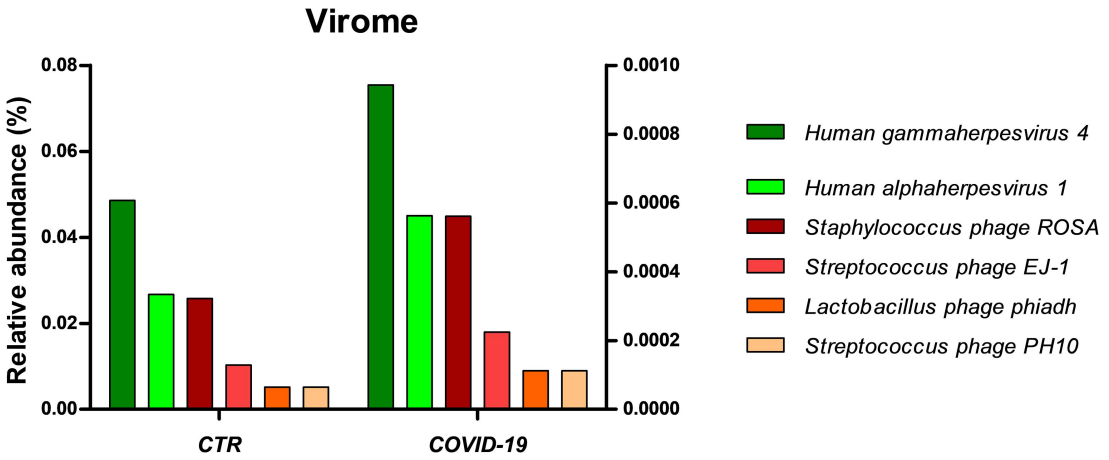


FIGURE 6 | Virome profile in the oral cavity of control (CTR) and COVID-19 subjects. The results are expressed as relative abundance (%). Left *y* axis refers to Human gammaherpesvirus 4 and Human alphaherpesvirus 1 (green bars), whereas right *y* axis refers to the amount of the four detected bacteriophage (orange-red bars).

more common *Candida* and *Saccharomyces* genera. In this regard, significant differences in fungal community with a higher richness of fungal species were detected in HIV-infected compared to uninfected individuals (Mukherjee et al., 2014) and in HBV/HCV symptomatic patients, where the diversity of intestinal fungi was positively associated with disease progression (Chen et al., 2011). Oral mycetes may be increased in the mouth because of bacterial alterations, ultimately favoring SARS-CoV-2 infection, due to the increased inflammation originated by fungi enzymatic and catabolic/toxic activity in the mouth (Chen X. et al., 2020). Beyond the potential mechanisms underlying the cooperation between SARS-CoV-2 and fungi, the results suggest that it could be important to consider this component of HOM in the management of virus infection.

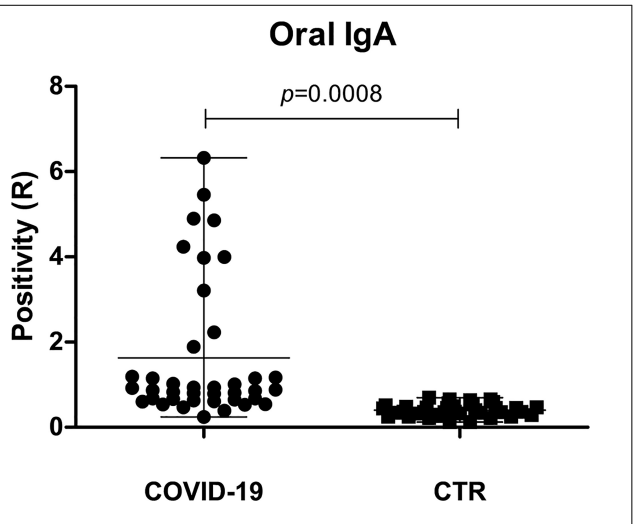


FIGURE 7 | Mucosal sIgA response in the oral cavity of COVID-19 and control (CTR) subjects. The positivity is expressed as the ratio (R) between the value detected in the sample and the threshold control value, following manufacturer's instructions; mean value with range is also shown.

Another non-bacterial HOM component that was augmented in COVID-19 patients was the viral one (from 0.07 to 1.12% of the total microbiome). HSV-1 and EBV herpesviruses were most present, and EBV coinfection was evidenced in about 30% of COVID-19 patients compared to only 5% of controls. In this regard, the HOM dysbiosis may have facilitated the activation/reactivation of oral viruses, and in turn, the high presence of herpesviruses infection/reactivation may further impair proper immune control (Jasinski-Bergner et al., 2020), thus potentially contributing to worse efficiency of the immune response against SARS-CoV-2. Consistent with this, EBV infection was detected in COVID-19 patients, associated with increased risk of severe COVID-19 symptoms and fatal outcome (Roncati et al., 2020; Chen et al., 2021), and correlated increased levels of IL-6 (Lehner et al., 2020). Similarly, alpha-herpesvirus (HSV-1, VZV) reactivation was observed, impacting the prognosis of COVID-19 patients (Le Balc'h et al., 2020; Hernandez et al., 2021). Thus, the presence of Herpesviridae infections in the oral cavity and their direct consequences deserve further investigation.

In parallel with the HOM profile, our work also characterized the local inflammatory and immune response as critical parameters to understand the evolution of the SARS-CoV-2 infection at the primary site of entry.

A hallmark of disease severity in COVID-19 is the uncontrolled inflammatory response, with the detection of IL-6, IL-17, TNFα, and GM-CSF at the serum/blood level (Chen G. et al., 2020; Mehta et al., 2020; Parra-Medina et al., 2020), the so-called "cytokine storm." Here we showed a significant increase of those cytokines in the oral cavity of COVID-19 patients, indicating the development of inflammation right at the entry site of the virus. It is noteworthy that the level of oral inflammation paralleled the symptom severity, pointing to the importance of oral conditions for the subsequent systemization of virus infection and inflammation cascade.

A still unanswered point in COVID-19 progression regards the development and role of the local immune response against

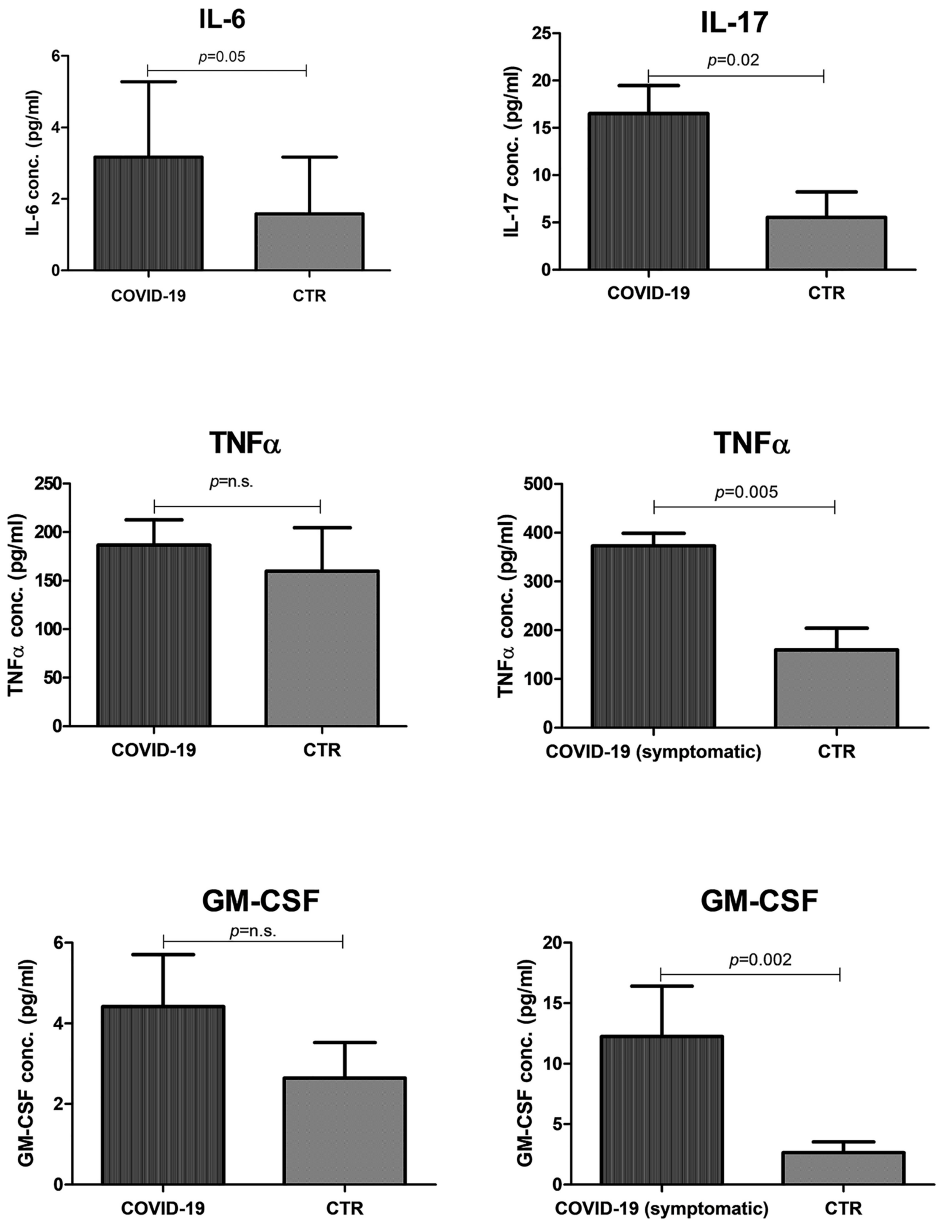


FIGURE 8 | Presence of pro-inflammatory cytokines/chemokines in the oral cavity of COVID-19 patients and controls (CTR). The results are expressed as the mean values ± SEM of the concentration (pg/ml) for each indicated cytokine.

SARS-CoV-2. Mucosal sIgA has long been known to be crucial in controlling viruses that enter the body via mucosal surfaces (Yan et al., 2002); sIgA were indeed found in the ocular fluid of at least 40% of COVID-19 patients (Caselli et al., 2020b), and microbiome composition is reportedly known to interact with and influence IgA response, in different anatomical niches including the nares (Salk et al., 2016; Grosserichter-Wagener et al., 2019; Pabst and Slack, 2020). Here, we demonstrate anti-SARS-CoV-2 sIgA in the oral cavity and that they are significantly more abundant in asymptomatic/paucisymptomatic COVID-19 patients (p = 0.02), suggesting that sIgA may be important in controlling virus penetration in the body.

The main limitation of our study is the number of enrolled subjects, who represented all the eligible subjects hosted at the enrolled center. The enrollment of a higher number of subjects, ideally in a multi-center study, may confirm the generalizability of the study results. A higher number of subjects would also enable us to stratify patients for age, thus providing a direct comparison of more homogeneous microbial populations, as the microbiome composition is dependent on the subject's age (Bourgeois et al., 2017; Caselli et al., 2020a). The relatively low number of recruited patients in our study also did not enable us to evidence a high statistically significant correlation between sIgA production and protection from severe COVID-19. Thus,

studying a higher number of patients may be of importance to ascertain this point, especially in developing effective prevention strategies and vaccines.

Overall, the data presented here suggest a correlation between HOM dysbiosis and individual susceptibility to SARS-CoV-2 severe infection, indicating an interplay between HOM profile (including mycobiome and virome), inflammation, and mucosal IgA response. If HOM alteration is the cause or effect of severe COVID-19, it is not currently possible to distinguish, because the presence of SARS-CoV-2 in the oral cavity may impact microbiome dysbiosis (Xiang et al., 2020; de Oliveira et al., 2021). On the other hand, connections between oral dysbiosis and post-viral complications have been reported, suggesting that improving oral health may reduce the risk of complications from COVID-19 (Sampson et al., 2020), thus supporting the hypothesis of a role of dysbiosis in the virus-induced disease. Toward this direction, recent studies reported that SARS-CoV-2 load can be reduced by the use of chlorhexidine mouthwashes (Yoon et al., 2020), supporting the use of antiseptics against coronavirus infection (Koch-Heier et al., 2021; Mateos Moreno et al., 2021), and clinical studies are developing accordingly (Carrouel et al., 2020) and hopefully will help to clarify this aspect.

These findings may be important in defining markers useful to predict the development of symptomatic COVID-19, and open new therapeutic opportunities addressed to balance HOM and inflammation to prevent the development of severe symptoms. In this direction, IL-6 inhibitors have been reported to reduce the odds of COVID-19 mortality (Sinha et al., 2021), and specific probiotic administration has been proposed to balance microbiome dysbiosis and prevent the development of virus-induced respiratory diseases (Wang et al., 2016) and may represent a possible intervention in COVID-19 patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB42999.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee Area Vasta Emilia Centro della Regione Emilia-Romagna (CE-AVEC): approval document no. 408/2020/Oss/UniFe, approved on April 21, 2020. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IS and MD'A analyzed the samples. CF and MF contributed to the design of the study, protocol for oral sampling, and interpretation of data. AP provided COVID-19 clinical samples and patient data. RM and GZ provided COVID-19 clinical samples and interpretation of clinical data. ML and CC provided control clinical samples. CC contributed to writing the manuscript. EC designed the study, elaborated the results, and wrote the manuscript. All authors read and approved the final manuscript.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Characterization of Supragingival Plaque and Oral Swab Microbiomes in Children With Severe Early Childhood Caries

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The human oral cavity harbors one of the most diverse microbial communities with different oral microenvironments allowing the colonization of unique microbial species. This study aimed to determine which of two commonly used sampling sites (dental plaque vs. oral swab) would provide a better prediction model for caries-free vs. severe early childhood caries (S-ECC) using next generation sequencing and machine learning (ML). In this cross-sectional study, a total of 80 children (40 S-ECC and 40 caries-free) < 72 months of age were recruited. Supragingival plaque and oral swab samples were used for the amplicon sequencing of the V4-16S rRNA and ITS1 rRNA genes. The results showed significant differences in alpha and beta diversity between dental plaque and oral swab bacterial and fungal microbiomes. Differential abundance analyses showed that, among others, the cariogenic species *Streptococcus mutans* was enriched in the dental plaque, compared to oral swabs, of children with S-ECC. The fungal species *Candida dubliniensis* and *C. tropicalis* were more abundant in the oral swab samples of children with S-ECC compared to caries-free controls. They were also among the top 20 most important features for the classification of S-ECC vs. caries-free in oral swabs and for the classification of dental plaque vs. oral swab in the S-ECC group. ML approaches revealed the possibility of classifying samples according to both caries status and sampling sites. The tested site of sample collection did not change the predictability of the disease. However, the species considered to be important for the classification of disease in each sampling site were slightly different. Being able to determine the origin of the samples could be very useful during the design of oral microbiome studies. This study provides important insights into the differences between the dental plaque and oral swab bacteriome and mycobiome of children with S-ECC and those caries-free.

Keywords: dental plaque, oral swab, bacteria, fungi, microbiota, machine learning, case-control, artificial intelligence

INTRODUCTION

The oral cavity harbors one of the most diverse microbial communities within the human body (Stearns et al., 2011). A variety of oral niches (non-shedding tooth surfaces, tongue, cheek, hard and soft palates, and gingival sulcus) provide different levels of oxygen, nutrients, salivary flow, and masticatory forces (Hall et al., 2017). Each of these different microenvironments allow the colonization of unique and adapted microbial communities. Therefore, it is expected that the microbial composition of each oral site differs significantly from each other.

Usually, the oral microbiota exists in a homeostatic balance with the host and contributes to the development of the immune system. However, once this balance is disturbed, some microbial species can overgrow and diseases associated with site-specific microbes such as periodontitis (subgingival microbiota), dental caries (supragingival microbiota), and oral candidiasis (oral mucosal and salivary microbiota) may occur (Lamont et al., 2018; Vila et al., 2020). Therefore, it is important to select the most appropriate site of sampling for the study and/or diagnosis of each oral infectious diseases. Recent studies have shown that the SARS-CoV-2 virus, which causes the coronavirus disease 19 (COVID-19), can be detected in saliva (Fernandes et al., 2020). It has been reported that salivary glands can be important reservoir of the virus (Xu et al., 2020b). Consequently, the presence of high SARS-CoV-2 viral load in saliva could make it a suitable diagnostic tool for COVID-19. Therefore, this also validates the importance of exploring different sampling options for diagnosis of infectious diseases (Fernandes et al., 2020; Sapkota et al., 2020; Xu et al., 2020a).

Since the nineteenth century, it is known that the oral microbes play a crucial role in the development of dental caries (Russell, 2009). However, the establishment of new technologies, such as next generation sequencing (NGS) and machine learning algorithms, has provided a unique opportunity to an enhanced understanding of the role of oral microbes (bacteria, fungi, and viruses) on caries development and progression.

As dental caries continues to be one of the most prevalent chronic diseases among children worldwide, there is a clear need for a deeper understanding of how oral microbial communities and their interactions could impact children's oral health. The terms early childhood caries (ECC) and severe ECC (S-ECC) were first introduced in the 1990s (Ismail and Sohn, 1999). ECC is described as any caries experience in the primary dentition of children younger than 6 years of age. S-ECC is the severe form of ECC and has an important effect on children's development and well-being (Pierce et al., 2019; Folayan et al., 2020).

We hypothesized that the microbial (bacterial and fungal) profile of dental plaque significantly differs from that of oral swabs, and because the dental biofilm is in closer contact with the tooth surface, it would provide a better prediction model for caries onset. To test this hypothesis, first we characterized the differences between the dental plaque and oral swab bacterial and fungal microbiota in children with S-ECC and those caries-free. Second, we analyzed which of those commonly used sampling sites (dental plaque and oral swab) would provide a better model for the classification of S-ECC vs. caries-free, using machine learning approaches. Third, we further evaluated whether the observed differences between the microbial profiles of the samples could be used for the differentiation between the sampling sites (dental plaque vs. oral swab) to assist researchers during the design of oral microbiome studies. This is one of the first studies to explore the oral microbiome profiles to classify oral sites.

Abbreviations: ASVs, Amplicon Sequence Variants; FDR, False Discovery Rate; HOMD, Human Oral Microbiome database; ITS1, Internal Transcribed Spacer 1; PCoA, Principal Coordinates Analysis; PERMANOVA, Permutational Multivariate Analysis of Variance using distance matrices; S-ECC, Severe Early Childhood Caries.

MATERIALS AND METHODS

Study Population

In this cross-sectional study, eighty children < 72 months of age were recruited between December 2017 and August 2018. Among those, 40 had S-ECC, according to the American Academy of Pediatric Dentistry definition (AAPD, 2020), and 40 were caries-free. Children with S-ECC were recruited at the Misericordia Health Centre (MHC), Winnipeg-MB, Canada, on the day of their full-mouth rehabilitative dental surgery under general anesthesia. Caries-free children were recruited from the community. Caries-free children had a dmft (cumulative score of the number of decayed, missing, or filled primary teeth) index equal to zero and had no incipient lesions. To confirm the caries-free status, a dental examination was performed by R.J.S. at the Children's Hospital Research Institute of Manitoba by means of visual/tactile examination using artificial light and no radiographs. Inclusion criteria: children less than 72 months of age who were caries-free (dmft = 0) or have been diagnosed with S-ECC (based on the American Academy of Pediatric Dentistry definition). Exclusion criteria: children older than 72 months of age, use of antibiotics, and children who did not satisfy the case definition of S-ECC.

Based on the power analysis published by La Rosa et al. (2012) at 5% significance level, with 40 samples per group and the average number of reads of 50,000 per sample our study would achieve a power > 97%. This study protocol was approved by the University of Manitoba's Health Research Ethics Board (HREB # HS20961–H2017:250) and by the MHC, Winnipeg, MB, Canada. Written informed consent was provided by the parents or legal caregivers (de Jesus et al., 2020). This work follows the STROBE guidelines checklist for cross-sectional studies (**Supplementary Table**).

Sample Collection

Due to the young age of the participants and their inability to spit saliva, oral swab samples were collected with a sterile polyester-tipped applicator (Fisher Scientific) by swabbing the buccal mucosa and anterior floor of the mouth under the tongue. The oral swabs were stored in RNAprotect Reagent (Qiagen, Cat. # 74324, Hilden, Germany) at −80°C until further analysis. Supragingival plaque samples were collected from all available tooth surfaces with a sterile interdental

brush (Agnello et al., 2017; de Jesus et al., 2020). They were dislodged into the RNAprotect Reagent (Qiagen, Cat. # 76506, Hilden, Germany) and stored at −80°C until further analysis. For simplicity, supragingival plaque samples are referred to as dental plaque.

DNA Extraction and 16S and ITS1 rRNA Amplicon Sequencing

Total DNA was extracted from 160 samples (80 oral swabs and 80 dental plaque samples) using QIAamp DNA mini kit (Qiagen, Hilden, Germany) following manufacturer's protocol. An additional enzymatic digestion step with lysozyme treatment (20 μg/ml lysozyme in a buffer containing 20 mM Tris HCl, pH 8; 1.2% Triton X 100; 2 mM EDTA) was performed before DNA extraction from dental plaque samples.

The total DNA was sent on dry ice to McGill University–Génome Québec Innovation Center (Montreal, Canada) for paired-end Illumina MiSeq PE250 sequencing. The primers 515F, (5′-GTGCCAGCMGCCGCG GTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), targeting the V4 hypervariable region of the bacterial 16S rRNA gene and the primers ITS1-30 (5′-GTCCCTGCCCTTTGTACACA-3′) and ITS1-217 (5′-TTTCGCTGCGTTCTTCATCG-3′), targeting the Internal Transcribed Spacer 1 (ITS1) of the fungal rRNA gene were used for amplification (Usyk et al., 2017; de Jesus et al., 2020).

Bioinformatics and Statistical Analysis

The sequences were received as demultiplexed, barcode removed, paired ends fastq files. The quality control analysis was performed with FastqC v0.11.8 (Andrews, 2010). The sequences were then imported and analyzed with QIIME2 2018.11 (Bolyen et al., 2019). The 16S pair-end sequences were quality trimmed, filtered to remove ambiguous and chimeric sequences, and merged using DADA2 implemented in QIIME2, resulting in the amplicon sequence variant (ASV) table (Callahan et al., 2016). The ITS1 pair-end sequences were trimmed using the Q2-ITSxpress QIIME2 plugin prior to the DADA2 step, with default parameters (Rivers et al., 2018). The taxonomic assignment of ASVs was performed using the Human Oral Microbiome Database (HOMD, version 15.1) for bacteria and the UNITE database (version 8.2; QIIME developer release) for fungi at 99% sequence similarity (Dewhirst et al., 2010; Agnello et al., 2017; Abarenkov et al., 2020b; de Jesus et al., 2020). Due to the presence of many fungal ASVs that were assigned only at kingdom level, further fungal ASV curation was performed with the R package LULU (Frøslev et al., 2017). The remaining ASVs assigned as *Fungi* at kingdom level only, or with unidentified phylum were manually assessed using the program BLASTN in NCBI (Zhang et al., 2000). The ASVs with non-fungal BLASTN results were discarded and the remaining were repeatedly assigned to new taxonomic assignments using different UNITE databases threshold levels (Abarenkov et al., 2020a,b,c) and taxonomy classification methods (q2-feature-classifier classify-sklearn and classify-consensus-blast) in QIIME2, as described previously (Martinsen et al., 2021). The data was imported into R using the R package "qiime2R" (version 0.99.13) and additional filtering was performed using "phyloseq" (version 1.30.0) to remove singletons and samples with less than 1,000 reads (McMurdie and Holmes, 2013; Bisanz, 2018; Depner et al., 2020). The ASV counts were then normalized using the cumulative-sum scaling (CSS) approach from the R package "metagenomeSeq" version 1.28.2 (Paulson et al., 2013).

The alpha diversity analyses (within-samples) were performed using the Chao1 and Shannon indices to estimate richness and diversity, respectively, using raw ASV count data from QIIME2 in "phyloseq". Pairwise comparisons of alpha diversity were done by the paired Wilcoxon signed rank test. Beta diversity measures were calculated on CSS normalized ASV data. This analysis was performed to compare the structure of the bacterial and fungal microbial communities between samples, using the permutational analysis of variance (PERMANOVA) test with 999 permutations in the R package "vegan" (adonis function; version 2.5.6) (Anderson, 2001). It was visualized using principle coordinate analysis (PCoA) with Bray-Curtis dissimilarity index in the R package "ggplot2" (version 3.3.3) (Beals, 1984; Wickham, 2016).

Differentially abundant species were identified using the DESeq2 negative binomial Wald test, controlling the false discovery rate (FDR) for multiple comparison, within "phyloseq" (Love et al., 2014). For this, the raw ASV counts were collapsed to the species level. For comparisons between dental plaque vs. oral swab, a paired DESeq2 analysis was performed. FDR adjusted $P < 0.05$ was considered significant.

Machine Learning Analysis

Machine learning methods were used to train multivariable classification models to identify the caries status, S-ECC and caries-free. To generate the machine learning models, taxonomic features were used in the form of ASV tables collapsed to species-level. For the classification, we used the workflow provided in "Siamcat," which provides a machine learning toolbox for metagenome analysis through state-of-the-art machine learning methods (Wirbel et al., 2019, 2021). The data were separately processed for fungi and bacteria and sample-wise relative abundance for the microbiome quantitative profiles was used as input data to maintain the uniformity.

To process the data in "Siamcat," features with a prevalence of less than five percent across samples were removed and the

TABLE 1 | Characteristics of study participants*.

	Caries status	
	S-ECC (*N* = 40)	**Caries-free (*N* = 40)**
Age (months), mean ± SD	45.6 ± 11.4	46.2 ± 14.2
Sex, n(%)		
Female	25 (62.5)	21 (52.5)
Male	15 (37.5)	19 (47.5)

**Other demographics of the study participants have been previously published (de Jesus et al., 2020).*

remaining features were normalized by centered log-ratio (CLR) transformation. The data was then prepared for cross-validation with eightfold and 5 repeats. After this, the models were trained using Lasso, Ridge, Elastic Net (Enet), and RandomForest classification methods in Siamcat, which uses the "mlr" package for machine learning based classification (Bischl et al., 2016). The models' performance for cross validation was evaluated using the area under the receiver operating characteristic (AUROC) value. To show the importance of the model features, the model feature weights were converted to relative weights and up to the top 20 features were selected, based on their median values, to generate a heatmap using the R package "ggplot2" (Wickham, 2016).

For the machine-learning based classification of plaque and swab samples, a pairwise sample analysis was performed using a boosting conditional logistic regression from R package "clogitboost," which takes the paired nature of the dental plaque and oral swab samples into account (Shi and Yin, 2015). The model was fitted using component-wise smoothing spline. The caries-free and S-ECC samples were divided into training and test sets using three-quarters of the data for training and the remaining for test in a way that paired samples for plaque and swab should be together in either training or test sets. For the features (species) selection in training dataset, we obtained the *p*-values from the differential abundance analysis described above. The top features selected by the *p*-values were used to train the classification models. Since we have only 30 independent samples in the training set, we considered only top 5, 10, 15, 20, and 25 features to build the model, respectively. The models' performance was evaluated using AUROC. Each of the trained models were then tested on the test set. The

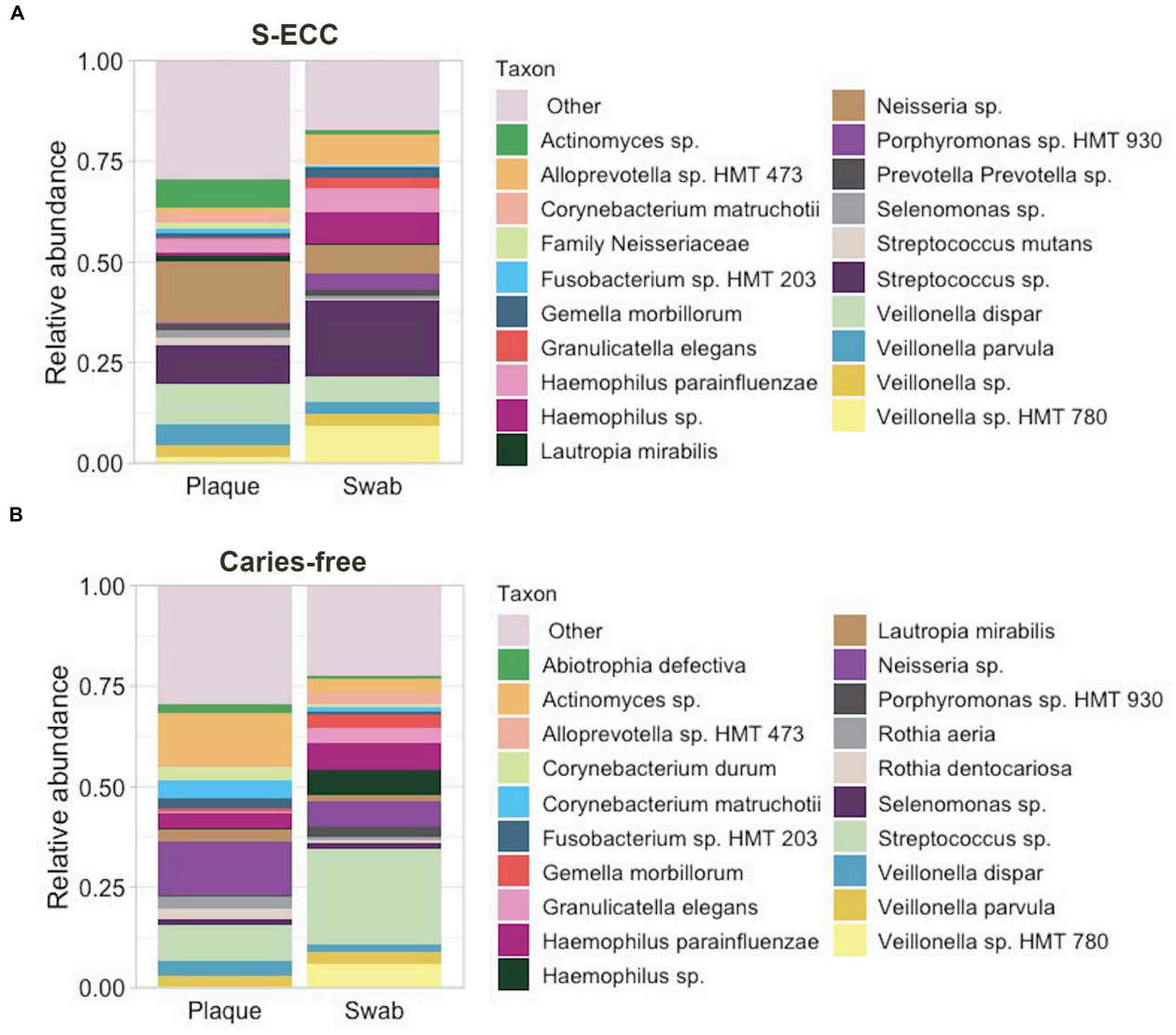

FIGURE 1 | Bacterial taxonomic profiles of dental plaque and oral swab. Relative abundance of the top 20 bacterial taxa in dental plaque and oral swab samples from **(A)** children with S-ECC and **(B)** caries-free children. "Other" indicates the taxa not individually shown. S-ECC, severe early childhood caries.

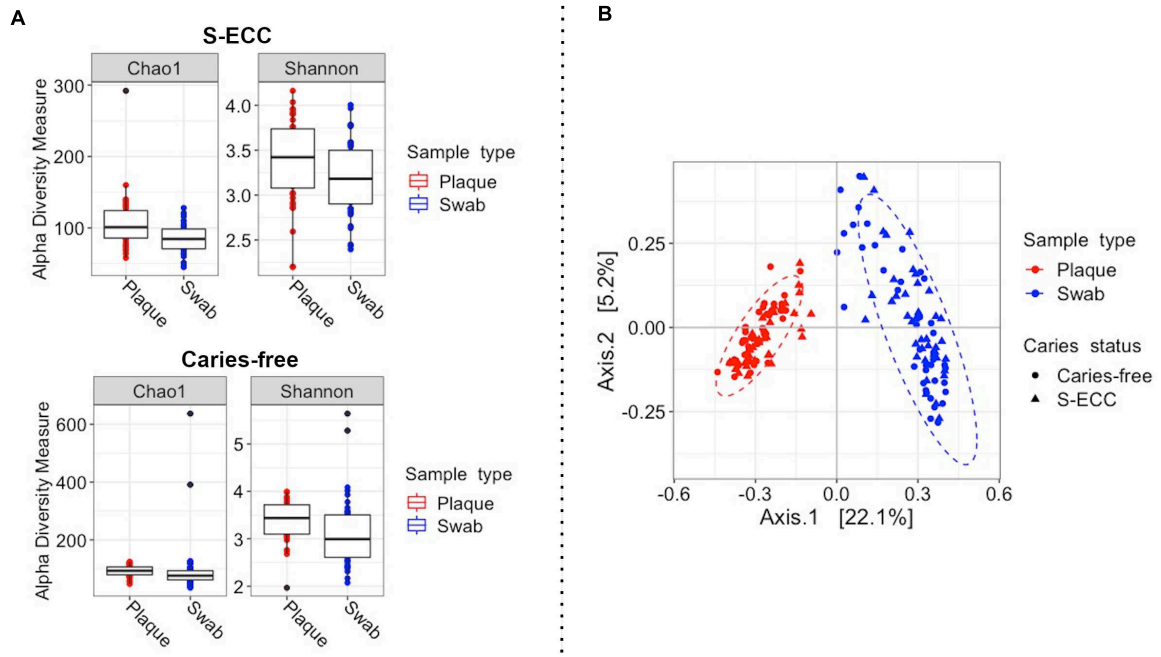

FIGURE 2 | Bacterial diversity of dental plaque and oral swab samples from children with S-ECC and those caries-free. **(A)** For alpha diversity (within-sample) the Shannon and Chao1 diversity and richness measures were calculated according to sample type in both caries-free and S-ECC groups. A significant difference between oral swab and dental plaque alpha diversity and richness was observed in both caries-free and S-ECC groups ($P < 0.05$, paired Wilcoxon test). **(B)** For beta (between-sample) diversity, Bray-Curtis distances were calculated, followed by principal coordinates analysis (PCoA). The plot shows the separation of samples according to sample type (pseudo-F = 40.4, $R^2 = 0.2$, $P = 0.001$, PERMANOVA accounting for the children's caries-status). The ellipses represent a 95% confidence level. S-ECC, severe early childhood caries.

training-test strategy/process was repeated for 30 iterations and the classification performance between caries-free and S-ECC samples were compared by the average of AUROC values from the 30 repeats.

RESULTS

Eighty children who fit the study criteria were recruited and 160 samples (80 dental plaque and 80 oral swabs) were collected. The **Table 1** shows some characteristics of the study participants. Additional information about the participants have been recently published (de Jesus et al., 2020).

Bacterial Community Analysis

After filtering out low quality and chimeric sequences, a total of 8,664,777 16S rRNA reads were obtained, with an average number of 54,154.9 reads per sample (160 samples). A total of 5,421 ASVs were assigned to 141 genera and 320 species. Overall, the most abundant phyla were *Firmicutes* (41.08%) and *Proteobacteria* (27.37%). In oral swabs, *Streptococcus* (overall: 21.81%; S-ECC: 19.22%; Caries-free: 24.41%) was the most abundant genus followed by *Veillonella* (overall: 17.03%; S-ECC: 21.65%; Caries-free: 12.40%) and *Haemophilus* (overall: 13.28%; S-ECC: 13.62%; Caries-free: 12.94%). In dental plaque, *Neisseria* (overall: 16.06%, S-ECC: 15.93%; Caries-free: 16.13%), *Veillonella* (overall: 13.66%; S-ECC: 19.54%; Caries-free: 7.73%), and *Streptococcus* (overall: 11.35%; S-ECC: 12.77%; Caries-free: 9.92%) were the most abundant genera. The taxonomic profile of the dental plaque and oral swab samples are shown in **Figures 1A,B**.

Bacterial alpha diversity (within samples) analysis showed a significant difference between oral swab and dental plaque alpha diversity (Shannon index, S-ECC: $P = 0.0034$; Caries-free: $P = 0.015$) and richness (Chao1 index, S-ECC: $P < 0.001$; Caries-free: $P = 0.025$) in both caries-free and S-ECC groups (**Figure 2A**). Bacterial beta (between-sample) diversity analysis showed a clear separation of samples according to sampling site, oral swab and dental plaque (pseudo-F = 42.71, $R^2 = 0.2$, $P = 0.001$, PERMANOVA accounting for the children's S-ECC status and the paired samples; **Figure 2B**). A significant difference in bacterial community was also observed between the S-ECC and caries-free groups (pseudo-F = 2.85, $R^2 = 0.014$, $P = 0.001$).

Figure 3A shows the relative abundance of the top 20 bacterial species across the subgroups. The differential abundance analysis revealed numerous species that were overabundant in dental plaque or oral swab samples within the S-ECC and caries-free groups (**Figures 3B,C**, adjusted $P < 0.05$, DESeq2). Interestingly, many species were significantly more abundant in dental plaque or oral swab in both S-ECC and caries-free groups. For instance, *Capnocytophaga* sp. oral taxon 326 (S-ECC: −10.83 log2fold change; Caries-free: −4.72 log2fold), *Kingella* sp. oral taxon 012 (S-ECC: −9.52 log2fold change; Caries-free:

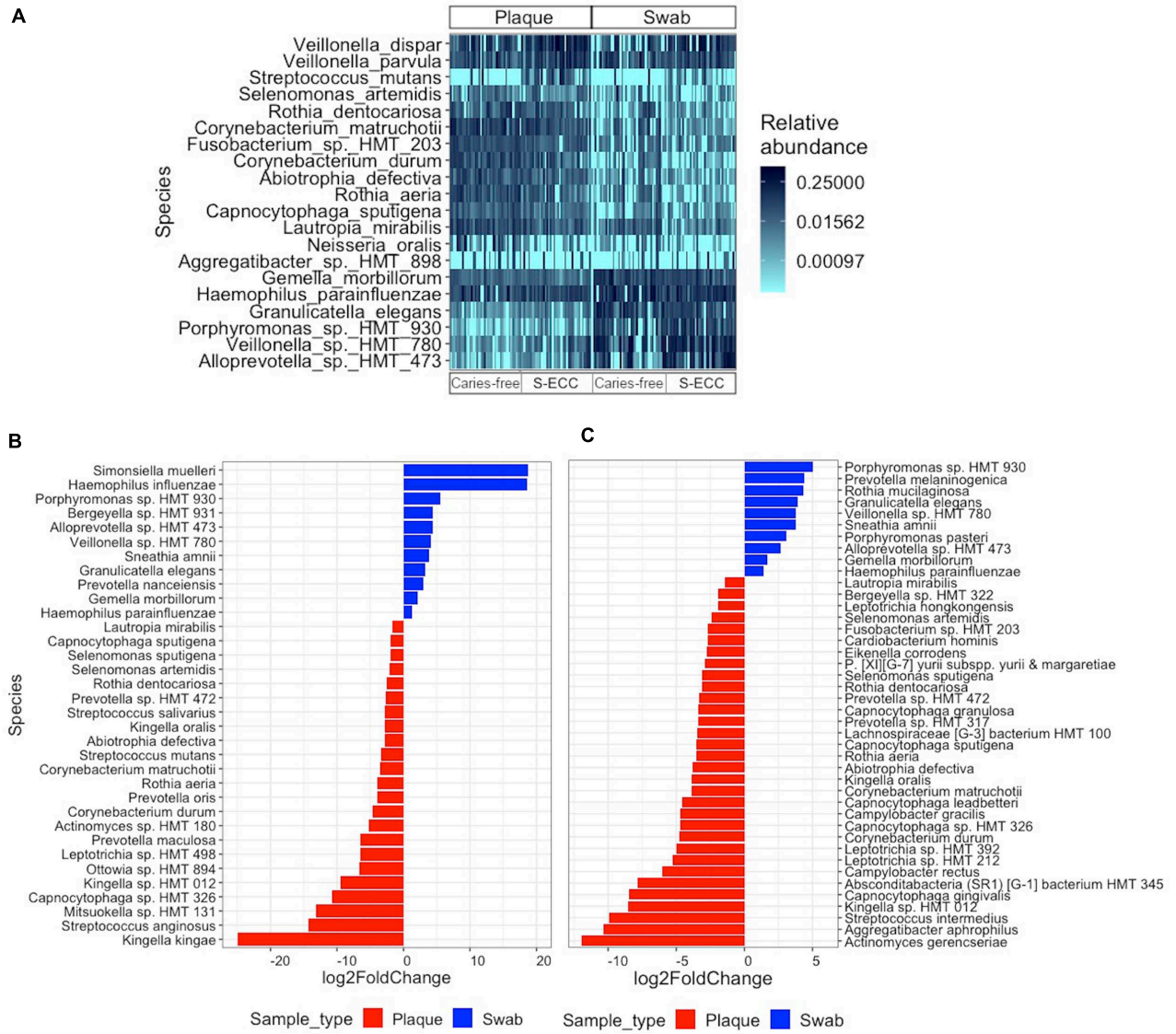

FIGURE 3 | Differential abundance of bacterial species. **(A)** Heatmap showing the relative abundance of the top 20 bacterial species identified in all dental plaque and oral swab samples. **(B,C)** Relative fold change in the abundance of bacterial species in **(B)** samples from children with S-ECC, and **(C)** samples from caries-free children, according to sample type. The differential abundance of the bacterial species was tested using the DESeq2 negative binomial Wald test. **(B,C)** All species listed have an FDR adjusted $P < 0.05$. S-ECC, severe early childhood caries.

−8.57 log2fold change), *Corynebacterium durum* (S-ECC: −4.71 log2fold change; Caries-free: −4.81 log2fold change), *Rothia aeria* (S-ECC: −3.93 log2fold change; Caries-free: −3.56 log2fold change), *Corynebacterium matruchotii* (S-ECC: −3.54 log2fold change; Caries-free: −3.90 log2fold), among others, were more abundant in dental plaque than oral swabs in both caries-free children and those with S-ECC. On the other hand, *Porphyromonas* sp. oral taxon 930 (S-ECC: 5.57 log2fold change; Caries-free: 5.01 log2fold change), *Alloprevotella* sp. oral taxon 473 (S-ECC: 4.35 log2fold change; Caries-free: 2.66 log2fold change), *Veillonella* sp. oral taxon 780 (S-ECC: 4.12 log2fold change; Caries-free: 3.79 log2fold change), *Sneathia amnii* (S-ECC: 3.86 log2fold change; Caries-free: 3.76 log2fold change) *Granulicatella elegans* (S-ECC: 3.26 log2fold change; Caries-free: 3.93 log 2fold change), and *Haemophilus parainfluenzae* (S-ECC: 1.26 log2fold change; Caries-free: 1.39 log2fold change) were more abundant in oral swabs in both caries-free and S-ECC groups. In children with S-ECC, the well-known cariogenic bacterium *Streptococcus mutans* was more abundant in dental plaque samples (−3.45 log2fold change, adjusted $P < 0.05$).

Within the oral swab samples, three species were more abundant in S-ECC compared to caries-free: *Veillonella dispar* (2.09 log2fold change), *Prevotella veroralis* (23.33 log2fold change), and *Neisseria bacilliformis* (24.58 log2fold change, adjusted $P < 0.05$, DESeq2). While *Lautropia mirabilis* (−1.41 log2fold change) was significantly more abundant in caries-free

TABLE 2 | Mean relative abundance of the top 20 most abundant fungal taxa.

	S-ECC		Caries-free	
Species	**Plaque**	**Swab**	**Plaque**	**Swab**
*Candida dubliniensis** #	47.76 ± 44.28	13.09 ± 25.16	0.01 ± 0.03	0.00 ± 0.002
Class Agaricomycetes* §	2.52 ± 12.30	11.06 ± 25.85	1.98 ± 6.94	7.21 ± 18.82
*Candida albicans**	9.51 ± 24.79	3.59 ± 14.27	5.16 ± 18.28	1.58 ± 6.65
Blumeria sp. §	3.1 ± 16.65	0.00 ± 0.00	15.08 ± 30.59	0.00 ± 0.00
Family Thelephoraceae*#	2.165 ± 5.35	0.001 ± 0.01	12.85 ± 26.20	1.14 ± 4.07
*Malassezia restricta** § #	0.29 ± 1.03	5.45 ± 18.25	5.55 ± 19.88	0.07 ± 0.38
Candida tropicalis#	3.90 ± 14.90	2.90 ± 9.77	0.00 ± 0.00	0.01 ± 0.04
*Trichosporon asahii** §	0.00 ± 0.00	1.34 ± 8.06	2.99 ± 17.14	0.09 ± 0.56
*Ramicandelaber taiwanensi** §	0.52 ± 1.64	0.09 ± 0.52	3.58 ± 7.00	0.05 ± 0.21
Fusarium sp.* §	0.58 ± 2.46	0.00 ± 0.00	3.01 ± 17.14	0.00 ± 0.00
Meyerozyma guilliermondii§	0.10 ± 0.59	0.00 ± 0.00	3.00 ± 17.14	0.00 ± 0.00
Exophiala radices	0.00 ± 0.00	0.00 ± 0.00	2.65 ± 15.46	0.00 ± 0.00
Candida parapsilosis	0.36 ± 2.16	0.02 ± 0.11	2.204 ± 12.75	0.00 ± 0.00
*Malassezia globosa**	0.00 ± 0.00	2.23 ± 13.41	0.03 ± 0.15	0.00 ± 0.00
Order Malasseziales§	0.27 ± 1.63	0.00 ± 0.00	2.06 ± 11.99	0.00 ± 0.00
*Stereum rugosum**	2.03 ± 12.17	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Phylum Rozellomycota§	0.17 ± 0.61	0.10 ± 0.58	1.65 ± 8.99	0.04 ± 0.08
Phylum Chytridiomycota§	0.26 ± 0.93	0.08 ± 0.34	1.24 ± 5.69	0.37 ± 0.71
Phylum Ascomycota§	0.01 ± 0.05	0.03 ± 0.13	0.55 ± 2.19	1.17 ± 5.02
*Wallemia tropicalis**§	0.01 ± 0.07	0.00 ± 0.001	0.001 ± 0.01	0.00 ± 0.00

**Adjusted $P < 0.05$ (DESeq2), dental plaque vs. oral swab in children with S-ECC.*
§ Adjusted $P < 0.05$ (DESeq2), dental plaque vs. oral swab in caries-free children.
#Adjusted $P < 0.05$ (DESeq2), S-ECC vs. caries-free in oral swab samples.
S-ECC, severe early childhood caries.

children's oral swabs (adjusted $P < 0.05$, DESeq2). The differences between the dental plaque microbial composition between children with S-ECC and those caries-free have been previously published (de Jesus et al., 2020). The proportion of bacterial and fungal ASVs assigned to different taxonomic levels are shown in **Supplementary Figure 1**.

Fungal Community Analysis

A total of 8,000,067 filtered ITS1 rRNA reads were obtained, with an average number of reads per sample of 50,000.42 (160 samples). The 622 ASVs where assigned to 63 genera and 59 species. After filtering, ten samples had low reads (<1,000) and were removed from the fungal analysis as well as their respective oral swab or dental plaque pairs, resulting in a total sample size of 140. Differential abundance analysis showed that among the top 20 most abundant fungal taxa, within the S-ECC group, *Stereum rugosum* (−29.03 log2fold change), *Fusarium* sp. (−29.14 log2fold change), *Trichoderma* sp. (−24.79 log2fold change), *Candida albicans* (−10.42 log2fold change), *C. dubliniensis* (−6.04 log2fold change) and others were enriched in dental plaque. While *Trichosporon asahii* (23.97 log2fold change), *Malassezia globosa* (20.36 log2fold change), *M. restricta* (14.9 log2fold change) and others were more abundant in oral swabs. Within the caries-free group, the class Agaricomycetes (13.63 log2 fold change) was more abundant in oral swab, while *Blumeria* sp. (−29.94 log2fold change), *Fusarium* sp. (−23.26 log2fold change), *Wallemia tropicalis* (−22.68 log2fold change), *Malassezia restricta* (−16.44 log2fold change) and others were more abundant in dental plaque. Within the oral swab samples, *Candida dubliniensis* (12.92 log2fold change), *Candida tropicalis* (24.99 log2fold change), and *Malassezia restricta* (24.14 log2fold change) were more abundant in children with S-ECC compared to caries-free controls (**Table 2**, adjusted $P < 0.05$, DESeq2). The results of the differential abundance analysis according to caries status in dental plaque (caries-free vs. S-ECC) have been published previously (de Jesus et al., 2020).

The fungal alpha diversity analysis showed a significant difference in Chao 1 diversity ($P < 0.001$, paired Wilcoxon test) in the caries-free group (**Figure 4A**). Fungal community (β-diversity) analysis also showed a significant difference between dental plaque and oral swab microbiomes (pseudo-F = 5.58, $R^2 = 0.04$, $P = 0.001$, PERMANOVA; **Figure 4B**). The fungal communities of samples from caries-free children and those with S-ECC also showed a significant difference (pseudo-F = 4.17, $R^2 = 0.03$, $P = 0.001$).

Machine Learning Analysis

We first evaluated the model performance using Lasso, Ridge, Elastic Net (Enet), and RandomForest methods to classify S-ECC vs. caries-free. Overall, the Ridge approach with default parameters provided the best classification accuracy while the other three methods provided similar AUROC values (data not shown). Hence, Ridge was the model of choice for further classification.

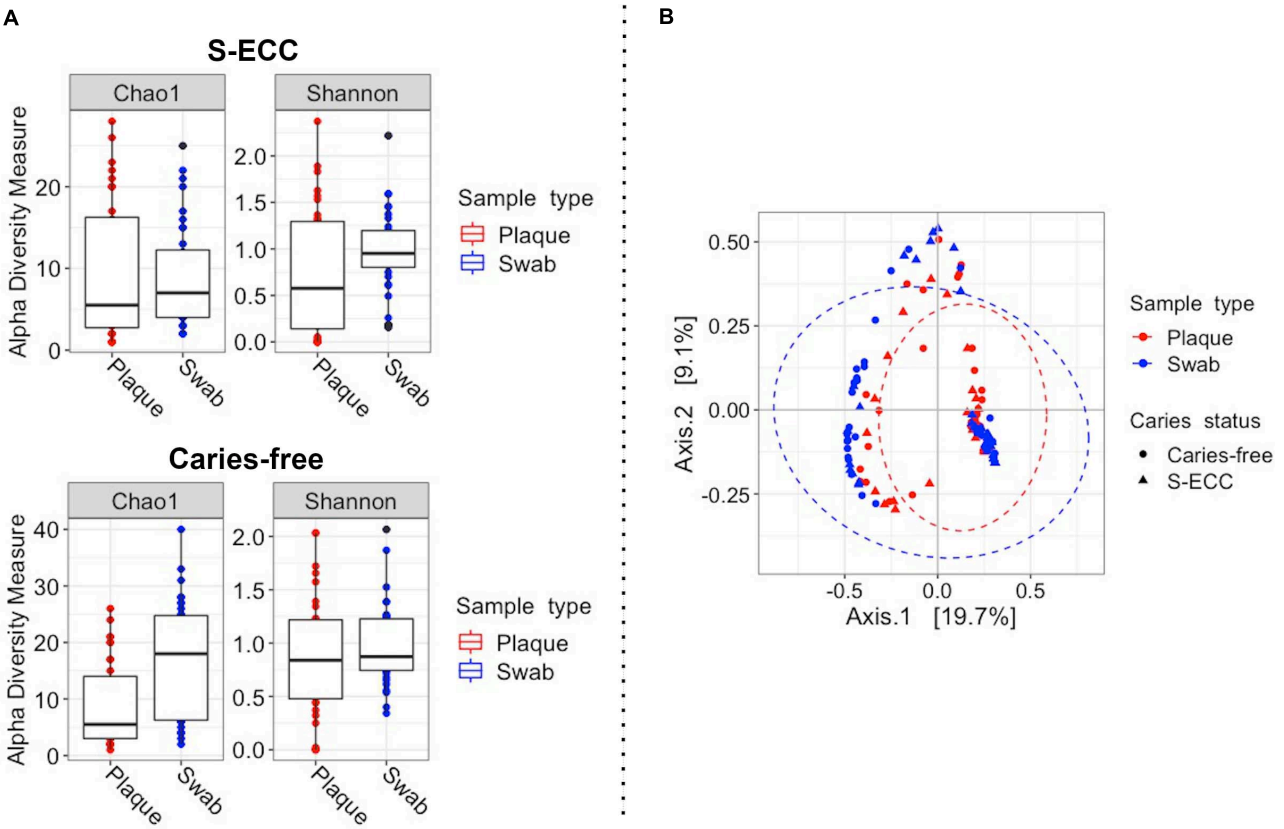

FIGURE 4 | Fungal diversity of dental plaque and oral swab samples. **(A)** For alpha diversity (within-sample) the Shannon and Chao1 diversity and richness measures were calculated according to sample type in both caries-free and S-ECC groups. A significant difference in richness was observed between the sampling sites in the caries-free group ($P < 0.001$, Chao1 index, paired Wilcoxon test). **(B)** For beta (between-sample) diversity, Bray-Curtis distances were calculated, followed by principal coordinates analysis (PCoA, pseudo-F = 11.58, $R^2 = 0.04$, $P = 0.001$, PERMANOVA). The ellipses represent a 95% confidence level. S-ECC, severe early childhood caries.

To evaluate which sampling site, dental plaque or oral swabs, would provide a better classification model for S-ECC vs. caries-free, the samples were grouped according to sampling site. The AUROC values obtained by the Ridge model with bacterial species were 0.92 and 0.91 for dental plaque and oral swab samples, respectively (**Figure 5A**). While, for fungal taxa, the AUROC values were 0.85 and 0.835, respectively (**Figure 5B**). The median relative feature weights used to predict the corresponding models and their ranks are shown in **Figures 5C,D**. Among the most important bacterial features for the S-ECC vs. caries-free classification model are *Gemella morbilorum*, *Lautropia mirabilis*, *Actinomyces* oral taxon 525 and *Capnocytophaga* oral taxon 336. While for fungi, *Mycosphaerella tassiana, Betamyces americae meridionalis, Wickerhamiella* sp. and *Cyberlindnera jadinii* were among the most important discriminatory fungal species.

To evaluate if it is possible to differentiate dental plaque samples from oral swab samples based on their bacterial and fungal profiles, both in caries-free and S-ECC groups, the samples were grouped according to caries status. The AUROC values were compared for the models built based on the top 5, 10, 15, 20, and 25 species selected through differential abundance analysis in the training set. For bacteria, in caries-free samples, the maximum AUROC value was 0.80 using 10 species while for S-ECC, the maximum AUROC value was 0.73 with 25 species. For fungi, the maximum AUROC was obtained by 10 species in caries-free samples and 5 in S-ECC samples (**Table 3**). The performance of paired analysis for different number of species is summarized in **Table 3**. It was notable that in site-based classification, in bacteria low number of species provide better classification in caries-free samples. While, for S-ECC samples high number of species are required for improving prediction. For fungi the classification was better with low number of species in both caries-free and S-ECC groups, which might be due to the low alpha diversity in the fungal samples.

DISCUSSION

In this study, first we confirmed that the bacterial and fungal community composition of dental plaque differed significantly from that obtained from oral swabs. Second, we investigated, using machine learning approaches, which sampling site would be the most appropriate to differentiate the oral microbial profile of children with S-ECC and those caries-free. Identifying the

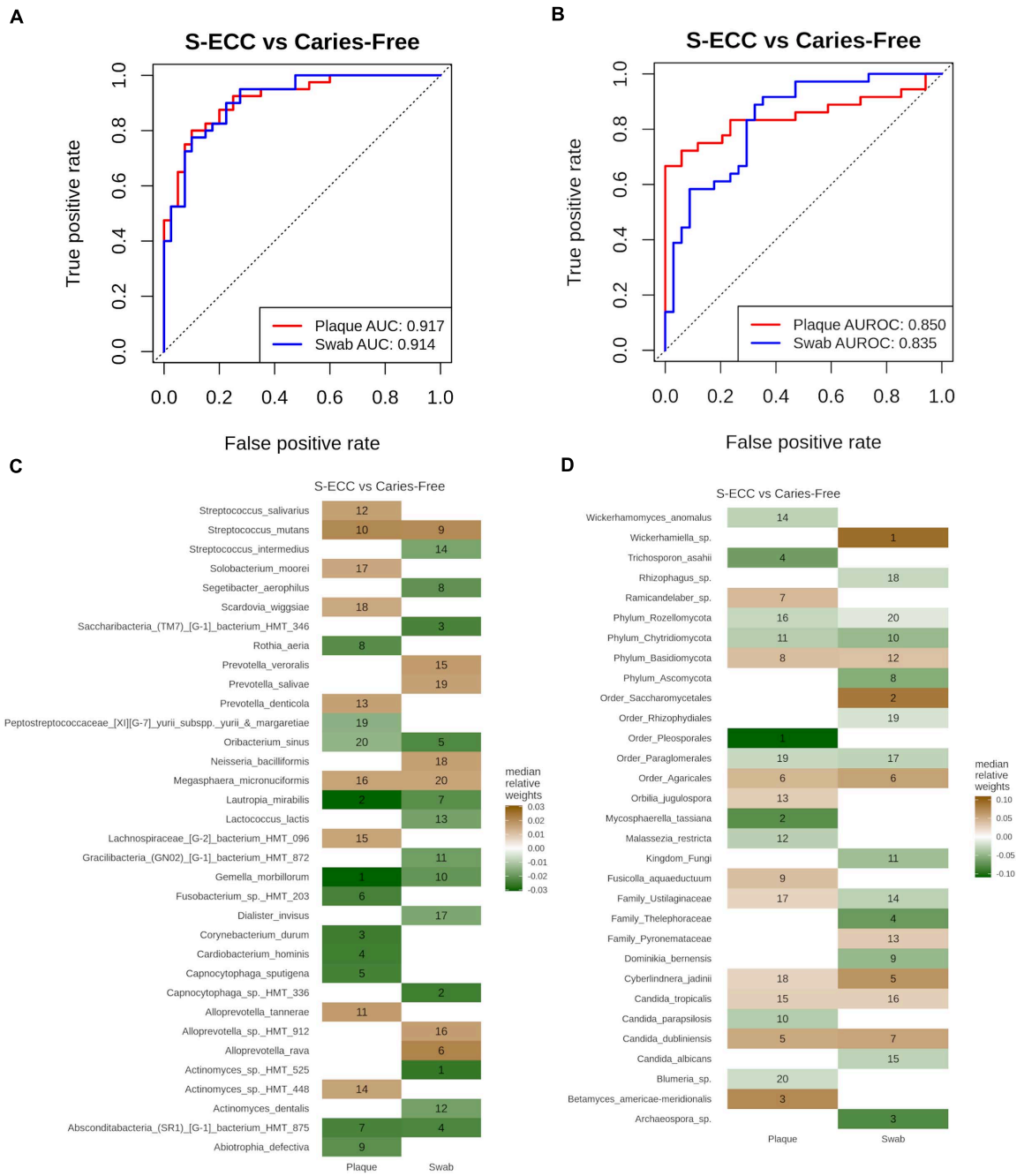

FIGURE 5 | Classification of S-ECC vs. caries-free. **(A,B)** Receiver operating characteristic (ROC) curve representing the cross-validation performance as for the classification of S-ECC and carries-free in **(A)** bacteria and **(B)** fungi using "Ridge" model in Siamcat. The area under the receiver operating characteristic curve (AUROC) represents the sample taken from dental plaques and oral swabs, by red and blue colors, respectively. AUROC values are shown in the bottom-right of the plot. **(C,D)** The relative feature weights used to predict the corresponding model. A maximum of 20 weights in each category were selected to plot on the heatmap and are marked with the ranking of the weights in the heatmap for bacterial **(C)** bacterial and **(D)** fungal taxa. The green color represents the features important in caries-free and brown is for S-ECC.

appropriate type of sample to be used is important to guide future caries association studies. Third, we evaluated whether it could be possible to predict the sampling site (dental plaque vs. oral swab) based on the microbial profile of the samples. Being able to determine the origin of the samples could be useful for the design of future microbiome studies. For instance, if researchers want to collect supragingival plaque, it would be useful to have a way of detecting if during sample collection the supragingival plaque got contaminated with subgingival plaque, as each of those should have unique microbial profiles.

TABLE 3 | Mean AUROC value for plaque vs. swab classification through conditional logistic regression.

	Bacteria		Fungi	
Species	**Caries-free**	**S-ECC**	**Caries-free**	**S-ECC**
5	0.77 ± 0.14	0.67 ± 0.12	0.62 ± 0.17	**0.73 ± 0.15**
10	**0.80 ± 0.13**	0.69 ± 0.15	**0.63 ± 0.18**	0.73 ± 0.16
15	0.73 ± 0.16	0.71 ± 0.17	0.62 ± 0.17	0.69 ± 0.16
20	0.71 ± 0.17	0.72 ± 0.17	0.63 ± 0.19	0.65 ± 0.18
25	0.72 ± 0.16	**0.73 ± 0.17**	0.62 ± 0.17	0.69 ± 0.18

The species column shows the number of species used in the classification and the mean AUROC values are provided with the standard deviation of 30 iterations of the training-test based prediction. The highest AUROC value of each group is bolded.

The oral microbiome is considered highly diverse, compared to other body sites. Although dental plaque, saliva and the buccal mucosa are in close contact, they have diverse microbial communities. The Human Microbiome Project (HMP), for instance, compared the diversity of microbes among five major body areas of 242 healthy individuals and showed that supragingival plaque has higher bacterial alpha diversity compared to the oral mucosa, which agrees with the results reported in the present study (The Human Microbiome Project Consortium, 2012). Hall et al. identified a significant difference between the microbial communities of supragingival plaque, saliva, and tongue samples from health subjects, demonstrating the existence of site-specific oral microbiomes (Hall et al., 2017).

Interestingly, while dental plaque showed increased bacterial alpha diversity compared to oral swabs the fungal alpha diversity showed an opposite pattern, with oral swabs displaying increased fungal alpha diversity. The higher fungal diversity observed in the oral swab may be associated with more fungal DNA of transient colonizers from the environment through mouth breathing and food intake (Xu and Dongari-Bagtzoglou, 2015; Diaz and Dongari-Bagtzoglou, 2021). Furthermore, most oral fungi are present at low biomass and may be difficult to detect in oral samples (Diaz and Dongari-Bagtzoglou, 2021). The above factor may explain why the number of observed fungal ASVs was lower than that of bacteria.

Streptococci was the most abundant bacterial genera in oral swabs, similar to what has been previously reported (Caselli et al., 2020). *Neisseria*, *Haemophilus* and *Veillonella*, found to be the most abundant in dental plaque or oral swab samples, have also been reported as highly abundant in different oral sites by previous studies (Huse et al., 2012; Caselli et al., 2020). *Streptococcus, Fusobacterium*, *Gemella*, and *Veillonella* have all been considered core OTUs in different oral sites (Huse et al., 2012; Hall et al., 2017). Here we showed site-specific differences in the abundance of certain species from these genera, with some being significantly more abundant in dental plaque compared to oral swabs or vice-versa. Among children with S-ECC, the known cariogenic bacterium *S. mutans* was significantly enriched in dental plaque samples compared to oral swabs. It also showed to be among the top 10 most important feature for the classification of S-ECC vs. caries-free in both dental plaque and oral swab samples. Other caries associated bacteria such as *Leptotrichia* spp. and *Selenomonas* spp. (Kalpana et al., 2020) were more abundant in dental plaque than oral swab samples from children with S-ECC.

Fungal species from the genera *Candida, Malassezia, Meyerozima,* and *Trichosporon,* were among the most abundant in dental plaque and oral swab, similarly to what has been reported in other studies (Shelburne et al., 2015; Baraniya et al., 2020; Robinson et al., 2020; Diaz and Dongari-Bagtzoglou, 2021). The differential abundance analysis showed a significant difference between *C. dubliniensis* and *C. tropicalis* in the oral swab of caries-free children and children with S-ECC. Those fungal species were also among the top 20 most important features for the classification of S-ECC vs. caries-free in oral swabs. *Candida* spp. are among the most abundant fungal species in the oral cavity and they are associated with different oral diseases (Peters et al., 2017; Diaz et al., 2019). *C. dubliniensis* has only recently been associated with dental caries in children (Al-Ahmad et al., 2016; de Jesus et al., 2020; O'Connell et al., 2020). Here we show that this fungus is not only highly abundant in the dental plaque of children with S-ECC, as previously reported, but it is also enriched in the oral swabs obtained from children with S-ECC compared to those caries-free.

In recent years, machine learning has become a commonly applied approach to early childhood oral health research (Peng et al., 2021). One of the challenges in microbiome data analysis is that the differential analysis methods generally lack the information about predictability. Thus, we used machine learning methods to identify site-specific taxonomic features in dental plaque and oral swabs. The results suggested that both dental plaque and oral swab samples provide a good model for S-ECC vs. caries-free classification. They also suggest that it is possible to differentiate dental plaque from oral swab samples using their microbial profiles. However, site-based classification through fungal species was not optimum in caries-free samples. This could be due to the small number of fungal species that significantly differed in abundance between dental plaque and oral swabs, as observed in the differential abundance analysis.

From our classification results for caries status, it appears that the models using the microbial composition of dental plaque or oral swabs were both able to discriminate between caries-free and S-ECC samples. However, it is important to notice that the species considered to be important for the classification of disease for each sampling site are slightly different. Based on the results from other machine learning models (Lasso, Enet, and RandomForest), we also observed that the choice of the model does not significantly affect the outcome of the analysis (data not shown).

The limitations of this study include, but are not limited to, the lack of information about the socio-economic status of the participants and the convenient sampling used for recruitment, which means that during recruitment the groups were only matched by caries status. As many factors may influence the oral microbial composition, the results of this study may not be generalizable to other populations with different age groups

and geographic locations. In this study, an additional enzymatic lysis step was used during DNA extraction from dental plaque samples to disrupt the dental plaque biofilm. Rosenbaum et al. compared the impact of using different DNA extraction methods, including the use of QIAamp DNA Mini Kit (Qiagen) with and without additional enzymatic lysis step, in the oral bacterial (16S rRNA) and fungal (ITS1 rRNA) microbiota. They showed that all tested DNA extraction methods were able to lyse Gram-positive bacterial species. They also reported no significant differences in bacterial and fungal diversity among DNA extraction methods (Rosenbaum et al., 2019). Other studies also found no significant effect of DNA extraction methods in the microbial composition of oral samples (Lim et al., 2017). Therefore, while we do not expect that the additional enzymatic lysis step significantly contributed to the differences observed between the dental plaque and oral swab microbiota, we cannot completely rule out the possible bias associated with the sample preparation on the analyses comparing dental plaque and oral swab microbiomes.

Currently, UNITE is the most commonly used database for taxonomic classification in mycobiome studies of different environments. However, there is an increased concern regarding the lack of taxonomic coverage on the available databases, which creates limitations to studies trying to characterize the human mycobiome (Nilsson, 2016). Here, a high proportion of fungal ASVs (37.14%) could not be classified to a meaningful taxonomic level beyond kingdom. As the reads passed through the quality control process, the observed high number of unclassified ASVs could be a limitation of the database used. Therefore, the construction of a curated ITS database specific for the oral mycobiome, as exists for the oral bacteriome, is urgently needed.

This is a cross-sectional study. Thus, based on our results it is not possible to determine when a significant oral microbial shift from a healthy to a diseased state occurs. Xu et al. performed a longitudinal study where they did a 1-year follow-up of caries-free 3-year-old children (Xu et al., 2018). The authors suggested that prior to any clinical sign of caries, there is a microbial shift that could potentially be used for the diagnosis and prevention of dental caries in young children. Therefore, future longitudinal studies aiming to further characterize the microbial shifts that precede the first clinical signs of dental caries are needed.

In summary, this study characterized the differences in microbial profiles of dental plaque and oral swab samples from children with S-ECC and those caries-free. Importantly, our machine learning results were able to predict the caries-status (S-ECC vs. caries-free) and sampling site (dental plaque vs. oral swab) based on the microbial profile of the samples. In the future, when data from related studies distinguishing oral sampling sites using microbiome profiles are available, we will perform the replication studies to validate our results.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA555320, PRJNA714139.

ETHICS STATEMENT

This study protocol was approved by the University of Manitoba's Health Research Ethics Board (HREB # HS20961–H2017:250) and by the MHC, Winnipeg, MB, Canada. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

VCJ and PC conceived the study. VCJ, MK, PH, and PC contributed to the design, data analysis, interpretation, and writing of the manuscript. VCJ, BAM, and RJS contributed to data acquisition. KD and RJS contributed to the design, data interpretation, and writing of the manuscript. KD, PH, RJS, and PC contributed to funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure 1 | Proportion of ASVs assigned to different taxonomic levels.

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ORIGINAL RESEARCH
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Characterization of the Oral Microbiome Among Children With Type 1 Diabetes Compared With Healthy Children

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Aim: Current microbiome profiling of type 1 diabetes mellitus (T1D) patients is mostly limited to gut microbiome. We characterized the oral microbiome associated with T1D in children after the onset of the disease and explored its relationship with oral physiological factors and dental status.

Methods: This cohort study comprised 37 children aged 5–15 years with T1D and 29 healthy children matched in age and gender. Unstimulated whole saliva was collected from diabetic and non-diabetic children, in the morning after brushing their teeth and a fasting period of at least 1 h before sampling. 16S rRNA gene-based analysis was performed by Powersoil Pro kit by Qiagen and Phusion High-Fidelity PCR Master Mix. Oral physiological and dental parameters studied included decayed, missing, and filled teeth index, salivary flow rate, and salivary pH, glucose, calcium, phosphate, and urea levels.

Results: Of the identified 105 different genera and 211 different species, the most abundant genera were *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, and *Neisseria*. *Streptococcus* was more abundant in T1D children. The diabetes group had 22 taxa at the genus level and 33 taxa at the species level that were not present in the control group and the control group exhibited 6 taxa at the genus level and 9 taxa at the species level that did not exist in the diabetes group. In addition, *Catonella*, *Fusobacterium*, and *Mogibacterium* differed between healthy and T1D subjects. Eight species and eight subspecies were significantly more abundant among healthy children than in T1D children. *Porphyromonas* and *Mogibacterium* genera were significantly correlated with salivary parameters. We found similarities between taxa revealed in the

present study and those found in gut microbiome in type 1 diabetes mellitus according to gutMDisorder database.

Conclusions: Salivary microbiome analysis revealed unique microbial taxa that differed between T1D children and healthy subjects. Several genera found in the saliva of T1D children were associated with gut microbiome in T1D individuals.

Keywords: type 1 diabetes, children, 16S rRNA gene sequencing, salivary microbiome, periodontitis

INTRODUCTION

Oral microbiome represents an important part of the human microbiome and can have detrimental consequences on both our general and oral health. The genetic setup of the host may affect the microbial composition and function, the activation of intrinsic and adaptive immunity, and susceptibility to various diseases (Zhou et al., 2020). Accumulating evidence links oral bacteria to several systemic diseases including diabetes (Genco et al., 2005). Type 1 diabetes (T1D), also known as insulin-dependent diabetes, is a chronic autoimmune-mediated disease in which the insulin-producing pancreatic beta cells are destroyed. Although it can be diagnosed at any age, it is one of the most common chronic diseases of childhood and adolescence (Maahs et al., 2010; Hong et al., 2017). T1D accounts for 5–10% of diabetic patients worldwide (Maahs et al., 2010; World Health Organization, 2018) and is the second most frequent autoimmune disease in childhood; its incidence has tripled in the last 30 years (de Groot et al., 2017). Worldwide, 1.1 million children and adolescents under the age of 20 live with T1D (International Diabetes Federation, 2019).

The increasing disease rate cannot be explained merely by genetic factors but implies that these changes are an outcome of interactions between the environment and predisposing genes (Siljander et al., 2019).

Diabetes is associated with several soft-tissue abnormalities in the oral cavity secondary to the disease that have a significant effect on the quality of life of diabetic patients (Ferizi et al., 2018). Patients with T1D are more susceptible to periodontal diseases and tooth loss and such problems might be aggravated with aging (Sadeghi et al., 2017). Quantitative and qualitative salivary changes in diabetics have also been confirmed (Angus and Richard, 2008).

The oral microbiome is known to vary in response to oral and systemic diseases (Simpson and Thomas, 2016). Diabetes has a significant impact on the gut microbial composition, stability, and connectivity, which in turn can alter the development of T1D by influencing the immune response of hosts (Han et al., 2018). Oral microbiome of adults has been implicated in the development of type 2 diabetes (T2D), but has been rarely explored in T1D. Long et al. (2017) analyzed the oral microbiome of T2D patients and discovered that the relative abundance of *Actinobacteria*, which associates with a lower risk of developing T2D, decreased (Long et al., 2017). On the contrary, a study in T1D subjects showed significantly higher abundance of taxa belonging to the phyla *Actinobacteria* and *Firmicutes*, including *Streptococcus* spp., *Actinomyces* spp., and *Rothia* spp. (de Groot et al., 2017).

The complex etiology of T1D is underlined by the fact that several years may pass between initial β-cell damage to manifestation of clinical diabetes (Størling and Pociot, 2017). Thus, early diagnosis of diabetes by targeting the microbiota at the latent period could potentially enable early treatment and postpone T1D development in children with β-cell autoimmunity.

The aim of the present study was to profile the salivary microbiome of children with T1D based on 16S ribosomal RNA (16S rRNA) gene community profiling, and to compare it with healthy children, while considering additional aspects of the oral environment. We also analyzed the impact of oral and salivary parameters including DMFT index, salivary flow rate, glucose, pH, calcium, phosphate, and urea on the salivary microbiome.

MATERIALS AND METHODS

Study Population

Ethical Considerations

All procedures performed were in accordance with the study protocol [ClinicalTrials.gov (NCT03908021)] that was approved by the Institutional Human Subjects Ethics Committee of Hadassah Medical Organization (0714-18-HMO). No compensation was provided for the participating patients. The study was conducted in the period from 2019 to 2020.

Because this was an initial study examining the differences in oral microbiome between children with T1D and non-diabetic children, no power calculation was performed. It was decided to collect saliva from all attendants to the division of Pediatric Endocrinology, Hadassah Medical Center, Hebrew University of Jerusalem, Israel, who met the inclusion criteria and were willing to participate in the study during a period of 1 year. Those children were matched in age and gender with healthy children attending the postgraduate program in Orthodontics of the Hebrew University–Hadassah Faculty of Dental Medicine. Control group saliva collection was terminated after a year.

The study was conducted on 66 children, including 37 with diabetes aged 5–15 years, during a routine follow-up visit at the Pediatric Endocrinology Clinic, Hadassah Hebrew University Medical Center (Jerusalem, Israel). All diabetic children were treated with but not with any other therapy at least a week prior to checkup. The control group, matched in age and gender, included

29 healthy children who were attending the Orthodontic Clinic at the same medical center. All healthy children were without functional orthodontic appliances and no history of drug therapy at least a week prior to checkup. Exclusion criteria for both groups were diseases other than T1D and known oral disease. All patients were medication free apart from insulin if needed at the day of sample collection and at least a week before.

Clinical Examination and Collection of Saliva Samples

Clinical dental health status was measured using the Decayed, Missing and Filled Teeth (DMFT) Index according to the WHO caries diagnostic criteria for epidemiological studies (World Health Organization, 1997). All dental examinations were performed by a single qualified dentist from the department of Pediatric Dentistry, Faculty of Dental Medicine, Hebrew University of Jerusalem, Israel, in accordance with the clinic checkup procedures.

Access to dental care, parents' dental education, and the quality of diet were provided through patients' and parents' interview.

Unstimulated whole saliva was collected from diabetic and non-diabetic children, in the morning after brushing their teeth and a fasting period of at least 1 h before sampling. The children were asked to spit saliva into a 15-ml sterile tube over a measured period of time and sufficient for salivary parameter measurement. Only the liquid of the saliva was allocated and collected for the analysis (He et al., 2015).

Before centrifugation, 350 μl of saliva was stored at −80°C for microbiome analysis, and pH and salivary glucose were determined. The saliva samples were then centrifuged at 1,500 RCF (relative centrifugal force) for 15 min at 4°C to reduce salivary debris and viscosity. Salivary calcium, phosphate, and urea were evaluated later in the supernatant fluid, stored at −20°C.

Measurement of Salivary Flow Rate, pH, and Glucose

The salivary flow rate was defined without the foam as the volume (in ml) of saliva secreted per minute of collection. Salivary pH was measured using color-coded pH-indicator strips (pH 0–14 Universal indicator, MQuant; Sigma-Aldrich, Israel). Glucose test strips (Medi-Test Combi 3A; Praxisdienst, Germany) measured salivary glucose.

Measurement of Salivary Calcium, Phosphate, and Urea

Salivary calcium, phosphate, and urea concentrations were calorimetrically measured from the stored clear salivary supernatant fluid and according to the manufacturer's instructions. The following kits were used, respectively: Calcium Colorimetric Assay Kit (MAK022—Sigma-Aldrich, St. Louis, MO 63103, United States), Phosphate Colorimetric Assay Kit (MAK030—Sigma-Aldrich), and Amplite Colorimetric Urea Assay Kit *Blue Color* (10058—AAT Bioquest, Sunnyvale, CA 94085, United States).

Microbiome Analysis and 16S Ribosomal RNA Gene-Based Analysis

DNA extraction was performed by the Powersoil Pro kit by Qiagen (47016), following the company's protocol, with mild modifications. All saliva samples were centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant fluid was discarded and the pellet was re-suspended in 800 μl of CD1 and then added to the PowerBead Pro tube. Samples were also treated in a bead beaten beater (TissueLyzer; QIAGEN) at 20 Hz for 10 min. 16S rRNA libraries were prepared according to the published protocol (Poyet et al., 2019) with mild modifications. First, qPCR was used to normalize template concentrations and determine the optimal cycle number needed for amplification of the V4 region of the 16S rRNA gene. In the qPCR, each sample was amplified in two 25-μl reactions using iTaq Universal SYBR Green Supermix (#17525124) and the primers 515 F (AATGATACGGCGA CCACCGAGATCTACACTATGGTAATTGT GTGCCAGCMG CCGCGGTAA) and 806rcbc0 (CAAGCAGAAGACGGCATAC GAGAT TCCCTTGTCTCC AGTCAGTCAG CC GGACTACH VGGGTWTCTAAT). Samples were quantified using the formula $1.75^{\Delta Ct}$. To minimize over-amplification, each sample was diluted to the lowest concentration sample, and the Ct value of this lowest concentration sample was used as the cycle number in the PCR reaction for library construction.

For library construction, four 25-μl reactions were prepared per sample using Phusion High-Fidelity PCR Master Mix with HF buffer (M0531L) and the primer 515F and 806R. Each sample was given a unique reverse barcode primer from the Golay primer set (see "Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms"; Caporaso et al., 2012). The replicates were then pooled and cleaned using Agencourt AMPure XP beads. Purified libraries were diluted 1:100 and quantified via qPCR, again using two reactions of 25 μl with iTaq Universal SYBR Green Supermix, but with the primers Read 1 and Read 2. The undiluted samples were normalized by way of pooling using the formula mentioned previously, and the pools were quantified by Qubit, as well as analyzed on the TapeStation. The pools were then normalized into a final pool based on the concentration calculated by Qubit, the average library size determined by TapeStation results, and the number of samples in the pool.

Final pools were sequenced on an Illumina MiSeq using the custom index 5′-ATTAGAWACCCBDGTAGTCCGGCTGA CTGACT-3′ and custom Read 1 and Read 2, mentioned previously, and using 30% PhiX.

16S Ribosomal RNA Analysis

All sequences passed fastQC using default parameters and had an average of 11,500 reads (with a minimum of 5,876 reads per sequence). BURST v0.99 (Al-Ghalith and Knights, 2017) was applied to the raw reads using default parameters, and with the burst_linux_DB12 database,[1] which is based on the RefSeq Targeted Loci Project.[2]

[1]https://github.com/knights-lab/BURST/releases/download/v0.99.7f/burst_linux_DB12

[2]https://www.ncbi.nlm.nih.gov/refseq/targetedloci/

Results were then divided according to taxonomic level. For family, genus, species, and subspecies level, a threshold of reads was set to 25. Thus, only taxa which had more than 25 reads throughout all the samples were further analyzed. Following the removal of the bacteria that did not meet the threshold, the relative abundance of each bacterium in each sample was re-normalized. A taxon was considered abundant if it had a relative abundance greater than 5% in at least one sample.

Statistically Significant Differential Taxonomic Analysis

To find differential bacterial taxa in which the relative abundance was statistically different between T1D patients and control subjects, we used MaAsLin 2 (Microbiome Multivariable Associations with Linear Models) (Mallick et al., 2021) multivariate linear regression along with an annotation of whether the sample belonged to a case or control. MaAslin2 results were considered statistically significant in case q-value < 0.25. Relevant taxa were then plotted using the ggplot2 package v3.3.3 (Wickham, 2016) in R v4.0.3. The plots include annotation of the coefficient, p-value, and q-value as calculated by MaAslin2.

Taxa Appearing Only in the Study or Control Group

To find bacteria at a certain taxonomic level that appeared only in one of the groups, we took the data table of the relevant taxonomic level and selected only the bacteria that were completely missing in one study group. The value represented by the axis refers to the sum of the relative abundances of the bacteria, within the samples of the axis's population.

Microbial Taxa Association With Various Variables

To find significant associations between various subject parameters and specific bacteria, the data of the relevant taxonomic level was given as input to MaAslin2 alongside a table containing the various metadata variables. Results were considered significant if they had a q-value smaller than 0.25.

Heatmap of Abundant Taxa

A genus was considered abundant if it had a relative abundance greater than 5% in at least one sample across all analyzed samples. This added up to 23 abundant genera in our analysis. The relative abundance of each genus within each sample was plotted into a heatmap, along with an annotation at the top of the plot designating if the sample belonged to a case or control. The heatmap plot was created using the pheatmap package v1.0.12 (Raivo, 2019) in R v4.0.3.

Alpha Diversity Analysis

Alpha diversity measurement was done using the Shannon diversity index that was calculated using the diversity function within the vegan v2.5.7 R package.[3] A Wilcoxon test was applied between the two population groups using the ggpubr v0.4 package.[4]

[3]https://CRAN.R-project.org/package=vegan

[4]https://CRAN.R-project.org/package=ggpubr

Principal Coordinate Analysis

Beta diversity was calculated using the Bray–Curtis dissimilarity index as calculated using the vegan 2.5.7 and ape 5.4.1 R packages (Paradis and Schliep, 2019).

Data Analysis

The average and standard error (SE) of DMFT index, salivary flow rate, pH, glucose, calcium, phosphate, and urea between the two groups were analyzed using Student's t-test and $p < 0.05$ was considered statistically significant.

Microbiome data were analyzed by the "Burst Analyzer" software (Burst-Analyzer—knights-lab)[5] and MaAslin2 comprehensive R package (Maaslin2—Bioconductor)[6] for efficiently determining specific genus and families in which considerable differences were found between study and control samples. Data visualization was performed by ggplot2 in R package (Wickham, 2016).

RESULTS

Population

The study group included 37 children with T1D (17 males) with a mean age (±SD) of 13 ± 2.69 years, and the control group included 29 (11 males) healthy children with a mean age (±SD) of 10 ± 2.38 years with no other relevant differences noted between the groups. All study group participants were using insulin since diagnosed as having T1D; 81.1% of them used insulin pumps with continuous delivery of short-acting insulin. The mean (±SD) time since diagnosis of diabetes was 2 ± 2.58 years. According to the patients' files, 70.3% of diabetic children were metabolically stable at the time of sample collection.

As looking into caries risk factors is beyond the scope of this preliminary study, we used only a general interview that is accepted for initial checkup in the department of Pediatric Dentistry, Faculty of Dental Medicine, Hebrew University of Jerusalem, Israel. A more comprehensive study that will address this issue is planned as a future project. Interviews revealed that children with T1D visited the dentist only when necessary, while children in the control group were orthodontic patients who kept high standards of oral care. The level of parents' education regarding dental care in T1D group was medium and low, whereas the control group dominated with the medium and higher levels of parents' education.

Salivary Microbiome

Sequencing Data

A total of 762,156 reads were obtained from sequencing with an average of ∼11.5 thousand reads per sample (ranging from 5,876 to 42,528 reads). Sequencing data passed quality check using FastQC[7] with default parameters. Following BURST taxonomic alignments 690,143 raw reads were mapped with an average of 10,456 reads per sample (ranging from 5,482 to 26,107 reads per

[5]https://github.com/knights-lab/BURST

[6]https://www.bioconductor.org/packages/release/bioc/html/Maaslin2.html

[7]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

sample). After removing bacteria with less than 25 reads across all samples at the genus level, 689,850 reads remained for further analysis with an average of 10,452 reads per sample (ranging from 5,478 to 26,106 reads). After similar filtering at the species level, 689,399 reads remained for further analysis with an average of 10,445 reads per sample (ranging between 5,474 and 26,101 reads per sample).

Microbiome Characterization

We found 105 different genera and 211 different species in the oral microbiome of the tested children. Most abundant genera in the saliva of both groups were *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, and *Neisseria*. We first wanted to check whether the bacterial communities of the two study populations were similar or not. Overall, there are no strong shifts between the two sample types, and the most abundant genera in the saliva of both groups are *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, and *Neisseria* (**Figure 1A**). Performing a principal coordinate analysis (PCoA) on these samples did not reveal any clear separation between the groups (**Figure 1B**). However, when we examined the microbial richness of each sample, we found that control samples had a significantly higher diversity (calculated using Shannon diversity index, **Figure 1C**).

We next searched for differential taxa between the T1D and control samples. Using a multivariate linear regression model, we identified eight differential species and three differential genera (see section "Materials and Methods). Eight species had significantly higher values among healthy children than in T1D children (**Table 1** and **Figure 2A**), and at the subspecies level, eight taxa were higher in the control group than T1D group

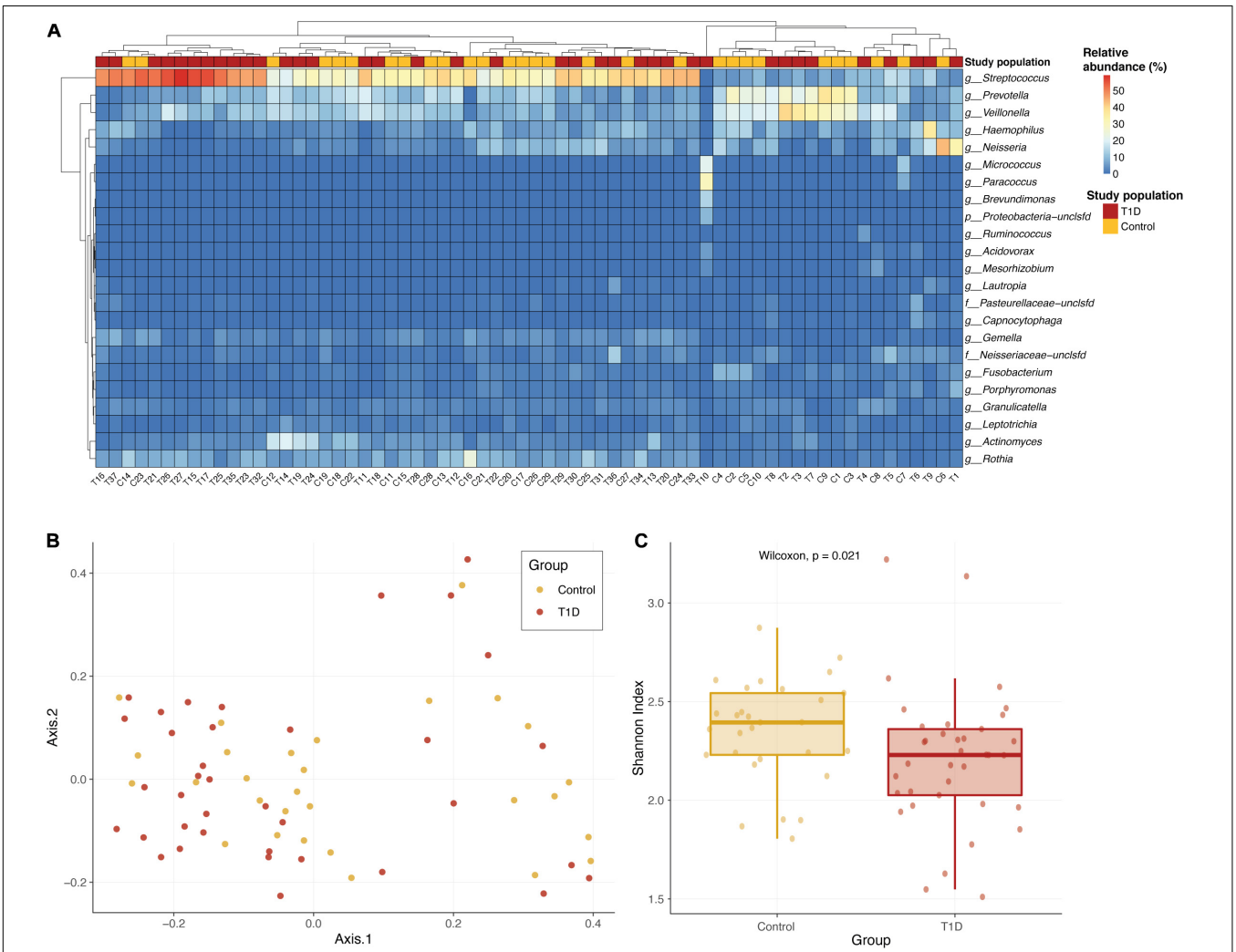

FIGURE 1 | Overall microbial composition in the two population groups. **(A)** Relative abundance of the most abundant genera in the saliva of T1D group (red) and the control group (yellow). The color of each cell in the heatmap is related to the abundance level of each genera per sample. **(B)** PCoA of the Bray–Curtis dissimilarity between study samples. Samples from T1D and controls are colored in red and yellow, respectively. Samples that are closer in their microbiome composition will be closer in this 2D plot. No clear distinction between the groups is identified. **(C)** Alpha diversity comparison of microbial communities of saliva samples from T1D group (red) and control group (yellow). Shannon diversity index was calculated as the metric for alpha diversity. The boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the vertical line inside the box defines the median. *P*-value calculated using Wilcoxon test.

TABLE 1 | Eight species and eight subspecies had significantly higher values among healthy children than in T1D children.

Species	*p*	*q*
Granulicatella-unclassified	0.00537	0.162
Mogibacterium-unclassified	0.00391	0.137
Alloprevotella rava	0.00154	0.118
Catonella morbi	0.00171	0.118
Fusobacterium periodonticum	0.00101	0.118
Oribacterium parvum	0.00701	0.185
Prevotella melaninogenica	0.00223	0.118
Prevotella pallens	0.00374	0.137
Subspecies	***p***	***q***
Granulicatella-unclassified	0.00537	0.165
Mogibacterium-unclassified	0.00390	0.140
Alloprevotella rava-unclassified	0.00154	0.120
Fusobacterium periodonticum-unclassified	0.00101	0.120
Catonella morbi atcc 51271	0.00171	0.120
Oribacterium parvum acb1	0.00701	0.188
Prevotella melaninogenica atcc 25845	0.00223	0.120
Prevotella pallens atcc 700821	0.00374	0.140

(**Table 1**). Three bacterial genera were higher in the control group than in T1D (**Figure 2B**) including *Catonella* ($p = 0.0017$, $q = 0.0894$), *Fusobacterium* ($p = 0.0007$, $q = 0.0798$), and *Mogibacterium* ($p = 0.0056$, $q = 0.1986$).

To clarify the relationship between the changes in the salivary microbiome and other salivary parameters, we analyzed the correlations between metadata and different microbes. We found that some physiological parameters (salivary pH and DMFT index) were associated with two genera of microbes (*Porphyromonas* and *Mogibacterium*; **Figure 2C**).

Finally, the diabetes group presented 22 taxa at the genus level and 33 taxa at the species level that were not presented in the control group, and the control group exhibited six taxa at the genus level and nine taxa at the species level that were not present in the diabetes group (**Figure 2D** and **Supplementary Table 1**).

The five most abundant genera in the T1D group were *Brevundimonas*, *Ruminococcus*, *Micrococcaceae-unclassified*, *Blautia*, and *Faecalibacterium* with sum of values 13.79, 5.60, 3.70, 2.57, and 2.0, respectively. The most abundant species in the T1D group were *Brevundimonas-unclassified*, *Micrococcaceae-unclassified*, *Lactobacillus salivarius*, *Ruminococcus bromii*, *Prevotella copri*, *Ruminococcus champanellensis*, *Faecalibacterium prausnitzii* with sum of values 13.79, 3.70, 3.65, 3.27, 2.43, 2.33, and 2.10, respectively. The most abundant genera in the control group were *Hymenobacter*, *Xanthomonadaceae-unclassified*, *Dietzia*, *Microbacterium*, and *Erythromicrobium* with sum of values 2.76, 0.50, 0.46, 0.43, and 0.34. The most abundant species in the control group were *Hymenobacter qilianensis*, *Flavobacterium columnare*, *Brevibacterium daeguense*, *Xanthomonadaceae-unclassified*, *Methanobrevibacter olleyae*, *Bacteroides vulgatus*, and *Dietzia-unclassified* with sum of values 2.76, 0.91, 0.76, 0.50, 0.47, 0.46, and 0.42.

Physiological Measures

Salivary Flow Rate

The average (±SE) of diabetic and healthy children were 0.50 ± 0.04 ml/min and 0.53 ± 0.03 ml/min, respectively, with no difference between the groups ($p = 0.47$) (**Table 2**).

Salivary pH. Salivary pH showed no difference between the two groups ($p > 0.05$), with an average (±SE) 6.88 ± 0.11 and 7.14 ± 0.10 of the experimental and control group, respectively.

Salivary Glucose

The percentage of salivary glucose concentrations showed 95 and 100% negative results in diabetic and healthy children, respectively (**Table 3**).

Salivary Calcium, Phosphate, and Urea

There were no differences in the average (±SE) values of salivary calcium (1.37 ± 0.11 and 1.12 ± 0.08 nmol/μl, $p = 0.10$), phosphate (4.72 ± 0.25 and 4.71 ± 0.22 nmol/μl, $p = 0.98$), and urea (4.30 ± 0.17 and 4.23 ± 0.18 nmol/μl, $p = 0.77$) in diabetic and healthy children, respectively.

Decayed, Missing, and Filled Teeth Index

Clinical examination showed higher caries incidence in diabetic children. The average (±SE) values with respect to DMFT index were 6.08 ± 0.61 and 3.76 ± 0.67 in the experimental and control group, respectively ($p < 0.05$). Furthermore, diabetic females had more tooth decay with no statistically significant difference between the groups (DMFT = 6.45 compared with 5.65 among diabetic males, $p = 0.52$). As reported from the data gathered while interviewing patients and parents, diabetic patients had poor quality diet, poor oral hygiene, less access to dental care, and less parents' dental education compared with the control group.

DISCUSSION

Only a limited number of studies have investigated the oral microbial composition of patients with T1D. We examined the oral microbiome in children with T1D and healthy children and found significant differences between the oral microbiota of diabetic children and the oral microbiota of healthy children. This is in accordance with de Groot et al. (2017) who found a markedly difference in oral microbiota in T1D (e.g., abundance of Streptococci) compared with healthy controls.

A recently published study by Pachoński et al. (2021) using classical methods routinely used in microbiological diagnostics confirmed quantitative and qualitative significant difference between the oral microbiome of children with T1D and healthy children. The present study used salivary samples, which according to Pachoński et al. (2021) are much more diverse than the samples they acquired with swab technique in the soft tissue of the oral cavity.

As in Pachoński et al. (2021)'s study, *Streptococcus* genus was also one of the largest groups of isolated microorganisms in the present study. However, we additionally found a large amount of *Prevotella*, *Veillonella*, *Haemophilus*, and *Neisseria*. Significantly higher number of bacteria from the *Streptococcus*

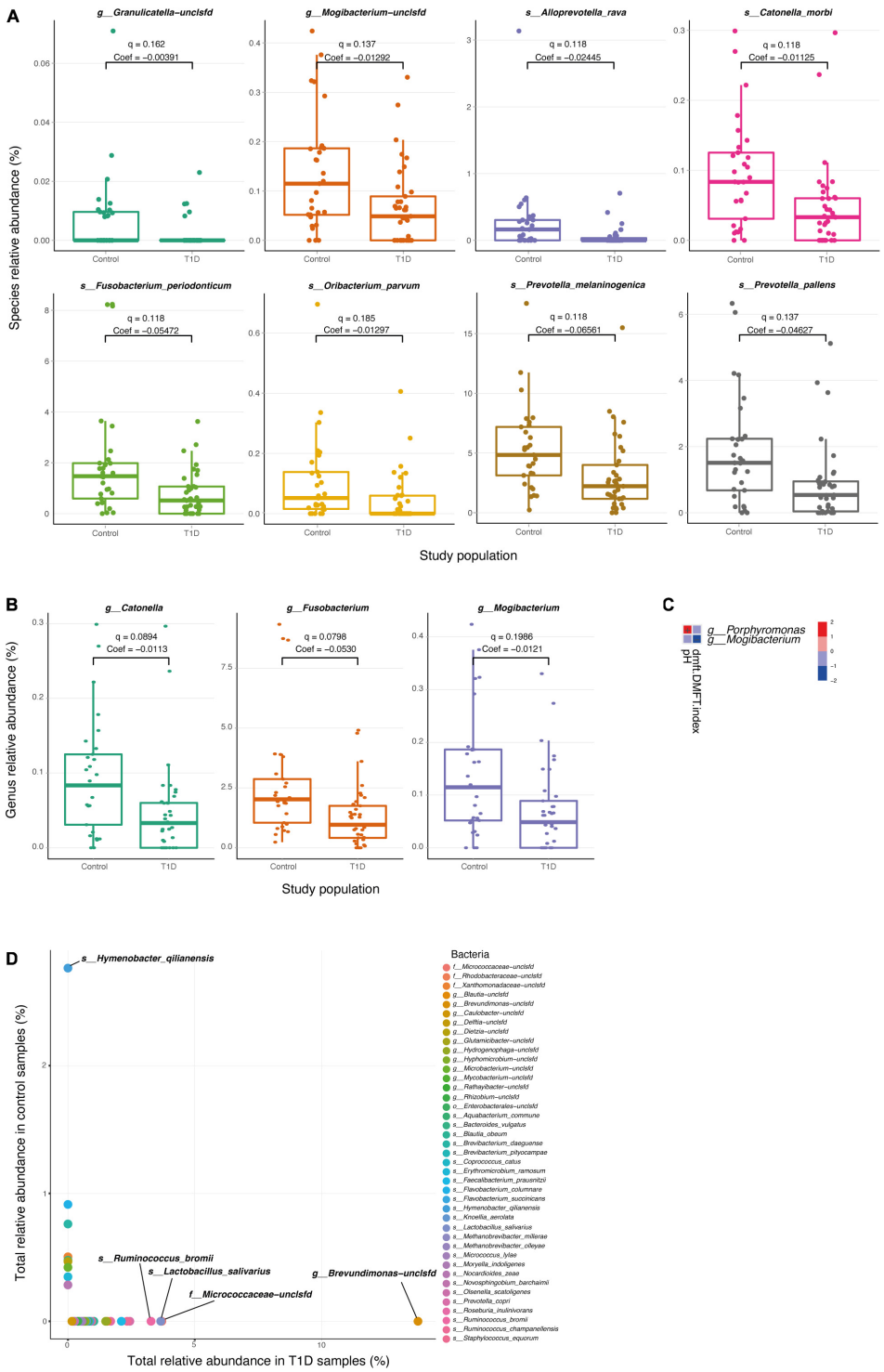

FIGURE 2 | Differential microbial taxa between T1D samples and controls. **(A,B)** Relative abundance of the significant taxa at the species **(A)** and genus **(B)** level, in T1D samples and control samples. *Q*-values and coefficients calculated using a multivariate linear regression model (MaAsLin, see section "Materials and Methods). The boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively) and the vertical line inside the box defines the median. **(C)** Significant associations between microbial taxa and clinical parameters of the oral cavity. Only salivary pH and DMFT index are shown in the vertical axis as significantly correlated parameters ($q < 0.25$), and two genera; *Porphyromonas* and *Mogibacterium*, are shown in the horizontal axis. The variations in color are the magnitude of correlation between both variables. Correlation coefficient values range between −1.0 and 1.0; a correlation coefficient that is greater than zero indicates a positive relationship between two variables, a value that is less than zero signifies a negative relationship between two variables. **(D)** Relative abundance species apparent in only one type of population. The horizontal axis represents the sum of the relative abundance of the bacteria in T1D samples. The vertical axis represents the sum of the relative abundance of the bacteria in control samples.

TABLE 2 | Salivary parameters in children with type 1 diabetes mellitus and healthy children.

Parameters	Diabetics		Non-diabetics		*P*-value
	N	Average ± SE	N	Average ± SE	
Salivary flow rate (ml/min)	37	0.50 ± 0.04	29	0.53 ± 0.03	0.47
DMFT index	37	6.08 ± 0.61	29	3.76 ± 0.67	0.01*
pH	37	6.88 ± 0.11	29	7.14 ± 0.10	0.24
Calcium (nmol/ml)	37	1.37 ± 0.11	29	1.12 ± 0.08	0.10
Phosphate (nmol/ml)	37	4.72 ± 0.25	29	4.71 ± 0.22	0.98
Urea (nmol/ml)	37	4.30 ± 0.17	29	4.23 ± 0.18	0.77

**Significant differences.*

genus were found in the group of children with well-controlled diabetes mellitus compared with healthy children in Pachoński et al. (2021) and in the present study. Our study shows that 16S rRNA gene-based analysis enables the identification of a very broad scope of organisms: 105 different genera and 211 different species. The present study was also able to establish a unique group of bacteria (taxon) at the genus level and at the species level that either appeared or were absent in the saliva of T1D children. In addition to species and subspecies, clinical microbiologists study bacterial genera and families, so we concentrated on the differences between healthy and diabetic children in those taxonomic groups. The present data exhibited significant increase of the genera *Catonella*, *Fusobacterium*, and *Mogibacterium* in the control group. At the species level, *Granulicatella* spp., *Alloprevotella rava*, *Catonella morbi*, *Fusobacterium periodonticum*, *Oribacterium parvum*, *Prevotella melaninogenica*, and *Prevotella pallens* were significantly more abundant in the control group, in addition to *Catonella morbi ATCC 51271*, *Oribacterium parvum ACB1*, and *Prevotella melaninogenica ATCC 25845* at the subspecies level. *Brevundimonas*, *Ruminococcus*, *Micrococcaceae* spp., *Blautia*, and *Faecalibacterium* were predominant genera only in the T1D group.

According to the literature, three of these subspecies—*Blautia*, *Ruminococcus* (of family Lachnospiraceae), and *Faecalibacterium*—were enriched in women with gestational diabetes (Crusell et al., 2018).

TABLE 3 | Salivary glucose percentage in children with type 1 diabetes mellitus and healthy children.

Parameters	Diabetics		Non-diabetics	
		Percentage		Percentage
Glucose	Negative*	95	Negative	100
	Normal*	5	Normal	0

**Using Medi-Test Combi 3A where the color fields correspond to the following ranges of glucose concentrations: neg. (yellow), neg. or normal (greenish), 2.8, 8.3, 27.8 ≥ 55.5 mmol/L.*

As reported in the Results section, several unique taxa were identified in T1D: *Lactobacillus salivarius*, *Ruminococcus bromii*, *Prevotella copri*, *Ruminococcus champanellensis*, and *Faecalibacterium prausnitzii*. The identification of those unique taxa can be partly clarified by the quality of diet consumed by diabetic patients, in addition to the increase of periodontal inflammation among T1D children (Novotna et al., 2015). Diet has an important role in composition and metabolism of the oral microbiome. We report poor-quality diet among T1D children, which is in line with previous reports (Patton, 2011) of high saturated fat consumption and low intake of fruits, vegetables, and whole grain foods. Such diets are rich in advanced glycation end products (AGEs), which are complex heterogeneous compounds derived from non-enzymatic glycation reactions. While dietary advanced glycation end products (dAGEs) are formed during industrial processing and home cooking, high plasma glucose, as in diabetes (Rhee and Kim, 2018), accelerates the formation of endogenous AGEs. AGE-modified proteins accumulate within the body and are thought to play a role in a number of age-related diseases including diabetes. Long-term glycemic control regime decreased AGE levels in patients with T1D (Kostolanská et al., 2009).

As the absorption of dietary AGEs is limited, the majority of protein-bound AGEs pass through the gastrointestinal tract to the colon, where they can serve as substrates for the gut microbiota. Conflicting evidence on how dietary AGEs influence the composition of the microbiome include reduced levels of *Prevotella copri*, found here to be predominant genera only in the T1D group in peritoneal dialysis patients who underwent a 4-week low-dAGE regime (Yacoub et al., 2017) and that an AGE-rich diet in rats reduced the abundance of *Ruminococcaceae* and *Alloprevotella*, genera that we found to be both predominant and exclusive to the T1D group. The same diet increased the levels of *Bacteroides* (found in our study only in the control group) (Qu et al., 2017).

Elaboration on the unique taxa found in T1d patients in our study:

(1) *Brevundimonas* spp. are non-fermenting Gram-negative bacteria considered of minor clinical importance infection. Many of these non-fermenting Gram-negative bacteria are opportunistic pathogens that affect patients suffering from underlying medical conditions (Ryan and Pembroke, 2018) including diabetes (Lee et al., 2011). In the oral cavity, *Brevundimonas diminuta* was detected in refractory periodontitis (Krishnan et al., 2017).

(2) *Blautia* and *Faecalibacterium prausnitzii* are members of the human gut microbiome producing butyrate as fermentation end product. A high concentration of butyrate could result in apoptosis in human gingival epithelial cells and play an essential role in the initiation of periodontitis (Guan et al., 2021). Abundances of *Faecalibacterium* were negatively correlated with HbA1c levels in T1D (Huang et al., 2018). Moreover, a relative overabundance of the genus *Blautia* was found in the gut microbiome in the prediabetes and progressive stage of T1D (Kostic et al., 2015).

(3) *Prevotella copri* is by far the most abundant member of the genus *Prevotella* inhabiting the human large intestines. *P. copri* is strictly dependent on a sugar source partly elucidating its detection in T1D individuals in our cohort, who reported poor quality and high sugar diet. In the oral cavity, the proportion of *P. copri* was relatively higher in T2D patients with periodontitis (Sun et al., 2020). Thus, the detection of *Brevundimonas*, *Blautia*, *Faecalibacterium prausnitzii*, and *P. copri* in the oral cavity of T1D children may be associated with altered periodontal state among the study group.

(4) *Ruminococcus* is a genus of gut microbiome. Experimental evidence has confirmed its significant difference in the gut of diabetic mice may contribute to the pathogenesis of T1D by decreasing FOXP3-positive regulatory T cells (Tregs) that protect against diabetes (Krych et al., 2015). *R. bromii* possesses an exceptional ability to colonize and degrade starch particles in the human colon (Crost et al., 2018). *R. champanellensis* is a cellulose-degrading bacterium from human gut microbiota, in which fermentable carbohydrates are required for growth of this species (Chassard et al., 2012). Thus, the presence of *Ruminococcus* species in the saliva of T1D children in our cohort might be due to high intake of starch and sugars fermented by these bacteria.

(5) *Lactobacillus* is an indigenous member of human gut and oral microbiota. *L. salivarius* was found to be more highly associated with caries in children than the other *lactobacilli* because it is acidogenic and can produce lactate, acetate, and hydrogen peroxide (Piwat et al., 2010). Thus, we suggest that the elevated caries incidence among T1D children could induce increase of *L. salivarius* in the study group.

When checking the taxa found in the present study against data on T1D in gutMDisorder,[8] a manually curated database of comprehensive dysbiosis of the gut microbiota (Cheng et al., 2020), the genus *Blautia* increased in the gut microbiome of T1D patient and was present only in the T1D group in our study. Genus *Haemophilus* and family *Veillonellaceae* were abundant in both groups in the present study compared with a decrease in the gut microbiome of T1D patients. *Fusobacteria* phyla were more abundant in the control group of the present study and were decreased in gut microbiome. *Porphyromonadacea* species were increased in the gut of T1D patients and appeared to be correlated with oral parameters in the present study. Genus *Prevotella* was abundant in both groups in the present study compared with a decrease in the gut microbiome of T1D patients. Genus *Bacteroides* was increased in T1D gut microbiome, but *Bacteroides vulgatus* was found only in the saliva of the control group in the present study. Being part of the typical westernized pattern, *Bacteroides*, *Faecalibacterium*, and *Prevotella* were the predominant genera in gut microbiota composition of both women with gestational diabetes and normal glucose regulation (Crusell et al., 2018). Although we find similarities between gut and oral microbiome, there appear to be multifaceted relations that are determined by the environment; genera *Porphyromonas* and *Mogibacterium* were correlated with both pH and DMFT index parameters and were both classified as microbial signatures of periodontitis in the oral microbiome (How et al., 2016; Hunter et al., 2016). There are studies that show a lower incidence of dental caries in diabetic children compared with their healthy peers (Orbak et al., 2008), differing from our study and from others (Ferizi et al., 2018) who presented a significantly higher DMFT index in children with T1D than that in the control. This can be related to the fact that in both studies children with T1D rarely visited the dentist. In addition, in the present study children in the control group were orthodontic patients who kept high standards of oral care.

No differences in unstimulated salivary flow rate and salivary glucose calcium and phosphate levels were detected between the two groups. Furthermore, no significant difference was observed between diabetic and healthy children with respect to salivary urea, in disagreement with López et al. (2003) who found greater salivary urea levels in T1D children than in controls.

Study Limitation

Like Pachoński et al. (2021), the present study was a preliminary one and was not aimed to link between quality of dental care and oral hygiene and differentiating the dental health status between the children with T1D and healthy controls. Differentiating the oral microbiome in this case will be targeted by a more specific future study. Because this was an initial study examining the differences in oral microbiome between children with type 1 diabetes and non-diabetes children no power calculation was performed. It was decided to collect saliva from all attendants to the division of Pediatric Endocrinology, Hadassah Medical Center, Hebrew University of Jerusalem, Israel, who met the inclusion criteria and were willing to participate in the study during a period of 1 year.

CONCLUSION

We have established a unique microbial taxon that either appeared or were absent in the saliva of T1D children.

Many of the bacteria identified belong to the gut microbiome, indicating the complex interplay between the oral and gut microbiome in the pathogenesis of T1D.

In addition, some microbial taxa were linked to other parameters in the oral cavity of T1D individuals, such as higher incidence of dental caries.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA BioProject, accession no: PRJNA759836.

[8]http://bio-annotation.cn/gutMDisorder/browse.dhtml

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Human Subjects Ethics Committee of Hadassah Medical Organization (0714-18-HMO). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MM, SF, DZ, DR, and DS conceived and designed the study. MN collected the samples. MN and AC performed laboratory assays. MY and NM performed bioinformatics analysis. MN and MM performed statistical analysis and wrote the draft of the manuscript. MM, DS, and MN interpreted the results. MM and DS supervised the work and revised and contributed to the final manuscript. DR contributed with resources and funding. All authors read and approved the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.756808/full#supplementary-material

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Oral Microbiota Is Associated With Immune Recovery in Human Immunodeficiency Virus-Infected Individuals

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The role of the oral microbiota in HIV-infected individuals deserves attention as either HIV infection or antiretroviral therapy (ART) may have effect on the diversity and the composition of the oral microbiome. However, few studies have addressed the oral microbiota and its interplay with different immune responses to ART in HIV-infected individuals. Salivary microbiota and immune activation were studied in 30 HIV-infected immunological responders (IR) and 34 immunological non-responders (INR) ($\geq$500 and $<$ 200 CD4 + T-cell counts/μl after 2 years of HIV-1 viral suppression, respectively) with no comorbidities. Metagenome sequencing revealed that the IR and the INR group presented similar salivary bacterial richness and diversity. The INR group presented a significantly higher abundance of genus *Selenomonas_4*, while the IR group manifested higher abundances of *Candidatus_Saccharimonas* and *norank_p_Saccharimonas. Candidatus_Saccharimonas* and *norank_p_Saccharimonas* were positively correlated with the current CD4 + T-cells. *Candidatus_Saccharimonas* was positively correlated with the markers of adaptive immunity CD4 + CD57 + T-cells, while negative correlation was found between *norank _p_Saccharimonas* and the CD8 + CD38 + T-cells as well as the CD4/CD8 + HLADR + CD38 + T-cells. The conclusions are that the overall salivary microbiota structure was similar in the immunological responders and immunological non-responders, while there were some taxonomic differences in the salivary bacterial composition. *Selenomona_4, Candidatus_Saccharimonas,* and *norank _p_Saccharimonas* might act as important factors of the immune recovery in the immunodeficiency patients, and *Candidatus_Saccharimonas* could be considered in the future as screening biomarkers for the immune responses in the HIV-infected individuals.

Keywords: HIV-1, oral microbiota, immunological responders, immunological non-responders, antiretroviral therapy

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Abbreviations: HIV, human immunodeficiency virus; ART, antiretroviral therapy; HAART, highly active antiretroviral therapy; INR, immunological non-responders; IR, immunological responders; SLE, systemic lupus erythematosus; RDs, rheumatic diseases; ALL, acute lymphoblastic leukemia; RA, rheumatoid arthritis; BMI, body mass index; MSM, men who have sex with men; LPS, Lipopolysaccharide; PCoA, Principal coordinate analysis; LDA, Linear discriminant analysis; LEfSe, linear discriminant analysis effect size; OUT, operational taxonomic unit; 16S rRNA, 16S ribosomal RNA; ROC, receiver operating characteristic; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; AZT, Zidovudine; TDF, Tenofovir Disoproxil Fumarate; 3TC, Lamivudine; EFV, Efavirenz; LPV/r, Lopinavir/ritonavir.

INTRODUCTION

The widespread use of potent antiretroviral therapy (ART) which has ability to achieve viral suppression and immune reconstitution has made human immunodeficiency virus (HIV) infection become a chronic manageable disease. ART can improve the immune function of HIV infected subjects, but significant individual difference exists in the extent of immunological recovery. Despite the persistent manifestation of virological suppression after receiving ART, HIV infected individuals with low increases of CD4 + T-cells are considered as immunological non-responders (INR), showing contrast to immunological responders (IR) (Cenderello and De Maria, 2016). An unanimously agreed definition of INR has not been reached by far. Therefore, the acceptable range of the prevalence of INR is from 10 to 40% in the cohorts (Yang et al., 2020). The most restrictive definition of INR refers to patients whose absolute CD4 + T-cells fail to reach 200 cells/μl with an undetectable plasma viral load after 2 years of receiving ART (Florence et al., 2003; Kaufmann et al., 2003; Tsukamoto et al., 2009).

Thanks to high-throughput sequencing, thorough and comprehensive studies of complex human microbiology have been conducted during the past years. In recent years, studies mainly focused on characterizing the impact of HIV infection on host–microbe interactions in the gut (Dillon et al., 2016) and some studies found that the gut microbiota are associated with immune recovery in HIV-infected patients (Ji et al., 2018; Lu et al., 2018; Xie et al., 2021). Oral microbiomes were also reported to have strong associations with human immune system functions, therefore, they were correlated with human immune system diseases such as rheumatic diseases (RDs), acute lymphoblastic leukemia (ALL), and HIV infection (Gao et al., 2018). Alterations in the oral microbiome distinguished individuals with rheumatoid arthritis (RA) from the healthy controls; correlations were shown between these alterations and clinical measures including immune response, and they could be used to stratify individuals based on their responses to the therapy, especially with microbial triggers being implicated in RA (Bellando-Randone et al., 2021). Furthermore, characterization of the oral microbiome in ALL patients demonstrated a structural imbalance of the oral microbiota, indicating the importance of immune status in shaping the structure of the oral microbiota. Although valuable insights on immune status have been presented by these studies, it is surprising that studies which focus on the role of the oral microbiome and their relationship with immune response in HIV are relatively rare. Few research addresses the overall oral microbiota structure in HIV infection; to our knowledge, the oral microbiota dysbiosis and the interaction with different immune responses to ART is still poorly defined. This study aims to evaluate the microbial composition in the salivary samples collected from HIV-infected immunological non-responders and immunological responders. We hypothesize that the compositional changes of the salivary microbiota could be associated with different immune responses of HIV-infected individuals receiving ART. The study adopted 16S ribosomal RNA (rRNA) targeted sequencing and flow cytometry to explore the oral microbiome and their relationship with immune activation in patients who are immunodiscordant and who are immunoconcordant. The compositional changes of salivary microbiota and their association with different immune responses in HIV-infected patients with ART is characterized for the first time in this study.

MATERIALS AND METHODS

Recruitment of Subjects

64 HIV-infected individuals who were diagnosed by the Disease Control and Prevention Center of Zhejiang Province (30 immunological responders and 34 immunological non-responders) were all recruited from the HIV clinic of the First Affiliated Hospital of Zhejiang University from November 2015 to October 2017. All subjects start ART during the chronic phase of HIV infection. In this study, IR and INR were defined as patients who with the average of the last two CD4 + T-cell counts/μl equal or is more than 500 or less than 200 and after 2 years of receiving complete viral suppression therapy, respectively. The selection excluded candidates with either one of the following conditions: below 18 years old; showing opportunistic infection symptoms; infected with hepatitis B or C; having history of using antibiotics, immunosuppressive regimen, probiotics, prebiotics, or symbiotics in the past 6 months; BMI higher than 30; showing oral active inflammation. None of the patients had obvious symptoms of oral mucosal diseases and periodontal disease (redness, swelling, and bleeding) when the clinical samples were collected. However, formal dental examinations were not performed to rule out the mild periodontal symptoms.

Ethics Statement

This study conforms to the ethical norms of the 1975 Helsinki Declaration. The research protocol was approved by the Institutional Review Committee of The First Affiliated Hospital of Zhejiang University on October 7, 2015. All participants provided written informed consents before participating in the study. All the data used for analysis were anonymized.

Salivary Samples Collection and DNA Extraction

Participants were required to refrain from eating, drinking, smoking before saliva collection. The amount of each salivary sample collected from participants before their clinic visit was 5 ml. The samples were stored in sterile containers of −80°C until DNA extraction by QiaAmp DNA Mini Kit (QIAGEN, Hilden, Germany) following instructions of the manufacturer. NanoDrop (Thermo Fisher Scientific) was used to determine the concentration and purity of DNA as well as 1.0% agarose gel electrophoresis for the integrity and size of DNA. After the procedures above, the DNA samples were frozen at −20°C for further analysis.

16S Ribosomal RNA Gene Sequencing

The bacterial 16S rRNA gene high-throughput sequencing was conducted by Shanghai Majorbio Bio-Pharm Technology Co.,

Ltd. (Shanghai, China). The bacterial 16S rRNA gene sequences spanning the variable regions V3–V4 were amplified using the primer 338F (5′- ACTCCTACGGGAGGCAGCAG-3′), and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) as previous recorded (Xie et al., 2021). The amplicons were extracted from 2% agarose gels, purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using QuantiFluor™-ST (Promega, United States) based on the guidelines of the manufacturer's protocol. In equimolar amounts, purified amplicons were sent to paired-end sequencing (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, United States).

Bacterial Translocation, Viral Load, and Flow Cytometry

Sera samples of immunological responders and non-responders were collected to measure the bacterial translocation markers. Following the standard protocols, human Lipopolysaccharides (LPS) ELISA Kit (CUSABIO; Wuhan, China) and Human soluble CD14 (sCD14) ELISA Kit (MultiSciences, Hangzhou, China) were used to test plasma LPS and sCD14. Flow cytometry and Cobas Amplicor (Roche Molecular Systems Inc., Branchburg, New Jersey, United States) were used to quantify CD4 + /CD8 + T-cells and HIV-1 RNA, respectively. Fresh anticoagulated whole blood was used to quantify the expressing markers of immune activation (CD25 +, CD38 +, HLADR +, or CD38 + /HLA-DR +) of CD4 + and CD8 + T-cells and immune senescence (CD57 +) by BD FACS Canto II flow cytometer (BD Biosciences, California, United States). The antibodies needed during the experiment were purchased from Biolegend (San Diego, CA), including CD3-FITC, CD4- PerCP/Cy5.5, CD8-Brilliant Violet 510™, CD38-Brilliant Violet 421, CD25-PE, HLA-DR-APC/Fire™ 750, and CD57-allophycocyanin (APC).

Bioinformatics and Statistics

The 16S rRNA high-throughput sequencing raw fastq files were demultiplexed and quality-filtered by QIIME (version 1.9.1).[1] Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using UPARSE.[2] The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier (version 2.2)[3] and compared with the SILVA rRNA database[4] with the confidence threshold being 70%. After eliminating the interference sequence, alpha diversity estimator calculations were performed using Mothur v.1.30.2. Phylogenetic beta diversity measures, such as Bray-Curtis distance metrics analysis; the representative sequences of OTUs were used for each sample, respectively. Principal Coordinates analysis (PCoA) was performed to visualize the microbial communities following the distance matrices, as well as Linear discriminant analysis effect size (LEfSe) on Galaxy to calculate bacteria taxa with significantly different abundances between groups (Segata et al., 2011). In this study, alpha values for the factorial Kruskal Wallis at 0.05 and linear discriminant analysis (LDA) effect size threshold of 2.0 for discriminative features were applied for all bacteria that were discussed. The calculation of correlations between the variables were conducted with Spearman's rank-correlation analysis. Spearman correlation matrix with p-adjust lower than 0.05 and ρ-value above 0.2 were used to filter strong correlations. The discriminatory function of the biomarkers was evaluated through calculating the area under the receiver operating characteristic (ROC) curve (AUC) using pROC of R package. The comparisons between groups were conducted through the Chi-square test, Independent-Samples T-test, Wilcoxon rank sum test and Mann-Whitney U-test in the R package and SPSS 21.0 software (SPSS Inc., Chicago, IL, United States). Differences were considered significant when $P < 0.05$.

[1] http://qiime.org/install/index.html

[2] http://drive5.com/uparse/

[3] http://rdp.cme.msu.edu/

[4] http://www.arb-silva.de

RESULTS

General Clinical Features of the Patients

The cross-sectional study subjects included 30 immunological responders and 34 immunological non-responders with HIV infection. The characteristics such as gender, age, body mass index (BMI), and smoking status are relatively matched between the two groups (**Table 1**). The MSM transmission route rate is 53.3% vs. 44.1% ($p = 0.461$).

ART medications were composed by two Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a ritonavir-boosted protease inhibitor (PI). No differences are observed in the duration of ART and the type of ART drugs between the IR and the INR groups ($p = 0.193$ and $p = 0.821$). The HIV RNA viral load levels were considered as undetectable (<20 copies/ml) in all samples of the patients. In the IR group,

TABLE 1 | Clinical characteristics data summary.

Characteristics	IR (n = 30)	INR (n = 34)	P-value
Age, (years), mean ± SD	36.97 ± 9.91	38.59 ± 9.45	0.506
Gender male/female	27/3	33/1	0.224
BMI, mean ± SD	21.31 ± 2.60	20.67 ± 2.58	0.368
Mode of transmission, MSM, N (%)	16 (53.3%)	15 (44.1%)	0.461
Smoking, N (%)	0 (0%)	1 (2.94%)	0.096
HAART months (mean ± SD)	37.60 ± 13.21	33.61 ± 10.20	0.193
Ongoing ART regimen, N (%)			
NNRTI-based	27 (90.0%)	30 (88.2%)	0.821
PI-based	3 (10.0%)	4 (11.8%)	0.821
Nadir CD4 + T cell count, /mm^3, median (IQR)	298 (200, 371)	42.5 (13, 140.3)	**<0.001**

Continuous variables were compared using Independent-Samples t-test or the Mann-Whitney U-test. Categorical variables were compared using Chi-square test or Fisher's exact test. BMI, body mass index; MSM, men who sex with men; NNRTI, Non-nucleoside reverse transcriptase inhibitors; PI, Protease inhibitor; IR, immunological responders; INR, immunological non-responders. Bold values indicate P < 0.05.

TABLE 2 | Salivary microbiota 16S rRNA gene high-throughput sequencing data summary.

Characteristics	IR (n = 30)	INR (n = 34)	P-value
Sobs index[#]	268.93 ± 45.29	262.79 ± 40.93	0.716
Shannon index[#]	3.56 ± 0.27	3.50 ± 0.29	0.471
Simpson index[#]	0.06 ± 0.02	0.07 ± 0.02	0.245
ACE[#]	306.14 ± 49.96	295.87 ± 44.66	0.497
Chao 1 index[#]	310.13 ± 54.65	301.49 ± 48.64	0.568
Good's coverage (%)[#]	99.85 ± 0.04	99.84 ± 0.03	0.404

[#]*Indicate the diversity and richness was calculated after the reads number of each sample were equalized. IR: immunological responders; INR: immunological non-responders.*

the number of Nadir CD4 + T cell is greatly higher than that in the INR group (298 vs. 42.5, $p < 0.001$) (**Table 1**).

Salivary Microbiota Analysis in the Immunological Responders and Immunological Non-responders Groups

To characterize the salivary microbiota composition, 3,884,775 high-quality16S rRNA sequences were obtained from all 64 participants. An average length of 445 bp and an average of 60,700 sequences per sample were adopted for further analysis. Rarefaction was conducted on the OTU table to 27,986 reads per sample to avoid any methodological artifacts. Specifically, 504 OTUs in the IR group and 503 OTUs in the INR group were defined at a similarity level of 97%. The diversity (Sobs index, Simpson's index of diversity and Shannon index) and richness (Chao1, ACE estimator and Good's coverage) of the salivary microbiota in each group at the OTU level were analyzed, and the IR group present similar Alpha and Beta diversity compared to the INR group (**Table 2**). It can be seen from the principal coordinate (PCoA) analysis by Bray-Curtis matrices that there is no significant difference between the two groups (PERMANOVA, pseudo-F: 1.64608, R^2 = 0.02586, p = 0.109, **Figure 1**). Visualization of the relative abundances of dominant taxa at the genus level in the oral microbiome is presented in **Figure 2**. In order to identify the key phylotypes responsible for the difference in distinguishing the saliva microbiota between the two groups, linear discriminant analysis (LDA) effect size (LEfSe) was performed and the threshold was 2. The INR group present a significantly higher abundance of genus *Selenomonas_4*, while the IR group has higher abundances of genus *Candidatus_Saccharimonas, norank _p_Saccharimonas,*

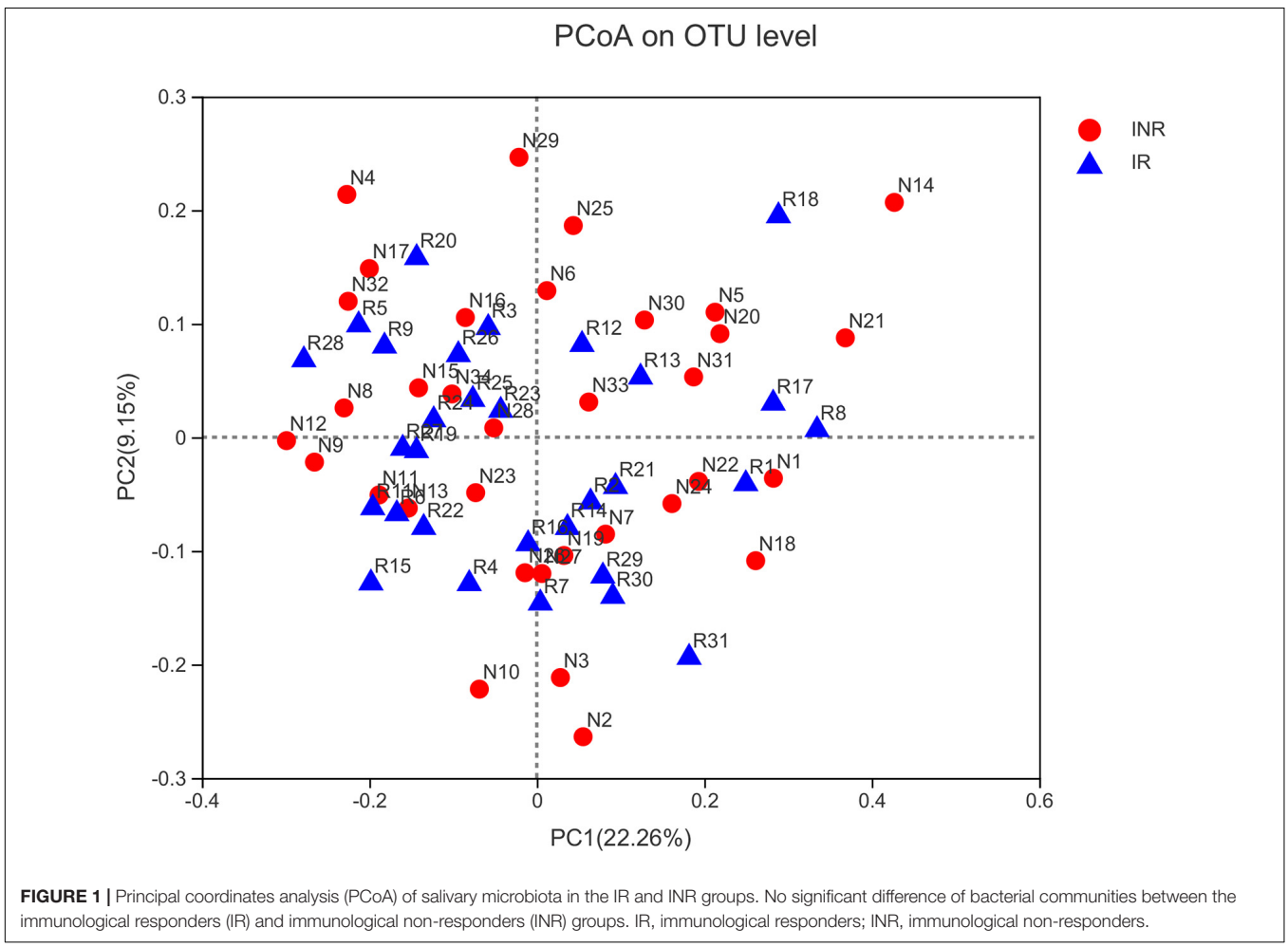

FIGURE 1 | Principal coordinates analysis (PCoA) of salivary microbiota in the IR and INR groups. No significant difference of bacterial communities between the immunological responders (IR) and immunological non-responders (INR) groups. IR, immunological responders; INR, immunological non-responders.

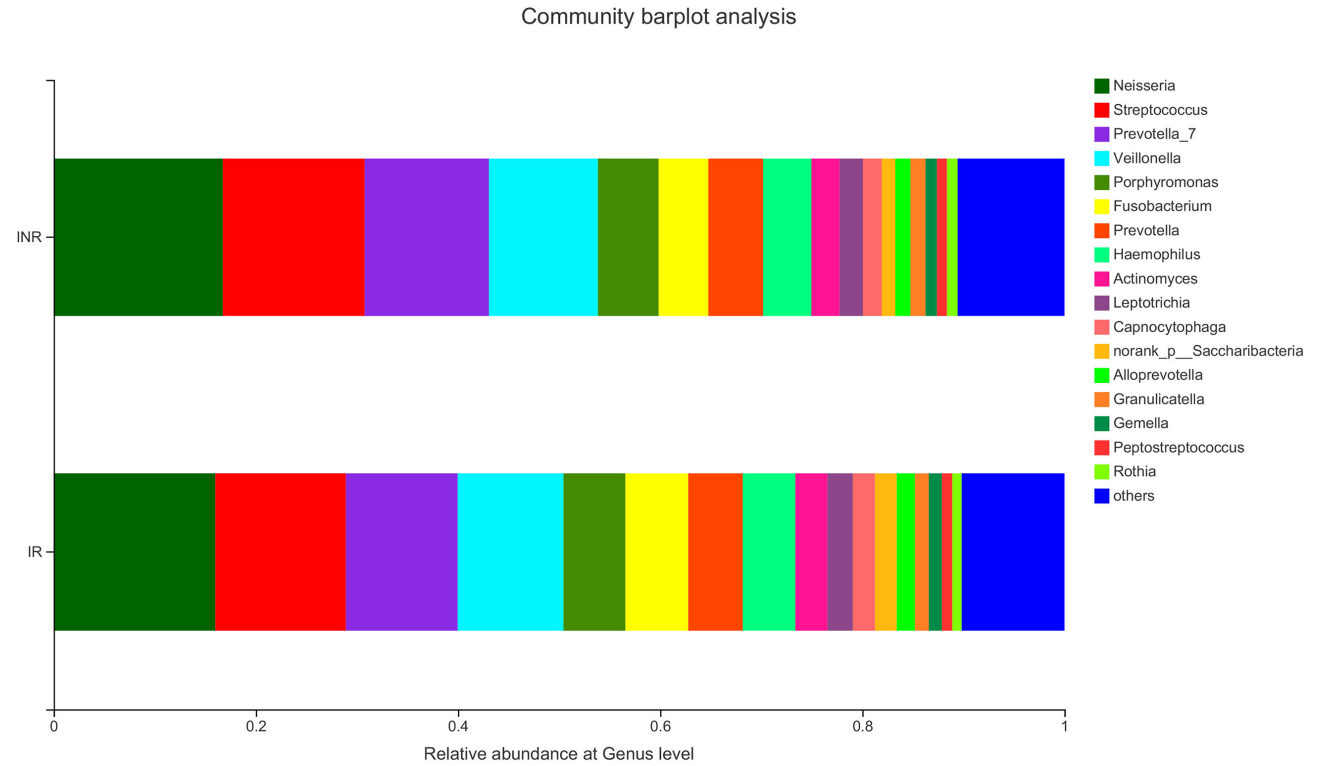

FIGURE 2 | The salivary microbiota composition at genus level in the IR and INR groups. IR, immunological responders; INR, immunological non-responders.

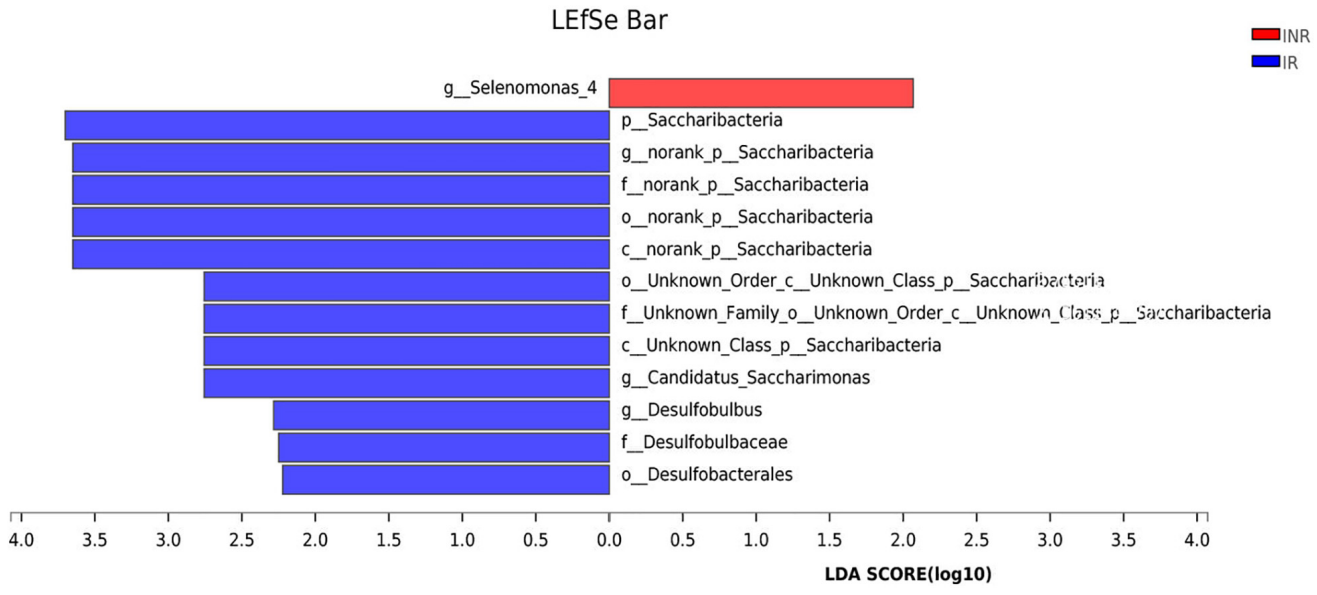

FIGURE 3 | Linear discriminative analysis (LDA) effect size (LEfSe) analysis in the IR and INR groups. LDA scores for the significant taxa in the immunological non-responders (INR) group are represented on the positive scale (red), and LDA-negative scores represent enriched taxa in the immunological responders (IR) group (blue). IR, immunological responders; INR, immunological non-responders.

and *Desulfobulbus* (**Figure 3**). The Wilcoxon Rank Sum test was also used to detect the taxa with significant differences in the relative abundances between the two groups (using confidence interval method). The abundance of *Saccharimonas* is more abundant in the IR group than the INR group at the phylum level (**Figure 4A**). Compared with the INR group, the abundances of

genus *Candidatus_Saccharimonas* and *norank_p_Saccharimonas* are dramatically increased in the IR group, while the abundance of genus *Selenomonas_4* is dramatically decreased in the IR group (**Figure 4B**).

Comparison of Adaptive Immunity and Bacterial Translocation Markers in the Immunological Responders and Immunological Non-responders Groups

As expected, the amount of the current CD4 + T-cell counts and the CD4/CD8 ratio in the INR group is lower than those in the IR group ($p < 0.001$). The proportion of the CD8 + CD38 + T-cell and the CD8 + CD57 + T-cell is significantly higher in the INR group than those in the IR group ($p = 0.032$ and $p = 0.001$). The proportion level of the CD4 + immune activation (CD4 + T-cell by the expression of CD25 +, HLA-DR +, CD38 +, or HLA-DR + /CD38 +) shows similarity in the INR and IR groups. Lipopolysaccharide (LPS), which is commonly used as the major antigens driving chronic immune activation, is significantly higher in the INR group compared with that in the IR group ($p = 0.027$) (**Table 3**).

Associations Between Oral Microbiome and Adaptive Immunity

The Spearman's correlation test was used to investigate the correlation between the relative abundance of the different genera and adaptive immunity markers. The *p*-value was corrected by FDR, *p*-adjust lower than 0.05 with ρ-value above 0.2 were considered relevant and showed in **Figure 5**. As the abundance of genus *Selenomonas_4* is low in the two groups, the multiple correlation analyses were not performed. *Candidatus_Saccharimonas* and *norank _p_Saccharimonas* are positively correlated with the current CD4 + T cells (*p*-adjust = 0.033 and 0.025, respectively). *Candidatus_Saccharimonas* is positively correlated with the markers of the adaptive immunity CD4 + CD57 + T-cells (*p*-adjust = 0.049), while *norank _p_Saccharimonas* is negatively correlated with the CD8 + CD38 + T-cells and the CD4/CD8 + HLADR + CD38 + T-cells, respectively (*p*-adjust = 0.001 and 0.032, respectively) (**Figure 5**). To explore the potential function of the saliva microbiome for discriminating the IR and INR status, a random forest model was created based on the microbiome and the top 5 genus was shown in **Figure 6A**. The ROC analysis shows that *Candidatus_Saccharimonas* could be used to discriminate the IR from the INR group [ROC-plot AUC value of 0.7 (95% CI, 0.56–0.83), **Figure 6B**].

DISCUSSION

It is well-known that there is a dynamic interaction between the host and the microbiota, which is a major factor of people's health (Dethlefsen et al., 2007). Current available studies have confirmed that discernible alterations of the composition of the salivary microbiota are inherent to a range of systemic disorders. Studies on oral microbiomes of human immune system diseases such as RA, ALL, and HIV have also indicated that the oral microbiota is strongly related to the immune responses in immunodeficiency patients (Acharya et al., 2017; Corrêa et al., 2017; Annavajhala et al., 2020; Bellando-Randone et al., 2021). Furthermore, the hypothesis that oral microbiota is associated with different immune responses to ART is supported by the high frequency of opportunistic oral infections in HIV-infected patients and its association with CD4 + T cells levels (Berberi and Noujeim, 2015). As stated, the role of the oral microbiota played in HIV-infected patients deserves much attention because the diversity and composition of the oral microbiome can be changed by HIV infection or by ART (Heron and Elahi, 2017); however, the results are highly variable. Previous studies have observed the generally similar structure of salivary microbiota as well as the differences in the relative abundances of several bacterial taxa between HIV-infected subjects and uninfected controls (Li et al., 2014; Kistler et al., 2015; Mukherjee et al., 2018; Lewy et al., 2019). However, significant differences were also found by several studies in the saliva bacterial communities between HIV-infected and uninfected individuals (Hegde et al., 2014; Mukherjee et al., 2014; Beck et al., 2015; Goldberg et al., 2015; Kistler et al., 2015; Li et al., 2021). What's more, some studies reported significant distinctions in the prevalence and the distribution of the saliva bacterial communities among HIV-infected individuals before and after the antiretroviral therapy (Li et al., 2014; Kistler et al., 2015; Mukherjee et al., 2018). Few studies have addressed the interaction of oral microbiota with different immune responses to ART received by the HIV-infected individuals. In this paper, we demonstrate the oral microbiota structure and its relationship with immune response in HIV infection.

A previous study indicated that after receiving 24 weeks of ART, the salivary microbiome in the three HIV-infected participants with persistently low CD4 + T-cell count had significantly higher bacterial richness and Shannon diversity; when compared to those with CD4 counts that remained or recovered to greater than 200 cells/μl. Several taxa with different abundancies, such as *Porphyromonas* species, discriminated between the baseline and the posttreatment samples; this suggested that the salivary microbiome can be an important factor in the CD4 + T-cell count recovery after ART in the study (Presti et al., 2018). However, the major limitation of this study is the insufficient number of studied subjects and the short time period of patients receiving ART. Thus, more studies on larger cohorts are necessary for a better understanding of the potential roles of different immune responses to ART on oral microbiome. Making the most use of the non-invasive and unsuspicious functions of saliva sampling, we collected the salivary samples from the HIV-infected immunological non-responders and immunological responders and studied the salivary microbiome using high-throughput sequencing technology. To our knowledge, this is the first study that has fully utilized the next-generation sequencing technology to characterize and compare the community composition of the salivary microbiota in the higher number of HIV-infected immunological responders and non-responders. These findings are particularly important given that the adaptive immunity markers assessments were performed on all participants, offering

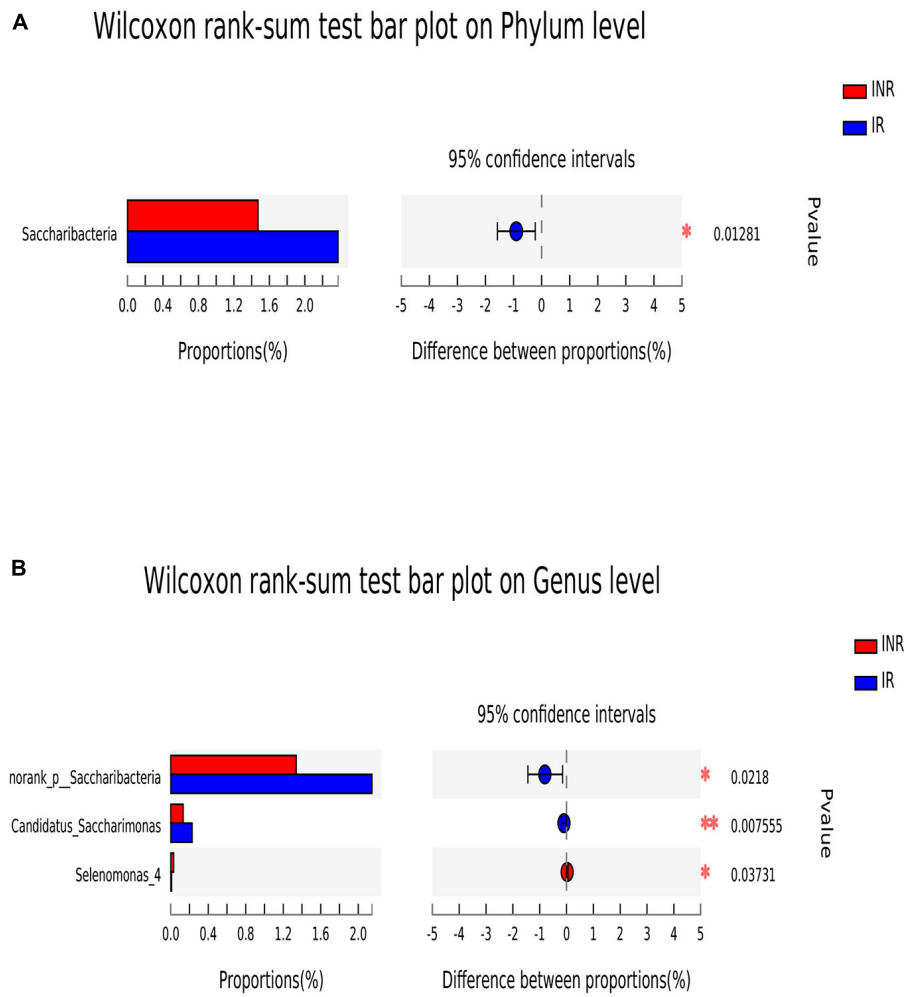

FIGURE 4 | The difference of saliva microbial taxa between the IR and INR groups at the phylum and genus levels. The Wilcoxon Rank Sum test was performed to detect taxa with significant differences in relative abundances at the phylum **(A)** and the genus levels **(B)** between the two groups (using confidence interval method). IR, immunological responders; INR, immunological non-responders. $^{*}P < 0.05$ and $^{**}P < 0.01$.

TABLE 3 | Comparison of adaptive immunity and bacterial translocation markers.

Characteristics	IR (*n* = 30)	INR (*n* = 34)	*P*-value
Current CD4 + T cell count,/mm^3, median (IQR)	597 (466.5, 666.5)	209 (163.3, 303)	**<0.001**
Current CD4 + /CD8 + T-cell ratio	0.9 (0.5, 1)	0.4 (0.2, 0.5)	**<0.001**
%CD4 + HLADR + CD38 +	7.2 (4.5, 10)	7.8 (4.7, 12.4)	0.418
%CD4 + CD25 +	1.1 (0.6, 1.5)	0.8 (0.3, 1.6)	0.388
%CD4 + CD57 +	2.3 (0.7, 4.1)	1.1 (0.2, 2.9)	0.083
%CD8 + CD38 +	31.6 (25.7, 39.2)	44.1 (28.0, 58.0)	**0.032**
%CD8 + HLADR + CD38 +	17.8 (13.1, 26.5)	23.7 (12.4, 35.7)	0.388
%CD8 + CD57 +	13.4 (10.5, 19.8)	21.7 (14.2, 34.5)	**0.001**
LPS (pg/ml, mean ± SD)	64.7 (50.6, 104.4)	90.1 (65.5, 152)	**0.027**
sCD14 (pg/ml, mean ± SD)	2052.1 (1797.7, 2413.5)	2319.8 (1878.1, 2654.4)	0.169

IR, immunological responders; INR, immunological non-responders. Bold values indicate P < 0.05.

an opportunity to evaluate the relationship between the salivary microbiota and the immunologic markers in the immunological responders and non-responders.

The IR group presented similar salivary bacterial richness and diversity when compared with the INR group in this study. A similar Alpha diversity of salivary bacterial community was

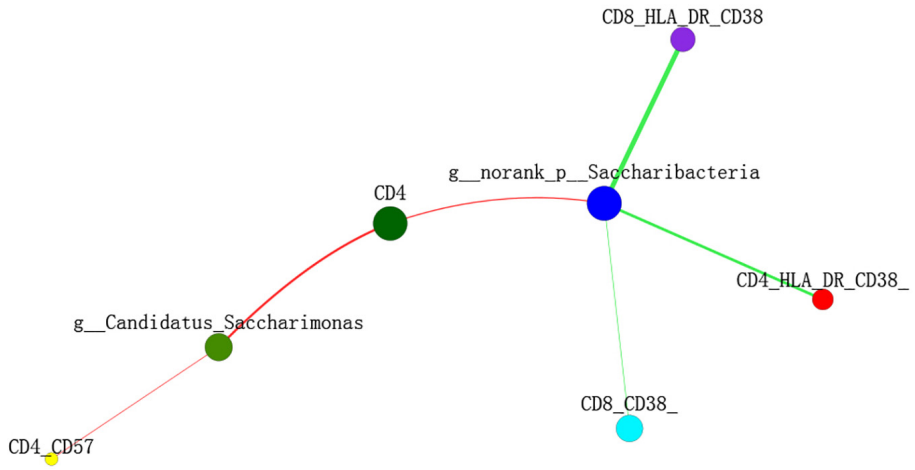

FIGURE 5 | Two-way correlation network analysis between the saliva microbiota and the adaptive immunity markers. Some adaptive immunity markers are correlated with the specific genera of saliva microbiota. Positive correlation is shown in the red line, negative correlation is shown in the green line. Spearman's correlation was used, and associations with *p*-adjust lower than 0.05 and ρ-value above 0.2 were considered relevant.

also described in the HIV-infected IR (n = 18) and INR (n = 9) individuals in a previous study (Jiménez-Hernández et al., 2019). While another study reported that the three HIV-infected participants with persistently low CD4 + T-cell counts had significantly higher salivary bacterial richness and Shannon diversity after their 24 weeks of ART, when compared with the participants whose CD4 + T-cell counts higher than 200 cells/μl (Presti et al., 2018). It claimed that the HIV infection and highly active antiretroviral therapy (HAART) had significant effects on salivary microbial colonization and composition, and *Selenomonas* noticeably increased after HAART (Li et al., 2014), which were reported to be depleted in the oral microbiome of the HIV-associated periodontitis (Noguera-Julian et al., 2017). For patients with systemic lupus erythematosus (SLE), increased numbers of *Selenomonas* were directly correlated with the elevated levels of inflammatory cytokines IL-6, IL-17, and IL-33 (Corrêa et al., 2017). In this study, when we visualized the relative abundances of dominant taxa at the genus level in the oral microbiome, we found that the INR group presented a significantly higher abundance of genus *Selenomonas_4*. Gut *Candidatus_Saccharimonas* was reported decreased in rats acute necrotizing pancreatitis (Chen et al., 2017), which indicated that *Candidatus_Saccharimonas* did play an important role in maintaining normal intestinal functions. A previously published study stated that the relative abundance of gut *Candidatus_Saccharimonas* was negatively correlated with the expression levels of cadherin-11, IL-17α, and TLR2 in the adjuvant-induced arthritis rat model (Huang et al., 2019). In this study, the IR group had higher abundances of genus *Candidatus_Saccharimonas* and *norank _p_Saccharimonas* when compared with the INR group. In addition, *Candidatus_Saccharimonas* had positive correlation with the CD4 + T-cells and CD4 + CD57 + T-cells, while *norank _p_Saccharimonas* was positively correlated with the CD4 + T-cells but negatively correlated with the CD8 + CD38 + T-cells and CD4/CD8 + HLADR + CD38 + T-cells. While host biomarkers were subjected to the individual biological variations, oral microbiome was relatively conserved among unrelated individuals. Recent advances of saliva analysis have played a key role in the definitions of biomarkers for the diagnosis, prognosis, and the treatment of human immune system diseases (Bellando-Randone et al., 2021). Expansion of specific microbial consortia in the saliva may act as imprints of the underlying immuno-inflammatory processes, especially in HIV. This study indicated that *Selenomona, Candidatus_Saccharimonas,* and *norank _p_Saccharimonas* might all played important roles in the immune recovery of the immunodeficiency patients, and *Candidatus_Saccharimonas* could be considered in the future as screening biomarkers for immune responses in HIV-infected individuals, leading to the future design of effective individualized treatment strategies such as probiotics for the immunological non-responders. However, a previous study on the effect of the prebiotic modulation of the salivary microbiota in HIV-infected patients with diverse immunopathogenesis stated that: *Streptococcus anginosus* was in correlation with the CD4 + T cells, *Veillonella parvula* with the CD4 + CD25 + T cells, and *Prevotella pallens, Prevotella copri, and Prevotella nigrencens* with the markers of adaptive immunity such as CD4 + CD25 + T cells, CD4 + CD57 + T cells, and CD4 + HLADR + CD38 + T cells, respectively (Jiménez-Hernández et al., 2019). The inconsistent results may be caused by the different number of subjects in the two studies and their different definitions of INR. More studies need to be carried out to determine the precise cause of this inconsistency.

This study provides a better understanding of the oral microbial profiles and their relationship with the immune responses in the immunocompromised patients. Although the correlations between the salivary microbiota and the biomarkers of adaptive immunity were observed, we could not establish a causal influence between the oral microbiome and the immune

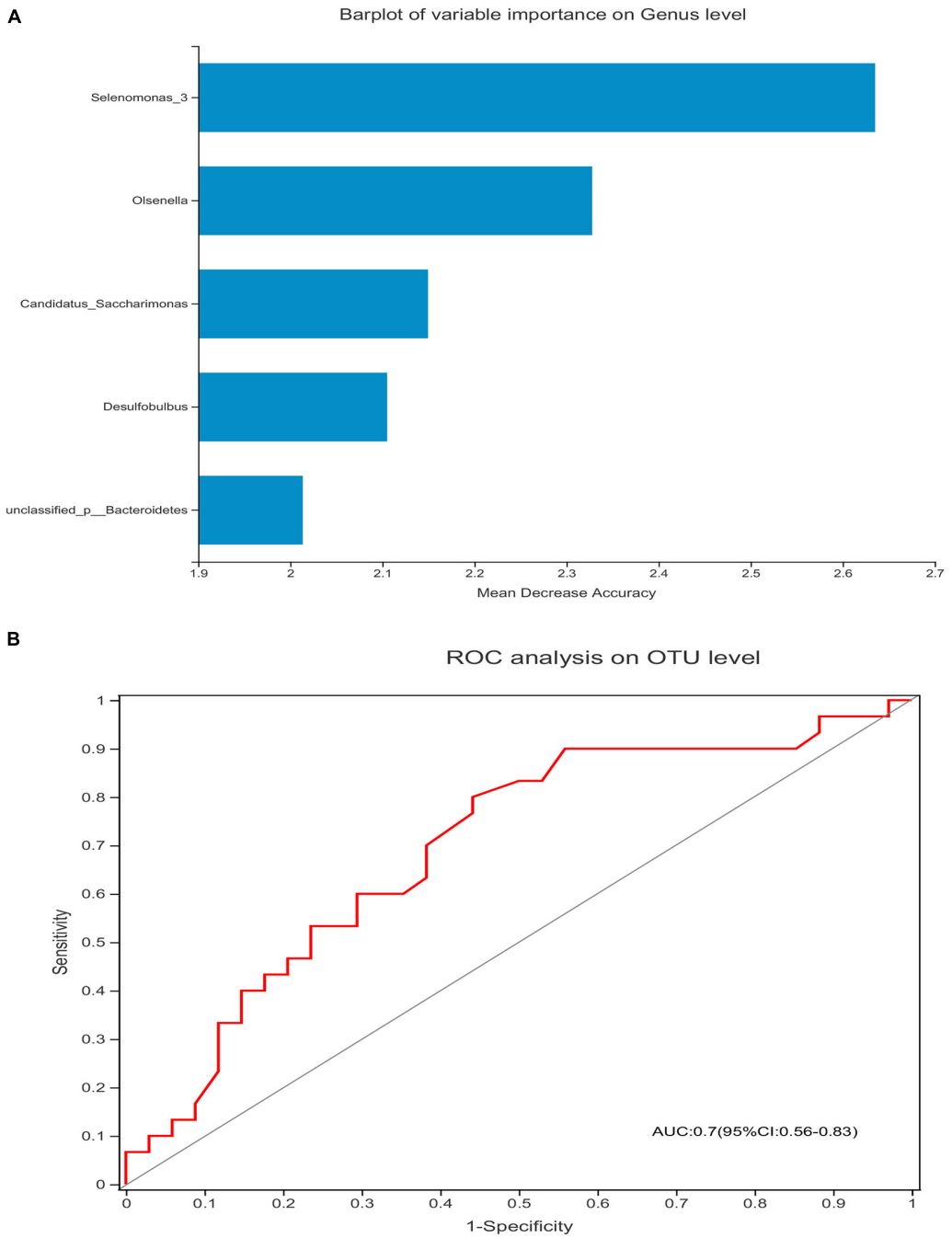

FIGURE 6 | *Candidatus_Saccharimonas* could be used to discriminate the IR from the INR group. Barplot of variable importance on the genus level **(A)** and the receiving operational curve analysis were performed **(B)** [area under curve (AUC) = 0.7]. Diagonal lines represent random classifications (AUC = 0.5).

system in the HIV-infected patients. Thus, further research on the exact mechanisms involved in the interaction between the immune system and oral microbiota is still required. We find the low number of participants, absence of control group, and cross-sectional analysis caused limitations to the explanation of our findings. Intra-person variability will need larger longitudinal studies with control group, which should involve plaque collection and the observation of the clinical and environmental changes that account for intra-person variability over time. Apart from taxonomic characterization, there should be more studies on identifying bacterial pathways and the resulting metabolites that promote disease and immunity.

In summary, this study focuses on the overall oral microbiota structure and its interactions with different immune responses

to ART. While there were some taxonomic differences in the salivary bacterial composition, the results suggested that the overall structure of the salivary microbiota in the immunological responders was similar with those that were in the immunological non-responders. *Selenomona_4, Candidatus_Saccharimonas,* and *norank_p_Saccharimonas* might act as important factors of the immune recovery in the immunodeficiency patients, and *Candidatus_Saccharimonas* could be considered in the future as screening biomarkers for immune responses in the HIV-infected individuals.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database and is accessible with the following link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA744583?reviewer=3gmrc9a67n88rhe2gr9ehub4ok.

ETHICS STATEMENT

The research protocol was approved by the Institutional Review Committee of The First Affiliated Hospital of Zhejiang University on October 7, 2015. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YX participated in the designing of the study and wrote the manuscript. JS performed the statistical analysis and revised the manuscript. JS and CH collected the biopsy samples and carried out the experiment. BR and BZ participated in the designing and reviewing of the manuscript. All authors read through and approved the final manuscript.

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Can the Salivary Microbiome Predict Cardiovascular Diseases? Lessons Learned From the Qatari Population

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Background: Many studies have linked dysbiosis of the gut microbiome to the development of cardiovascular diseases (CVD). However, studies assessing the association between the salivary microbiome and CVD risk on a large cohort remain sparse. This study aims to identify whether a predictive salivary microbiome signature is associated with a high risk of developing CVD in the Qatari population.

Methods: Saliva samples from 2,974 Qatar Genome Project (QGP) participants were collected from Qatar Biobank (QBB). Based on the CVD score, subjects were classified into low-risk (LR < 10) ($n = 2491$), moderate-risk (MR = 10–20) ($n = 320$) and high-risk (HR > 30) ($n = 163$). To assess the salivary microbiome (SM) composition, 16S-rDNA libraries were sequenced and analyzed using QIIME-pipeline. Machine Learning (ML) strategies were used to identify SM-based predictors of CVD risk.

Results: *Firmicutes* and *Bacteroidetes* were the predominant phyla among all the subjects included. Linear Discriminant Analysis Effect Size (LEfSe) analysis revealed that *Clostridiaceae* and *Capnocytophaga* were the most significantly abundant genera in the LR group, while *Lactobacillus* and *Rothia* were significantly abundant in the HR group. ML based prediction models revealed that *Desulfobulbus, Prevotella*, and *Tissierellaceae* were the common predictors of increased risk to CVD.

Conclusion: This study identified significant differences in the SM composition in HR and LR CVD subjects. This is the first study to apply ML-based prediction modeling using the SM to predict CVD in an Arab population. More studies are required to better understand the mechanisms of how those microbes contribute to CVD.

Keywords: CVD, salivary microbiome, precision medicine, machine learning, QGP

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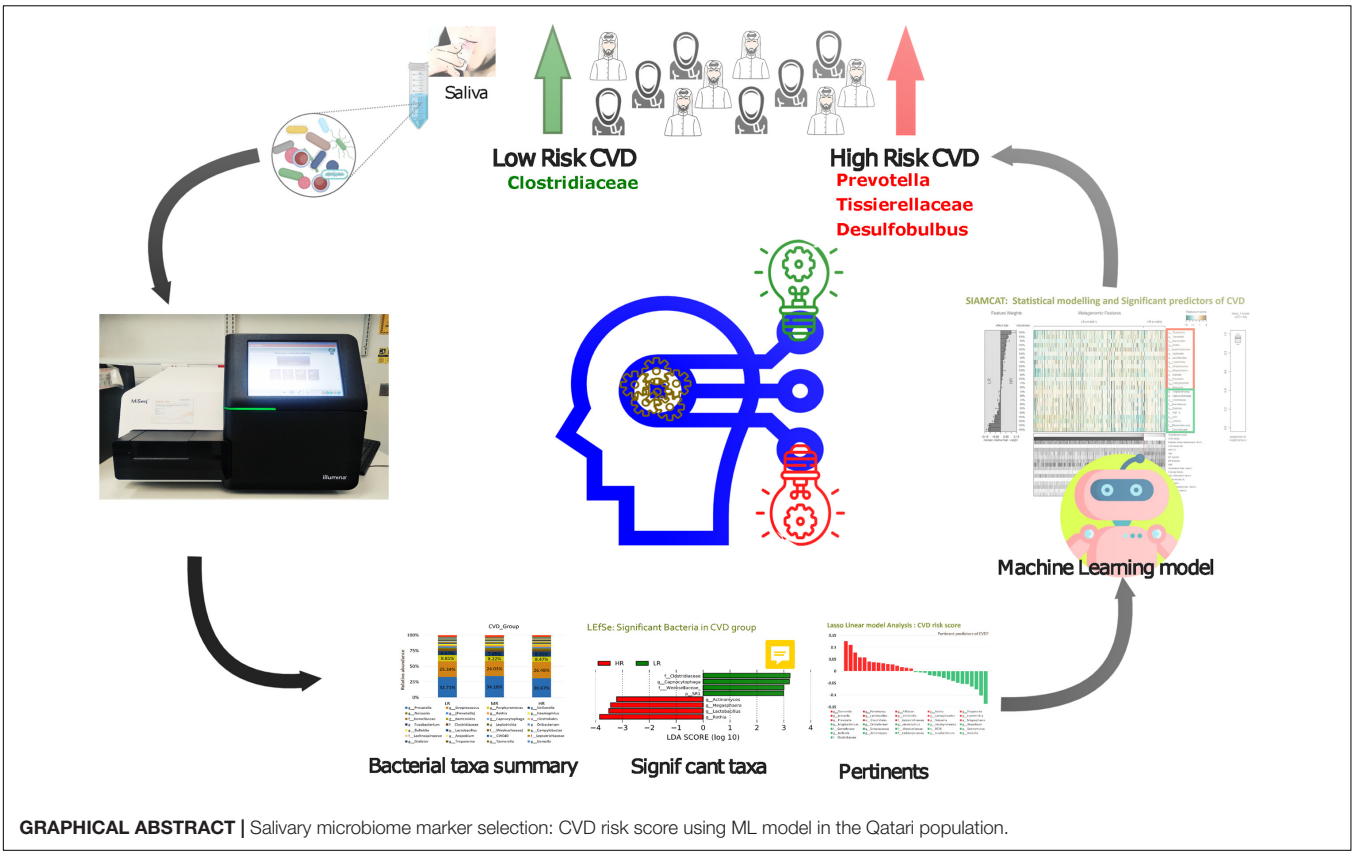
INTRODUCTION

Non-communicable Diseases (NCDs) are the leading cause of death globally (Allen et al., 2017). According to the World Health Organization [WHO] (2013) report, the global burden of non-communicable diseases (NCDs) raised to 82% by 2020. The most common NCDs are cardiovascular diseases (CVD), cancer, respiratory disorders, and diabetes (Balakumar et al., 2016).



GRAPHICAL ABSTRACT | Salivary microbiome marker selection: CVD risk score using ML model in the Qatari population.

CVD comprises coronary heart disease, heart failure, stroke, rheumatic heart disease, and cardiomyopathies among others (Caldwell et al., 2019). CVD is the leading cause of death, claiming about 17.9 million deaths annually and increasing worldwide (Lear et al., 2017; Al-Shamsi et al., 2019).

In Qatar, NCDs are the leading cause of death for the past 10 years (Al-Kaabi and Atherton, 2015) with the CVD mortality rates reaching 8.3 per 100000 MOPH (2020). In addition, the 2006-World-Health-Survey revealed that the Qatari population suffers from various predisposing factors to CVD such as obesity (28.8%), high cholesterol (24.7%), diabetes (16.7%), and hypertension (14.4%) Haj Bakri and Al-Thani (2012).

In the past decade, advances in the multi-omics technologies have enhanced our chances to discover novel biomarkers (Olivier et al., 2019). Blood-based biomarkers are considered invasive, there is an urgent need to use non-invasive samples such as saliva to develop new disease biomarkers. In addition, the advance in Next-Generation Sequencing platforms (NGS) has enabled us to assess the human microbiome with an unprecedented resolution and depth. Using the human microbiome composition to identify disease biomarkers is the next chapter of precision medicine (Morganti et al., 2019; Zhong et al., 2021).

The human microbiome (HM) comprises trillions of bacteria, viruses, protozoa, and fungi that reside in and on our body surfaces (Amon and Sanderson, 2017). The HM is complex, dynamic, ubiquitous, and shows striking variability from one individual to another and between various body sites (Ursell et al., 2012; Aagaard et al., 2013). The HM has a wide array of roles ranging from digestion, protection from pathogens, immune-regulation, and metabolites production (Marchesi et al., 2016). The oral cavity harbors more than 700 diverse microorganisms and is considered the second most diverse site after the gut (Deo and Deshmukh, 2019). In healthy subjects, the core salivary microbiome (SM) includes genera *Streptococcus, Veillonella, Neisseria,* and *Actinomyces* (Zaura et al., 2009, 2014). In a large-scale population-based Japanese study, the authors showed that the SM is dominated by *Streptococcus, Neisseria, Rothia, Prevotella, Actinomyces, Granulicatella, Haemophilus,* and *Porphyromonas* (Yamashita and Takeshita, 2017). Our previous study aiming to characterize the salivary microbiome composition in the Qatari population (Murugesan et al., 2020) showed that *Bacteroidetes, Firmicutes, Actinobacteria,* and *Proteobacteria* were the common phyla, with *Bacteroidetes* being the most predominant (Murugesan et al., 2020). Dysbiosis in the SM is associated with oral diseases (Mashima et al., 2017; Davis et al., 2020) and systemic diseases like obesity, diabetes, and CVD (Wade, 2013; Kholy et al., 2015; Cortez et al., 2019).

Advances in Machine Learning (ML) technologies, an essential branch of artificial intelligence, have enabled researchers to build prediction biomarker models for various diseases such as arthritis, diabetes, and inflammatory bowel disease (Jamshidi et al., 2019; Aryal et al., 2020; Kohli et al., 2020). On the other hand, few studies have trained ML models using the gut microbiome profiles to identify predictors of atherosclerosis

and CVD (Aryal et al., 2020; Liu et al., 2020) and none have used the SM so far.

This study aims to identify whether a predictive salivary microbiome signature is associated with a high risk of developing CVD in the Qatari population. We integrated the phenotypic, clinical, and microbiome data, and we identified SM-biomarkers associated with an increased risk to CVD using ML models.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Institutional Review Board (IRB) of Sidra Medicine under (protocol #1510001907) and by Qatar Biobank (QBB) (protocol #E/2018/QBB-RES-ACC-0063/0022. All study participants signed an informed consent before sample collection. All experiments were performed under the approved guidelines.

Clinical Data

We collected de-identified saliva samples, phenotypic and clinical data from a total of 2,974 participants enrolled in the Qatar genome project (QGP). QGP included any adult who is either a Qatari national or long-term resident (lived in Qatar for at least 15 years) and can contribute to QBB around 3 h of their time for answering all the questionnaires, complete measurements, imaging and fitness assessments, in addition to providing all the samples required including saliva. In the pilot phase, the cohort consisted of 1,432 males and 1,542 females (**Table 1**). Each subject's anthropometric and blood parameters were established by analyzing body mass index (BMI), total protein content, hemoglobin, albumin, ferritin, calcium, iron, vitamin-D, high or low-density lipoprotein cholesterol (HDL, LDL), triglycerides, and glucose levels.

Calculation of Cardiovascular Diseases Risk Score

Cox proportional-hazards regression has been used to evaluate the risk of developing CVD over 10-years. The CVD-risk score for 2974 patients was estimated using sex-specific multivariable factors consisting of age, total-Cholesterol, HDL, systolic blood pressure (BP), hypertension treatment, smoking, and diabetes status (HbA1C $\geq$ 6.5%, and participants who confirmed having diabetes). D'Agostino et al. (2013) adapted the regression coefficient for the functions from earlier analysis. This method uses the following equation:

$$\hat{p} = 1 - S_0(t)^{\exp(\sum_{i=1}^{p} \beta_i x_i - \sum_{i=1}^{p} \beta_i \bar{x}_i)}$$

Where $S_0(t)$, baseline survival at follow-up time t (here $t = 10$ years); β_i, estimated regression coefficient (log hazard ratio that is measured for all risk functions and sex-specific); x_i, log-transformed value of the ith risk factor; i, corresponding mean, p, number of risk factors.

Sample Collection

Qatar Biobank collected saliva samples according to standard procedure. They organized to collect 5 mL of spontaneous, whole, unstimulated saliva into a 50 mL sterile DNA-free Falcon tube from each participant by spitting. The samples were divided into 0.4 mL aliquots and stored at −80 C until further analysis. The aliquots were received from QBB for total salivary DNA extraction.

DNA Extraction and 16S rRNA Gene Sequencing

The total salivary DNA was extracted using automated QIAsymphony protocol (Qiagen, Hilden, Germany), following the Manufacturer's instructions. DNA purity was evaluated by the A260/A280 ratio using a NanoDrop 7000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), and the DNA integrity was checked on a 1% agarose by gel electrophoresis.

The V1–V3 regions of the 16S rRNA gene were amplified using Illumina NextEra XT library preparation Kit (FC-131-1002). Step 1 PCR is performed using 10 ng of template DNA for 50 μL PCR reaction using 2X Phusion Hot Start Ready mix (Thermo Fisher Scientific^{TM}). The following thermal cycling conditions were used: 5 min of initial denaturation at 94°C; 25 cycles of denaturation at 94 C for 30 s, annealing at 55°C for 30 s, extension at 72 C for 30 s; and a final extension at 72 C for 5 min. According to the Manufacturer's instructions, the amplified PCR products of approximately 550 bp in size was purified using AgenCourt AMPure XP magnetic beads (Beckman Coulter). Purified PCR products of STEP 1 was used as template for amplification of STEP 2 NextEra index PCR using thermocycling conditions of 5 min of initial denaturation at 94°C; 8 cycles of denaturation at 94 C for 30 s, annealing at 55°C for 30 s, extension at 72 C for 30 s; and a final extension at 72 C for 5 min. These PCR products were purified using AgenCourt AMPure XP magnetic beads and purified products were pooled in equimolar concentrations. High-throughput sequencing was performed using an Illumina MiSeq 2 × 300 platform following the manufacturer's instructions.

16S rRNA Sequencing Data Analysis

Demultiplexed sequence data were revised for quality control using FastQC (Andrews, 2010). PEAR tool was used to merge both forward and reverse sequence reads of respective samples (Zhang et al., 2014), and sequence reads of quality score <20 were discarded. All merged reads were trimmed to 160 bp > Reads < 500 bp using the Trimmomatic tool (Bolger et al., 2014). Trimmed FASTQ files were converted into FASTA files. Demultiplexed FASTA files were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) v1.9.0 pipeline (Caporaso et al., 2010; Murugesan et al., 2020). Operational taxonomic units (OTUs) were generated by aligning against the Greengenes database (Version: 13_8) with a confidence threshold of 97% (DeSantis et al., 2006).

Statistical Taxonomic and Diversity Analyses

Linear Discriminant Analysis Effect Size (LEfSe) (Segata et al., 2011) was used to find differentially abundant taxa between the studied categories. Alpha diversity measures including Chao1, Observed, Shannon, and Simpson indices were calculated with R-phyloseq package (McMurdie and Holmes, 2013). The alpha

TABLE 1 | Clinical parameters of the study cohort.

	LR (*N* = 2491)	MR (*N* = 320)	HR (*N* = 163)	*P*-value
Male (*N* = 1432)	1184	161	87	<0.001[a]***
Female (*N* = 1542)	1307	159	76	<0.001[a]***
CVD score	2.78 ± 2.48	13.89 ± 2.75	31.76 ± 11.87	<0.001[b]***
BMI	28.37 ± 5.86	30.51 ± 4.76	31.18 ± 5.80	<0.001[b]***
Age	35.11 ± 10.22	50.89 ± 7.15	55.87 ± 8.14	<0.001[b]***
APT	33.82 ± 2.97	33.82 ± 2.97	33.13 ± 3.05	0.011[b]*
Albumin (gm/L)	44.30 ± 3.31	44.16 ± 3.16	43.14 ± 3.59	0.001[b]**
Alkaline phosphatase (U/L)	70.02 ± 20.66	75.71 ± 21.32	76.39 ± 21.70	<0.001[b]***
ALT (GPT) (U/L)	22.02 ± 16.54	28.67 ± 16.15	27.72 ± 15.11	<0.001[b]***
AST (GOT) (U/L)	19.89 ± 16.80	21.08 ± 7.83	20.39 ± 7.41	<0.001[b]***
Calcium (mmol/L)	2.29 ± 0.08	2.30 ± 0.095	2.32 ± 0.10	<0.001[b]***
Cholesterol total (mmol/L)	4.92 ± 0.93	5.37 ± 1.11	5.44 ± 1.28	<0.001[b]***
C-Peptide (ng/mL)	2.14 ± 1.30	2.88 ± 2.22	2.83 ± 1.38	<0.001[b]***
Creatinine (μmol/L)	65.24 ± 13.90	74.04 ± 13.91	77.71 ± 19.86	<0.001[b]***
Dihydroxy VitD Total (ng/mL)	17.65 ± 11.46	19.57 ± 11.35	19.13 ± 9.43	<0.001[b]***
Ferritin (mcg/L)	65.02 ± 105.93	109.76 ± 96.33	124.33 ± 101.1	<0.001[b]***
Fibrinogen (gm/L)	3.29 ± 0.68	3.40 ± 0.65	3.48 ± 0.67	0.001[b]**
Folate (nmol/L)	20.64 ± 7.51	22.42 ± 7.25	22.82 ± 7.44	<0.001[b]***
Free thyroxine (pmol/L)	12.96 ± 1.89	12.73 ± 1.85	12.82 ± 1.46	0.006[b]**
Glucose (mmol/L)	5.18 ± 1.50	6.71 ± 2.91	7.92 ± 3.79	<0.001[b]***
HbA1C	5.40 ± 0.83	6.28 ± 1.56	7.14 ± 1.95	<0.001[b]***
HDL-Cholesterol (mmol/L)	1.43 ± 0.38	1.19 ± 0.30	1.12 ± 0.29	<0.001[b]***
Hemoglobin (gm/dL)	13.44 ± 1.79	14.59 ± 1.44	14.45 ± 1.56	<0.001[b]***
Insulin (mcunit/mL)	12.31 ± 14.90	19.03 ± 27.04	16.25 ± 12.89	<0.001[b]***
INR	1.05 ± 0.09	1.01 ± 0.09	1.00 ± 0.10	<0.001[b]***
Iron (μmol/L)	14.92 ± 6.71	16.59 ± 5.75	16.18 ± 5.74	<0.001[b]***
LDL-Cholesterol (mmol/L)	2.96 ± 0.87	3.29 ± 1.20	3.37 ± 1.18	<0.001[b]***
Potassium (mmol/L)	4.36 ± 0.37	4.44 ± 0.38	4.51 ± 0.42	<0.001[b]***
Total protein (gm/L)	73.67 ± 3.90	73.26 ± 3.82	73.15 ± 3.81	0.083[b]
Triglyceride (mmol/L)	1.16 ± 0.69	1.81 ± 1.18	1.94 ± 1.15	<0.00[b]***
Urea (mmol/L)	4.21 ± 1.25	4.75 ± 1.21	5.07 ± 1.84	<0.001[b]***

APT, activated partial thromboplastin time; BMI, body mass index; INR, International Normalization Ration, PT, prothrombin time; TSH, thyroid stimulating Hormone; TIBC, total iron binding capacity.
[a]Chi-square test, [b]Kruskal–Wallis test.
**P-value < 0.05, **P-value < 0.01, ***P-value < 0.001.*

diversity statistical significance was calculated using Mann–Whitney test through Minitab-17 (2010). *P*-values less than 0.05 were considered statistically significant. Differences in the beta diversity were presented as principal coordinate analysis using QIIME. Analysis of similarities (ANOSIM) was used to calculate the distance matrix difference between the categories using Bray-Curtis metric (Caporaso et al., 2010).

Supervised Machine Learning Modeling

We applied four statistical ML methods for regularization and feature selection based on penalized least squares (**Figure 1B**). The methods are the Least Absolute Shrinkage and Selection Operator (Lasso), Smoothly Clipped Absolute Deviation Penalty (Zou and Li, 2008) (SCAD), Elastic Net (Zou and Hastie, 2005) (ENet), and Minimax concave penalty (Zhang, 2010) (MCP). The methods differ by the mathematical properties of the corresponding penalties: Lasso and ENet use convex penalties, while MCP and Scad use concave penalties. We applied two transformations to the abundance-counts as in: a binary transformation (Binary), and a variance-stability transformation (Arcsin), while the CVD-score outcome was log-transformed (Dong et al., 2020). Analyses were performed using the R-packages glmnet (Hastie and Qian, 2014) and ncvreg (Breheny, 2020). The graphics were generated using the R-packages ggplot2, RVenn, and ggpubr (Wickham, 2011; Akyol, 2019; Kassambara, 2020). We randomly split the data 50-times into a training set (80%) on which the predictive-models were build and a test-set (20%) on which we tested the performance of each model. Optimal tuning parameters were chosen *via* 10-fold cross-validation.

RESULTS

Demographic and Clinical Parameters of the Study Population

The study population was composed of 2,974 Qatari participants. The cohort was classified into three CVD groups as low-risk (LR) (CVD score < 10), moderate-risk (MR) (CVD score: 10–20), and high-risk (HR) (>20), as described in the section

"Materials and Methods." As a result, 2491 participants were LR, 320 were MR, and 163 were HR (**Table 1**). The average participant's age in the HR group (55.87 ± 8.14 years) was significantly higher than those in the MR (50.89 ± 7.15 years) and LR (35.11 ± 10.22 years) groups (**Table 1**). Moreover, the BMI was significantly higher in the HR group than in the MR and LR groups (**Table 1**). In addition, among the blood parameters tested, Alkaline phosphatase, Calcium, Total-Cholesterol, LDL, Creatinine, Ferritin, Fibrinogen, Folate, Glucose, HbA1C, Urea, and Triglycerides were significantly higher in the HR group (**Table 1**).

The Salivary Microbiome Composition Reveals Signatures for Cardiovascular Diseases

After stratifying the study cohort based on the CVD risk score, we assessed the SM composition in all subjects. Then, we compared the compositional changes between different study groups. A diagram that summarizes the study design is shown in **Figures 1A,B**. The microbial sequence data generated from all the participants revealed 22 bacterial phyla, 46 classes, 87 orders, 173 families, and 390 genera. *Bacteroidetes, Firmicutes, Actinobacteria*, and *Proteobacteria* were the most abundant phyla observed in the saliva samples collected from the Qatari subjects, covering approximately 90% of total microbial abundance (**Figure 2A**). In addition, our data showed that *Streptococcus, Prevotella, Porphyromonas, Granulicatella, and Veillonella* represent the salivary core microbiome members at the genus level (**Figure 2B**).

Differential Salivary Microbial Taxa Between the High-Risk and Low-Risk-Cardiovascular Diseases Groups

After assessing the study cohort's SM, LEfSe analysis compared the salivary microbiome compositions in the LR, MR, and HR (**Figure 3**). Our data indicated that *Capnocytophaga* and *Clostridiaceae* were significantly abundant in the LR group compared to the HR group ($p < 0.0001$). In contrast, *Lactobacillus* and *Rothia* were significantly enriched in the HR group ($p < 0.0001$) (**Figure 3A**) in comparison to the LR group. *Clostridiaceae* and *Porphyromonas* were significantly increased in the LR group than MR group. *Neisseria* and *Capnocytophaga* were greatly enriched in the MR group than HR group (**Figures 3B,C**).

Alpha and beta diversity measures were calculated to assess the changes in diversity among groups (**Supplementary Figure 1**). Alpha diversity parameters revealed no significant differences observed between all groups (**Supplementary Figure 1A**). We then performed beta diversity analysis to assess the divergence in the community composition between the groups using the Bray-Curtis distance metric (**Supplementary Figure 1B**). We showed that the salivary microbiome in HR and MR were

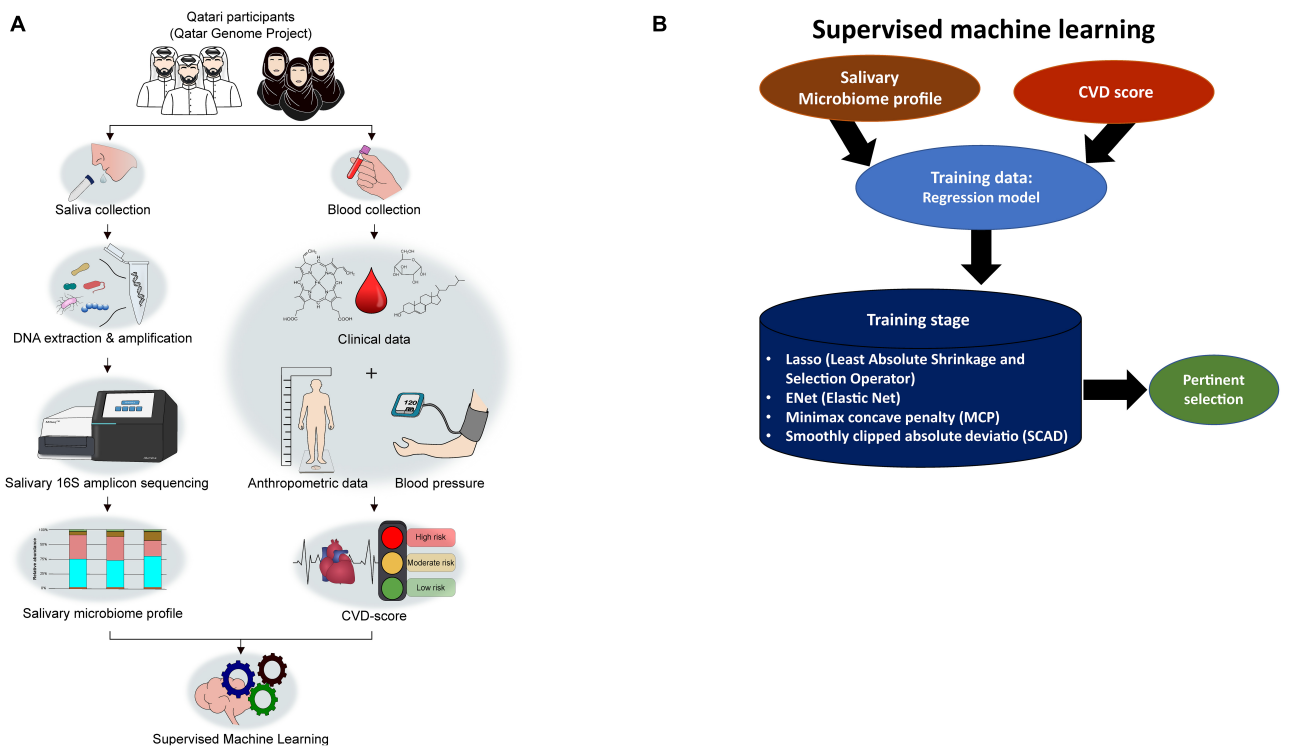


FIGURE 1 | Overall study design from participant recruitment to SM-based CVD marker selection. **(A)** The study workflow. **(B)** Strategies applied in Supervised machine learning (ML) to select pertinents.

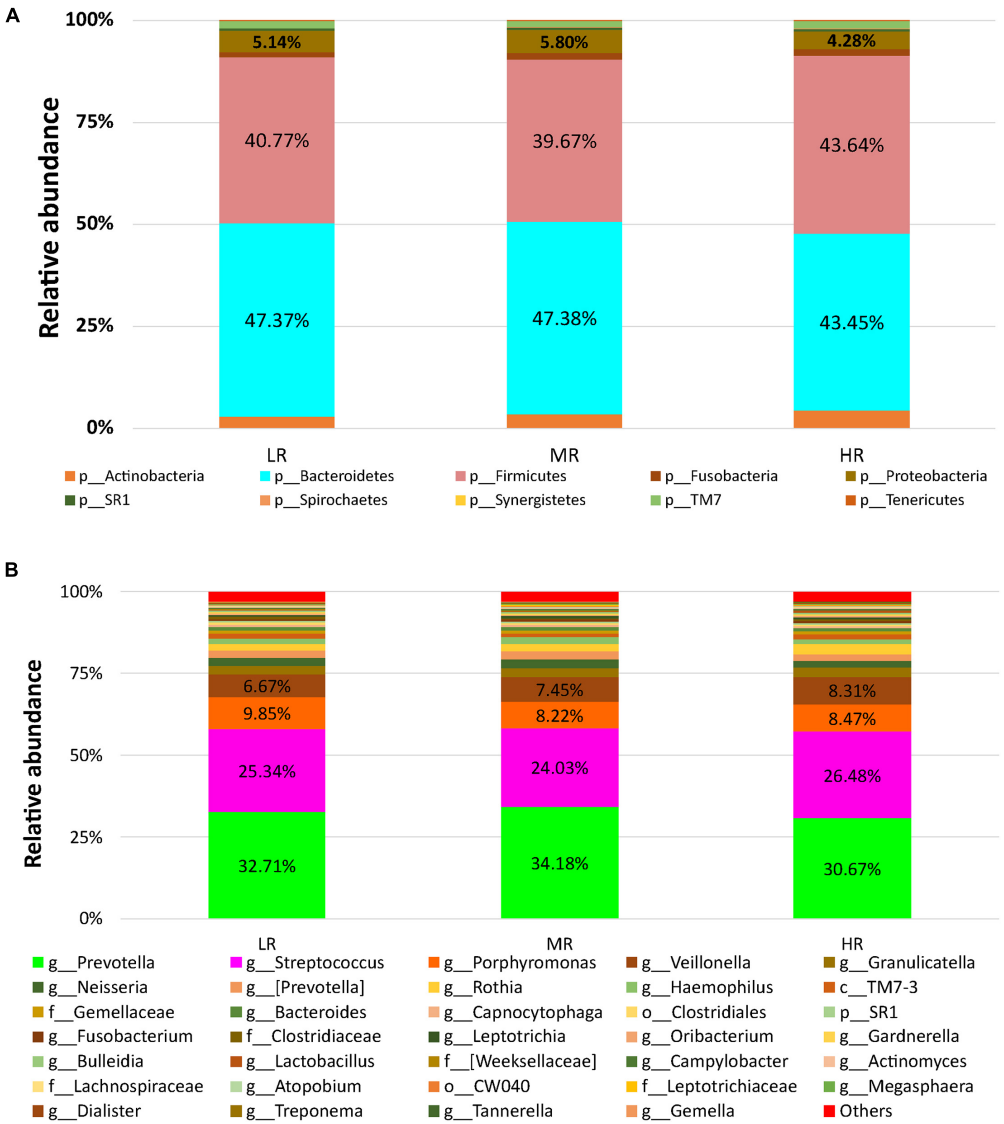


FIGURE 2 | The salivary microbiome composition of CVD risk groups. *Y*-axis shows % of relative abundance of the microbiome; *X*-axis indicates the microbial abundance in LR, MR, and HR groups; **(A)** phylum level; **(B)** genus level.

not significantly dissimilar from the LR group (p = 0.085) (**Supplementary Figure 1B**).

Identification of Pertinent Salivary Microbial Markers Associated With the Cardiovascular Diseases Score Using Machine Learning Models

The apparent differences between the study groups using alpha and beta diversity measures were not identified due to the significant sample size differences and imbalance. In this study, the participants were selected from the QGP Cohort, who provided saliva samples exclusively. QBB collected the biosamples from all volunteers as a sampling of Qatari population without focusing on CVD risk-based recruitment. We decided to use regression-based ML selection of pertinent SM biomarkers to avoid bias based on the sample size. The data were split 50-times randomly, using the four feature selection techniques, and the whole dataset was used without any exclusion (**Figure 1B**).

To search for pertinent variables, we focused on the abundances of SM selected at least 80% of the time among the 50-random splits of the data and the four feature selection techniques as described in the section "Materials and Methods." Our results are shown in **Figure 4**. Seven microbes were selected at least 80% of the time using the binary and Arcsin transformations by all the ML methods (Lasso, SCAD, ENet, and MCP) (**Figures 4A,B**). Three microbes were presented at all the tested models and both transformations (**Figures 4C,D**). In comparison, four microbes were specific to the binary transformation and four were particular to the Arcsin transformation (**Figure 4D**).

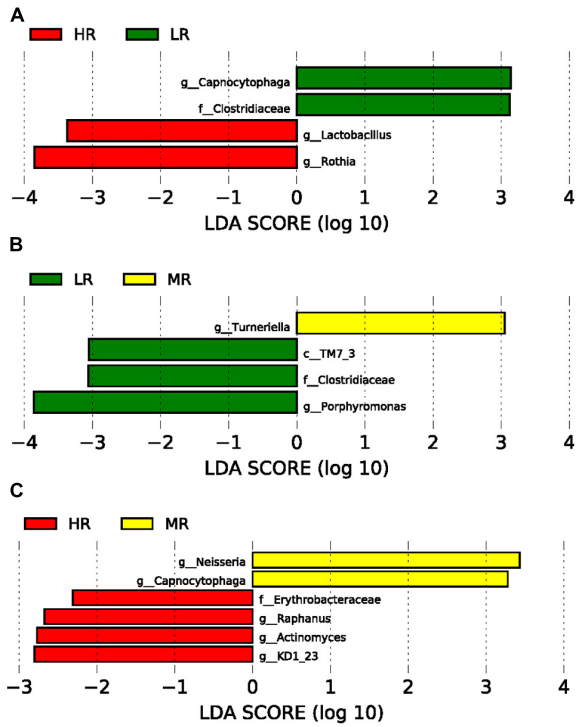


FIGURE 3 | Graphs of linear discriminant analysis (LDA) scores for differentially enriched bacterial genera among the groups. **(A)** LR (green) vs. HR (red) groups; **(B)** LR (green) vs. MR (Yellow) groups; **(C)** HR (red) vs MR (Yellow) groups.

The common microbes were *Prevotella, Tissierellaceae,* and *Desulfobulbus* (**Figure 4D**). To better understand how these microbes affect the CVD-score, we counted the sign of the regression coefficients number of times, Positive, Negative, or Zero (**Figure 4E**). From this analysis, the three microbes mentioned above contribute to an increase in the CVD score (**Figure 4E**). At the same time, our data showed that an increase in *Clostridiaceae* level contributed to a decrease in CVD-score (**Figure 4F**). Assessment using the Mean squared error (MSE) method disclosed that binary transformation has better prediction accuracy than Arcsin (**Figure 4G**).

DISCUSSION

The need for practical, non-invasive tools for predicting and preventing CVD risk has led to concerted research efforts in recent years to identify and characterize biomarkers associated with the disease as a step forward toward precision medicine. In addition, recent studies on the microbiome have enlightened its role in human health and disease (Solbiati and Frias-Lopez, 2018). Despite that, the diversity of the gut microbiome is affected by several factors like gender, ethnicity, age, and environmental factors; it was found to be associated with many diseases, including CVD and IBD using ML-models (Gulden, 2018; Chang and Kao, 2019). However, the potential use of the SM composition in assessing CVD is still lacking.

This study evaluated whether the SM composition can predict a high risk for developing CVD in a diverse Qatari population. Using a large cohort of 2,974 Qatari participants and based on the CVD risk score, we showed for the first time that the SM composition in LR and HR individuals is different (LefSe analysis). A significant SM alteration was observed between LR, MR, and HR groups (**Figures 3A–C**). Furthermore, *Capnocytophaga* and *Clostridiaceae* were significantly enriched in the LR group (**Figure 3A**). While no studies are addressing the role of *Capnocytophaga* in health and disease, a study among Japanese patients showed that non-ischemic heart failure is associated with lower levels of *Clostridiaceae* (Katsimichas et al., 2018). In line with our findings, a significant reduction of *Clostridiaceae* was observed in the HR-CVD group in the Qatari population (**Figures 3A,4D,F**).

Moreover, our data showed that *Lactobacillus* and *Rothia* were enriched in the HR group compared to the LR group (**Figure 3A**). Similarly, a study aiming to utilize the gut microbiome as a diagnostic marker of coronary artery disease (CAD) in the Japanese population has revealed that *Lactobacilli* were more abundant in patients with CAD than their matching controls (Emoto et al., 2017). On the other hand, *Rothia,* a nitrate-reducing bacterium, was enriched in hypertensive patients (Wang et al., 2021).

Next, we employed a novel approach of regression-based machine learning by combining the entire dataset of 16S rDNA sequencing data with ML models to identify the potential predictors of HR CVD without stratifying the cohort to mask the bias due to sample size differences among groups. We found that three microbes (*Prevotella, Tissierellaceae,* and *Desulfobulbus)* were represented by binary and Arcsin transformations and different training model techniques. Those were associated with high CVD-score (**Figure 4**). The Bogalusa Heart Study aimed to associate the lifetime CVD risk among the participants using the gut microbes revealed that the genus *Prevotella* was significantly enriched in the CVD HR participants (Kelly et al., 2016). Also, the role of gut microbiome in Chinese CVD patients with cardiac valve calcification revealed that *Prevotella* is a potential pathogen that is positively correlated with LDL (Liu et al., 2019). Moreover, hypertensive rats had a significant increase of *Tissierellaceae* in the gut microbiome (Sherman et al., 2018). Furthermore, *Tissierella soehngenia* was more abundant in rats with acute myocardial infarction than in the control groups (Wu et al., 2017). *Tissierellaceae* produces trimethyl amino N-oxide (TMAO), a known microbial metabolite associated with heart attack, stroke, and chronic kidney disease (Al-Obaide et al., 2017). Our study showed that *Desulfobulbus* – sulfidogenic bacterium (Devkota et al., 2012) has a positive regression coefficient with CVD scores in both trained models (**Figures 4C,D**). The elevated level of *Desulfobulbus* is known to trigger proinflammatory cytokines in patients with rheumatoid arthritis and periodontitis (Eriksson et al., 2019). Moreover, its abundance is positively correlated with age rendering it an excellent predictor to diagnose systemic diseases like diabetes and CVD (Tomas et al., 2012).

To our knowledge, this study is the first to demonstrate the promising potential of artificial intelligence *via* ML modeling for a convenient prediction screening of CVD based on the SM

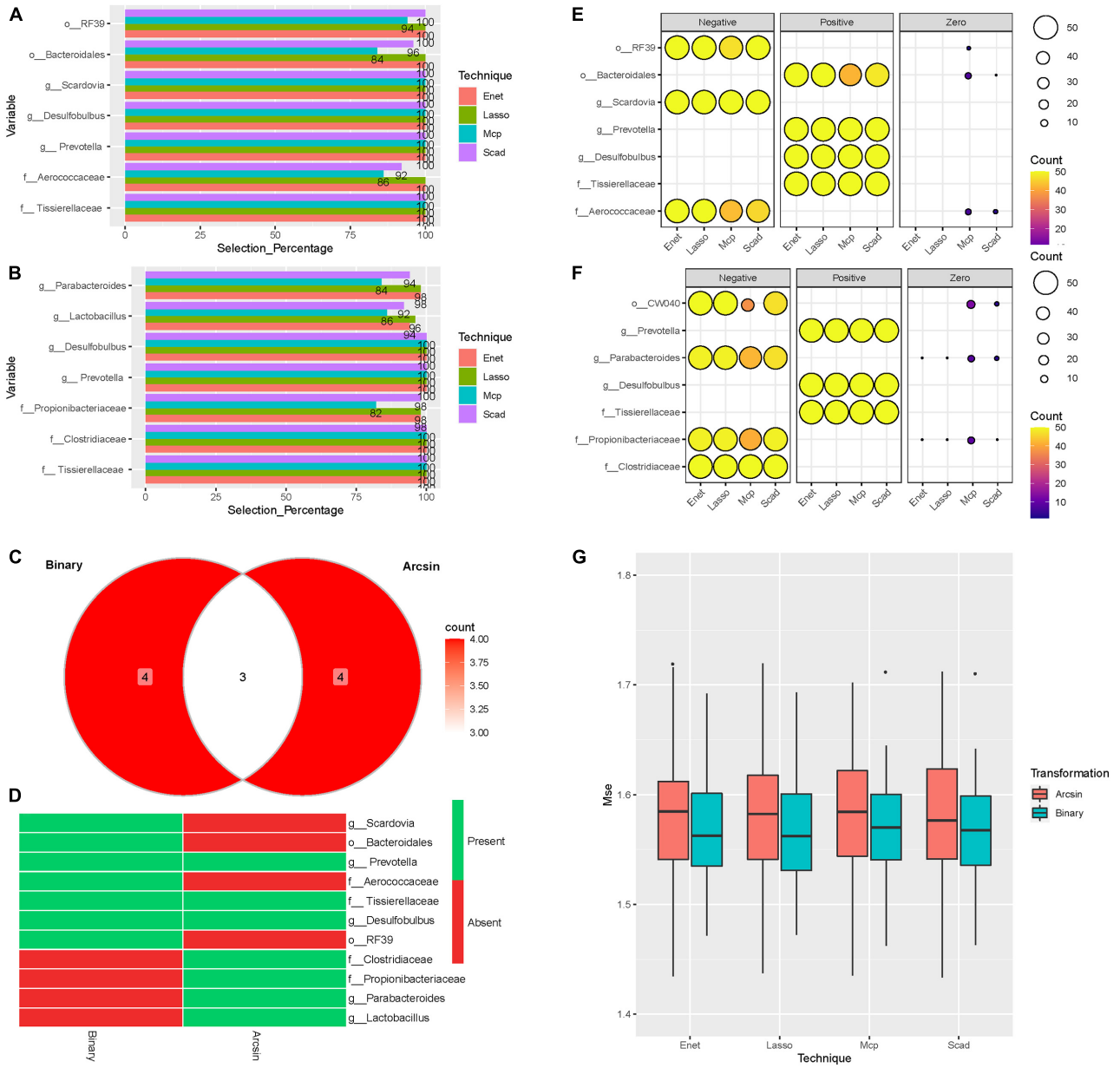


FIGURE 4 | Machine learning models. Barplots representing the selection percentages of the microbes selected at least 80% of the time by the four methods over the 50 random splits of the data. **(A)** Binary transformation. **(B)** Arcsin transformation. **(C)** Venn Diagram showing the number of microbes. **(D)** Heatmap [presence (green)/absence (red)] of selected microbes using Binary and Arcsin transformations. **(E)** Balloon plot representing sign counts of the regression coefficients: Binary transformation **(F)** Arcsin transformation. The size of circles represents the number of splits. The color represents the number of counts. **(G)** Box plots of the MSE for the four-methods and the two transformations applied to the microbiome abundance data. Each point of the boxplot represents the MSE on the test-set.

composition in the Arab population. While most ML strategies based on the health records (including age, sex, smoking habit, systolic BP, total cholesterol, HDL, cholesterol, BP treatment, and diabetes), fewer studies used gut microbiome profiles to predict IBD and CVD with an AUC of ≈0.70 and 0.90, respectively (Masetic and Subasi, 2016; Weng et al., 2017; Aryal et al., 2020; Tsoi et al., 2020; Manandhar et al., 2021). A pilot study of Japanese patients with atherosclerotic cardiovascular disease (ACVD) revealed that SM could be used as an optimal marker of ACVD with an AUC of 0.933 (Kato-Kogoe et al., 2021). It is a promising finding to enable the discovery of non-invasive biomarkers that can predict the risk of the disease before it occurs. This study is novel, and the outcomes will be a step toward developing new biomarkers for early non-invasive testing aiming to reduce the CVD burden. The main limitation of this study is the single time point recruitment of the participants without any follow-up on the participants, in addition to the imbalance in the sample size between the groups. This study mainly focuses on the

SM shift with a change in CVD-score. In this study, we did not consider the other confounding factors such as chronic diseases like diabetes, arthritis, and hypertension and their treatment, which can also influence the SM shift.

Further studies are warranted to confirm our findings and the potential use of these microbial signatures as diagnostic or prognostic markers. In addition, more investigation of these biomarkers for their mechanistic and pathophysiological evidence could be helpful in the personalized approach to treat CVD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA781451).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of Sidra Medicine under (protocol #1510001907) and Qatar Biobank (QBB) (protocol #E/2018/QBB-RES-ACC-0063/0022). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SAK designed the study, obtained funds for the project, reviewed the data, and finalized the manuscript. SM processed the samples, analyzed the data, and wrote the initial draft. AT and DB calculated the CVD scores and reviewed the data and the manuscript. ME analyzed the data using ML techniques. All authors reviewed and accepted the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.772736/full#supplementary-material

Supplementary Figure 1 | (A) Alpha diversity measures for the LR, MR, and HR groups. **(B)** Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances of SM.

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Characterization of Oral *Enterobacteriaceae* Prevalence and Resistance Profile in Chronic Kidney Disease Patients Undergoing Peritoneal Dialysis

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Chronic Kidney Disease (CKD) is a growing public-health concern worldwide. Patients exhibit compromised immunity and are more prone to infection than other populations. Therefore, oral colonization by clinically relevant members of the *Enterobacteriaceae* family, major agents of both nosocomial and dialysis-associated infections with frequent prevalence of antibiotic resistances, may constitute a serious risk. Thus, this study aimed to assess the occurrence of clinically relevant enterobacteria and their antibiotic resistance profiles in the oral cavity of CKD patients undergoing peritoneal dialysis (CKD-PD) and compare it to healthy controls. Saliva samples from all the participants were cultured on MacConkey Agar and evaluated regarding the levels of urea, ammonia, and pH. Bacterial isolates were identified and characterized for antibiotic resistance phenotype and genotype. The results showed that CKD-PD patients exhibited significantly higher salivary pH, urea, and ammonia levels than controls, that was accompanied by higher prevalence and diversity of oral enterobacteria. Out of all the species isolated, only the prevalence of *Raoultella ornithinolytica* varied significantly between groups, colonizing the oral cavity of approximately 30% of CKD-PD patients while absent from controls. Antibiotic resistance phenotyping revealed mostly putative intrinsic resistance phenotypes (to amoxicillin, ticarcillin, and cephalothin), and resistance to sulfamethoxazole (~43% of isolates) and streptomycin (~17%). However, all isolates were resistant to at least one of the antibiotics tested and multidrug resistance isolates were only found in CKD-PD group (31,6%). Mobile genetic elements and resistance genes were detected in isolates of the species *Raoultella ornithinolytica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, and *Enterobacter*

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asburiae, mostly originated from CKD-PD patients. PD-related infection history revealed that *Enterobacteriaceae* were responsible for ~8% of peritonitis and ~16% of exit-site infections episodes in CKD-PD patients, although no association was found to oral enterobacteria colonization at the time of sampling. The results suggest that the CKD-induced alterations of the oral milieu might promote a dysbiosis of the commensal oral microbiome, namely the proliferation of clinically relevant *Enterobacteriaceae* potentially harboring acquired antibiotic resistance genes. This study highlights the importance of the oral cavity as a reservoir for pathobionts and antibiotic resistances in CKD patients undergoing peritoneal dialysis.

Keywords: chronic kidney disease, oral microbiome, oral dysbiosis, peritoneal dialysis, *Enterobacteriaceae*, *Raoultella ornithinolytica*, antibiotic resistance

INTRODUCTION

Chronic kidney disease (CKD) is an increasing global health issue, defined as decreased kidney function shown by a glomerular filtration rate of less than 60 ml/min/1.73 m^2, or markers of kidney damage, or both, for a duration of at least 3 months (Webster et al., 2017; Benjamin and Lappin, 2021). CKD is commonly progressive and when patients reach end-stage renal disease, they may require renal replacement therapies, such as hemodialysis, peritoneal dialysis (PD) or kidney transplantation (Murdeshwar and Anjum, 2021). CKD patients exhibit increased mortality and morbidity, especially associated with cardiovascular events (Webster et al., 2017; Benjamin and Lappin, 2021).

The decrease in kidney function leads to the accumulation of substances in the body that in normal conditions would be eliminated in the urine (Lisowska-Myjak, 2014). These substances, known as uremic toxins, build up inside the organism and cause alterations in various body sites, including the oral cavity, with higher levels of urea, ammonia and pH being commonly detected in the saliva of CKD patients (Gupta et al., 2015; Simões-Silva et al., 2017). These changes are thought to be major selective factors responsible for altering the oral microbiome, which has shown significant association with CKD status (Simões-Silva et al., 2017, 2018; Hu et al., 2018).

On the other hand, it has been hypothesized that the oral microbiome may contribute in some extent to the progression of CKD. Three mechanisms have been proposed that link oral infection, especially periodontitis, to secondary systemic effects (Li et al., 2000; Simões-Silva et al., 2018). These include the generalized spread of infection from the oral cavity as a result of transient bacteremia; generalized injury resulting from circulating toxins of oral microbial origin; and systemic inflammation as a result of immunological activation induced by oral microorganisms (Li et al., 2000; Simões-Silva et al., 2018).

Among other complications, the accumulation of uremic toxins in the body leads to a chronic inflammatory status which can cause the suppression of lymphocytes and cell-mediated immunity, making CKD patients more prone to infections than other populations (Gupta et al., 2015). In fact, infectious episodes persist as the main weakness of PD programs, with peritonitis and exit-site/tunnel infections remaining as the most common and relevant concerns for these patients (Mujais, 2006; Liakopoulos et al., 2017). While Gram-positive agents (mainly *Staphylococcus* spp. and *Streptococcus* spp.) are responsible for the majority of infections, Gram-negative bacteria (mainly *Pseudomonas* spp. and members of the family *Enterobacteriaceae*) are more likely to provoke more severe infections with poor outcomes (Li and Chow, 2011; Liakopoulos et al., 2017).

The *Enterobacteriaceae* family is of particular relevance in end-stage renal disease patients. Aside from being recognized agents of nosocomial infections, *Enterobacteriaceae* are responsible for 10 to 12% of all peritoneal dialysis-associated peritonitis, constituting a significant risk for patients on PD programs (Jain and Blake, 2006; Szeto et al., 2006). Regarding the oral microbiome, enterobacteria are frequent inhabitants of periodontal pockets (Gonçalves et al., 2007), potentially contributing to generalized inflammation and disease progression in these patients. Furthermore, *Enterobacteriaceae* are critical level pathogens regarding antibiotic resistance phenotypes. The mechanisms of antibiotic resistance in these bacteria may be intrinsic due to the presence of features encoded by chromosomal genes related with functions such as antibiotic inactivating enzymes and non-enzymatic paths (efflux pumps, permeability or target modifications) that are observed in all or most of the members of a given bacterial species (Ruppé et al., 2015). In contrast, acquired antibiotic resistance is due to gene mutation or, more frequently, to incorporation of genes received through mobile genetic elements, such as plasmids or phages that often harbor genes encoding ß-lactamases, aminoglycosides modifying enzymes, or non-enzymatic mechanisms (Cox and Wright, 2013).

Antimicrobial resistant enterobacteria are quite prevalent in CKD patients and are the cause of serious drug-resistant infectious episodes (Wang et al., 2019). Probably because of frequent hospitalizations and frequent need for antibiotic therapy, patients with CKD tend to have more infections by drug resistant bacteria than people with normal kidney function (Fysaraki et al., 2013; Salloum et al., 2020). In 2014, outpatient hemodialysis facilities in the United States reported that, out of the isolates identified as agents of bloodstream infections and exit-site-related infections, 17.8% of *Escherichia coli* and 14.6% of *Klebsiella* spp. isolates were resistant to cephalosporins and 4.8% of *Enterobacter* spp. isolates were resistant to carbapenems (Nguyen et al., 2017). In another study conducted

in a hemodialysis unit in a hospital in Algeria, 100% of *K. pneumoniae* isolates associated with catheter-related infections produced extended spectrum beta-lactamase and were resistant to at least gentamicin (Sahli et al., 2017). Regarding peritoneal dialysis, in PD patients who developed dialysis-associated peritonitis between 2002 and 2011, 54.3% of Enterobacteriaceae isolates were resistant to third-generation cephalosporins and 76.1% were resistant to fluoroquinolones (ciprofloxacin), and all of them produced extended spectrum beta-lactamase (Prasad et al., 2014). This data shows that a large percentage of enterobacteria responsible for dialysis-related infections in CKD patients also exhibit resistance to one or more antibiotic drugs.

Taking into consideration the above mentioned, this work aimed to assess the prevalence and antibiotic resistance profile of clinically relevant enterobacteria in the oral cavity of CKD patients undergoing PD.

MATERIALS AND METHODS

Participant Recruitment

Chronic kidney disease patients undergoing peritoneal dialysis (CKD-PD, $n = 44$) followed at the outpatient clinic of the Nephrology Department of Centro Hospitalar Universitário de São João were invited to participate in this study. Exclusion criteria for this group included inability to give informed consent, pregnancy, recent history of infection (less than 3 months), recent history of antibiotic therapy (less than 3 months), and severe acute illness. Samples were collected according to patient attendance to the clinic over a period of 4 months. Relevant clinical and demographic information was gathered for each individual through a semi-structured interview and by reviewing the patient's computerized clinical reports. The occurrence of PD-related infectious episodes associated with species from the *Enterobacteriaceae* family was retrieved from clinical records from the date of entrance in the PD program until the moment of oral sample collection.

The control group was recruited from the student body of the Faculdade de Medicina Dentária da Universidade do Porto ($n = 37$). Relevant clinical and demographic information was gathered through a semi-structured interview. Exclusion criteria for the control group included inability to give informed consent, pregnancy, recent history of infection (less than 3 months), recent history of antibiotic therapy (less than 3 months), and compromised oral health (occurrence of gum disease, such as gingivitis or periodontitis, and a caries index including decayed, missing or filled teeth superior to 5). Controls had no history of chronic or systemic diseases (such as diabetes, hypertension, obesity, allergies, autoimmune diseases, etc.), no history of genetic disorders, no history of renal disease, and had not been diagnosed for any other health condition.

The protocols for the study were approved by both the Ethics Committee for Health of Centro Hospitalar Universitário de São João (approval number 200/18) and the Ethics Committee for Health of Faculdade de Medicina Dentária da Universidade do Porto (approval number 12/2019) and followed the 1964 Helsinki declaration and its later amendments; all recruited participants gave their written informed consent.

Sample Collection

Unstimulated whole saliva was collected *via* the spitting method (Navazesh, 1993) into sterile containers, at least 1 h after eating or tooth brushing. Prior to the collection, the subjects did a water mouthwash to minimize typical dry-mouth sensation and to clean the oral cavity. Saliva collection began after swallowing the residual saliva present in the mouth and allowing newly produced saliva to accumulate. Part of the saliva samples (~1 ml) were stored at −20°C until biochemical analysis and the remaining volume was kept refrigerated for microbiological evaluation.

Saliva Biochemical Analysis

The biochemical analysis was carried out using a sequential injection analysis system with potentiometric detection for the determination of urea and ammonia in saliva samples as previously described (Thepchuay et al., 2020), and the pH was measured using pH strips (5.0–8.0, Duotest, Germany and 1–14, Merck, Germany).

Oral Microbiota Isolation and Identification

Up to 2 h after collection, refrigerated saliva samples were serially diluted with sterile 0.9% NaCl solution and were plated in triplicate in MacConkey Agar (VWR Chemicals BDH, Belgium). This culture medium was used in order to target non-fastidious non-strict anaerobic Gram-negative species. After aerobic incubation at 37°C for 48 h, the total number of colonies was counted and the results were expressed in colony-forming units per mL of saliva (CFU/ml).

From each saliva sample, all colonies with distinct appearances were reisolated in the same medium, which resulted in the selection of 2 to 3 isolates per individual saliva sample. Additionally, urease production was tested by incubating colonies on Urea Broth (Panreac, Spain) at 37°C for 24 h. Isolates were then stored in Brain Heart Infusion broth (BHI; Biolab Zrt., Hungary) supplemented with 10% glycerol at −80°C and were later identified by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS, Bruker MALDI Biotyper).

The MALDI-TOF MS analysis was conducted directly from fresh (<24 h) bacterial colonies according to manufacturer's instructions. When necessary, formic acid 70% was added to samples to improve identification. Only isolates which obtained an identification score value equal or higher than 2.0, corresponding to probable identification to species level, were considered properly identified.

Antibiotic Resistance Phenotype and Genotype

Antibiotic resistance phenotype was determined using the Kirby–Bauer disk-diffusion method to test the following antibiotics: amoxicillin (AML, 25 μg), gentamycin (GEN, 10 μg),

TABLE 1 | CKD-PD patients' clinical information, including etiology of CKD, residual renal function, blood pressure, time on PD, and PD-related infection history.

	CKD-PD patients
Etiology of CKD (%)	
Diabetic nephropathy	25.0%
Hepatorenal polycystic disease	20.5%
Glomerular disease	13.7%
Urologic (nephrectomy, neoplasia, chronic PNC)	9.1%
Chronic interstitial nephritis	4.5%
Nephroangiosclerosis	2.3%
Vasculitis	2.3%
Unknown	22.7%
Residual renal function (mL/min)	5.9 ± 4.0
Blood pressure (mmHg)	
Systolic	140 ± 18
Diastolic	80 ± 10
PD vintage (months)	31.7 ± 30.2
Infection history[a] (%)	
Peritonitis	27.3%
By *Enterobacteriaceae*	2.3%
Exit-site infections	45.5%
By *Enterobacteriaceae*	9.1%

Results are shown in prevalence (%) or mean ± SD. [a]Prevalence of infections refers to PD patients that had at least one infection episode from the date of entrance in the PD program until the moment of oral sample collection. CKD, chronic kidney disease; PD, peritoneal dialysis; CKD-PD, chronic kidney disease patients undergoing peritoneal dialysis.

TABLE 2 | Demographic information and biochemical salivary parameters of healthy controls and CKD-PD patients.

	Controls	CKD-PD patients	*p* value
Age (years)	19.7 ± 1.2	55.9 ± 11.0	<0.001
Sex (male %)	18.9%	65.9%	<0.001
Saliva Biochemistry			
pH	6.7 ± 0.3	8.1 ± 0.8	<0.001
Urea (mg/dL)	20.4 ± 14.2	85.2 ± 46.2	<0.001
Ammonia (mg/dL)	16.1 ± 12.8	63.6 ± 38.1	<0.001

Results are shown in prevalence (%) or mean ± SD. CKD-PD, chronic kidney disease patients undergoing peritoneal dialysis.

ciprofloxacin (CIP, 5 μg), sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg), tetracycline (TET, 30 μg), cephalothin (CP, 30 μg), meropenem (MEM, 10 μg), ceftazidime (CAZ, 30 μg), ticarcillin (TIC, 75 μg), colistin (CT, 50 μg), sulfamethoxazole (SUL, 25 μg) and streptomycin (STR, 10 μg; Oxoid™, Hampshire, UK). Assays were conducted as recommended for *Enterobacteriaceae* (CLSI, 2017). As a quality control, *Escherichia coli* strain ATCC 25922 was included in all assays.

PCR-based gene screening (primers and PCR conditions described in **Supplementary Table S1**) used DNA extracts obtained from fresh pure cultures: bacterial colonies were resuspended in 50 μl of nuclease-free water and heated for 10 min at 95°C; subsequently, the mixture was refrigerated on ice for 5 min and centrifuged at 14 000 rpm for 5 min for sedimentation of cell debris; the supernatant was used as template for PCR reactions (Wiedmann-al-Ahmad et al., 1994). PCR gel electrophoresis pictures are presented in **Supplementary Figures S1–S9**. The screened genetic determinants included resistance determinants to β-lactams (bla_{TEM}, bla_{CTX-M}, bla_{OXA} and bla_{SHV}), sulfonamides (*sul1*) and quinolones (*qnrS*), and mobile genetic elements related with genetic recombination and gene acquisition, plasmid replicon types (incF, incHI1 and incHI2), representative of incompatibility groups circulating among *Enterobacteriaceae*, and class 1 integron integrase (*intI1*).

Data Analysis

All data analysis was carried out using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp, NY, United States). When appropriate, the chi-square independence test was used to analyze hypotheses regarding the categorical variables, using continuity correction for 2×2 Tables. T-test for independent samples was used for the continuous variables, using Levene's test for equal variances. In order to evaluate potential confounders like age and sex, binary logistic regression (Enter method) was applied for the presence of *Raoultella ornithinolytica* as dependent variable and the type of participant as another covariate with bootstrapping technique with 95% of confidence. A significance level of 0.05 was considered.

RESULTS

Clinical Characterization of CKD-PD Patients

Clinical information relating to the CKD-PD patients is included in **Table 1**. The most prevalent etiology of CKD was diabetic nephropathy and, at the time of sampling, the average time on PD therapy was 32 months, ranging from 2 months up to 12.4 years. Regarding salivary biochemical parameters, pH, urea, and ammonia levels were all significantly higher in CKD-PD patients compared to healthy controls (**Table 2**).

Oral *Enterobacteriaceae* Prevalence

CKD-PD patients exhibited significantly higher microbial counts and *Enterobacteriaceae* prevalence (**Table 3**). *Enterobacteriaceae* diversity was also higher in CKD-PD patients, with only three species being isolated from healthy controls while seven species (two in common with the controls and an additional five) were isolated from the study group, from a total of eight isolated species (**Table 3**). Six of the total eight species tested positive for urease production (excluding *Escherichia coli* and *Klebsiella aerogenes*; **Table 3**). Out of all *Enterobacteriaceae* species identified, only *Raoultella ornithinolytica* exhibited significantly higher prevalence in CKD-PD patients, being absent from the oral cavity of healthy controls.

Because controls and CKD patients vary significantly in terms of age and gender, binary logistic regression was applied for the presence of *Raoultella ornithinolytica* as dependent variable containing three independent variables: sex, age and type of participants. The full model with bootstrapping technique

TABLE 3 | Bacterial counts in MacConkey Agar and prevalence of participants colonized by urease producing (*) and non-producing *Enterobacteriaceae* species.

	Controls	CKD-PD patients	*P* value
Counts, CFU/ml	$8.8\times10^3 \pm 1.5\times10^4$	$3.1\times10^4 \pm 4.9\times10^4$	0.008
Enterobacteriaceae prevalence, %	10.8%	43.2%	0.003
*Raoultella ornithinolytica**	0%	29.5%	0.001
*Klebsiella oxytoca**	0%	2.3%	>0.999
*Klebsiella pneumoniae**	5.4%	2.3%	0.878
Klebsiella aerogenes	0%	2.3%	>0.999
*Enterobacter asburiae**	2.7%	4.5%	>0.999
Escherichia coli	2.7%	0%	0.930
*Citrobacter koseri**	0%	2.3%	>0.999
*Citrobacter freundii**	0%	2.3%	>0.999

*Results are shown in prevalence (%) or mean ± SD. *Species that tested positive for urease production. CKD-PD, chronic kidney disease patients undergoing peritoneal dialysis.*

TABLE 4 | Total number of PD-related infection episodes, including peritonitis and exit-site infections (ESI), and the infectious agent identified.

Infectious agent	PD-related infection episodes (n = 50)	
	Peritonitis (*n* = 13)	ESI (*n* = 37)
Others	12 (92.3%)	31 (83.8%)
Enterobacteria	1 (7.7%)	6 (16.2%)
Klebsiella pneumoniae	0	2 (5.4%)
Klebsiella oxytoca	1 (7.7%)	0
Enterobacter spp.	0	3 (8.1%)
Escherichia coli	0	1 (2.7%)

PD, Peritoneal dialysis.

containing all predictors was statically significant (χ^2 (3, N = 81) = 18.041, $p < 0.001$). According to the bootstrapping technique, only the type of participants (controls or CKD) made a unique statistically significant contribution ($p < 0.001$) in the model (CKD patients' coefficient, B = 20.299; CI95% = 17.675; 22.954). On the other hand, statistical significance was not attained for sex nor age, respectively ($p = 0.760$) and ($p = 0.956$).

Enterobacteriaceae-Related Infection History

During the complete period of PD therapy, around one quarter of the studied population had at least one peritonitis and around half of the patients had at least one ESI (**Table 1**). When one looks to the total number of infectious episodes (**Table 4**), *Enterobacteriaceae* were responsible for 7.7% of all peritonitis and for 16.2% of all ESI episodes, and the identified infectious agents include the genera *Klebsiella*, *Enterobacter* and *Escherichia*. No association was found between previous *Enterobacteriaceae* infectious agents and the oral enterobacteria colonization of the CKD-PD patients at the time of sampling.

Antibiotic Resistance Profiling

Enterobacteriaceae isolates ($n = 23$) obtained from different individuals exhibited mainly intrinsic antibiotic resistance phenotypes (CLSI, 2017; EUCAST, 2020; **Supplementary Table S2**). Specifically, resistance to amoxicillin (in all isolates of *Klebsiella* spp., one isolate of *Citrobacter freundii*, 2 out of 3 isolates of *Enterobacter* spp., and 12 out of 14 isolates from *R. ornithinolytica*), resistance to ticarcillin (2 out of 3 isolates of *Klebsiella* spp. and in 10 out of 14 isolates from *R. ornithinolytica*), and resistance to cephalothin (in all isolates of *Enterobacter* spp.) were observed. In addition to intrinsic antibiotic resistance, sulfamethoxazole (in *Enterobacter asburiae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Raoultella ornithinolytica* isolates), and streptomycin (in *Enterobacter asburiae* and *Raoultella ornithinolytica* isolates) resistances were also observed. Only 6 isolates (2 isolates of *Enterobacter asburiae* and 4 of *Raoultella ornithinolytica*) exhibited multidrug resistance, all of them originated from CKD-PD patients. These results are depicted in **Supplementary Table S2**. Overall, out of the 23 *Enterobacteriaceae* isolates, about 83% were resistant to amoxicillin, 52% showed resistance to ticarcillin, 43% to sulfamethoxazole, 35% to ciprofloxacin and 17% to streptomycin. 100% of the isolates were resistant to at least one of the antibiotics tested. The percentage of sensitivity to the studied antibiotics is presented in **Supplementary Figure S10**.

Among the genes screened, neither the antibiotic resistance genes bla_{TEM}, bla_{OXA} and *qnrS*, nor the plasmid replicon types incHI1 and incHI2 were detected. The other genetic determinants were observed in one *Escherichia coli* isolate from a healthy control (*sul1*); in 2 of the 3 *Enterobacter asburiae* isolates from CKD-PD patients and controls (*sul1*, $n = 1$ and *sul1* and *intI1*, $n = 1$); in the *Klebsiella oxytoca* isolate from a CKD-PD patient ($bla_{CTX\text{-}M}$, normally associated with the plasmid replicon type incF); in 2 isolates of *Klebsiella pneumoniae* from controls (bla_{SHV}, $n = 2$, probably intrinsic in this species, and *sul1*, $n = 1$); and in 4 of the 14 *Raoultella ornithinolytica* isolates from CKD-PD patients (*sul1* and *intI1*, $n = 3$ and *intI1*, $n = 1$). These results are depicted in **Supplementary Table S2**. Overall, the bla_{SHV} gene alone or with *sul1* and $bla_{CTX\text{-}M}$ with replicon type incF were detected in 4.3% of isolates. The *sul1* gene alone was found in 8.7% of isolates and *sul1* with *intI1* were found in 17.4% of isolates. None of the tested genes were detected in approximately 57% of isolates. The prevalence of these genetic elements is presented in **Supplementary Figure S11**.

DISCUSSION

Overall, our results show that the accumulation of uremic toxins in patients with advanced CKD and the consequent changes in the oral milieu (such as the increase in salivary urea, ammonia and pH) may indeed act as selecting factors on the oral microbiome, leading to an oral dysbiosis with proliferation of pathobionts in the oral cavity. In fact, 6 of the 8 species identified in **Table 3** tested positive for urease production. Thus, it was observed that more urease-producing species were found in the oral cavity of CKD patients in comparison to the oral cavity of healthy controls, which is expected to be due to the previously reported adaptation of the microbiome to the altered conditions of the oral milieu, evidenced in **Table 2** (Hu et al., 2018; Rysz et al., 2021).

The increased prevalence of *Enterobacteriaceae* spp. is significantly relevant in CKD patients. While enterobacteria are only occasionally detected in the oral cavity of healthy individuals, colonization is much more prevalent in immunocompromised patients, people who come in contact with hospital environments frequently, and people suffering from hyposalivation, oral lesions, and systemic disease, a scenario that fits CKD patients (Peleg and Hooper, 2010; Posse et al., 2017). Perhaps, all these reasons also contributed to the higher prevalence of *Enterobacteriaceae* in the oral microbiota of our CKD patients as well as to the fact that *Enterobacteriaceae* were responsible for approximately 8% of the peritoneal dialysis-associated peritonitis and 16% of exit-site infections in our group of patients, highlighting the clinical relevance of our results.

The increased prevalence of *Raoultella ornithinolytica* in CKD patients undergoing PD is a particularly significant finding. Formerly classified as *Klebsiella ornithinolytica*, *R. ornithinolytica*, a histamine-producing aquatic-commensal enterobacteria, is a virulent pathogen of community-acquired and emerging nosocomial infections, particularly associated with invasive procedures (Seng et al., 2016; Hajjar et al., 2018, 2020). *R. ornithinolytica* is able to occasionally survive in human saliva and a case report has pointed it as a cause of primary peritonitis in humans (Sibanda, 2014; Seng et al., 2016), thus aggravating the risk of its presence in the oral cavity of patients undergoing peritoneal dialysis. Regarding the oral microbiome, the high rate of colonization by *Raoultella ornithinolytica* is a very interesting finding in itself, since this species is still not described in the Human Oral Microbiome Database.[1]

Although no association was found between previous *Enterobacteriaceae* infectious agents in the group of CKD patients and oral colonization by enterobacteria at the time of sampling, the difficulty in the identification of *R. ornithinolytica* and common misidentification as belonging to the *Klebsiella* genus should be considered, as a misidentification of the infectious agent is a possibility (Ponce-Alonso et al., 2016). Additionally, the hypothesis of an oral-peritoneal association regarding infection cannot be discarded without further studies, especially when all the *Enterobacteriaceae*-related infectious episodes reported in these patients occurred at least 1 year prior to the collection of the samples used in this study.

It is important to note that all the species identified in this study are clinically relevant and constitute potential cause of infection, with many being associated with PD-related infections (Barraclough et al., 2009; Li and Chow, 2011). Although contamination concerns for patients undergoing peritoneal dialysis typically tend to focus on contamination from external origins, the oral microbiome may be a major source of contamination both through external and internal routes (Li and Chow, 2011; Smith and Nehring, 2021). Bacteria inhabiting the oral cavity can translocate into the bloodstream as a result from routine daily activities, such as tooth brushing or invasive dental procedures, becoming opportunistic infectious agents in distant body sites such as the peritoneum (Smith and Nehring, 2021). In fact, the risk associated with the presence of pathogenic bacteria in the oral-nasal cavities of CKD patients is already recognized, which is why these patients are frequently screened for nasal carriage of *Staphylococcus aureus* as a way to prevent infection and the efficacy of prophylactic intranasal antibiotics for the treatment of confirmed nasal carriage of *S. aureus* has been tested in several prospective studies (Grothe et al., 2014; Li et al., 2016). Furthermore, the presence of these bacteria in the oral cavity can stimulate the immune system and contribute to the inflammatory state in CKD, which then leads to cardiovascular events, the main cause of death in these patients (Li et al., 2000; Webster et al., 2017; Höfer et al., 2021).

The oral cavity has also recently been highlighted as a potential reservoir of antibiotic resistance genes (Roberts and Mullany, 2010; Jiang et al., 2018; de Sousa Moreira Almeida et al., 2020). Resistance against amoxicillin was observed in 83% of isolates, although in these cases it is possibly intrinsic (Cox and Wright, 2013). However, putative acquired genetic elements related with antibiotic resistance were observed in 10 out of 23 oral isolates, 3 from healthy individuals (*sul1* in *Escherichia coli* and bla_{SHV} in *Klebsiella pneumoniae*) and 7 from CKD patients (*sul1*, *intI1*, bla_{CTX-M} and incF in *Enterobacter asburiae*, *Raoultella ornithinolytica* and *Klebsiella oxytoca*). Curiously, the 5 isolates harboring the gene *intI1* and the one exhibiting the high mobile replicon type incF and bla_{CTX-M} genes all originated from CKD patients. These observations raise the interest to investigate the vulnerability of some population groups to be colonized by bacteria harboring acquired antibiotic resistance genes and mobile genetic elements (de Sousa Moreira Almeida et al., 2020). Overall, regarding the resistance phenotype, 100% of the *Enterobacteriaceae* isolates were resistant to at least one of the antibiotics tested and only 6 isolates, all originating from CKD-PD patients, exhibited multidrug resistance. Genetic elements related to resistance were also detected in approximately 43% of isolates. Antibiotic-resistant enterobacteria are known agents of dialysis-associated infections in CKD patients, frequently originating serious life-threatening complications (Wang et al., 2019). Therefore, it is important to keep screening isolates from CKD patients for antimicrobial resistances, in order to plan efficient therapeutic interventions in case of infection.

[1]http://www.homd.org/

Our study presents several strengths and some limitations. The fact that the control and CKD groups differ in age and gender may constitute a limitation of our study, although a binary logistic regression was applied using the prevalence of *Raoultella ornithinolytica* (the only member of the *Enterobacteriaceae* family with a significantly different prevalence between groups) as the dependent variable and sex, age, and type of participants as independent variables, confirming that only the type of participants (controls or CKD-PD) made a unique statistically significant contribution. Moreover, though ideally the groups should display similar demographic characteristics, several studies have reported that the oral microbiome does not suffer significant alterations throughout adulthood, with no significant differences being reported by gender or age groups (adults and young adults) at species level. Alterations in the healthy oral microbiota are only significant before adulthood or after the age of 65–70 years-old (Crielaard et al., 2011; Belstrøm et al., 2014; Lira-Junior et al., 2018; Verma et al., 2021). As so, the healthy young adults constituting the control group in this study are nevertheless representative of healthy reference values. Regarding methodology, although MALDI-TOF MS is a universally used method that has been routinely used in clinical microbiology (He et al., 2010; Tsuchida et al., 2020) and is considered extremely adequate for the identification of enterobacteria (Richter et al., 2013), it presents some limitations, as is the example of the inability to differentiate *Escherichia coli* from *Shigella* species (Devanga Ragupathi et al., 2017).

When one looks to the study strengths, firstly, it highlights how the uremic state in CKD can cause a dysbiosis of the oral microbiome and lead to a proliferation of enterobacteria, which are clinically relevant pathogens for patients on PD programs. Additionally, the identification of *Raoultella ornithinolytica* as a colonizer of the oral cavity is of extreme importance. The isolation of *R. ornithinolytica* from saliva samples has been reported before in the literature (Heggendorn et al., 2013; Derafshi et al., 2017), but never in such a high prevalence as in this study and never as an oral colonizer of CKD patients. It is important to keep in mind that this is an emerging species both in terms of infectious episodes in humans and in terms of resistance to antibiotic agents (Seng et al., 2016; Hajjar et al., 2020), two factors that make *R. ornithinolytica* a clinically relevant cause of concern, not only to CKD patients, but to the general population. The profiling of *Enterobacteriaceae* isolates in terms of antibiotic resistance, both regarding resistance phenotypes and the presence of genetic elements, is another strong point of this paper. As mentioned before, antibiotic-resistant enterobacteria are responsible for a significant percentage of infections in CKD patients (Prasad et al., 2014; Nguyen et al., 2017; Sahli et al., 2017; Wang et al., 2019), which makes their screening crucial for the clinical follow-up of said patients, given it can provide information for efficient antibiotic prescription in case of infection. Of note that infections are a major problem in peritoneal dialysis programs, leading to patients' deaths or exclusion from peritoneal dialysis programs (Li et al., 2016). Therefore, the search of predisposing factors that lead to peritonitis and catheter-related infections in CKD-PD patients is of outmost importance. Finally, our study also contributes to the profiling of antibiotic resistance genes in the oral microbiome. In recent years, the oral cavity has been highlighted as a reservoir for resistance genes (de Sousa Moreira Almeida et al., 2020) and the profiling of these genes provides extremely relevant information for dentistry and the treatment of oral infections.

CONCLUSION

Our results suggest that the accumulation of uremic toxins in CKD patients induces alterations in the oral milieu, which, in turn, exert selective pressure on the oral microbiome, leading to its dysbiosis with significantly increased prevalence of urease-producing enterobacteria, specifically of *Raoultella ornithinolytica*, an opportunistic enterobacteria. Antibiotic resistance phenotyping revealed mostly intrinsic resistances, although resistances to sulfamethoxazole and streptomycin were also detected. Multidrug resistance was exclusively found in CKD-PD patients isolates. These results, together with the detection of acquired antibiotic resistance genes, alert to the possibility of the oral microbiota as a potential reservoir of resistance genes.

Enterobacteriaceae are known opportunistic pathogens of CKD patients, and this CKD-induced oral dysbiosis can potentially lead to an increase of resistant infectious and inflammatory episodes, contributing, in turn, to the progression of the disease and to its mortality rate. For all these reasons, studying the oral microbiome and its associated resistome and comprehend its role in infection episodes and in the progression of systemic diseases is of extreme relevance to ensure the health and well-being of CKD patients undergoing peritoneal dialysis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee for Health of Centro Hospitalar Universitário de São João: (approval number 200/18) and Ethics Committee for Health of Faculdade de Medicina Dentária da Universidade do Porto: (approval number 12/2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM, AR, and BS-M contributed to conception and design of the study. CC, AM-R, CC, NS, LP, CF and AG contributed to data acquisition. CC, AM-R, CC, CF, RM, AR, CM and BS-M contributed to data analysis and interpretation. CC, CF,

AA, RM, AR, CM and BS-M performed the statistical analysis. CC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Saliva Quantification of SARS-CoV-2 in Real-Time PCR From Asymptomatic or Mild COVID-19 Adults

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The fast spread of COVID-19 is related to the highly infectious nature of SARS-CoV-2. The disease is suggested to be transmitted through saliva droplets and nasal discharge. The saliva quantification of SARS-CoV-2 in real-time PCR from asymptomatic or mild COVID-19 adults has not been fully documented. This study analyzed the relationship between salivary viral load on demographics and clinical characteristics including symptoms, co-morbidities in 160 adults diagnosed as COVID-19 positive patients recruited between September and December 2020 in four French centers. Median initial viral load was 4.12 $\log_{10}$ copies/mL (IQR 2.95–5.16; range 0–10.19 $\log_{10}$ copies/mL). 68.6% of adults had no viral load detected. A median load reduction of 23% was observed between 0–2 days and 3–5 days, and of 11% between 3–5 days and 6–9 days for the delay from onset of symptoms to saliva sampling. No significant median difference between no-symptoms vs. symptoms patients was observed. Charge was consistently similar for the majority of the clinical symptoms excepted for headache with a median load value of 3.78 $\log_{10}$ copies/mL [1.95–4.58] ($P < 0.003$). SARS-CoV-2 RNA viral load was associated with headache and gastro-intestinal symptoms. The study found no statistically significant difference in viral loads between age groups, sex, or presence de co-morbidity. Our data suggest that oral cavity is an important site for SARS-CoV-2 infection and implicate saliva as a potential route of SARS-CoV-2 transmission.

Keywords: SARS-CoV-2, COVID-19, saliva, viral load, virus isolation, real-time reverse transcription PCR, infectivity, quantitative

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INTRODUCTION

The fast spread of COVID-19 is related to the highly infectious nature of SARS-CoV-2. The disease is suggested to be transmitted through saliva droplets and nasal discharge (Li Y. et al., 2020). Indeed, SARS-CoV-2 is found in nasopharyngeal secretions, and its viral load is consistently high in the saliva, mainly in the early stage of the disease (Sapkota et al., 2020). In addition to saliva secreted

by the major or minor salivary glands, saliva samples also contain secretions from the nasopharynx or from the lungs through the action of cilia lining the airway (Huang et al., 2021). Saliva can have potential applications in the context of COVID-19 by direct detection of the virus, quantification of the specific immunoglobulins produced against it, and for the evaluation of the non-specific, innate immune response of the patient (Ceron et al., 2020). Up to date, several cross-section and clinical trial studies published support the potential of detecting SARS-CoV-2 RNA in saliva as a biomarker for COVID-19, providing a self-collection, non-invasive, safe, and comfortable procedure (Caixeta et al., 2021; Carrouel et al., 2021).

Most published RT-qPCR assays for the diagnosis of SARS-CoV-2 are qualitative (Han et al., 2021). Considering the variability of viral load between and within patients, quantification of absolute viral load directly from pure raw saliva is important for COVID-19 diagnosis and monitoring (Vasudevan et al., 2021). Quantification of viral load has other objectives compared to screening. It gives an indication of the degree of contagiousness, provides guidance on the interest to predict contagiousness of patients and hence to guide epidemiological decisions, to evaluate different samples from different anatomical locations, to predict the patient prognosis and assess disease progression (Jacot et al., 2020). In this context, evidence reports on the salivary viral load or duration of viral detection or infectivity of COVID-19 to trace the disease and to implement strategies aimed at breaking the chain of disease transmission are particularly important (Huang et al., 2021). Overall, the studies were of low-to-moderate quality given that most of the included studies comprised case series and case reports at the early stages of the pandemic (Nasserie et al., 2021). Furthermore, given the number of studies analyzing the Chinese population, it is quite possible that the results cannot be generalized to other populations, especially to asymptomatic cases linked to 80% of disease transmission (Walsh et al., 2020). There is consensus that these results should be viewed with caution and should be confirmed by larger, more robust studies. It is clear that more research is needed on this topic to correlate viral load with symptoms and clinical outcomes (Mahallawi et al., 2021).

Hart et al. (2021) showed that about 65% of virus transmission occurs before symptoms develop. Thus, individuals circulate in the general community before their infections are detected (Hart et al., 2021). To better understand SARS-CoV-2 infectiousness before symptoms develop or with mild symptoms, we analyzed salivary viral load at the moment of disease. The purpose of this study is to outline the salivary SARS-CoV-2 viral load over the course of the infection in asymptomatic or mild COVID-19 adults.

MATERIALS AND METHODS

Study Design and Subjects

This analysis was a part of the randomized controlled trial BBCovid protocol published (Carrouel et al., 2020) and registered at ClinicalTrials.gov (NCT04352959). One hundred and sixty subjects diagnosed as COVID-19 positive patient were recruited between September and December 2020 in four French hospital centers. The study protocol was reviewed and approved by the National Ethics Committee "Committee for the Protection of Persons South Mediterranean III," France (2020.04.11 sept _20.04.06.46640). Written informed consent was obtained from all enrolled individuals. The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization–Good Clinical Practice guidelines.

The inclusion criteria were: (i) age 18–85 years-old, (ii) RT-PCR positive COVID-19 nasopharyngeal swab, (iii) asymptomatic or mild clinical symptoms for less than 8 days.

The exclusion criteria were: (i) pregnancy, (ii) breastfeeding, (iii) risk of infectious endocarditis, (iii) use of mouthwash regularly (more than once a week), (iv) inability to comply with protocol, (v) lack of written agreement, (vi) unable to answer questions, and (vii) uncooperative patients.

Collecting of Demographic Data and Clinical Characteristics of Subjects

During the inclusion visit, demographic data (age, sex) and clinical characteristics (co-morbidities, symptoms, date of apparition of symptoms. . .) were collected from the subjects. Reporting of the nature of the symptoms was recorded at the time of the viral load measurement. An electronic medical record (e-CRF) permitted to record all these informations.

Quantification of SARS-CoV-2 Salivary Load

Saliva Sampling

At 9 a.m., each subject collected one salivary sample with an accredited health care professional using the Saliva Collection System kit (Greiner Bio-one, Kremsmünster, Austria). Only one sample was collected from each adult. Firstly, with the saliva extraction solution, the subject rinsed the oral cavity for 2 min. Secondly, the subject spit into a saliva-collection beaker, without coughing or scraping to clear the throat, to collect the non-stimulated and pure saliva (between 1 and 3.5 mL). Finally, the saliva sample transferred to a sterile tube and stored at 4°C until analysis.

Quantification of SARS-CoV-2 Viral Load by Real-Time RT-PCR

RNA was extracted from 200 μL of saliva sample using the NucliSens easyMAG instrument (bioMérieux, Marcy-l'Etoile, France), according to the manufacturer's guidelines. The RNA was eluted in 50 μL of water and used as a template for real-time (rt) RT-PCR.

Real-time RT-PCR assays were performed with the Invitrogen Superscript^{TM} III Platinum One-Step qRT-PCR system (Invitrogen, Illkirch, France). The mix was composed of 5 μL of extracted RNA, 1 μL of Superscript III RT/Platinum Taq Mix, 12.5 μL of 2X reaction buffer, 0.4 μL of a 50 mM magnesium sulfate solution, 1 μL of RdRp-IP2 forward primer (0.4 μM), 1 μL of RdRp-IP2 reverse primer (0.4 μM), 1 μL of

RdRp-IP4 forward primer (0.4 μM), 1 μL of RdRp-IP4 reverse primer (0.4 μM). The primer and probe sequences correspond to the RdRp-IP2 and the RdRp-IP4 assays designed at The Institut Pasteur to target a section of the RdRp gene (nt 12621-12727 and 14010-14116 positions) based on the sequences of SARS-CoV-2 (NC_004718) made available on the Global Initiative on Sharing All Influenza Data data-base on 11 January 2020 (**Table 1**). These primers and probes were manufactured by Eurofins (Genomics, Germany).

The assays were performed on a Quant Studio 5 (Thermo Fisher Scientific, Dardilly, France) with the following program: 55°C for 10 min (reverse transcription), followed by 95°C for 2 min, and then 45 cycles of 95°C for 3 s and 58°C for 30 s. Each run included three negatives' samples bracketing unknown samples during RNA extraction, two positive controls, and one negative amplification control. When a sample was positive for RdRp-IP4, the quantification of the number of RNA copies was performed according to a scale ranging from 10^2 to 10^6 copies per μL. The SARS-CoV-2 viral load in saliva was calculated as the number of RNA copies per mL of saliva.

Statistical Analysis

SPSS Windows 20.0 (IBM, Chicago, IL, United States) was used for the descriptive statistics median values and interquartile range (IQR) and mean values with SD for the quantitative variables and percentages for categorical variables. One-way ANOVA was used to compare the differences of median between groups in a univariate analysis. Binary logistic regression analysis was used to model the relationship between viral load values as the dependent variable and the other parameters were entered individually as independent variables (adjusted for age and gender). The detailed statistical methods are indicated in the table footnotes. All data were considered statistically significant when $p < 0.05$.

RESULTS

Demographic Data and Clinical Characteristics of Subjects

The sex, the age and the clinical assessments of the study group are summarized in **Table 2**. The sample consisted of 160 subjects (55.97% of females and 44.03% of males) with a mean age of 43.62 ± 15.56 years. One co-morbidity was declared by 22.15% of subjects. The median time between symptoms onset and the positive nasopharyngeal RT-PCR was 4 days [IQR 3–5]. The median number of symptoms per subjects was 4 [IQR 2–5]. The most frequent symptoms described by 52.87% of subjects were the cough and the headache followed by myalgia in 48.41% of subjects. Fever was reported in 41.40% of subjects; anosmia in 40.13% of subjects; ageusia in 38.22% of subjects; dyspnea in 12.74% of subjects and; gastro intestinal symptoms in 8.92% of subjects. The absence of symptoms was only observed in 8.92% of subjects. For symptomatic subjects, the saliva sample was collected in median 6 days [IQR 5–7] after the onset of symptoms.

Quantification of SARS-CoV-2 Viral Load According to Demographics and Clinical Characteristics of Participants

Median initial viral load was 4.12 $\log_{10}$ copies/mL (IQR 2.95–5.16 $\log_{10}$ copies/mL, range 0–10.19 $\log_{10}$ copies/mL). The first quartile (Q1) corresponded to a viral load starting at 2.95 $\log_{10}$ copies/mL, whereas the second (Q2) corresponded to a viral load starting at 4.12 $\log_{10}$ copies/mL, and the third (Q3) corresponded to a viral load starting at 5.16 $\log_{10}$ copies/mL.

The SARS-CoV-2 salivary viral load according to demographics and clinical characteristics of participants are described in **Table 3** and **Figures 1**, **2**. The median SARS-CoV-2 salivary viral load increased not significantly with age groups from 3.82 $\log_{10}$ copies/mL (18–34 years) to 4.31 $\log_{10}$ copies/mL (55–77 years). Salivary viral load was not associated with sex. The presence of co-morbidity or not, did not significantly modify the SARS-CoV-2 salivary viral load. Same observation was done for the time between RT-PCR and symptom onset, the delay from symptom onset to saliva sampling and for the number of symptoms per participants. Patients with symptoms demonstrated a median initial viral load of 4.12 $\log_{10}$ copies/mL (IQR 2.95–5.16; range 0–10.19 $\log_{10}$ copies/mL), while the no-symptoms patients had indices of 4.01 $\log_{10}$ copies/mL (IQR 0.56–5.75 $\log_{10}$ copies/mL, range 0–6.24 $\log_{10}$ copies/mL).

Determination of Factors Associated With SARS-CoV-2 Salivary Viral Load

The analysis of the mean difference of SARS-CoV-2 salivary load was reported in **Table 4**. For the delay from onset of symptoms

TABLE 1 | RT-PCR for the detection of SARS-CoV-2: primers and probes used.

Name	Sequences (5′–3′)	PCR product	References
RdRp gene/nCoV_IP2			
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	108 pb	CNR*
nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT		CNR*
nCoV_IP2-12669bProbe(+)	[5′]HEX-AGATGTCTTGTGCTGCCGGTA-[3′]BHQ-1		CNR*
RdRp gene/nCoV_IP4			
nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	107 pb	CNR*
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG		CNR*
nCoV_IP4-14084Probe(+)	[5′]Fam—TCATACAAACCACGCCAGG—[3′]BHQ-1		CNR*

**CNR: National Reference Center for Respiratory Viruses, Institut Pasteur, Paris, France.*

TABLE 2 | Baseline characteristics of enrolled patients with Coronavirus Disease 2019.

Variable	
Gender, n/N* (%)	
Male	70/159 (44.03%)
Female	89/159 (55.97%)
Age (years)	N = 158
Mean ± SD	43.62 ± 15.56
Median [IQR]	43 [30–55]
Age (class), n/N* (%)	
18–34 years	52/158 (32.91%)
35–54 years	65/158 (41.14%)
55–77 years	41/158 (25.95%)
Co-morbidity, n/N* (%)	33/149 (22.15%)
Time between positive RT-PCR and symptom onset (days)	N = 153
Mean ± SD	3.93 ± 1.46
Median [IQR]	4 [3–5]
Time between positive RT-PCR and symptom onset (class), n/N* (%)	
0–2 days	27/153 (17.65%)
3–5 days	108/153 (70.59%)
6–8 days	18/153 (11.76%)
Number of symptoms per subjects	N = 157
Mean ± SD	3.71 ± 2.27
Median [IQR]	4 [2–5]
Number of symptoms per subjects (class), n/N* (%)	
None	14/157 (8.92%)
1–4	40/157 (25.48%)
5–6	65/157 (41.40%)
7–9	38/157 (24.20%)
Symptoms, n/N* (%)	
None	14/157 (8.92%)
Fever	65/157 (41.40%)
Cough	83/157 (52.87%)
Dyspnea	20/157 (12.74%)
Headache	83/157 (52.87%)
Myalgia	76/157 (48.41%)
Gastro symptoms	14/157 (8.92%)
Anosmia	63/157 (40.13%)
Ageusia	60/157 (38.22%)
Delay from symptom onset to saliva sampling (days)	N = 141
Mean ± SD	5.55 ± 1.62
Median [IQR]	6 [5–7]
Delay from symptom onset to saliva sampling (class), n/N* (%)	
0–2 days	7/141 (4.96%)
3–5 days	57/141 (40.43%)
6–9 days	77/141 (54.61%)

n, Number of subjects, N, Total number of subjects.
**n/N, Number of subjects/Total number of subjects.*

to saliva sampling, the SARS-CoV-2 salivary load continuously decreased with the of the days. A median load reduction of 23% was observed between 0–2 days and 3–5 days, and of 11% between 3–5 days and 6–9 days. However, no significant difference between the groups was detected.

TABLE 3 | Association of viral Load with demographics and clinical parameters for all enrolled participants: N: number of subjects.

Variable	N	Viral load, median [95% CI]	*p*-value
Sex			0.290[a]
Male	70	4.48 [2.99–5.54]	
Female	89	4.02 [2.18–4.69]	
Age			0.290[b]
18–34 years	52	3.82 [2.89–4.57]	
35–54 years	65	4.14 [2.72–5.72]	
55–77 years	41	4.31 [2.94–5.62]	
Co-morbidity			0.856[a]
No	116	4.12 [2.95–5.15]	
Yes	33	4.12 [3.01–5.5]	
Time between positive RT-PCR and symptom onset			0.637[b]
0–2 days	27	4.33 [2.62–5.09]	
3–5 days	108	4.13 [2.94–5.48]	
6–8 days	18	4.08 [0.81–4.82]	
Delay from symptom onset to saliva sampling			0.206[c]
0–2 days	7	5.63 [3.91–5.88]	
3–5 days	57	4.34 [2.75–5.47]	
6–9 days	77	3.86 [2.96–4.72]	
Number of symptoms per subjects			0.573[b]
1–4	40	3.85 [2.16–4.75]	
5–6	65	4.29 [3.4–5.49]	
7–9	38	3.93 [2.98–5.16]	
None	14	4.01 [0.56–5.75]	
Symptoms			
Fever			0.504[a]
No	92	4.07 [2.6–5.01]	
Yes	65	4.24 [3.04–5.47]	
Cough			0.097[a]
No	74	3.83 [2.05–4.74]	
Yes	83	4.14 [3.03–5.48]	
Dyspnea			0.627[a]
No	137	4.12 [2.75–5.17]	
Yes	20	4.13 [2.77–5.04]	
Headache			0.004[a]
No	74	3.78 [1.95–4.58]	
Yes	83	4.51 [3.32–5.59]	
Myalgia			0.721[a]
No	81	4.14 [2.25–5.02]	
Yes	76	4.10 [2.98–5.29]	
Gastrointestinal symptoms			0.212[a]
No	143	4.12 [2.95–5.17]	
Yes	14	3.88 [0–4.57]	
Anosmia			0.126[a]
No	94	4.29 [2.95–5.5]	
Yes	63	3.78 [2.7–4.54]	
Ageusia			0.147[a]
No	97	4.26 [2.94–5.49]	
Yes	60	3.78 [2.71–4.59]	

[a]*Mann-Whitney test.*
[b]*Kruskal-Wallis test.*
[c]*ANOVA test.*

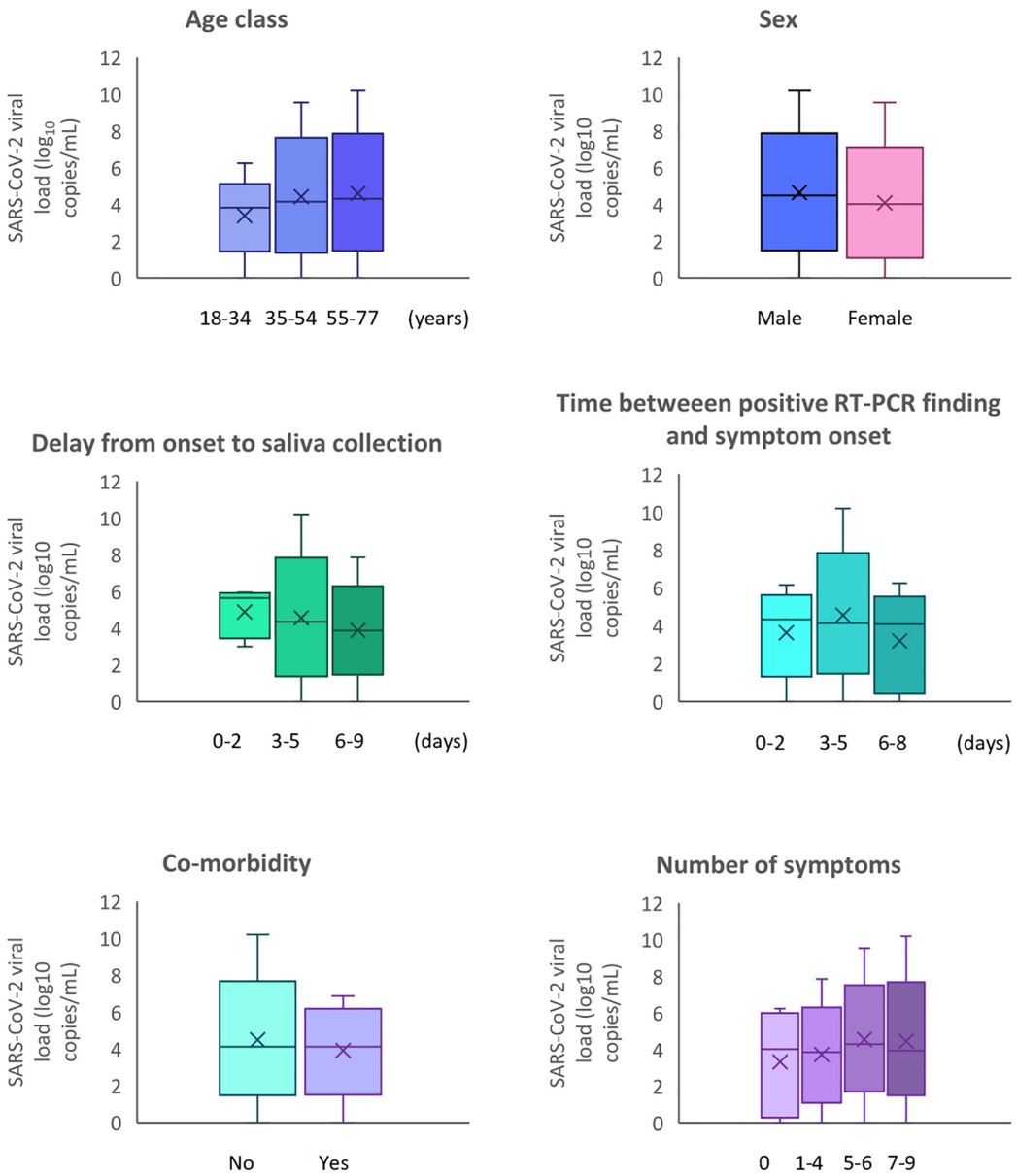

FIGURE 1 | SARS-CoV-2 viral load according to demographics and clinical characteristics of participants.

Concerning the time between positive RT-PCR and symptom onset, a non-significant decrease of −0.12 log_{10} copies/mL [IQR −0.34–0.10] was observed. First, an increase 0.26 log_{10} copies/mL [IQR −0.67–1.18] was observed at 3–5 days compared with 0–2 days. Then, a decrease of −0.39 log_{10} copies/mL [IQR −1.70–0.92] was observed at 6–8 days.

Between no-symptoms patients with an initially salivary SARS-CoV-2 load of 4.01 log_{10} copies/mL [IQR 0.56–5.75] and patients with symptoms (**Table 3**), there was no significant median difference in the viral load (**Table 4**). However, COVID-19 adults declaring 5–6 or 7–9 symptoms have a higher viral median difference charge of 0.56 log_{10} copies/mL [IQR −0.71–1.83] and 0.50 log_{10} copies/mL [IQR −0.85–1.85] respectively.

The salivary SARS-CoV-2 viral load was consistently similar for the majority of the clinical symptoms concerned even if SARS-CoV-2 salivary load increased in presence of fever 0.24 log_{10} copies/mL [IQR −0.46–0.93], cough 0.58 log_{10} copies/mL [IQR −0.10–1.26], dyspnea 0.50 log_{10} copies/mL [IQR −0.53–1.53] and myalgia 0.19 log_{10} copies/mL [IQR −0.50–0.88]. With a median difference increase of 1.04 log_{10} copies/mL [IQR 0.37–1.71], headache in confirmation of symptomatic cases influence significantly the salivary viral load ($P < 0.003$) (**Table 4**). The patients without headache had a mean salivary viral load of 3.78 log_{10} copies/mL [IQR 1.95–4.58] whereas patients with headache had a mean salivary viral load of 4.51 log_{10} copies/mL [IQR 3.32–5.59] (**Table 3**).

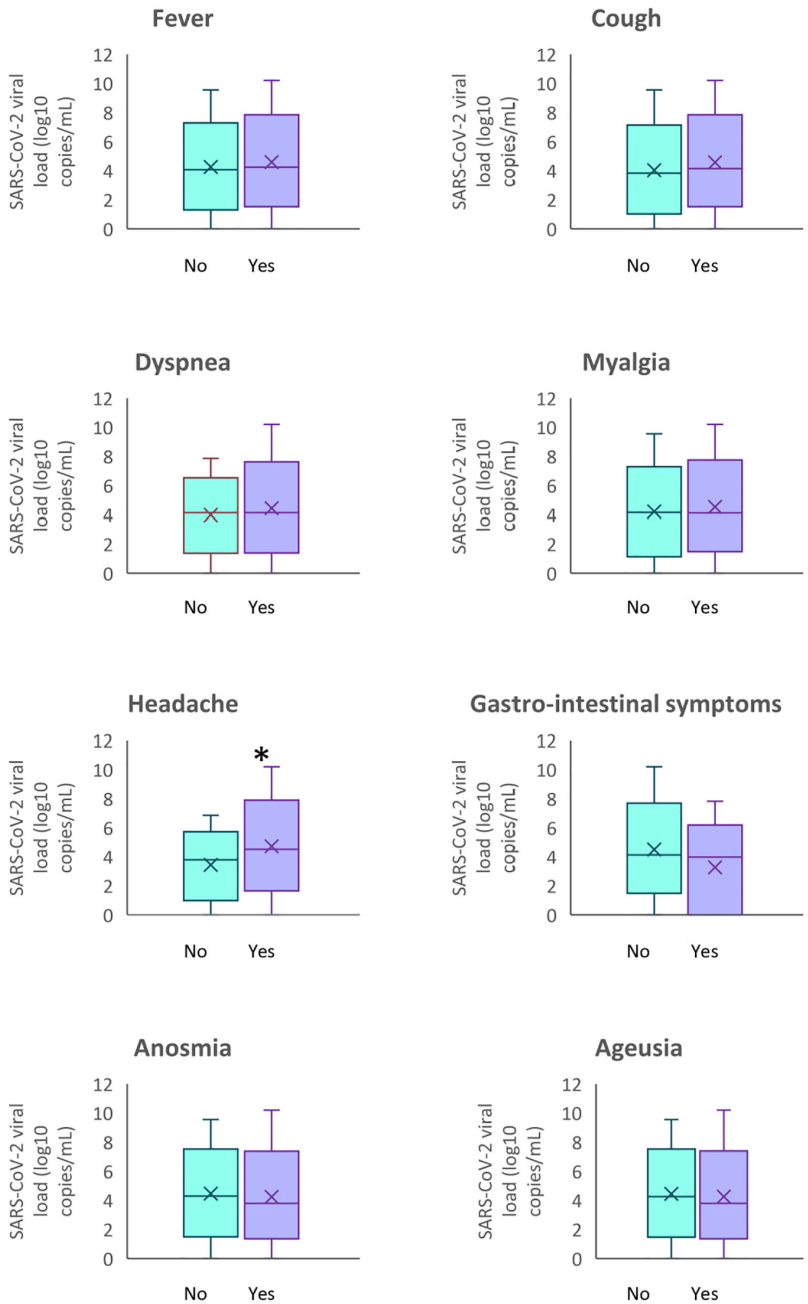

FIGURE 2 | SARS-CoV-2 viral load according to the symptoms of participants. *$p < 0.05$.

In multivariate logistic regression analysis, the relation between salivary viral load and headache and GI symptoms remained significant after adjustment for age and sex ($P = 0.004$ and $P = 0.043$, respectively) (**Table 5**).

DISCUSSION

The present study was designed to explore the quantification of SARS-CoV-2 in pure saliva of adults with asymptomatic to mild COVID-19. This robust multicenter observational trial, including 160 individuals, is the first, to our knowledge, to target this specific population—mean age of patients was 43.62 ± 15.56 years- and to describe the sociodemographic and clinical characteristics of the salivary viral load. The load SARS-CoV-2 value of the whole sample ranged from 0.00 to 10.19 with a median of 4.12 $\log_{10}$ copies/mL (IQR 2.95–5.16 $\log_{10}$ copies/mL), 27/159 patients (16.98%) had no charge. Between no-symptoms patients with an initially salivary SARS and patients with symptoms, there was no significant

TABLE 4 | Association of viral load with sex, age, symptoms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: univariable analysis.

Variable	References level	Class level	Mean difference [95% CI]	*p*-value
Age	CV		0.02 [−0.01–0.04]	0.179
Age (class)	18–34	35–54	0.40 [−0.40–1.20]	0.330
		55–77	0.42 [−0.48–1.32]	0.359
Gender	Male	Female	−0.60 [−1.28–0.08]	0.084
Co-morbidity	No	Yes	−0.01 [−0.83–0.81]	0.974
Time between positive RT-PCR and symptom onset	CV		−0.11 [−0.35–0.13]	0.354
Time between positive RT-PCR and symptom onset (class)	0–2 days	3–5 days	0.26 [−0.67–1.18]	0.583
		6–8 days	−0.39 [−1.70–0.92]	0.560
Delay from symptom onset to saliva sampling	CV		−0.12 [−0.34–0.10]	0.283
Delay from symptom onset to saliva sampling (class)	0–2 days	3–5 days	−0.87 [−2.55–0.81]	0.312
		6–9 days	−1.31 [−2.96–0.35]	0.124
Number of symptoms per subjects	CV		0.08 [−0.07–0.24]	0.275
Number of symptoms per subjects (class)	None	1–4	0.02 [−1.31–1.36]	0.973
		5–6	0.56 [−0.71–1.83]	0.387
		7–9	0.50 [−0.85–1.85]	0.468
Symptoms				
None	Yes	No	−0.39 [−1.60–0.81]	0.522
Fever	No	Yes	0.24 [−0.46–0.93]	0.504
Cough	No	Yes	0.58 [−0.10–1.26]	0.097
Dyspnea	No	Yes	0.50 [−0.53–1.53]	0.341
Headache	No	Yes	1.04 [0.37–1.71]	0.003
Myalgia	No	Yes	0.19 [−0.50–0.88]	0.589
Grasto-intestinal symptoms	No	Yes	−0.98 [−2.17–0.22]	0.112
Anosmia	No	Yes	−0.29 [−0.99–0.41]	0.423
Ageusia	No	Yes	−0.25 [−0.95–0.46]	0.497

CV: continuous variable.

TABLE 5 | Association of viral load with sex, age, symptoms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: Multivariable Analysis.

Variable	References level	Class level	Mean difference [95% CI]	*p*-value
Age	CV		0.01 [−0.01–0.04]	0.188
Gender	Male	Female	−0.56 [−1.23–0.11]	0.102
Symptoms				
Cough	No	Yes	0.40 [−0.28–1.08]	0.250
Headache	No	Yes	1.02 [0.34–1.7]	0.004
Grasto-intestinal symptoms	No	Yes	−1.22 [−2.39—0.05]	0.043

CV, continuous variable.

median difference in the viral load. Collectively, these data show that the oral cavity is an important site for SARS-CoV-2 infection and implicate saliva as a potential route of SARS-CoV-2 transmission.

While self-collected saliva is a realistic alternative option for the diagnosis of COVID-19, there are no significant studies in the literature evaluating the salivary viral load of adults with asymptomatic, to mild COVID-19. If Huang et al. (2021) suggest two possible sources for SARS-CoV-2 in saliva: an acellular fraction from infected glands making virus *de novo* and a cellular fraction from infected and shed oral mucosa, little is known about the correlation between viral load and age, gender, symptoms, and comorbidity. The first study quantifying the salivary load of SARS-CoV-2 included 12 hospitalized patients with laboratory-confirmed COVID-19, with a median age of 62.5 years. SARS-CoV-2 was detected in 91.7% of throat saliva samples from COVID-19 patients, and the number was as high as $1–2 \times 10^8$ infectious copies per milliliter (To et al., 2020). Observational studies are subject to a number of different biases (Accorsi et al., 2021). Lecture of meta-analysis of diagnostic results from saliva (Moreira et al., 2021) reveals that no studies met our objective and methodological criteria.

So, studies included different subgroups categorized by gradation of disease severity, primarily in patients who developed severe disease admitted to intensive care during their hospital stay, and both inpatients with moderate disease symptoms (Alteri et al., 2020; Jeong et al., 2020; Kam et al., 2020; Kim et al., 2020; Lai et al., 2020; Pan et al., 2020; Pujadas et al., 2020; To et al., 2020; Yoon et al., 2020; Yu et al., 2020; Abasiyanik et al., 2021; Chua et al., 2021; Han et al., 2021;

Hasanoglu et al., 2021). Then, participants were included with a diverse range of COVID-19 disease severity, including in majority cases hospitalized, and individuals with resolved infection. So, different diagnosis methods were used in self-collected saliva as nucleic acid amplification testing, transcription mediated amplification, reverse transcription loop-mediated isothermal amplification, TRIzol-based RNA extraction, despite quantitative reverse transcription PCR is the diagnostic standard for SARS-CoV-2 (Nagura-Ikeda et al., 2020). Several papers used the naive Ct values from qualitative RT-PCR as a quantitation unit or use the ΔCt values with incorrect quantitation unit (Shen et al., 2020; Zou et al., 2020). Saliva collection protocols for included studies were assessed for differences with respect to asking patients to collect pure saliva or to cough or clear their throat before submission of enhanced sample likely mixed sputum and saliva specimen or deep throat saliva specimen or requesting the patients submit "drool" or "spit" (Carrouel et al., 2020; Khiabani and Amirzade-Iranaq, 2021).

With reference to viral load during infection in asymptomatic or mildly affected adults with COVID-19, we observed that viral load is generally high, although asymptomatic viral loads are not statistically different from symptomatic viral loads, tending, however, to be lower. These results highlight the potential infectivity of saliva although the possible threshold of transmissibility is not specified and there is no standard reference in this regard. In particular, these results suggest that expelled oral droplets containing infectious virus and infected cells may be a source of airborne transmission of SARS-CoV-2. SARS-CoV-2 transmission from people who are either asymptomatic or mild has implications for prevention. Social distancing measures will need to be sustained at some level because droplet transmission from close contact with people with asymptomatic and mild infection occurs (Bazant and Bush, 2021). Easing of restrictions will, however, only be possible with wide access to testing, contact tracing, and rapid isolation of infected individuals.

The mean age of patients in our study was 43.6 years. We found that gender and age are not factors affecting viral load. According to published data, younger patients would be more likely to be asymptomatic than older patients (Li Y. et al., 2020). Studies have shown that both older age and male gender are associated with severe disease (Qian et al., 2020). To et al. (2020) found similar results to Zheng et al. (2020) and both concluded that older age is associated with higher viral load. Mahallawi et al. (2021) studied the viral load of lower and upper respiratory tract specimens from 3,006 COVID-19 positive patients. They found no statistically significant difference between age groups, while the viral load was statistically significantly higher in women than in men.

Specific observational studies as well as modeling work have shown that the infection can be asymptomatic or paucisymptomatic (causing little or no clinical manifestations) in 30–60% of infected individuals, especially in young adults and adults (Plucinski et al., 2021). Asymptomatic individuals were the source for 69% (20–85%) of all infections (Emery et al., 2020). Saliva from asymptomatic individuals contains infectious virus. Surprisingly, the viral load was found to be significantly similar between asymptomatic and symptomatic patients infected with SARS-CoV-2 ($p = 0.573$). Since the beginning of the pandemic, there has been controversy about the infectivity of asymptomatic patients. With the aforementioned limitation on the robustness of existing studies which are based on considerably smaller data sets, most studies demonstrate more rapid viral clearance in asymptomatic individuals than in symptomatic ones (McEvoy et al., 2021). A large representative sample with longitudinal data has shown that both symptomatic and asymptomatic patients are often characterized by a similar amount of virus at the onset of infection (Lavezzo et al., 2020). It is reported that approximately 40–45% of patients infected with SARS-CoV-2 will remain asymptomatic (Oran and Topol, 2020). Additionally, individuals with mild, non-specific, asymptomatic symptoms are difficult to identify and quarantine (Han et al., 2021). The ambiguity surrounding both asymptomatic and presymptomatic conditions is highlighted. They are described as suggestive, and both inconclusive. Because of the high risk of silent spread by asymptomatic individuals, it is imperative that screening programs include those without symptoms (Oran and Topol, 2020; Almadhi et al., 2021).

Screening from symptoms could prioritize tests and increase diagnostic sensitivity (Lan et al., 2020). However, in our study, salivary load quantification is not discriminated on the number and nature of reported symptoms except for headache. General non-respiratory symptoms (eye pain, muscle pain, general malaise, fever, headache, and extreme fatigue), although not very specific, are the strongest independent predictors of positive tests (Tostmann et al., 2020). As a non-specific symptom, headache can occur not only in COVID-19 but also in other viral diseases. Therefore, headache alone may not raise suspicion of SARS-CoV-2 infection although headache was 1.7 times more common in patients with COVID-19 respiratory viral infection than in those with non-COVID-19 respiratory viral infection with $p = 0.04$ (Correia et al., 2020; Mutiawati et al., 2021). The presence of gastrointestinal symptoms associated with salivary viral load in COVID-19 patients raises questions about the impact of COVID-19 infection on the quality of life of at-risk subjects. It is quite plausible to observe the development of several gastro-intestinal symptoms induced by SARS-CoV-2 infection in patients, ranging from nausea, vomiting, and diarrhea to loss of appetite and abdominal pain (Yusuf et al., 2021).

Chemosensory dysfunctions, especially hyposmia, anosmia, hypogeusia, and ageusia, are one of the major symptoms of SARS-CoV2 infection (Srinivasan, 2021). Self-reported loss of smell and taste is a better prognosticator than other symptoms such as cough, fatigue, or fever for predicting symptomatic infection (Mastrangelo et al., 2021). Our results indicate that SARS-CoV-2 levels in saliva do not correlate with taste alterations.

First limitation of our study is that it focused on the second wave of the epidemic in the second half of 2020. Adaptation of salivary quantification guidelines in relation to newly detected SARS-CoV-2 variants is necessary. At the time of the study the variant circulating in France was predominantly the UK variant (B.1.1.7) and not the Delta variant (B.1.617.2) which is now detected in the majority of cases. Thus, it is likely that the results would be different if the study were conducted now since the delta variant has different characteristics (Mlcochova et al., 2021).

Secondly, we also clearly explained the ambiguity surrounding asymptomatic vs. presymptomatic status. We describe them as suggestive, not conclusive. While measuring viral load can be useful in clinical practice, a positive RT-qPCR result does not necessarily mean that the person is still infectious or still has significant disease. The RNA could be from a non-viable virus and/or the amount of live virus could be too low to allow transmission (Trunfio et al., 2021). Contacts of a carrier with a high viral salivary load may have a higher risk of acquiring infection, but to date there is no evidence that acquired infection will be more likely to be symptomatic or severe (Li R. et al., 2020; Hasanoglu et al., 2021; van Kampen et al., 2021).

Strengths of our study is that, although *post hoc* studies have revealed the importance of SARS-CoV-2 corona virus 2 transmission from both asymptomatic and mildly symptomatic cases, the virologic basis for their infectivity remains largely unquantified (Jones et al., 2021). Despite PCR method do not measure infectious virus, our study produces original and robust quantitative data applied to an ambulatory adult population susceptible to be the prime actor of transmission until effective vaccines have been distributed widely.

CONCLUSION

Our results raise the possibility that the oral cavity is an important site for SARS-CoV-2 infection and implicate saliva as a potential route of SARS-CoV-2 transmission. This result may have public health implications if enhanced saliva samples are used for asymptomatic screening. Considering oral SARS-CoV-2 infection and the ease of saliva for transmission, it remains critical to further understanding of the dominant modes of viral spread across the spectrum of asymptomatic, pre-symptomatic and symptomatic individuals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee for the Protection of Persons South Mediterranean III, France. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FC, DB, and CD proposed the original study idea and designed the trial and study protocol. DB and FC contributed to the data interpretation and wrote the first draft of the manuscript. FC, CD, and HP verified the data. EG, AE, ML, and JD were responsible for the site work including the recruitment, follow up and data collection. HP monitored the trial. DB did the main analysis. CD, EG, and JD contributed to the revision of the manuscript. All authors reviewed and accepted the manuscript before submission.

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REVIEW
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Oral *Prevotella* Species and Their Connection to Events of Clinical Relevance in Gastrointestinal and Respiratory Tracts

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Prevotella is recognized as one of the core anaerobic genera in the oral microbiome. In addition, members of this genus belong to microbial communities of the gastrointestinal and respiratory tracts. Several novel *Prevotella* species, most of them of oral origin, have been described, but limited knowledge is still available of their clinical relevance. *Prevotella melaninogenica* is among the anaerobic commensals on oral mucosae from early months of life onward, and other early colonizing *Prevotella* species in the oral cavity include *Prevotella nigrescens* and *Prevotella pallens*. Oral *Prevotella* species get constant access to the gastrointestinal tract via saliva swallowing and to lower airways via microaspiration. At these extra-oral sites, they play a role as commensals but also as potentially harmful agents on mucosal surfaces. The aim of this narrative review is to give an updated overview on the involvement of oral *Prevotella* species in gastrointestinal and respiratory health and disease.

Keywords: anaerobic bacteria, commensalism, dysbiosis, inflammation, microbiology, *Prevotella*, systemic disease, taxonomy

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INTRODUCTION

Anaerobic bacteria constitute a significant part of oral microbial communities. In the oral cavity, Bacteroidetes is one of the major phyla and *Prevotella* its largest genus (Dewhirst et al., 2010). This genus, which has expanded significantly during the past decades, consists of gram-negative, strictly anaerobic, mainly short rod-shaped bacteria. In healthy adults, detection rates of *Prevotella* organisms are high in saliva and dental plaque (Keijser et al., 2008; Xu et al., 2015). In saliva, richness of the diversity within this genus is especially high (Keijser et al., 2008). At the species level, however, the majority of data currently available deals with *Prevotella intermedia* and/or *Prevotella nigrescens* due to their clinical relevance in oral pathologies but also ignorance of commensals. A study looking for intraoral distribution of bacterial species in 225 systemically healthy individuals showed *Prevotella melaninogenica* in high proportions in saliva and at the dorsum and lateral sites of the tongue (Mager et al., 2003).

In the context of this review, human habitats exposed to oral bacteria to be colonized outside the oral cavity are presented in **Figure 1**. There are obvious differences in the microbial communities between body habitats and between individual metabolic niches (Costello et al., 2009; Segata et al., 2012). At the genus level, *Prevotella* is frequent and widespread all over on the surfaces of the human body; however, a species-level identification methodology is necessary for revealing whether

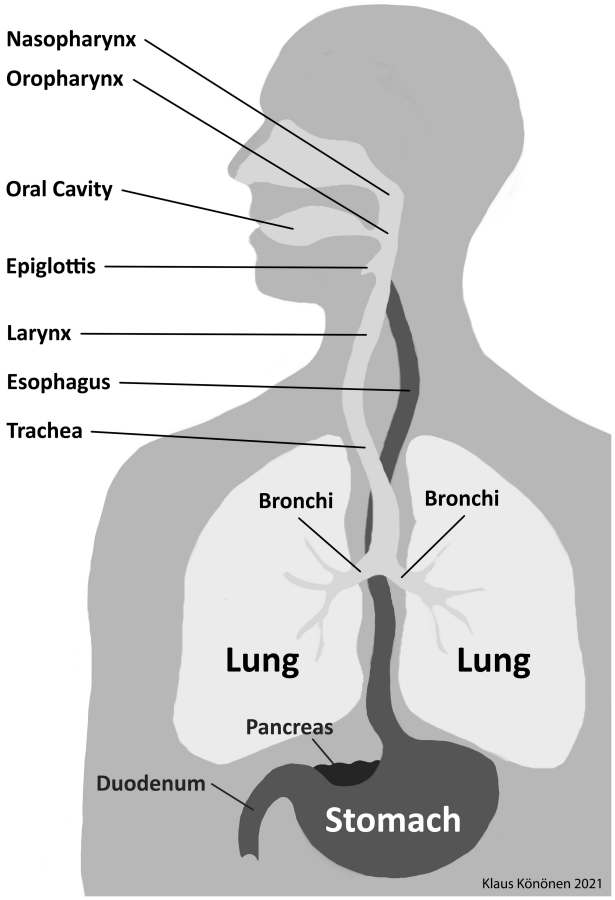

FIGURE 1 | The anatomy and potential habitats in the aerodigestive tract in humans.

same species colonize throughout the gastrointestinal tract and whether their relative abundance varies among the habitats as well as inter-individually (Segata et al., 2012; Schmidt et al., 2019). *Prevotella* strains present in saliva are of particular interest. Due to constant saliva swallowing of approximately 1.5 L daily (Humphrey and Williamson, 2001), this oral fluid is the most plausible vehicle for oral microorganisms and their biologically active components to be translocated to other parts of the digestive tract (Schmidt et al., 2019). Oral bacteria surviving to pass acidic circumstances of the stomach get access to the small intestine and colon, where they can interfere with intestinal bacteria (Schmidt et al., 2019; Kitamoto et al., 2020). At the genus level, *Prevotella* is a common colonizer of these distant habitats. However, at the species level, distinct bacterial populations occupy the oral and intestinal microenvironments. Also immune cells involved in chronic inflammatory processes in the mouth end up via saliva to the gut, sometimes resulting in pathological consequences (Byrd and Gulati, 2021).

In the respiratory tract, potential routes for bacterial translocation from the oral cavity include (micro)aspiration, in particular, and hematogenous spread. Indeed, recent studies have shaken our views on the oral source of bacteria for the composition of microbial communities of the lower respiratory tract (Bassis et al., 2015; Dickson et al., 2016). Currently, *Prevotella* is considered one of the major genera colonizing mucosal surfaces of the aerodigestive tract, including the lungs, which were long seen as a sterile site in the human body.

In this narrative review, the purpose is to give an updated overview on the presence of oral *Prevotella* species as members of the gastrointestinal and respiratory microbiota and on their involvement in diseases at these sites above the waistline.

TAXONOMICAL OVERVIEW OF ORAL *PREVOTELLA*

In 1990, the genus *Prevotella* was described by reclassifying a group of moderately saccharolytic and bile-susceptible *Bacteroides* species as members of this novel genus (Shah and Collins, 1990). The name *Prevotella* came after A. R. Prévot, who was a French microbiologist with pioneering expertise in anaerobic bacteriology. Most of these reclassified *Prevotella* organisms were recovered from the oral cavity of humans, *P. melaninogenica* being the type species of the genus. At that time, pigment production of colonies on blood agar was seen as an important feature to divide the organisms into pigmented and non-pigmented *Prevotella* species (Jousimies-Somer et al., 2002). In addition to *P. melaninogenica*, the pigmented group included *Prevotella loescheii*, *Prevotella denticola* (part of strains), *P. intermedia*, and *Prevotella corporis*, their color intensity varying from light brown to black. Some years later, the pigmented group within *Prevotella* was expanded by two phylogenetically closely related species, *P. nigrescens* (Shah and Gharbia, 1992) and *Prevotella pallens* (Könönen et al., 1998). During the 1990s, there was a notable research interest in black-pigmented gram-negative anaerobes, including *Porphyromonas* and *Prevotella* species, due to their link to various pathologies in humans (Finegold et al., 1993).

After the creation of the genus *Prevotella* and reclassification of moderately saccharolytic *Bacteroides* as *Prevotella* species by Shah and Collins (1990), there has been a great expansion of the genus with novel species, most of them of oral origin. **Table 1** presents the validly published *Prevotella* species that have been primarily isolated from the oral cavity: 12 species formerly classified as *Bacteroides* and 18 novel *Prevotella* species described after 1990. On the other hand, a couple of reclassifications were made; while the former *Mitsuokella dentalis* is now *Prevotella dentalis* (Willems and Collins, 1995), *Prevotella tannerae* was removed to a novel, closely related genus *Alloprevotella*, also including a novel oral species, *Alloprevotella rava* (Downes et al., 2013). Two species with a distant phylogeny, *Prevotella heparinolytica* and *Prevotella zoogleoformans*, remain "on the waiting list" to be removed from the genus *Prevotella* (Dewhirst et al., 2010).

In addition to the above-mentioned species principally isolated from the oral cavity, several novel *Prevotella* species have been described based on a few strains, or even a single strain, from various clinical specimens. Origins of these strains were as follows: two *Prevotella amnii* strains from amniotic fluid

TABLE 1 | Validly published *Prevotella* and *Alloprevotella* species with the primary isolation from the human oral cavity.

Year/Reference	Former *Bacteroides* sp. reclassified as *Prevotella* sp.	Year/References	Novel *Prevotella* and *Alloprevotella* spp.	Comments
Shah and Collins, 1990	*P. melaninogenica* (type species)	Shah and Gharbia, 1992	*P. nigrescens*	
	P. buccae	Moore et al., 1994	*P. enoeca* *P. tannerae*	
	P. buccalis	Willems and Collins, 1995	*P. dentalis*	*Mitsuokella dentalis*
	P. denticola	Könönen et al., 1998	*P. pallens*	
	P. heparinolytica	Sakamoto et al., 2004	*P. salivae* *P. shahii*	
	P. intermedia	Sakamoto et al., 2005a	*P. multiformis*	
	P. loescheii	Sakamoto et al., 2005b	*P. multisaccharivorax*	
	P. oralis	Downes et al., 2005	*P. baroniae* *P. marshii*	
	P. oris	Downes et al., 2007	*P. maculosa*	
	P. oulorum	Downes et al., 2008	*P. histicola*	
	P. veroralis	Downes et al., 2009	*P. micans*	
	P. zoogleoformans	Sakamoto et al., 2010	*P. aurantiaca*	
		Downes et al., 2010	*P. saccharolytica*	
		Downes and Wade, 2011	*P. fusca* *P. scopos*	
		Downes et al., 2013	*A. rava* *A. tannerae*	*Alloprevotella* gen. nov., reclassification of *P. tannerae*

(Lawson et al., 2008), eight *Prevotella bergensis* strains from skin and soft tissue infections (Downes et al., 2006), one *Prevotella brunnea* strain from a wound at foot (Buhl et al., 2019), one *Prevotella colorans* strain from a wound (Buhl et al., 2016), three *Prevotella jejuni* strains from the jejunum of a celiac child (Hedberg et al., 2013), three *Prevotella nanceiensis* strains from bronchial fluid, lung abscess, or blood (Alauzet et al., 2007), one *Prevotella pleuritidis* strain from pleural fluid (Sakamoto et al., 2007), one *Prevotella timonensis* strain from breast abscess (Glazunova et al., 2007), and one *Prevotella vespertina* strain from an abscess locating at the upper respiratory tract (Buhl and Marschal, 2020). With this limited information given, the knowledge of their preferred habitat remains open.

Three former *Bacteroides* species, reclassified as *Prevotella bivia*, *Prevotella corporis*, and *Prevotella disiens* (Shah and Collins, 1990) come mainly from the human urogenital tract but there are also occasional recoveries from the mouth. In the current literature, several novel *Prevotella* species from stool specimens have been described as colonizers of the colon. Of those, *Prevotella copri* has drawn remarkable attention due to its potential beneficial effects on human well-being (Tett et al., 2021), although its connections to chronic inflammatory conditions of the gut have been recognized, too (Ley, 2016).

Besides biochemical and physiological testing, methods for *Prevotella* classification have traditionally included DNA–DNA hybridization, measuring of G + C content, and multilocus enzyme electrophoresis, however, classification gradually changes toward genomic methods. Full-sequencing of the 16S rRNA gene is a routine, but also comparison to whole-genome sequence databases is available and has been used in recent *Prevotella* descriptions (Buhl et al., 2016, 2019; Buhl and Marschal, 2020). Tett et al. (2021) recently described genomic characteristics of 25 named human *Prevotella* species, among those 17 species with genomes of multiple oral isolates in the analysis. Varying genome lengths between 2.37–4.26 Mb and G + C contents between 36.4–56.1% as well as the high number of core genes speak for the wide diversity within this genus.

PREVOTELLA IN BACTERIAL COMMUNITIES OF THE DIGESTIVE TRACT

Prevotella as Members of the Core Microbiota in the Gastrointestinal Tract

Concerning the alimentary tract from the oropharynx downward above the waistline, it was for long considered of being without a resident microbiota until advanced molecular methods allowed to challenging these earlier beliefs, which were based on negative cultures by routine microbiology methods from the esophagus and stomach (Pei et al., 2004; Bik et al., 2006). Pei et al. (2004) tested their hypotheses on the presence of indigenous bacteria on esophageal mucosa and on their fastidious nature, i.e., mainly being uncultivable. Biopsy specimens from the distal esophagus of four healthy individuals were examined with 16S rDNA sequencing-based techniques, and the results showed Bacteroidetes as the second most common phylum and *Prevotella* as the second most common genus after *Streptococcus*. *P. pallens* was among the 14 bacterial taxa found in all individuals, while many other named *Prevotella* species were also recovered: *Prevotella veroralis* from three individuals, *P. intermedia* and *P. melaninogenica* from two individuals, and *P. denticola*,

P. nigrescens, and *Prevotella oris* each from one individual. In addition, several not-yet-named *Prevotella* clones were among the findings (Pei et al., 2004). To increase the understanding of the esophageal microbiome and its function in the host, Deshpande et al. (2018) studied the esophageal microbiome of over 100 individuals, with special emphasis on age, gender, proton pump inhibitor use, host genetics, and development of esophageal disease. They demonstrated that the esophageal microbiome clusters into functionally distinct bacterial community types. Cluster 3 was dominated by *P. melaninogenica* and *P. pallens*, and cluster 2 by mitis group streptococci, while cluster 1 was an intermediate type with respect to abundances of *Streptococcus* and *Prevotella*, indicating their ratio of being a significant factor in defining esophageal community types (Deshpande et al., 2018). It was shown that various, distinct pathways are increased in each community type. Age was observed to affect relative abundances within these two major genera; age positively correlated with *Streptococcus parasanguinis* (but not with mitis group streptococci), whereas age had an inverse correlation with *P. melaninogenica* (but not with *P. pallens*). Interestingly, there was a co-exclusion relationship between mitis group streptococci and *Prevotella* species across all esophageal disease stages (Deshpande et al., 2018).

Bik et al. (2006) collected gastric biopsy specimens from 23 adults, suffering from symptomatic upper gastrointestinal disease and being positive or negative for *Helicobacter pylori*. Advanced sensitive methods were used to reveal the bacterial composition of the gastric microbiota and the impact of *H. pylori* on its composition. There was a much larger bacterial diversity in gastric biopsies than was expected, but the sequence collection was also different from those of the mouth and esophagus presented in other studies. Interestingly, especially Bacteroidetes phylotypes were often absent from *H. pylori*-positive individuals (Bik et al., 2006). However, several recognized oral *Prevotella* species (and several *Prevotella* clones) were detected in individuals who were *H. pylori*-negative by conventional methods. Among the findings were, in descending order in their abundance, *P. melaninogenica* and *P. pallens*, in particular, and few *P. oris*, *P. intermedia*, *P. nigrescens*, *Prevotella oralis*, *Prevotella oulorum*, and *P. denticola*. It was emphasized that bacterial DNA may not indicate the presence of resident bacteria but, instead, could reflect the presence of bacterial cell remnants or transient flow of these bacteria in the specimens (Bik et al., 2006). Recently, it was confirmed that *H. pylori*, if present, dominates the bacterial community composition of the stomach, lowering the diversity and evenness of phylotypes (Schulz et al., 2018). Similarly to the study by Bik et al. (2006), two genera, *Streptococcus* and *Prevotella*, proved to dominate in bacterial communities of the upper gastrointestinal tract in *H. pylori*-negative individuals. By analyzing saliva, and stomach and duodenal aspirates and biopsies from a cohort of 24 individuals with chronic gastritis by high-throughput sequencing, Schulz et al. (2018) identified the oral cavity (saliva) of being the main source of active bacteria for the gastric microbiota, indicating a continuous migration of oral bacteria through the upper gastrointestinal tract. In individuals without *H. pylori*, no difference was seen in relative abundance of *Prevotella* and *Alloprevotella* between saliva and stomach aspirates, while reduced abundance for both genera was detected between the stomach and duodenum. In general, no significant difference at the genus level was observed in oral communities between individuals with or without *H. pylori*, but three phylotypes, among those *P. oris*, had significantly higher abundance in individuals without *H. pylori* (Schulz et al., 2018). Each individual harbored own specific bacterial communities throughout the upper gastrointestinal tract.

Saliva has been suggested as a key driver for the composition of bacterial communities in various habitats of the upper digestive tract, and as another key driver, a shared epithelial lining of their mucosae (Segata et al., 2012). Vasapolli et al. (2019) looked thoroughly for bacterial community changes in 21 healthy individuals throughout their gastrointestinal tract, including a wide selection of specimens: saliva, stomach (antrum and corpus), duodenum, terminal ileum, ascending and descending colon, and feces. They demonstrated that bacterial communities detected in samples from saliva, stomach, and duodenum formed 1 cluster and those from the lower gastrointestinal tract another cluster, while fecal communities differed from mucosa-associated findings of the gut (Vasapolli et al., 2019). The highest phylotype richness, including the genus *Prevotella*, was found in saliva, staying rather steady across the upper body sites, whereas in samples from lower parts of the body, a significantly reduced phylotype richness was observed. Among the phylum Bacteroidetes, abundance of *Prevotella* organisms present in saliva gradually decreased downward up to the duodenum, the decrease being drastic thereafter, whereas an opposite occurred to abundance of *Bacteroides* organisms (Vasapolli et al., 2019). In line were the results of a thorough culture-based study, performed in a homologous group of beagle dogs (Mentula et al., 2005), showing clear differences between jejunal and fecal samples and where a unique bacterial composition for each dog was found in small-intestinal fluid with a few species only at a time and fluctuating counts. Smoking seems to be a lifestyle factor affecting the mucosa-associated microbiota of the duodenum; in current smokers, a significantly reduced abundance of the phylum Bacteroidetes and the genus *Prevotella* was observed, and this reduction was only partially restored after quitting of smoking (Shanahan et al., 2018). *P. nanceiensis* was suggested to be a discriminatory species for previous smokers.

A few *Prevotella* species isolated from feces are considered having their habitat in the intestine. According to the current knowledge, especially *P. copri* is both a prevalent and abundant organism in the gut [see an excellent review by Tett et al. (2021)]. Interestingly, lifestyle factors, diet in particular, have a significant impact on its abundance. In non-Westernized populations with diets rich of carbohydrates and fibers, the *P. copri* complex is a common inhabitant of the gut, while different bacterial taxa dominate in populations with Western-type diets. On one hand, the *P. copri* complex seems to possess beneficial metabolic effects on human health but, on the other hand, potential detrimental effects may also exist (Ley, 2016; Tett et al., 2021). The impact of diet on oral *Prevotella* species is not known.

Prevotella Involvement in Gastrointestinal Diseases

In the human esophagus, gastroesophageal reflux disease is a rather common pathological condition, where the relaxation of the sphincter muscle at the lower esophagus allows acidic stomach contents frequently flow back into the esophagus and further into the pharynx[1]. This constant leakage irritates the epithelial surface, causing reflux symptoms or esophagitis, which may lead to Barrett's esophagus with a disturbed epithelium structure. The potential role of changes in the esophageal and/or salivary microbiota has been studied and, in this context, the genus *Prevotella* is of interest (Liu et al., 2013; Kawar et al., 2021). Bacterial communities of the distal esophagus were examined at the phylum and genus levels in biopsy samples collected from Japanese patients with either normal esophagus, reflux esophagitis, or Barrett's esophagus (Liu et al., 2013). The study revealed Bacteroidetes among the major phyla, and the proportions of *Prevotella* clones in the samples were 3, 5, and 12%, respectively. Of the six patients in each group, the number of *Prevotella*-positive patients was three, four, and six, respectively. A distinct bacterial composition seen between the groups was assumed to indicate an association with an esophageal health status and disease type (Liu et al., 2013). Recently, Kawar et al. (2021) compared the bacterial composition of salivary samples collected from reflux patients treated with proton pump inhibitors to that from non-medicated reflux patients and healthy controls. Abundant taxa present in the samples from the latter two groups differed considerably, including reduced abundances of *P. melaninogenica* and *P. pallens* in saliva of reflux patients. It was assumed that a lowered pH in the oral cavity of non-medicated patients explains this difference, since no significant difference was found between saliva of medicated reflux patients and healthy controls (Kawar et al., 2021).

Bacterial compositions of the stomach and duodenum were examined in 98 Korean patients with symptomatic gastritis (Han et al., 2019). It was demonstrated that the gastric and duodenal microbiota differ from each other. Symptom scores only weakly correlated with abundance of gastric *H. pylori* but, instead, correlated more strongly with the duodenal microbiota. An interesting gender-related finding was the higher relative abundance of *P. pallens* in the stomach of women than in that of men. There was a negative correlation with relative abundance of *P. pallens* and the severity of symptoms in the stomach. Positive correlations were found with *P. nanceiensis* and *A. rava* in the duodenum. According to the authors, other factors than *H. pylori* need to be taken into account in symptomatic gastritis (Han et al., 2019).

A novel *Prevotella* species was detected for the first time in a biopsy taken from the jejunum of a child with celiac disease and was named as *P. jejuni* due to its localization in this part of the small intestine (Hedberg et al., 2013). However, this species seems to be a common inhabitant of the oral cavity and especially in saliva, similarly to its close relative, *P. melaninogenica* (own unpublished data). Alterations in the salivary microbiota and metabolome in celiac children, who had been under glutein-free diet at least for 2 years, were examined and compared to those of healthy control children (Francavilla et al., 2014). The number of total cultivable anaerobes differed between the groups, with reduced amounts found in children with celiac disease. Members of the phylum Bacteroidetes, among those *P. nanceiensis*, dominated in saliva, with increased abundance in celiac children. It was concluded that the diet change of 2 years may not be enough to restore the salivary microbiota (Francavilla et al., 2014).

Although the relation of the oral microbiota to inflammatory bowel diseases (IBD) is still controversial, there is evidence on their connection to dysbiotic bacterial communities in saliva (Said et al., 2014; Xun et al., 2018; Qi et al., 2021). Dysbiosis with an increased relative abundance of Bacteroidetes, in turn, causes an elevated inflammatory response, which is also seen in saliva as increased levels of cytokines like interleukin-1β (Said et al., 2014). No alterations were observed in the richness and diversity of bacterial communities between saliva from IBD patients and healthy controls, whereas the bacterial composition in saliva varied (Said et al., 2014; Qi et al., 2021). While abundance of *Streptococcus* decreased, that of *Prevotella* (besides *Veillonella*) and *P. melaninogenica* significantly increased in both Crohn's disease and ulcerative colitis patients. The genus *Prevotella* was suggested as a potential indicator related to Crohn's disease (Qi et al., 2021). Moreover, the dysbiotic salivary composition contributed to aggravated immune disorders in IBD patients.

Prevotella in Cancers of the Digestive Tract Above the Waistline

In the esophagus, cancer types differ depending on the geographical location; in the East, squamous cell carcinoma (SCC) dominates, whereas adenocarcinoma is more common in Western countries (Sung et al., 2021). In the first study to examine bacterial infection as a background factor for esophageal SCC and to identify bacteria that could predict the cancer prognosis, the genus *Prevotella* came up as a potential prognostic indicator for this cancer type (Liu et al., 2018). Patients with lymph node metastasis had higher abundance of *Prevotella* and *Treponema* than patients without metastatic findings, while increased abundance of *Prevotella* combined with *Streptococcus* affected survival rates, predicting poor prognosis. Alterations of the esophageal microbiota have also been studied in connection to Barrett's esophagus and esophageal adenocarcinoma (Lopetuso et al., 2020). The former condition with changes in the epithelial structure with acid stress and inflammation is considered to expose to metaplastic mucosa. Again, abundance of *Prevotella* was significantly increased, however, species-level shifts showed decreased proportions of *P. melaninogenica* in samples from Barrett's esophageal mucosa with or without cancer but increased proportions of unclassified *Prevotella*. *P. histicola* was common on mucosae of Barrett's esophagus, while *P. nigrescens* proportions on metaplastic mucosa were slightly increased (Lopetuso et al., 2020). The authors reported a co-exclusion association between *Streptococcus* and *Prevotella*; a significant reduction in relative abundance of *Streptococcus* and

[1]https://www.hopkinsmedicine.org/gastroenterology_hepatology/_pdfs/esophagus_stomach/gastroesophageal_reflux_disease.pdf

corresponding increase in abundance of *Prevotella* were observed on mucosae of both Barrett's esophagus and adenocarcinoma.

A decade ago, bacterial communities of the stomach in gastric cancer patients were described for the first time by molecular techniques and compared to those in dyspeptic patients with normal mucosa as controls (Dicksved et al., 2009). The second most dominant phylum proved to be Bacteroidetes, composing mainly of *Prevotella* taxa; among known species, *Prevotella multiformis*, *P. nigrescens*, *P. oris*, and *A. tannerae* were recognized in gastric cancer patients. It can be speculated whether changed conditions due to an increased use of acid-reducing drugs or neoplastic mucosa would allow the colonization of atypical bacteria in the stomach and progression of cancer (Dicksved et al., 2009; Coker et al., 2018). Coker et al. (2018) investigated mucosal biopsy specimens collected from superficial gastritis, atrophic gastritis, intestinal metaplasia, and gastric cancer patients, and detected highest abundance of several oral bacteria, among those *P. intermedia* and *P. oris*, in gastric cancer samples. It was emphasized, however, that it remains to be elucidated in targeted studies whether bacteria enriched are passengers or drivers of carcinogenesis (Coker et al., 2018).

Poor oral hygiene is suggested as a moderate risk factor for pancreatic cancer (Huang et al., 2016). This may be connected to increased amounts of microbial biofilms on oral surfaces and activation of host inflammatory response. In a recent study, a large variety of samples from the gastrointestinal tract, including oral (saliva and swabs from tongue, buccal mucosa, and gingiva), upper intestinal (duodenum tissue and swabs from jejunum and bile duct), and pancreatic (tumor or normal tissue and pancreatic duct) samples were examined to clarify the microbiota in patients with pancreatic cancer or other diseases of the pancreas or the foregut (Chung et al., 2021). *Streptococcus*, *Veillonella*, and *Prevotella* were the most shared genera between oral and gut or pancreatic samples, and *P. veroralis* among the most frequently shared species. Amplicon Sequence Variants had some overlaps between the close sites within the mouth and within the pancreas. In co-abundance analyses, distinct strain-level cluster patterns were observed among microbial findings in buccal swabs, saliva, duodenal tissue, jejunal swabs, and pancreatic tumor tissue. In the latter sample site, *P. nigrescens* was found among dominating species in one cluster (Chung et al., 2021).

PREVOTELLA IN BACTERIAL COMMUNITIES OF THE RESPIRATORY TRACT

Prevotella as Members of the Core Microbiota in the Respiratory Tract

A gradual maturation of the early microbiota of the lower respiratory tract occurs within less than 2 months after birth in full-term infants (Pattaroni et al., 2018). Three distinct colonization patterns were recognized in tracheal aspirates and were explained by distinct microenvironments in preterm and term infants. Of those, a mixed microbial profile consisted of a balanced composition of six genera, including *Streptococcus* and *Neisseria* as keystone genera, and anaerobic *Prevotella*, *Veillonella*, *Porphyromonas*, and *Fusobacterium*. This combination stayed stable across the first year of life (Pattaroni et al., 2018). Interestingly, this is a typical bacterial composition for the early microbiota established in the mouth during the first year of life (Könönen et al., 1999; Könönen, 2005). It also resembles the composition of the lung microbiota of adults during health (Bassis et al., 2015).

Similar interacting consortia as seen in the oral cavity (but not in the nasal cavity) can be observed in the lower respiratory tract, even though relative abundance and diversity richness are lower in the lungs (Bassis et al., 2015). Indeed, the oropharynx is considered the principal origin for the lung bacteriome during health (Bassis et al., 2015; Dickson et al., 2016). At the genus level, *Prevotella* is consistently among the core bacterial communities of the respiratory tract (Bassis et al., 2015; Einarsson et al., 2019). However, methodologies assessing the bacterial taxa at the species level, and even at the strain level, are necessary for discovering their source and role as beneficial, harmless or potentially harmful members of the bacterial communities at a specific body site. Noteworthy is that the *Prevotella* genus accommodates a high number of species with distinct clinical significance.

Valuable, detailed species-level oropharyngeal data assessed by in-depth sequencing are available from a study where tonsillar crypts in 2- to 4-year-old children and young adults were examined during recurrent tonsillitis and were compared to tonsillar crypts in children with tonsillar hyperplasia but without inflammation and those in healthy adult controls (Jensen et al., 2013). *Streptococcus* and *Prevotella* were found in all 20 samples from children and high *Prevotella* abundance was observed. Recurrent tonsillitis associated with a shift in the bacterial composition, especially with increased *P. melaninogenica/P. histicola* in adults. Typical oral *Prevotella* species, including *Prevotella buccae*, *P. dentalis*, *P. denticola*, *Prevotella fusca*, *Prevotella micans*, *P. oralis*, *P. oris*, *P. pallens*, *Prevotella salivae*, and *P. veroralis*, were more abundant in adults but *Prevotella saccharolytica* in children. *P. intermedia* was absent, except for a healthy adult with a high quantity. The recoveries of *P. nanceiensis* and *P. pleuritidis* from the oropharynx give support for their oral habitat. These study results indicate that a core microbiome, with a few significant genera, is present in tonsillar crypts regardless of individuals' age and health status (Jensen et al., 2013).

Recently, a potential link between the microbial community composition and lung homeostasis was examined by analyzing bronchoalveolar lavage samples from a longitudinally followed post-transplant study population (Das et al., 2021). Although the lung microbiota proved to be highly variable, there were a few bacterial taxa with high prevalence and/or abundance, and the majority of them were either obligate or facultative anaerobes. Among the most prevalent species were *P. melaninogenica*, *Veillonella atypica*, *Veillonella dispar*, *Streptococcus mitis*, and *Granulicatella adiacens* (all typical recoveries from the oral cavity). The microbiota was categorized into four distinct compositional states (pneumotypes) where the balanced pneumotype represented a diverse bacterial community, resembling that in the oropharynx and including

P. melaninogenica, which occurred in 97.4% of the samples (Das et al., 2021). In addition, this pneumotype had a high immune-modulatory activity and preserved lung stability.

Although oral bacteria get access to proximal airways via microaspiration, their growth at high densities is prevented by the continuous mucociliary clearance (Bassis et al., 2015). A recent study using a mouse model (Wu et al., 2021) demonstrated that the episodic aspiration of oral commensals, such as *P. melaninogenica*, *Veillonella parvula*, and *S. mitis*, leads to dysbiosis and low-dose inflammation in the lower airways; the consequence is a reduced susceptibility to pathogenic *Streptococcus pneumoniae* via activation of pulmonary Th17 cells. The shift in the human lung microbiome from the phylum Bacteroidetes in health to Gammaproteobacteria in disease indicates that *Prevotella*-activated Th17 response is an essential part of the pathogen recognition and suppression system of a healthy lung environment (Huffnagle et al., 2017). Anaerobic bacteria, especially *Prevotella*, are frequent recoveries from clinical respiratory specimens; however, understanding of their contribution to lung diseases is not clear yet.

Prevotella Involvement in Acute Diseases of the Respiratory Tract

Along with high research interest targeted to the COVID-19 pandemic, an increasing number of reports on the potential involvement of oral bacteria in the disease persistence and treatment outcome are available in the current literature. Interestingly, also *Prevotella* organisms, being analyzed from salivary, oropharyngeal, and bronchoalveolar lavage samples examined for SARS-CoV-2, have come up recently in this context. The microbiome and SARS-CoV-2 viral loads in saliva were compared between hospitalized COVID-19 and control patients (Miller et al., 2021). Although no significant difference in their bacterial compositions was found, the abundance of some taxa associated with the viral load in saliva; here, of special interest is enriched *P. pallens* but reduced *P. denticola* and *P. oris* in saliva of COVID-19 patients (Miller et al., 2021). Xiong et al. (2021) examined the difference in the microbial composition between SARS-CoV-2-positive and -negative pharyngeal swab samples collected from symptomatic patients with cough and fever. A significantly reduced species richness was seen in COVID-19 samples. The top-3 genera enriched were *Streptococcus, Prevotella,* and *Campylobacter*. Changes in abundance were seen also for several *Prevotella* species, such as *P. denticola*, *P. oris*, *P. jejuni*, *P. intermedia*, *P. melaninogenica*, *P. fusca*, and *P. scopos*, which were among the 37 species distinctive for the healthy and diseased individuals, and most of them separated the symptomatic COVID-19 and non-COVID groups from each other (Xiong et al., 2021). A dysbiotic oropharyngeal microbiota with gram-negative commensals, considered pathobionts due to their lipopolysaccharide production, has been connected to a so-called long-COVID disease (Haran et al., 2021). Among 164 patients with various types of symptoms, increased abundances of several *Prevotella* species were among the top predicting taxa from swabs of the posterior oropharynx: *P. denticola*, *P. nigrescens*, *P. histicola*, and *P. oulorum* in the patient group with ongoing symptoms, and *P. denticola*, *P. melaninogenica*, *P. jejuni*, and *P. nigrescens* in the determined long-COVID group (Haran et al., 2021). Sulaiman et al. (2021) examined a hospitalized cohort of 589 critically ill COVID-19 patients, characterizing their lung microbiome from bronchoalveolar lavage samples. Especially interesting were the findings of two oral commensals, *P. oris* and *Mycoplasma salivarium*, among the most dominant, functionally active microbial taxa. While *M. salivarium* was enriched in the deceased and >28-day mechanically ventilated groups, *P. oris* was enriched in the ≤28-day group. It was suggested that dissimilar microbial pressures related to host factors could explain the difference between the groups (Sulaiman et al., 2021). These studies indicate that the microbiome of the host plays a role in COVID-19 outcome.

Oral bacteria present in saliva can promote aspiration pneumonia via colonizing on mucosal surfaces, affecting immune response of epithelial cells, and producing proinflammatory cytokines and degradative enzymes, but dispersal via hematogenous route is also an option (Scannapieco, 2021). Older age and supine position as well as poor oral hygiene increase the risk for aspiration of bacteria from the oral cavity to lower parts of the respiratory tract (Hasegawa et al., 2014; Scannapieco, 2021). In pneumonias as well as pleural empyema, both anaerobes and oral bacteria, which can be missed by conventional culture, are more frequent findings by molecular methods (Yamasaki et al., 2013; Dyrhovden et al., 2019; Aoki et al., 2021). In community-acquired pneumonia, approximately 8% of bronchoalveolar lavage specimens from 64 hospitalized pneumonia patients were positive for *Prevotella/Alloprevotella*, three with *P. veroralis*, and *P. melaninogenica* and *A. tannerae* one each (Yamasaki et al., 2013). These findings came from mild cases, however, their pathogenic role remained unknown. Attempts to recover bacterial taxa regardless of their expected pathogenicity or quantity from respiratory specimens revealed *Prevotella* species in the majority of 17 aspiration pneumonias and eight lung abscesses (Aoki et al., 2021). Only occasional *Prevotella* recoveries, including *P. buccae*, *P. oris*, *P. pleuritidis*, and *A. tannerae*, came from 27 pleural empyema with poorly described etiology but potentially of oral origin (Dyrhovden et al., 2019).

Prevotella Involvement in Chronic Diseases of the Respiratory Tract

Chronic diseases of the airways are characterized by a reduced capability of eliminating microbes (Dickson et al., 2016). This may allow a persistent colonization of opportunistic pathogens, such as *Pseudomonas aeruginosa* or *Haemophilus influenzae*, with harmful consequences for respiratory health. During exacerbation, there are acute periods resulting in both local and systemic inflammation and worsened lung function. Due to culture-independent methods and increased research interest in the role of anaerobic bacteria and their function, it has been shown that dysbiotic bacterial compositions are, indeed, involved in inflammation of the respiratory airways (Dickson et al., 2016; Huffnagle et al., 2017). The presence of *Prevotella* in the lower respiratory tract is related to the Th17 activation and

differentiation, as defined by Th17-chemoattractant chemokine concentrations and STAT3 expression, respectively (Segal et al., 2016). However, the role of *Prevotella*-mediated Th17 activation in the maintenance of lung health is not fully elucidated yet. A recent mouse-model study demonstrated that the episodic aspiration of oral commensals (*P. melaninogenica*, *V. parvula*, and *S. mitis*) leads to dysbiosis and low-dose inflammation in lower airways, decreasing the susceptibility to the respiratory pathogen *S. pneumoniae* via activation of pulmonary Th17 cells (Wu et al., 2021).

Reduced abundances of the phylum Bacteroidetes and the genus *Prevotella* have been observed in the oropharynx of patients with asthma and chronic obstructive pulmonary disease (COPD) as well as in bronchial washings in COPD patients (Hilty et al., 2010; Park et al., 2014; Einarsson et al., 2016). A recent study examined pediatric asthma-associated alterations in the respiratory microbiota connected to host metabolism and responses, showing that several *Prevotella* species were enriched in the control group as well as *P. pallens* and *Prevotella* oral taxon 306 having an inverse correlation with total and allergen-specific IgE levels (Chiu et al., 2020). In the oropharynx of 13 adult asthma patients, *Prevotella* proved to be the most abundant genus and *P. melaninogenica*, *P. pallens*, and *P. nigrescens*, in descending order, most abundant *Prevotella* species (Lopes Dos Santos Santiago et al., 2017). Although *P. melaninogenica* and *S. mitis/S. pneumonia*e were the most abundant species, the bacterial compositions did not differ from those found in non-asthmatic individuals. As regards COPD, a recent study characterizing a potential association between the microbiota and risk for exacerbation or airflow limitation revealed significantly reduced proportions of *P. histicola*, *Gemella morbillorum*, and *Streptococcus gordonii* in sputum of patients with high risk of COPD exacerbation (Yang et al., 2021). The authors assumed that this kind of alteration in the resident microbiota to a dysbiotic direction could enhance inflammation in respiratory mucosae. In a mouse model, *P. melaninogenica*, *P. nanceiensis*, and *P. salivae* strains were shown to induce COPD-like symptoms via activation of neutrophils and elevating cytokine expression in a TLR-2 dependent manner (Larsen et al., 2014). An interesting finding was that only whole cells but not lipopolysaccharide of *Prevotella* initiated these symptoms, indicating that TLR-4 does not take part in the cellular response against *Prevotella* seen in healthy airways of humans. In a previous study, the authors demonstrated that the same *Prevotella* strains were able to reduce the expression of *Haemophilus*-induced human dendritic cell IL-12p70, but not IL-23 and IL-10 expressions (Larsen et al., 2012). Later, it was demonstrated that *Prevotella* outer membrane proteins (OMPs) are responsible for the Th17 development, activation, and IL-17B and IL-17A expression, which eventually promote pulmonary fibrosis (Yang et al., 2019). Noteworthy is that OMPs of *Prevotella* contain lipopolysaccharides and lipoproteins, which stimulate IL-17 expression via the TLR-Myd88 signaling pathway (Yang et al., 2019). These results indicate that commensal *Prevotella* organisms not only contribute to the aggravation of airway immune response, but also enable the regulation of pathogen-induced immune response.

Also in other chronic diseases of the lower respiratory tract, like bronchiectasis and cystic fibrosis, *Prevotella* is among the predominant findings (Tunney et al., 2013; Renwick et al., 2014; Muhlebach et al., 2018; Einarsson et al., 2019). For example, a multi-center study, including over 200 participants with age ranging from childhood to mid-adulthood and with different genetic and geographic backgrounds, examined whether there is a link between strict anaerobes and the severity of cystic fibrosis (Muhlebach et al., 2018). Across all ages, *Streptococcus* and *Prevotella* had the highest detection rates in sputum samples, 82 and 51%, respectively. Contrasting to the high prevalence of *Prevotella*, its abundance appeared to be low. Interestingly, the presence of anaerobes associated with phenotypically milder disease, whereas *Pseudomonas* (*P. aeruginosa*), the typical pathogen in cystic fibrosis, associated with severe disease (Muhlebach et al., 2018). Recently, Einarsson et al. (2019) assessed microbial community structures within the airways and clarified how various taxa are distributed in communities representing health or chronic disease (here: bronchiectasis and cystic fibrosis). The "core" community was composed of members of the genera *Streptococcus*, *Veillonella*, *Prevotella*, *Granulicatella*, and *Fusobacterium*, while skewed community structures were found in cystic fibrosis and bronchiectasis samples. Notably, anaerobic bacteria, e.g., *Prevotella* and *Veillonella*, proved to affect the variance within the airways, interacting with opportunistic lower airway pathogens (Einarsson et al., 2019).

Some species-level data are available on *Prevotella* in cystic fibrosis. A recent multi-center study (O'Connor et al., 2021), looking for bacterial communities in bronchoalveolar lavage fluid of 63 diseased and 128 control individuals from infancy to young adulthood, demonstrated the *S. mitis* group (52%) and *P. melaninogenica* (44%) as being the most prevalent bacterial taxa. However, distinct abundance patterns were recognized between the study groups; while the abundance of the *S. mitis* group was high regardless of age in controls, *Staphylococcus aureus* dominated in cystic fibrosis. Low abundance of *P. histicola* was found in part of diseased and control individuals across the age spectrum, and interestingly, *P. oris* was detected at high abundance in some diseased individuals (O'Connor et al., 2021). Based on the comparison of bronchoalveolar fluid and oropharyngeal swab samples, overall diversity of the upper and lower airway microbiome is similar in clinically stable children with cystic fibrosis (Renwick et al., 2014). However, bacterial communities in lower airways significantly differed between cystic fibrosis and control children; while *P. veroralis* was absent in cystic fibrosis, it was common in controls. Pulsed-field gel electrophoresis patterns were produced in a study targeting to reveal the degree of clonal similarity of 42 *Prevotella* isolates collected from sputum samples during stable, exacerbated, and post-exacerbation periods (Gilpin et al., 2017). Initial sampling was performed during clinical stability, and the *Prevotella* findings included *P. denticola*, *P. histicola*, *P. melaninogenica*, *P. nigrescens*, and *P. salivae*. Seven isolates could not be definitely identified but remained as *P. veroralis/P. histicola* or *P. melaninogenica/P. histicola*. Genotyping analysis allowed recognizing similar banding patterns (genotypes) during the

follow-up. It was suggested that, instead of repeated acquisition, a persistent colonization of *Prevotella* species had occurred in patients with cystic fibrosis (Gilpin et al., 2017). In an experimental study, cystic fibrosis bronchial epithelial cells were exposed to *P. histicola* or *P. nigrescens* (Bertelsen et al., 2021). Both strains were able to induce disrupted NF-κB(p65) and MAPK activations via suppressing TLR-4 and stimulating TLR-2 in epithelial cells. Infection with a *P. nigrescens* strain induced only low levels of p65-mediated inflammation compared to inflammatory response of a *P. aeruginosa* strain from the same patient (Bertelsen et al., 2020). The authors speculated that by TLR-2 signaling, and by reducing TLR-4 release and IL-6 and IL-8 production, *Prevotella* may inhibit the growth of the major pathogen, *P. aeruginosa*, and have a beneficial effect on immune response in the lungs affected by cystic fibrosis.

SUMMARY

Due to increased research interest in commensal bacteria in humans, there is now considerable evidence on the complex nature of commensal bacterial communities in the lower airways as well as gastrointestinal tract above the waistline, including the esophagus, stomach, and upper part of the small intestine. It is obvious that oral members of the genus *Prevotella* play an important role in health and disease at these body sites. In the gastrointestinal tract, the presence of *Prevotella* may influence, positively or negatively, the severity of disease, such as reflux disease, gastritis, IBD, and different cancer types. In the respiratory tract, current research has brought information on the potential involvement of oral bacteria, including some *Prevotella* organisms, in COVID-19 persistence and treatment outcome. As regards *Prevotella* species in chronic respiratory diseases, the current data report on reduced abundance of anaerobes, especially *Prevotella*, which indicates a disruption of homeostatic respiratory microbiota, potentially exposing to lung disease progression. To date, there is only a limited number of mechanistic studies to explain the relation between specific *Prevotella* species involved in diseases in the respiratory and gastrointestinal tracts. The wide intra-genus variation and distinct properties of individual species within the genus *Prevotella* call for further studies on oral *Prevotella* species and their involvement inside and outside the oral cavity to clarify their impact on human health and disease.

AUTHOR CONTRIBUTIONS

EK conceptualized the manuscript and was responsible for the content dealing with microbes. UKG was responsible for immune-inflammatory aspects. Both authors read and approved the final manuscript.

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Human Microbiota in Esophageal Adenocarcinoma: Pathogenesis, Diagnosis, Prognosis and Therapeutic Implications

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Esophageal adenocarcinoma (EAC) is one of the main subtypes of esophageal cancer. The incidence rate of EAC increased progressively while the 5-year relative survival rates were poor in the past two decades. The mechanism of EAC has been studied extensively in relation to genetic factors, but less so with respect to human microbiota. Currently, researches about the relationship between EAC and the human microbiota is a newly emerging field of study. Herein, we present the current state of knowledge linking human microbiota to esophageal adenocarcinoma and its precursor lesion—gastroesophageal reflux disease and Barrett's esophagus. There are specific human bacterial alternations in the process of esophageal carcinogenesis. And bacterial dysbiosis plays an important role in the process of esophageal carcinogenesis *via* inflammation, microbial metabolism and genotoxicity. Based on the human microbiota alternation in the EAC cascade, it provides potential microbiome-based clinical application. This review is focused on novel targets in prevention, diagnosis, prognosis, and therapy for esophageal adenocarcinoma.

Keywords: microbiota, esophageal adenocarcinoma, Barrett's esophagus, gastroesophageal reflux disease, microbial therapy

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INTRODUCTION

Esophageal cancer (EC) is the seventh most common cancer with an estimated 604, 000 new cases worldwide in 2020. It is also the sixth leading cause of cancer death with an estimated 544,000 deaths in 2020 (Sung et al., 2021). The age-standardized 5-year net survival of the EC patients was in the range of 10–30% between 2010 and 2014, except in Japan and Korea. In many countries, the age-standardized 5-year net survival trends increased by 6–10% from 2000 to 2014 (Allemani et al., 2018). Tackling the global burden of the EC is one of the major challenges in this century.

There are two main distinctive histological subtypes that account for more than 95% of EC, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). Generally, ESCC occurs in the upper two-thirds of the esophagus, whereas EAC typically occurs in the lower third of the esophagus (Arnold et al., 2020). In recent decades, the incidence rate of EAC in the United States has increased to 7.2 per 100,000 populations, while the incidence rate of ESCC has been sharply decreasing (Simard et al., 2012). From 1999 to 2008, the incidence rate of EAC showed an increasing trend

in all races, except for American Indian or Alaska native, whose average annual percent change was −0.1. Although increased progressively during 1992 through 2007, 5-year relative survival rates for EAC were poor (Simard et al., 2012). Gastroesophageal reflux disease (GERD) and obesity have been identified as strong risk factors for EAC. Tobacco smoking and alcohol consumption might facilitate EAC development. In contrast, weight loss, estrogens, dietary fiber, and vegetable intake might protect against its development (Coleman et al., 2018). These risk factors provided clues for the primary prevention of EAC, thus public health interventions to modify them are advisable (Lagergren and Lagergren, 2013; Thrumurthy et al., 2019). Our knowledge of the human microbiota has expanded exponentially with the development of novel molecular methods, especially metagenome sequencing. Much of the current literature on cancer pays particular attention to the human microbiota (Plottel and Blaser, 2011). Accumulating evidence suggests that human microbiota contributes to colorectal cancer, gastric cancer, liver cancer, lung cancer and breast cancer (Schwabe and Jobin, 2013). Besides, the human microbiota is widely regarded as a potential co-factor for the development of EAC and its precursor Barrett's esophagus (BE) (Peters et al., 2017; Quante et al., 2018).

Herein, we present the current state of knowledge linking human microbiota to esophageal adenocarcinoma, with a primary focus on its potential clinical applications.

HUMAN MICROBIOTA

The human genetic makeup is virtually identical. Different from the human genome, the metagenome of the human microbiome shows greater variability (Lloyd-Price et al., 2016). The human microbiota is a highly individual, complex, and dynamic community in each healthy individual (Consortium, 2012; Gilbert et al., 2018). There are 10–100 trillion symbiotic microorganisms and 500–1000 species of bacteria in the human body, whereas the number of sub-species could be far more (Turnbaugh et al., 2007). Even in the same person, it will be extraordinarily different from before. Besides, there are diverse archaea, fungi, and viruses colonizing in the human body, although the current understanding of them remains limited. The digestive tract is the largest microbial habitat in the human body, which has the largest number of microbes and the most kind of species (Gupta et al., 2017). The gastrointestinal microbiota has three main ways of colonization: in the epithelial mucosa, in digest particles, and suspension solution (Dominguez-Bello et al., 2019). Investigators have been devoted to identifying the core microbiota, which is characterized by a group genera of being found in all populations regardless of their geographical location, ethnic background or residence. A population-level analysis reported a 14-genera core microbiota (*Lachnospiraceae*, *Ruminococcaceae*, *Bacteroides*, *Faecalibacterium*, *Blautia*, *Roseburia*, *Erysipelotrichaceae*, *Coprococcus*, *Dorea*, *Clostridiaceae*, *Hyphomicrobiaceae*, *Clostridiales*, *Veillonellaceae*, *Clostridium* XIVa) by assessing human fecal samples (Falony et al., 2016).

Given the well-established carcinogenesis that *Helicobacter pylori* had in gastric cancer and human papillomavirus had in cervical cancer, human microbiota was starting to be considered as a key factor that influences both human health and disease in the past decade (Bashan et al., 2016). Along with the deep-going of the research, in addition to special pathogens, the imbalance of normal microbiota can also cause diseases, such as allergy and psoriasis. Studies in colon cancer animal models have revealed evidence for tumor-promoting effects of the microbiota dysbiosis. There is a significant decrease in the number of tumors with the treatment of wide-spectrum antibiotics (Schwabe and Jobin, 2013). In addition, microbial diversity is associated with disease status. It is well established that type 2 diabetes and inflammatory bowel disease have low intestinal microbial diversity, as well as cervical intraepithelial neoplasia and bacterial vaginosis have high vaginal microbial diversity (Fredricks et al., 2005; Consortium, 2012; Qin et al., 2012; Mitra et al., 2015; Proctor, 2019). The mechanisms by which the human microbiota is involved in carcinogenesis primarily includes inflammation, immunity, metabolism, genomic integration, and genotoxicity (Scott et al., 2019). As an example, Gram-negative bacteria could acquire carcinogenic ability by producing genotoxin (He et al., 2019). Consequently, Microbiome Wide Association Studies, including DNA sequencing, metabolomics, proteomics, and computation, are providing potential microbiome-based screening tools, diagnostic markers, and adjuvant therapies (Kåhrström et al., 2016). It links microbial community structure and metabolites with disease status, which will lead clinical researches to a new field in the future.

HUMAN MICROBIOTA ALTERNATION IN THE ESOPHAGEAL ADENOCARCINOMA CASCADE

Esophageal Dysbiosis in the Esophageal Adenocarcinoma Cascade

Unlike the oral cavity, stomach, or intestine, the esophagus has its unique microbiota. A total of 41 genera belonging to six phyla of bacteria colonizing in the normal distal esophageal were identified (Pei et al., 2004). Six phyla consisted of *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and TM7. And top five genera were *Streptococcus*, *Prevotella*, *Veillonella*, *Rothia*, and *Megasphaera*. Furthermore, shotgun sequencing identified that there were not only abundant bacteria but also a relatively low abundance of viruses and eukaryotes in the esophagus, such as betaherpesvirus 7 and *Candida glabrata* (Deshpande et al., 2018). The esophageal microbiota is classified mainly into three main community types, and it has been proved significant differences across the three types. Among them, the predominant genus is *Streptococcus* in type 2 and it is *Prevotella* in type 3. Type 1 is an intermediate type between type 1 and type 2, which is composed of not only *Streptococcus* and *Prevotella*, but also increased abundances of *Haemophilus* and *Rothia* (Deshpande et al., 2018). Although there is no statistical difference in the

total amount of microbial DNA among normal esophagus, reflux esophagitis (RE), and BE, the microbial communities are different among them. By detecting bacterial populations of the distal esophagus, the percentage of Bacteroidetes in the normal esophagus, RE, and BE increased successively, but the percentage of Proteobacteria was detected successively (Liu et al., 2013). The normal esophageal mucosa had higher levels of Gram-positive *Firmicutes* and *Actinobacteria* compared to RE, BE, and EAC (Zhou et al., 2020). The microbe composition of esophagus samples including low-grade dysplasia (LGD), high-grade dysplasia (HGD), EAC, and healthy controls, were analyzed by 16S DNA sequencing. The top five different microbial taxa in abundance at the phylum level were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*. Compared with controls, phylum *Planctomycetes* and genus *Balneola* were decreased across disease groups, especially in HGD and EAC. And phylum *Crenarchaeota* was similarly decreased (Peter et al., 2020). The influence of age, host genetics and disease status on the esophageal microbiome has been identified. In support of these findings, a prospective study showed age was positively associated with the relative abundance of *Streptococcus* and negatively associated with relative abundance of *Prevotella melaninogenica* by using amplicon sequencing from 106 subjects. Deshpande et al. (2018) (Elliott et al., 2017) demonstrated a connection between host genetics and the composition of the esophageal microbiome with the help of MicrobiomeGWAS. Although the disease did not affect the global taxonomic composition of the esophageal microbiome, increasing Gram-negative bacteria taxa were found in esophageal carcinogenesis, which was only appearing in the disease states (Deshpande et al., 2018).

In order to avoid the interference of microorganisms in other parts of the digestive tract, investigators put forward various methods. At earlier stages of research on esophageal microbial colonization, esophageal biopsy and aspiration specimen measurement were applied in analyzing esophageal microbial composition. By Yang's preliminary statistics of previous cultivation-independent studies on esophageal microbiota, the number of bacterial species detected by biopsy samples ranged from 7 to 166 (Yang et al., 2014). And they found enrichment of *Streptococcus* on esophageal microbiota. In another research, a total of 18 species were isolated from normal esophageal mucosa, while only three genera were detected in esophageal aspirate specimens, including *Lactobacilli*, *Streptococci*, and yeasts (Macfarlane et al., 2007). For patients with Barrett's esophagus, the highest relative proportions were *Anaerococcus*, *Streptococcus*, and *Alloicoccus* in the esophagus, while the highest relative proportions were *Fusobacterium*, *Prevotella*, and *Dialister* in the uvula (Okereke et al., 2019). Recently, the microbial communities of EAC samples were examined by means of Cytosponge. EAC tissues had decreased microbial diversity, including a reduction of Gram-positive taxa (*Granulicatella*, *Atopobium*, *Actinomyces*, and *Solobacterium*) as well as Gram-negative taxa (*Veillonella*, *Megasphaera*, and *Campylobacter*) compared with healthy controls (Elliott et al., 2017). These studies confirmed that decreased microbial diversity and altered microbial composition may play a significant role in the EAC cascade.

Oral Dysbiosis in the Esophageal Adenocarcinoma Cascade

The oral cavity is the initial part of the digestive tract. It consists of oral lips, cheek, palate, teeth, tongue, and salivary gland. Microorganisms inhabit the available surface of oral cavity, such as the surfaces of teeth, tongue and mucosal membranes (Lamont et al., 2018). Thus, polymicrobial communities which inhabit the oral cavity have unique biogeography. The Human Microbiome Project (HMP) collected the specimens of 15 to 18 body sites from over 200 individuals. Seven of body sites were taken from the mouth including buccal mucosa, keratinized attached gingiva, hard palate, saliva, tongue and two surfaces along with the tooth. Segata et al. (2012) analyzed sub-gingival plaques, supra-gingival plaques, stool and oral specimens from the HMP. They demonstrated that the microbial communities of the tongue are similar to saliva and the microbial communities of buccal mucosa are similar to keratinized attached gingiva and hard palate, while the microbial communities of sub-gingival and supra-gingival plaque were distinct from others. The site-specialist hypothesis for oral microbiota was proposed that there was a prime habitat for oral microbiota where most of oral microorganisms grew and divided (Mark Welch et al., 2019). Besides, microbial compositions in the oral cavity and esophagus are similar but essentially different (**Figure 1**). Dong et al. (2018) collected oral samples from saliva, tongue dorsum and supragingival plaque, as well as esophageal samples from upper, middle and lower of the esophagus. There were 594 genera subjected to 29 phyla in the esophagus and 365 genera subjected to 29 phyla in the oral cavity. Both of them detected high relative abundances of bacteria, including *Streptococcus*, *Neisseria*, *Prevotella*, *Actinobacillus*, and *Veillonella*. The predominant genus in the esophagus was *Streptococcus*, while the predominant genus in the oral cavity was *Neisseria*.

It is well-established that oral microbiota has a close association with many oral diseases, such as periodontitis, tooth reduction, dental caries. In addition to these diseases, oral microbiota alteration has been suggested to play an important role in diabetes, rheumatoid arthritis, chronic obstructive pulmonary diseases, cardiovascular diseases, and cancer (Gupta et al., 2017; Bourgeois et al., 2019b; Sun et al., 2020; Tuominen and Rautava, 2021). In particular, the relative abundance of *Porphyromonas gingivalis* in patients with digestive tract cancer (tongue/pharyngeal cancer, EC, gastric cancer, colorectal cancer) was higher than that in healthy controls (Kageyama et al., 2019). Other studies have reported the relationship between oral microbiota and EAC. On the one hand, a prospective study showed that a history of periodontal disease and tooth loss was associated with a 43% and 59% increased risk of EAC over 22–28 years of follow-up (Lo et al., 2021). On the other hand, the salivary bacterial diversity was significantly higher in EC patients than that in healthy controls (Kageyama et al., 2019). And a case–control study in China showed a significant shift in oral microbiota between the EC patients

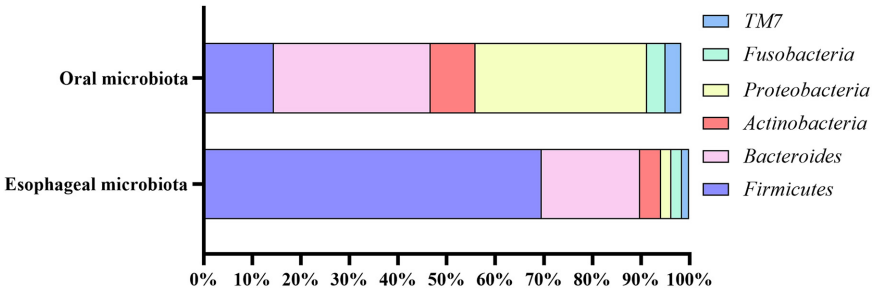

FIGURE 1 | The predominant phyla of esophageal microbiota and oral microbiota. The top six most abundant phyla of esophageal microbiota consisted of *Firmicutes* (69.60%), *Bacteroides* (20.20%), *Actinobacteria* (4.30%), *Proteobacteria* (2.20%), *Fusobacteria* (2.20%), *TM7* (1.40%); And the top six most abundant phyla of oral microbiota consisted of *Proteobacteria* (35.34%), *Bacteroides* (32.20%), *Firmicutes* (14.48%), *Actinobacteria* (9.26%), *Fusobacteria* (3.76%), *TM7* (3.25%).

and the healthy participants. By detecting salivary microbial communities, EC patients had a higher relative abundance of phylum *Firmicutes*, class *Negativicutes*, order *Selenomonadales*, family *Veillonellaceae*, and genus *Prevotella*, and a lower relative abundance of phylum *Proteobacteria*, class *Betaproteobacteria*, order *Neisseriales*, family *Neisseriaceae*, and genus *Neisseria* in contrast with healthy individuals (Zhao et al., 2020). Moreover, another research showed that BE patients had a higher relative abundance of *Firmicutes* and a lower relative abundance of Proteobacteria in saliva compared to patients without BE (Snider et al., 2018), which was in accordance with the EC patients. All of these researches support a link between oral microbiota and EAC development.

HUMAN MICROBIOTA IN ESOPHAGEAL ADENOCARCINOMA

Determination of the variation in human microbiota between health and disease is crucial to understanding the biases that occur in disease. There were many studies of the esophageal microbiota alteration in EAC. One prior case–control study found altered microbial communities in esophageal carcinogenesis, notably increases in *Proteobacteria* and reductions in *Firmicutes*. Besides, two families, *Verrucomicrobiaceae* and *Enterobacteriaceae*, became increasingly in HGD and EAC (Snider et al., 2019). Similarly, Zaidi and colleagues found a high prevalence of *Escherichia coli* in EAC and BE patients, while it was lacking in the tumor adjacent normal epithelium. All these indicated that the shift toward *Enterobacteriaceae* in esophageal carcinogenesis was not accidental. According to the research of the esophageal microbiome, there is a reduction of *Streptococcus* and an increase of *Prevotella* in EAC compared with healthy controls (Lopetuso et al., 2020). Zhou and colleagues discovered a unique esophageal microbiota in EAC subjects. Compared with normal esophageal, there were abundant *Proteobacteria* and *Firmicutes*, mostly like *Staphylococcus aureus*, *Streptococcus infantis*, *Moryella* sp. and *Lactobacillus salivarius*, and rare *Actinobacteria* (*Rothia mucilaginosa*) in the EAC esophageal microbiota (Zhou et al., 2020). Most of them were lactic acid-producing bacteria. As is well established, sustained high lactate level could promote angiogenesis, immune escape, cell migration and metastasis, thus supporting the tumorigenesis and progression (San-Millán and Brooks, 2017). The authors proposed that increased lactic acid-producing bacteria in the esophageal may work as one of the factors contributing to the development of the EAC. Additionally, there is a high prevalence of *Candida albicans* and *Candida glabrata* in more than half of the human EAC samples (Zaidi et al., 2016), which suggests the existence of fungal microbiota in the esophagus.

Esophageal adenocarcinoma has been studied extensively in relation to the esophageal microbiota, but relatively insufficiency so with respect to microbiota at other sites of the human body. A prospective study examined the relationship between EAC and oral microbiota. In mouthwash samples, there was a high amount of *Tannerella forsythia*, *Actinomyces cardiffensis*, *Veillonella* oral taxon 917, and *Selenomonas* oral taxon 134 was associated with higher EAC risk, whereas a low amount of *Prevotella nanceiensis*, *Corynebacterium durum*, *Streptococcus pneumoniae*, *Lachnoanaerobaculum umeaense*, *Solobacterium moorei*, *Oribacterium parvum*, *Neisseria flavescens*, *Neisseria sicca*, and *Haemophilus* oral taxon 908 was associated with lower EAC risk (Peters et al., 2017). If these results turn out to characterize the shift with the progression of EAC, rather than simply correlative, they demonstrate potential prevention *via* protecting against microbial exposure.

HUMAN MICROBIOTA IN GASTROESOPHAGEAL REFLUX DISEASE AND BARRETT'S ESOPHAGUS

Gastroesophageal reflux disease was regarded as a risk factor for EAC and BE was established as the precursor lesion of EAC. It is of momentous significance to clarify the human microbiota of GERD and BE for the EAC researches. An early study by Yang in 2009 found the potential link between

alterations in the human distal esophageal microbiome and reflux-related disorders. The bacterial communities of 34 patients were checked after biopsies of the distal esophagus by 16S rRNA gene sequencing. The authors classified the human esophageal microbiome into two types according to the results of gene analysis. The type I esophageal microbiome was more relevant to the normal esophagus, while the type II esophageal microbiome was more relevant to the abnormal esophagus. The type I microbiome had a higher mean abundance of *Streptococcus*, while the type II microbiome had a higher level of microbial diversity and a higher average proportion of Gram-negative bacteria. They also concluded that the type II microbiome was mainly composed of Gram-negative anaerobes or microaerophiles, including *Veillonella*, *Prevotella*, *Neisseria*, *Haemophilus*, *Rothia*, *Granulicatella*, *Campylobacter*, *Fusobacterium*, *Porphyromonas*, and *Actinomyces*. The predominant organisms shifted from Gram-positive aerobic bacteria to Gram-negative anaerobic bacteria (Yang et al., 2009).

Similarly, increasing evidence has supported a shift toward some specific Gram-negative bacteria in the EAC cascade. It was reported that Gram-negative organisms colonizing the esophageal mucosa, especially *Campylobacters*, became increasingly in GERD and BE compared with healthy control groups (Blackett et al., 2013). Other studies found that there was a shift away from *Firmicutes* and toward Gram-negative *Fusobacteria*, *Sphingomonas*, *Proteobacteria* and an unclassified species of *Campylobacter* in BE compared to controls (Snider et al., 2019; Zhou et al., 2020). Of note, Lopetuso and colleagues found that the relative abundance of *Streptococcus* and *Granulicatella* decreased in the EAC mucosa compared with BE mucosa, with the relative abundance of *Prevotella* increased correspondingly. The authors considered EAC as an extreme dysbiotic perturbation of microbiota in BE mucosa which consisted largely of Gram-negative bacteria (Lopetuso et al., 2020). In summary, alteration of the human microbiota in EAC cascade was presented as decreased microbial diversity and enrichment of Gram-negative bacteria in esophagus as well as increased microbial diversity and enrichment of *Firmicutes*, *Tannerella forsythia*, *Actinomyces cardiffensis* in oral cavity (**Figure 2**).

HUMAN MICROBIOME AS POTENTIAL DIAGNOSTIC BIOMARKERS AND SCREENING TOOLS FOR ESOPHAGEAL ADENOCARCINOMA

Current screening tools have respective advantages and disadvantages. The gold-standard technique of EC and preinvasive lesions is endoscopy with adequate targeted biopsies. However, this method cannot be used extensively due to the time and expense. Esophageal tissue samples including sponges and inflatable balloons have good specificity but lack sensitivity (Lao-Sirieix and Fitzgerald, 2012). It has been confirmed some specific pathogens could promote the development of EC, while other pathogens could be a protective factor against the reduced risk of EC. As a result, some biomarkers have enormous potential as diagnostic biomarkers and screening tools for EAC (**Table 1**). Finding out a biomarker with excellent sensitivity and specificity is the key to extending the biomarker detection application field.

With the increase in antibiotic treatment in the mid-twentieth century, infections of *Helicobacter pylori* began to decline, then the incidence of esophageal adenocarcinoma and eosinophilic

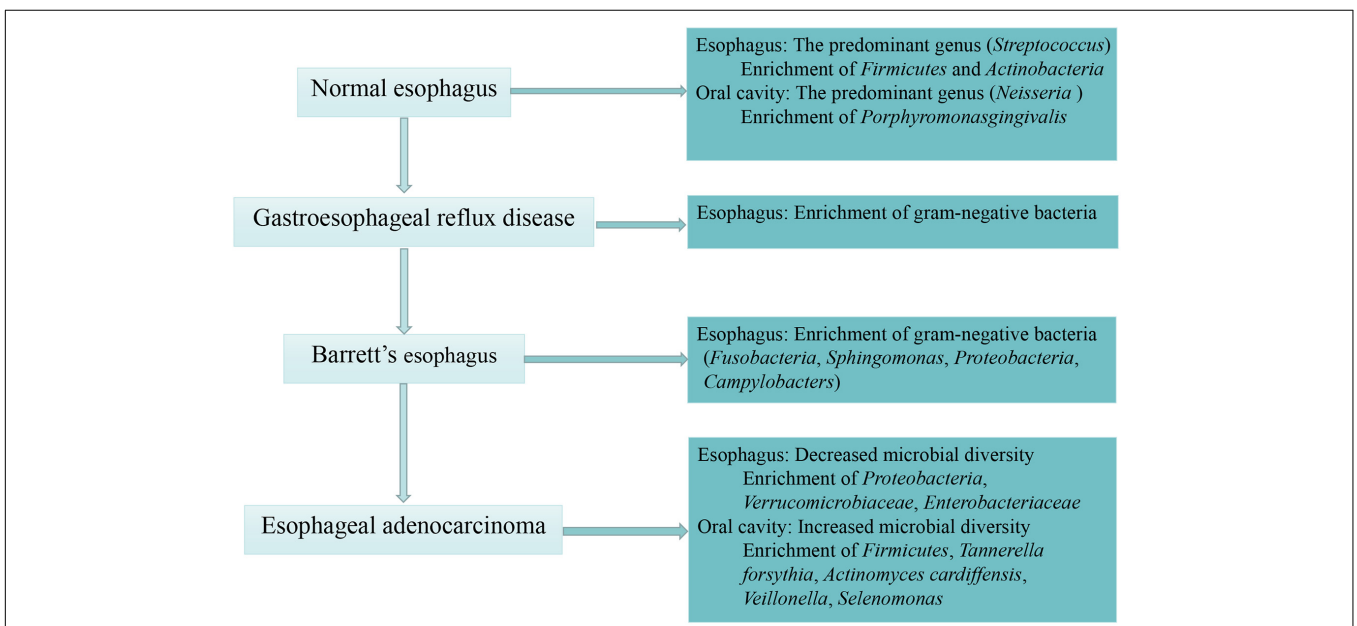

FIGURE 2 | The process of esophageal carcinogenesis and associated human microbiota. This figure describes specific human bacterial alternations in the normal esophagus, esophageal adenocarcinoma and its precursor lesion—gastroesophageal reflux disease and Barrett's esophagus.

TABLE 1 | Human microbiota studies for esophageal adenocarcinoma.

Study	Population(s)	Study sample size	Study period	Study platform	Sample type	Main findings	Tool type
Lopetuso et al., 2020	Rome	BE (*n* = 10); EAC (*n* = 6); controls (*n* = 16)	2020	16S rRNA	Esophageal mucosa	*Prevotella*, *Veillonella*, and *Leptotrichia* had higher abundance in EAC than that of CTRL, while *Streptococcus* had lower abundance.	Diagnosis
Zhou et al., 2020	Australia	RE (*n* = 20); BE (*n* = 17); EAC (*n* = 6); controls (*n* = 16)	2020	16S rRNA	Esophageal mucosa	Compared with CTRL, there was a reduction of *Actinobacteria* in EAC, with an increase of *Firmicutes* and *Proteobacteria*.	Diagnosis
Peters et al., 2017	America	EAC (*n* = 81); controls (*n* = 160)	2017	16S rRNA	Mouthwash samples	The abundances of species *Tannerella forsythia* were positive correlated with risk of EAC, while the abundances of the genus *Neisseria* and the species *Streptococcus pneumoniae* were inversely correlated with risk of EAC	Diagnosis
Snider et al., 2019	United States	LGD (*n* = 6); HGD (*n* = 5); BE (*n* = 14); EAC (*n* = 4); controls (*n* = 16)	2019	16S rRNA	Saliva samples	There was a shift toward *Enterobacteriaceae* and *Akkermansia muciniphila*, while away from *Firmicutes* in patients with HGD and EAC relative to controls	Diagnosis
Zhao et al., 2020	China	EC (*n* = 39); controls (*n* = 51)	2020	16S rDNA	Saliva samples	*Prevotella* was enriched in EC, while *Neisseria* was decreased.	Diagnosis
Peter et al., 2020	United Kingdom	IM (*n* = 10); LGD (*n* = 10); HGD (*n* = 10); EAC (*n* = 12); controls (*n* = 10)	2020	16S rDNA	Esophageal mucosa	The abundance of the phylum *Planctomycetes* and the archaean phylum *Crenarchaeota* in EAC was significantly lower than that in CTRL	Diagnosis
Deshpande et al., 2018	Australia	EoE (*n* = 1); GERD (*n* = 29); GM (*n* = 7); BE (*n* = 5); EAC (*n* = 1); CTRL (*n* = 59)	2018	16S rRNA; 18S rRNA; shotgun sequencing	Esophageal mucosa; esophageal brushings	An enrichment of Gram-negative bacteria associated with the oral cavity and microbial lactic acid production in the EAC cascade	Diagnosis
Elliott et al., 2017	United Kingdom	ND (*n* = 20); BE (*n* = 23); EAC (*n* = 19); CTRL (*n* = 20)	2017	16S rRNA	Esophageal mucosa; esophageal brushes; Cytosponge samples	*Lactobacillus fermentum* was enriched in EAC compared with controls	Diagnosis
Kawasaki et al., 2020	Japan	EC (*n* = 61); CTRL (*n* = 62)	2020	PCR	Subgingival dental plaque; saliva samples	The prevalence of *Tannerella forsythia*, *Streptococcus anginosus*, and *Aggregatibacter actinomycetemcomitans* was positively related to the presence of EC with high odds ratios, respectively	Diagnosis
Rajendra et al., 2013	Australia	BE (*n* = 77); BD (*n* = 35); EAC (*n* = 27); CTRL (*n* = 122)	2013	PCR; immunohistochemistry;	Esophageal mucosa; tumor specimens	High activity of *human papillomavirus* was strongly association with worse disease severity	Prognosis
Yamamura et al., 2016	Japan	EC (*n* = 325)	2016	PCR	Tumor specimens; tumor adjacent normal specimens	*Fusobacterium nucleatum* in EC was related to higher tumor stage and poor prognosis in the patients after the esophagus carcinoma resection	Prognosis

BEM, esophageal metaplastic samples; BE, Barrett's esophagus; IM, intestinal metaplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia; BD, Barrett's dysplasia; EC, esophageal cancer; EAC, esophageal adenocarcinoma; CTRL, healthy control samples; PCR, polymerase chain reaction.

esophagitis rises (May and Abrams, 2018). The large-scale pooled analysis found that *Helicobacter pylori* infection varied directly as the odds of BE and inversely proportional to the odds of GERD (Wang et al., 2018). However, Aghayeva and colleagues retrospectively analyzed cases in Azerbaijan, a high-prevalence region, and highlighted that there is no difference between the prevalence of *Helicobacter pylori* in BE and control group cases. The authors concluded that neither BE nor dysplasia is inversely associated with the prevalence of *Helicobacter pylori* (Aghayeva et al., 2019). Similarly, the hypothesis of the Swedish nationwide population-based cohort study was confirmed by calculating the standardized incidence ratios (SIRs), which were equal to the observed number of individuals in the *Helicobacter pylori* eradication cohort over the expected number of individuals in the Swedish background population. This study found that there is no evidence a gradually increased risk of BE or EAC is linked with *Helicobacter pylori* eradication treatment in spite of the increasing SIRs of BE and EAC after *Helicobacter pylori* eradication treatment (Doorakkers et al., 2020). Whether *Helicobacter pylori* infection influences EAC and its precursor is still a debatable point. However, it has been noted that *Helicobacter pylori* infection promotes Ki-67 expression in BE.

According to a meta-analysis with 1243 samples, Ki-67 showed a reasonable diagnostic odds ratio of 5.54, sensitivity of 82% and specificity of 48% in identifying high-risk patients of EAC in BE group (Altaf et al., 2017). In addition to *Helicobacter pylori*, other human microbiota-associated biomarkers may be reasonably efficient in EAC screening and diagnosis. As a result of significantly increased abundance of *Prevotella* at the genus level and family level that covered all samples, Zhao and colleagues indicated that *Prevotella* was may be used in the early prediction or prevention of EC (Zhao et al., 2020). Overall, *Prevotella* and Ki-67 may play an important role in the personalized precision diagnosis of EAC.

Several studies have also implicated periodontal pathogens as potential diagnostic biomarkers for EAC. As mentioned above, Peters and colleagues indicated that *Tannerella forsythia* was strongly related to EAC. They observed that the increased abundance of *Tannerella forsythia* was correlated to the higher risk of EAC, while the decreased abundance of Neisseria and *Streptococcus pneumoniae* was correlated to the lower risk of EAC (Peters et al., 2017). Similarly, the prevalence of *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* with EC patients was significantly higher in the subgingival plaque compared with healthy controls (Kawasaki et al., 2020). It is now well accepted that both *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* are Gram-negative periodontal pathogens that might contribute to the pathogenesis of periodontitis (Sharma, 2010; Gholizadeh et al., 2017). A previous study suggested a possible correlation between *Aggregatibacter actinomycetemcomitans* and the increasing risk of pancreatic cancer (Fan et al., 2018). An oral microbiome-based model containing a relative abundance of *Streptococcus*, *Lautropia*, and *Bacteroidales* discriminated between BE patients and controls with the ROC of 0.94, the sensitivity of 96.9%, and the specificity of 88.2% (Snider et al., 2018). Our findings suggest that periodontal pathogens, like *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans*, may be utilized as biomarkers for detecting EAC-associated changes in the human microbiota.

HUMAN MICROBIOTA FOR CLINICAL PROGNOSIS ANALYSIS OF ESOPHAGEAL ADENOCARCINOMA PATIENTS

The late presentation of symptoms and the aggressiveness of EAC results in poor prognosis (Coleman et al., 2018). Interestingly, *human papillomavirus* (HPV)-related biomarkers in pre-cancer lesions can become an important prognostic indicator of EAC. A previous study has demonstrated that head and neck squamous cell carcinoma (HNSCC) patients with HPV-positive have a higher rate of overall survival and a lower risk of recurrence compared with HPV-negative patients (Ragin and Taioli, 2007; O'Rorke et al., 2012). Given the well-established impact that HPV status has on the prognosis of HNSCC, it is highly plausible that HPV-related EAC would show a similar prognosis. A prospective study has identified that high-risk HPV with transcription activity is associated with BD and EAC. Biopsy samples were used for HPV DNA determination *via* PCR and viral transcriptional activity determination *via* E6/7 oncogene mRNA expression and $p16^{INK4a}$ immunohistochemistry. Compared with BE and controls, the proportion of HPV DNA-positive, $p16^{INK4a}$ positivity and oncogene expression in Barrett's dysplasia (BD) and EAC was significantly higher (Rajendra et al., 2013). The authors emphasized that HPV was strongly relevant to BD and EAC but irrelevant to BE and controls, which suggested the role of HPV in the pathogenesis of tumors. Based on preliminary studies, a retrospective case–control study assessed HPV-related biomarkers [retinoblastoma protein (pRb), cyclin D1 (CD1), Ki-67, and minichromosome maintenance protein (MCM2)] to estimate the prognostic value on the patients with BD and EAC. The authors found low expression of CD1 with a good prognosis in EAC (Rajendra et al., 2020). In contrast to HPV-negative patients, HPV-positive patients with low expression of CD1, high expression of MCM2, low expression of pRb, high expression of p16 and positive status of E6 and E7 mRNA had improved disease-free survival, suggesting HPV-positive EAC and HPV-negative EAC are two distinct diseases, exactly as in HNSCC (Rajendra et al., 2018).

Recently many studies about the relationship between *Fusobacterium nucleatum* and gastroenteric cancer have been reported. Yamamura and colleagues found the new application of *Fusobacterium nucleatum* DNA status in prognosis prediction in EC. The relative amounts of *Fusobacterium nucleatum* DNA were significantly higher in tumor tissue compared with adjacent normal tissue. The cancer-specific survival and OS were significantly shorter in *F. nucleatum*-positive individuals than that in *Fusobacterium nucleatum*-negative individuals. Similarly, the cancer-specific mortality was significantly higher in *Fusobacterium nucleatum*-positive individuals than that in *Fusobacterium nucleatum*-negative individuals. Thus, we consider this periodontal bacteria can be used for the clinical prognosis of the EC as an indicator (Yamamura et al., 2016).

A Japanese study has revealed that the presence of oropharyngeal allopatric flora was an independent predictive factor of post-esophagectomy pneumonia. The authors divided 675 patients into three groups by categorization of oropharyngeal flora, including indigenous flora (Ind group), antibiotic-sensitive microbes only (Allo-S group) and antibiotic-resistant microbes (Allo-R group). Compared with the Ind group, the incidence of postoperative pneumonia in the Allo-S and Allo-R groups increased markedly and the survival in the Allo-R group significantly decreased (Yuda et al., 2020). Hence, it is anticipated that we can prevent post-esophagectomy pneumonia from the classification of the oral microbiome someday.

POTENTIAL MECHANISMS OF MICROBE-MEDIATED ESOPHAGEAL CARCINOGENESIS

The molecular mechanisms by which the human microbiota could initiate and drive tumorigenesis have always been the focus.

Genomic integration, genotoxicity, inflammation, immunity and metabolism are major mechanisms (Lv et al., 2019; Scott et al., 2019). Given the well-established impact that the composition of human microbiota and its activity mediated inflammation and genotoxicity in tumorigenesis of many cancers, such as colon cancer, liver cancer and pancreatic cancer (Cani and Jordan, 2018), many investigators were making their attempts to elucidate the mechanism of human microbiota during carcinogenesis of EC, including metabolites, genotoxicity, inflammation and immune dysregulation. Here, we review the main microbiota-associated mechanisms which have been under extensive research in esophageal carcinogenesis (**Figure 3**). However, despite considerable evidence to suggest significant changes in human microbiota following EC, it remains to be determined whether these changes have a causal effect or are only correlative in nature.

The Human Microbiota, Inflammation and Esophageal Carcinogenesis

Specific Pathogens

Sustained infection or non-infectious factors may lead to various pro-inflammatory and oncogenic mediators in the process of chronic inflammation, eventually resulting in tumor promotion (Khandia and Munjal, 2020). Data from several studies suggested that *Campylobacters* may play an important role in the process of inflammation and esophageal carcinogenesis. With the dominant change of the appearance of *Campylobacters* during the disease states, Blackett and colleagues found IL-18 expression significantly increases in both GERD and BE colonized subjects compared with non-colonized subjects (Blackett et al., 2013). IL-18 is a multifunctional cytokine that induces pro-inflammatory cytokine expression and is associated with anti-tumor immunity. Many studies implicate that the serum IL-18 levels of EC patients were significantly higher than the control group, deficiency of IL-18 can aggravate the progression and development of EC and IL-18 signaling is strongly associated with BE and EAC (Diakowska et al., 2006; Babar et al., 2012; Li et al., 2018). The *Campylobacters* consist almost entirely of *Campylobacter concisus*, which virtually only appearing in the disease states. The cell culture model of Barrett's cell lines reported a marked increase and a time-dependent manner in the expression of pro-inflammatory mediators (IL-18 and TNF-α) and tumor suppressor gene (p53) in co-culture with *Campylobacter concisus* (Mozaffari Namin et al., 2015b). By means of a comprehensive analysis of *Campylobacter* species, a new viewpoint that *Campylobacter* species modulated the host inflammatory response, and then, it initiated the EAC cascade was presented theoretically

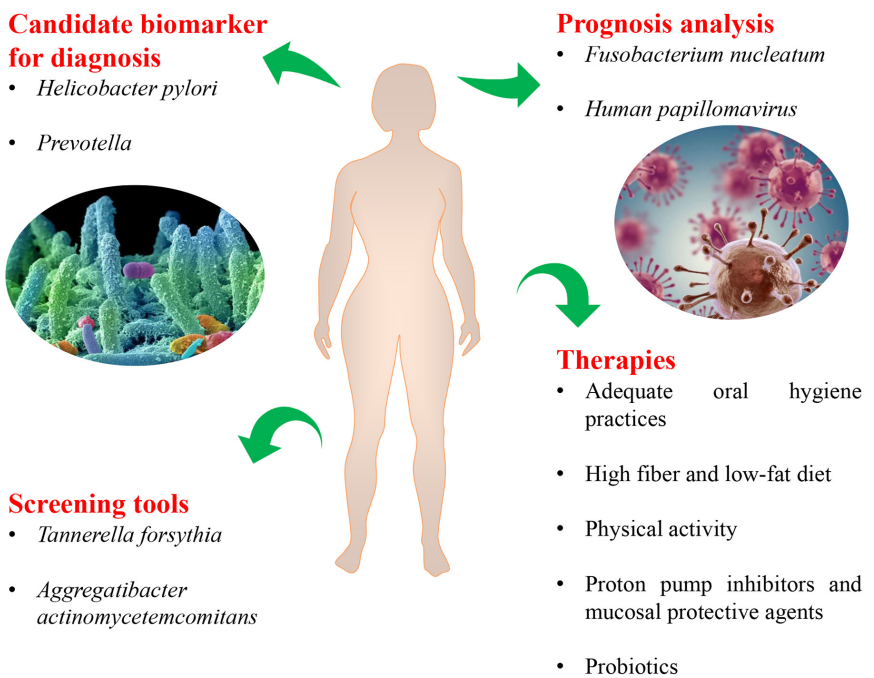

FIGURE 3 | The molecular mechanisms of microbe-mediated in EAC carcinogenesis. On the basis of the known contribution of human microbiota in esophageal carcinogenesis, the main mechanisms included inflammation, metabolism, and genotoxicity. Alteration of human microbiota in EAC showed a shift toward Gram-negative bacteria. Some specific pathogens, such as *Campylobacter concisus*, *Helicobacter pylori*, and *Escherichia coli*, involved in the process of inflammation and EAC cascade by regulating the expressions of toll-like receptors (TLRs) and pro-inflammatory mediators such as TNF-α, IL-18, COX-2, prostaglandins. And the components of Gram-negative bacteria activated the NLRP3 inflammasome and NF-κB pathway. Besides, detrimental metabolites, such as hydrogen sulfide, products of protein fermentation and bile acid metabolism, could play an important role in the initiation and progression of EAC. Some Gram-negative bacteria produced the cytolethal distending toxin (CDT), which could induce DNA damage and trigger EAC carcinogenesis. LPS, lipopolysaccharides; NF-κB, nuclear factor kappa B; TLRs, toll-like receptors; CDT, cytolethal distending toxin; TNF-α, tumor necrosis factor-α; IL, interleukin; COX, cyclooxygenase; PG, prostaglandin; p53, tumor protein 53; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing protein 3.

(Kaakoush et al., 2015). Previous research has indicated that the colonization of *Helicobacter pylori* in the esophagus increased the incidence of BE and EAC (Liu et al., 2011). Subsequently, some researchers have implicated the possible role of *Helicobacter pylori* in the malignant progression of the esophagus by promoting the expression of gastrin, COX-2, prostaglandins and Ki-67 (Kountouras et al., 2019; Doorakkers et al., 2020). Similarly, investigators also explained the association between *Enterobacteriaceae* infection and esophageal carcinogenesis has been proposed. The expression of toll-like receptors (TLRs) 1–3, 6, 7, and 9 significantly increases in EAC rats (Zaidi et al., 2016), demonstrating an *Escherichia coli*-related esophageal carcinogenesis. As mentioned previously, *Campylobacters*, *Helicobacter pylori* and *Escherichia coli* seemed to be specifically involved in EAC cascade through pro-inflammatory cytokine expression. Nevertheless, it is not yet clear whether there is a causality between specific pathogens and EAC.

Microbial Metabolites

In addition to specific pathogens, human microbiota could trigger carcinogenesis as an integrated community. A quintessential example should be cited that microbiota dysbiosis and host–microbiota interactions seemed to promote colorectal tumorigenesis (Schwabe and Jobin, 2013). The metabolites play an important role in the initiation and progression of cancer. Protective metabolites are represented by short-chain fatty acids. And detrimental metabolites are represented by hydrogen sulfide, products of protein fermentation and bile acid metabolism (Louis et al., 2014). Evidence suggests that human microbiota contributes to esophageal tumorigenesis, not only *via* the inflammation of specific pathogens but also *via* the influence of its metabolome (Louis et al., 2014). Some bacteria produced certain compounds which might be a carcinogen. Bile acid metabolism is one of the most important microbial metabolism. Researchers found that chronic exposure to bile acids might result in esophageal carcinogenesis through over-expression of glucose-6-phosphate dehydrogenase and active nuclear factor-kB (NF-κB) (Munemoto et al., 2019). The toll-like receptor-4 ligand, named LPS, is produced by Gram-negative bacteria. LPS can activate the NOD-like receptor protein 3 inflammasome and NF-κB pathway. The esophageal microbiome, dominated by Gram-negative bacteria, might contribute to materializing the inflammation-mediated carcinogenesis in BE by LPS *via* relaxing the lower esophageal sphincter and delaying gastric emptying (Yang et al., 2012; Nadatani et al., 2016; Lv et al., 2019). This provides new evidence about the molecular mechanisms underlying the association between LPS and esophageal carcinogenesis.

The Human Microbiota, Genotoxicity and Esophageal Carcinogenesis

Genotoxicity refers to structural DNA damage (Scott et al., 2019). A multitude of Gram-negative bacteria mainly including *Escherichia coli*, *Actinobacillus actinomycetemcomitans*, Campylobacters and *Helicobacter pylori* could produced the cytolethal distending toxin (CDT), which could induce DNA damage and promote cancer (Nesiæ et al., 2004; He et al., 2019). Certain species within *Enterobacteriaceae* produced a DNA-alkylating genotoxin so that led to DNA damage, which might accelerate tumor progression (Wilson et al., 2019). *Helicobacter pylori* is prescribed for class I carcinogen. *Helicobacter pylori* toxin cytotoxin-associated gene A induced oxidative DNA damage and modulated the host inflammatory response in gastric carcinogenesis (Wroblewski et al., 2010). It continues to be controversial whether *Helicobacter pylori* influences the canceration course of esophageal. The study of esophageal epithelial cell transfection has demonstrated that *Helicobacter pylori* infection led to the up-regulated expression of microRNA-212-3p targeted COX2 and miR-361-3p targeted CDX2 through the translation inhibition of miRNAs, which contributed to the phenotypic transformation of esophageal epithelial cells (Teng et al., 2018).

THE HUMAN MICROBIOTA-BASED THERAPIES IN ESOPHAGEAL CANCER

The therapeutic principle of esophageal cancer is based on individualized comprehensive treatment. In fact, surgery combined with radiotherapy and chemotherapy has become the mainstay of clinical treatment for EC (Stahl et al., 2010). It is now well established that several healthy behaviors are helpful for cancer prevention, including a healthy diet, physical activity, weight control and alcohol consumption limit (Rock et al., 2020). In addition, some interventions related to the altered human microbial composition may become the new adjuvant treatment in EC, such as proton pump inhibitors, probiotics, mucosal protective agents, and chlorhexidine mouth rinse.

Oral Hygiene

A large body of published research has consistently demonstrated poor oral hygiene was associated with a higher risk of cancers, such as oral cancer (Deng et al., 2021), gastric cancer (Zhang et al., 2021), colorectal cancer (Wang et al., 2021). Based on the outcomes of two case–control studies, poor oral hygiene was an important risk factor for EC (Mmbaga et al., 2020; Poosari et al., 2021). And patients who received dental prophylaxis had a reduced risk of EC (Lee et al., 2014). The data highlighted the importance of adequate oral hygiene practices, which could be a simple means to prevent various cancers (Yano et al., 2021). The interdental brush is a form of toothbrush which could be inserted between the teeth in order to remove plaque (Worthington et al., 2019). Denis and colleagues demonstrated that toothbrushing and interdental brushing can decrease the number of oral bacteria in particular those who were associated with periodontal disease (Bourgeois et al., 2019a). The individuals may benefit from the daily use of toothbrushing and interdental brushing. Previous research has argued that interdental brush reduces interdental bleeding compared with manual toothbrush (Bourgeois et al., 2016). As for the frequencies of toothbrushing, it is suggested that toothbrushing twice daily for 2 min in order to prevent periodontal disease (Sälzer et al., 2020). Additionally, a randomized controlled trial analyzed the oral and esophageal microbiota and gene expression of the esophagus before and after

treatment of chlorhexidine mouth rinse. The authors identified significant alterations in the oral and esophageal microbiota and demonstrated that the alterations of the esophageal microbiota could be closely related to changes in gene expression of the esophagus, suggest the clinical application of mouth rinse treatment in EC (Annavajhala et al., 2020).

Diet

Diet and nutrition are the major areas of interest within the prevention of chronic diseases and cancer. A healthy diet should include nutritious food, whole grains, fiber-rich legumes, a variety of vegetables and fruits (Rock et al., 2020). Several dietary patterns are representative, including Mediterranean, Dietary Approaches to Stop Hypertension, Okinawa and vegetarian diets. Many bioactive nutrients of these diets have played an effective role in the epigenetic modification and maintaining the balance of intestinal microbiota (Divella et al., 2020). Mediterranean diet (MD) is internationally regarded as a "long life" diet (Mentella et al., 2019; Martinon et al., 2021). The composition of Okinawa and Dietary Approaches to Stop Hypertension diets is similar to MD. Recent studies have shown that MD is associated with a decreased cancer mortality risk (Molina-Montes et al., 2020). Analyses indicated the decreased risk of gastro-intestinal cancer was associated with a vegetarian diet (Tantamango-Bartley et al., 2013). A low-fiber, high-fat, and high-refined-sugar diet might be responsible for the declining diversities (Lloyd-Price et al., 2016). Nevertheless, diet therapy is expected to be universally accepted low-risk and patient-friendly intervention to prevent chronic diseases and even cancer among the population, just as vaccines prevent flu. New therapeutic strategies of the EC could be proposed by targeted dietary intervention. Enteric pathogenic bacteria boosted their growth and pathogenicity by exploiting some short-chain fatty acids, microbiota-derived sources of carbon, and other nutrients (Bäumler and Sperandio, 2016). Host diet has a profound effect on the composition of the gut microbiota and its metabolites. Nobel and colleagues found a negative correlation between fiber intake and the relative abundance of Gram-negative bacteria, most notably *Betaproteobacteria* (Nobel et al., 2018). This study provided new evidence about the potential mechanisms underlying the association between dietary fiber and esophageal microbiome composition. Current consensus suggests that the risk of EAC could decrease after a reduction in total dietary fat, saturated fat, and cholesterol (Thrumurthy et al., 2019). Data from a study suggested that participants with reduced microbial gene richness presented more higher aberrant metabolism and low-grade inflammation, and weight-loss dietary intervention may succeed in improving these changes (Cotillard et al., 2013). Similarly, Münch and colleagues indicated that a high-fat diet led to the alterations of gut microbiota which accelerated inflammation and esophageal carcinogenesis in the mouse model which was irrelevant to obesity (Münch et al., 2019). In the future, a high fiber and low-fat diet may be helpful to prevent EC.

Physical Activity

Several studies have documented that exercise contributes to the human gut microbiota alternation (Shukla et al., 2015; Allen et al., 2018). Long-term regular exercise lead to higher diversity and significant shifts of major bacterial taxa in human gut microbiota, especially a higher relative abundance of the genus *Akkermansia* (Clarke et al., 2014). In addition, the role of physical activity in cancer prevention has received increased attention across a number of disciplines in recent years. There are consistent evidence that physical activity plays an important role in preventing cancer. An American roundtable report found that physical activity can reduce the risk of seven types of cancer including EAC (Patel et al., 2019). Aerobic exercise and muscle strength training before esophagectomy is useful for reducing the rates of postoperative respiratory complications in EC patients (Akiyama et al., 2021). Based on the preventive effect of exercise on EC, a study set it out to investigate the usefulness of evaluating the prognosis. The 6-min walking distance is a clinical examination gradually used to evaluate the prognosis of patients after surgery. It has the advantages of low cost and easy implementation. A retrospective cohort study has established that the 6-min walking distance is directly proportional to the overall survival in patients undergoing esophagectomy (Kondo et al., 2021).

Proton Pump Inhibitors and Mucosal Protective Agents

Proton pump inhibitors (PPIs) are used extensively for the full spectrum of gastric-acid-related diseases in clinic (Malfertheiner et al., 2017). It inhibits the activity of gastric H^+/K^+-adenosine triphosphatase, resulting in the inhibition of acid secretion from parietal cells (Rochman et al., 2021). Previous researches have established that long-term PPI use induces changes in the gut microbiota (Clooney et al., 2016; Malfertheiner et al., 2017). Compared with controls not using PPI, PPI users had decreased relative abundance of Gram-negative bacteria and increased relative abundance of *Streptococcus* (Snider et al., 2019). Changes in esophageal microbiota were observed before and after 8 weeks of PPI treatment. The predominant decreased taxa was *Comamonadaceae*, while the main increased taxa *were Clostridiaceae*, *Lachnospiraceae*, *Micrococcaceae*, *Actinomycetaceae*, *Gemellales*. As we discussed above, there was a shift toward Gram-negative bacteria in the EAC cascade. Although there is no direct evidence, PPI treatment may potentially benefit the patients with esophageal precancerous lesions (Amir et al., 2014). Similarly, mucosal protective agents are also applied extensively in the treatment of the gastric diseases (Haruma and Ito, 2003). In a murine Eosinophilic esophagitis (EoE) model, supplementation with *Lactococcus lactis* NCC 2287 attenuated esophageal eosinophilic inflammation (Holvoet et al., 2016). Recent research in a rat model suggests that rebamipide, a mucosal protective agent, can reduce BE development and alter the esophageal microbiome composition, in particular *Lactobacillus* and *Clostridium* (Kohata et al., 2015).

Probiotics

Probiotic is a major area of interest within the field of microbiotic therapy. Probiotics therapeutic tests showed a significant inhibitory effect on the expression of biomarkers that contribute

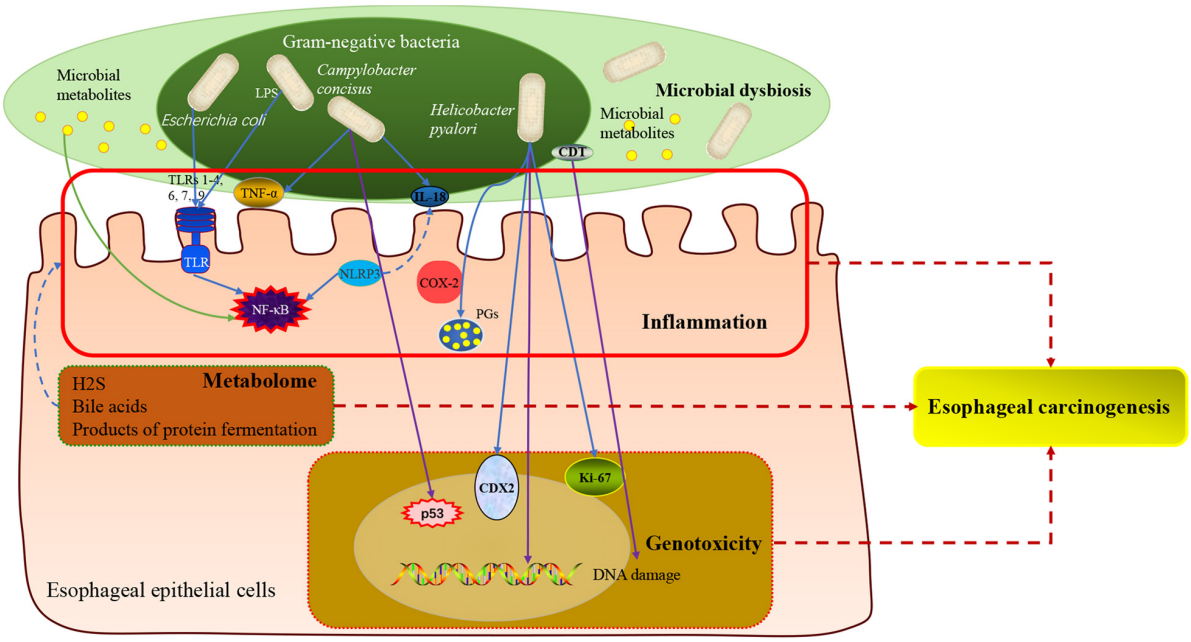

FIGURE 4 | The novel microbiota-related targets in screening, diagnosis, prognosis, and therapy for esophageal adenocarcinoma.

to BE transformation and indicated the possibility for the prevention of BE to EAC (Mozaffari Namin et al., 2015a). Besides, probiotics can be used to modulate the human microbiota in postoperative patients. A prospective trial evaluated the effect of probiotics on the prognosis of postoperative patients with EC (Lina et al., 2018). The result suggested that probiotics can reduce the rates of abdominal distension, constipation and gastric retention in postoperative patients with esophageal cancer.

CONCLUSION

The manuscript briefly summarizes our current knowledge regarding the relationship between human microbiota and the esophageal adenocarcinoma cascade. And it brings thinking from the fields of prevention, diagnosis, prognosis, and therapy for EAC (**Figure 4**). The current findings have identified decreased microbial diversity and altered human microbial communities in esophageal carcinogenesis, especially *Enterobacteriaceae*, *Campylobacters*, and acid-producing bacteria, periodontal pathogens (*Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans*). *Helicobacter pylori*, *Prevotella*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans* may be utilized as biomarkers for personalized precision diagnosis and screening of EAC. The expression of HPV-related biomarkers, the classification of the oral microbiome, and *Fusobacterium nucleatum* DNA status can become an important prognostic indicator of EAC. Notably, novel clinical interventions related to the human microbiota may also be used to treat EC, including adequate oral hygiene practices, a high fiber and low-fat diet, physical activity, PPI, mucosal protective agents and probiotics, which might benefit patients significantly. From this review, it emerged clearly that the human microbiota may impact the initiation and progression of EAC since it not only mediates inflammation and genotoxicity as specific pathogens, but also triggers detrimental metabolites as an integrated community. All mechanisms are not mutually exclusive and may be involved in tumorigenesis in a stage-specific and case-specific manner (Chen et al., 2017).

There were also certain limitations. Although preliminary studies have provided a comprehensive view of the role of the human microbiota in EAC development, information on causative effects on EAC cascade remained to be elucidated. This area needs more research to truly understand the complex mechanisms behind the impact of the human microbiota on tumorigenesis of EAC.

AUTHOR CONTRIBUTIONS

FP conceptualized the study, revised the manuscript, and supervised the study. WD drafted the manuscript and made the figures. LP and BY collected the literature and revised the manuscript. ZL improved the manuscript. All authors read and approved the final manuscript.

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Haemophilus pittmaniae and *Leptotrichia* spp. Constitute a Multi-Marker Signature in a Cohort of Human Papillomavirus-Positive Head and Neck Cancer Patients

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Objectives: Human papillomavirus (HPV) is a known etiological factor of oropharyngeal head and neck cancer (HNC). HPV positivity and periodontal disease have been associated with higher HNC risk, suggesting a role for oral bacterial species. Our objective was to determine oral microbiome profiles in HNC patients (HPV-positive and HPV-negative) and in healthy controls (HC).

Methods: Saliva samples and swabs of buccal mucosa, supragingival plaque, and tongue were collected from HNC patients (N = 23 patients, n = 92 samples) before cancer therapy. Next-generation sequencing (16S-rRNA gene V3–V4 region) was used to determine bacterial taxa relative abundance (RA). β-Diversities of HNC HPV+ (N = 16 patients, n = 64 samples) and HNC HPV– (N = 7 patients, n = 28 samples) groups were compared using PERMANOVA (pMonte Carlo < 0.05). LEfSe discriminant analysis was performed to identify differentiating taxa (Log LDA > 2.0). RA differences were analyzed by Mann–Whitney U-test ($\alpha = 0.05$). CombiROC program was used to determine multi-marker bacterial signatures. The Microbial Interaction Network Database (MIND) and LitSuggest online tools were used for complementary analyses.

Results: HNC vs. HC and HNC HPV+ vs. HNC HPV– β-diversities differed significantly (pMonte Carlo < 0.05). *Streptococcus* was the most abundant genus for HNC and HC groups, while *Rothia mucilaginosa* and *Haemophilus parainfluenzae* were the most abundant species in HNC and HC patients, respectively, regardless of antibiotics treatment. LEfSe analysis identified 43 and 44 distinctive species for HNC HPV+ and HNC HPV– groups, respectively. In HNC HPV+ group, 26 periodontal disease-associated species identified by LefSe had a higher average RA compared to HNC HPV– group. The significant species included *Alloprevotella tannerae*, *Fusobacterium periodonticum*, *Haemophilus pittmaniae*, *Lachnoanaerobaulum orale*, and *Leptotrichia* spp. (Mann–Whitney U-test, $p < 0.05$). Of 43 LEfSe-identified species in HPV+ group, 31 had a higher RA compared to HPV– group (Mann–Whitney U-test,

$p < 0.05$). MIND analysis confirmed interactions between *Haemophilus* and *Leptotrichia* spp., representing a multi-marker signature per CombiROC analysis [area under the curve (AUC) > 0.9]. LitSuggest correctly classified 15 articles relevant to oral microbiome and HPV status.

Conclusion: Oral microbiome profiles of HNC HPV+ and HNC HPV– patients differed significantly regarding periodontal-associated species. Our results suggest that oral bacterial species (e.g., *Leptotrichia* spp.), possessing unique niches and invasive properties, coexist with HPV within HPV-induced oral lesions in HNC patients. Further investigation into host–microbe interactions in HPV-positive HNC patients may shed light into cancer development.

Keywords: head and neck cancer, HPV, oral microbiome, next generation sequencing, *Leptotrichia* spp.

INTRODUCTION

Head and neck cancer (HNC) is the sixth most common cancer worldwide with over 95% comprising squamous cell carcinomas (SCCs) (Jemal et al., 2008; Kumarasamy et al., 2019). Head and neck SCCs are characterized by a locoregional development mainly diagnosed at an advanced stage of the disease, resulting in difficult treatment and eradication of both pre-neoplastic and neoplastic tissue (Carvalho et al., 2005; Ganci et al., 2015). Despite advancements in chemoradiation, ionizing radiation, and surgical resection techniques, HNC has an overall mortality rate of approximately 50% and is characterized by high recurrence rates (Carvalho et al., 2005). While triggers of HNC development have not been fully elucidated, two primary risk factors have been identified, namely, alcohol and tobacco consumptions (Božinović et al., 2019). Most recent studies have identified infection with human papillomavirus (HPV) as a third and more prominent cause of tumor formation (Božinović et al., 2019). HPV-associated SCCs represent the most common HPV-related cancer in the US and are classified by a new staging system for oropharyngeal cancers (National Cancer Institute[1]: van Gysen et al., 2019).

HPV-positive (HPV+) HNC patients are often younger and present with a more advanced cancer stage than HPV-negative (HPV–) HNC patients (Blitzer et al., 2014). It has been reported that the majority of HPV-associated HNCs are caused by HPV16, though more than 220 HPV serotypes have been identified (Tumban, 2019). Two HPV genes, E6 and E7, have been the matter of extensive research due to their role as oncogenes (Yim and Park, 2005). These genes are involved in multiple pathways such as transmembrane signaling, cell cycle regulation, and cell transformation (Yim and Park, 2005). E6 has been shown to promote degradation of tumor suppressor TP53 (Crook et al., 1991), while E7 is able to inhibit retinoblastoma protein (Pal and Kundu, 2020). Aside from E6 and E7, the E5 HPV gene promotes malignancy, has anti-apoptotic effects and plays a role in epidermal growth factor (EGF) receptor-regulated cell proliferation (Venuti et al., 2011).

There is a mounting body of evidence that a synergistic interaction between periodontal disease-associated pathogens and HPV exists. Indeed, a case–control study by Tezal et al. (2009) found that HPV+ tumors in 21 patients had a significantly higher alveolar bone loss mean and a fourfold increased risk for HPV+ tumor status for every millimeter of alveolar bone loss caused by periodontal disease.

Overall, without implying a causal effect, a link between oral microbiome dysbiosis and cancer has been suggested by several studies (Kudo et al., 2016; Hayes et al., 2018; Mohammed et al., 2018; Wu et al., 2018; Mougeot et al., 2020). For instance, *Fusobacterium nucleatum* was found overabundant in the oral cavity of patients with colon cancer and lymph node metastasis (Kudo et al., 2016). *F. nucleatum* might initiate oncogenic and proinflammatory responses that stimulate the growth of colon cancer cells (Kudo et al., 2016). Increased levels of blood serum antibodies against the oral bacterial species *Porphyromonas gingivalis* was associated with a twofold higher risk of pancreatic cancer when compared to healthy individuals (Mohammed et al., 2018).

Furthermore, the increased prevalence of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* was shown to initiate a Toll-like receptor signaling pathway predictive of pancreatic cancer in animal models (Mohammed et al., 2018). Higher levels of firmicutes and bacteroidetes also constitute a potential risk for gastric cancer (Wu et al., 2018). A study by Hayes et al. (2018) suggested an association between the oral microbiome and HNC.

HNC has also been associated with oral cavity diseases such as periodontal disease and dental caries (Michaud et al., 2017; Gasparoni et al., 2021). Periodontitis disrupts the normal oral microbial environment, thereby leading to dysbiosis. Dysbiosis can translate into an abundance shift of opportunistic species, like *P. gingivalis*, which produce several virulence factors resulting in the destruction of periodontal tissues (Rafiei et al., 2017). Chronic periodontitis can result in the release of proinflammatory cytokines from squamous cells, causing inflammation and possible decreased apoptosis (Gholizadeh et al., 2016). In a meta-analysis by Zeng et al. (2013) HNC cancer risk was found to be increased by 2.63-fold in patients with periodontitis.

Using 16S rRNA gene next-generation sequencing and computational approaches, the purpose of this study was to

[1]https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents/hpv-and-cancer

compare microbiome profiles of a limited cohort of HNC patients to those of healthy control subjects, and profiles of HNC HPV+ patients to those of HNC HPV– patients, within the HNC cohort. We also aimed to determine whether bacterial species differentiating HPV+ from HPV– HNC patients are associated with periodontal disease-associated species.

MATERIALS AND METHODS

Patient Recruitment

HNC patients with SCC [N = 30; 8 females, 22 males, age range = 23–75 years (SD = ± 12.02)] were recruited from the OraRad study (U01DE022939) (Brennan et al., 2017; Lalla et al., 2017). OraRad was a multicenter cohort study that collected longitudinal data on radiation-treated HNC patients at 6-month intervals for 2 years. Primary cancer site origin included the base of the tongue, tonsil, neck, tongue, and oral cavity.

Of 30 HNC patients, 23 were clinically classified as HPV+/− into 16 HPV+ and 7 HPV– patients. In addition, healthy control subjects (HC group) (N = 20; age range 24–84, SD = −12.93) were recruited through Atrium Health's Carolinas Medical Center, Charlotte, NC. Of 30 HNC patients, 11 had received antibiotic treatment within 2 weeks of sampling. No HC subject had received antibiotic treatment. The study was approved by the institutional review board, and all participants gave informed consent for the study.

Sample Collection

Saliva (S) samples and swab samples of buccal mucosa (B), supragingival plaque (P), and tongue (T) were collected from HNC patients, pre-cancer treatment at baseline, and from HC subjects. Saliva collection was performed while chewing unsweetened and unflavored gum (The Wrigley Company—Mars, Chicago, IL, United States) for a period of 2 min into a 50-ml conical BD falcon polypropylene centrifuge tube (Corning, Corning, NY, United States).

Buccal mucosal samples were subsequently collected by swabbing both sides of the buccal mucosa for 10 s each. Tongue samples were then obtained by swabbing a 1-cm^2 region on both sides of the mid-dorsal region of the tongue for 5 s. Finally, supragingival plaque samples were obtained by swabbing across the lateral surfaces of all maxillary and mandibular teeth at the junction of the tooth and gingiva. All swab collections were performed using OmniSwabs (GE Life Sciences-Buckinghamshire, United Kingdom).

Bacterial DNA Extraction, Processing, and Sequencing

Bacterial genomic DNA was extracted from oral samples using QIAamp DNA Mini Kit procedure (QIAGEN, Valencia, CA, United States) per manufacturers' instructions. During sample preparation, 50 ng of genomic DNA was used for PCR in which the 16S rRNA gene (V3–V4) region was amplified, followed by purification and processing methods as previously described (Caporaso et al., 2011). Next-generation sequencing was performed using the MiSeq v3 reagent kit and platform (Illumina, Inc., San Diego, CA, United States). To prepare for cluster generation and sequencing, libraries were denatured with NaOH and diluted with a hybridization buffer. Libraries then underwent heat denaturation prior to MiSeq sequencing. Total of 100 ng of each library was pooled together, run on a gel, gel-extracted, and run on a bioanalyzer for quantification. A total concentration of 4 nM of the library was then diluted, and 12 pM of the library was spiked with 20% PhiX. At least 5% PhiX was added as an internal control for low-diversity libraries. Identification of bacterial genera and species was performed using Human Oral Microbe Identification, HOMI*NGS*, which employs a ProbeSeq BLAST program for species/genera identification through recognition of the 16S rRNA gene (V3–V4 region) sequence reads (Caporaso et al., 2011; Mougeot et al., 2016). ProbeSeq loads raw sequence files into a cell array, looping through the array one sequence at a time searching for small sequence strings that 100% match an oligomer (partials are not considered matches). If a match is identified, a counter begins giving counts of the total number of probe-specific "hits." Hits are then accumulated by species/genera and sample.

The sequence reads were matched to 737 ProbeSeq taxon probes, i.e., to species probes (n = 620) or genus probes (n = 117) if not matched to a species probe, or were otherwise recorded as an unmatched read. Matched and unmatched probe count data were provided per taxon per patient as Excel spreadsheets. Species/genus probes containing zeros for all samples were removed from the dataset. Raw abundance data were then transformed into relative abundance (RA) data for further analysis.

Bioinformatic Analysis

α-Diversity

Shannon and Simpson indices were generated using $PRIMER_{v7}$ (PRIMER-E Ltd., Ivybridge, United Kingdom) (Clarke and Gorley, 2006), based on microbiome RA data. RA data of HC subjects (HC group: N = 20) were compared to the RA data of HNC patients including those with antibiotic treatment within 2 weeks of sampling (Grp-All: N = 30). RA data of HC group were also compared to RA data of HNC patients excluding those with antibiotic treatment (Grp-NoAB: N = 19). Subsequently, comparisons of HPV+ vs. HPV– HNC sub-cohorts were performed by including or excluding patients who received antibiotic treatment. Mann–Whitney U-tests were then used to determine significant RA comparisons ($\alpha = 0.05$) using $XLSTAT_{v2016.02.29253}$ (Data Analysis and Statistical Solution for Microsoft Excel, Addinsoft, Paris, France, 2017).

Permutational Multivariate Analysis of Variance

Patient subgroups used for permutational multivariate analysis of variance (PERMANOVA) included Grp-All (HNC: N = 30; HC: N = 20) and Grp-noAB (HNC: N = 19; HC: N = 20). Sub-analyses were performed based on the multiple sample site combinations "BPST," "BST," and "PST" which provided sufficient power in $PRIMER_{v7}$ program (PRIMER-E Ltd., Ivybridge, United Kingdom) (Clarke and Gorley, 2006) for all

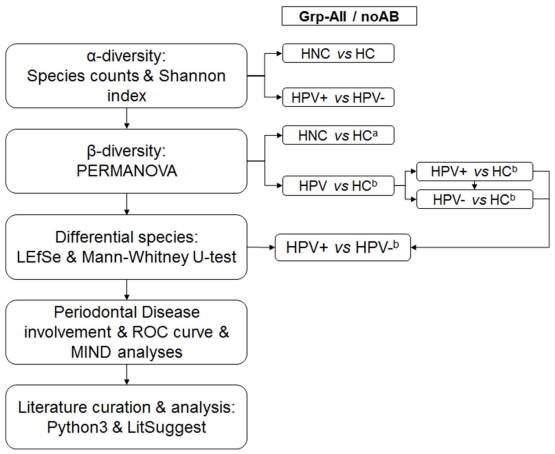


FIGURE 1 | Overall analytical strategy. α-Diversity was calculated for Grp-All HNC vs. HC (N = 30; N = 20) and HPV+ vs. HPV– (N = 16; N = 7) and for Grp-noAB HNC vs. HC (N = 19; N = 20) and HPV+ vs. HPV– (N = 12; N = 3). Sample sites consisted of different combinations of buccal (B), plaque (P), saliva (S), and tongue (T) samples. β-Diversity was calculated using PERMANOVA by two different analytical designs: (1) [a]Analytical design with "Diagnosis" and "Sample site" as fixed factors and "Antibiotic" as the nested random factor. Comparisons were Grp-All HNC vs. HC and Grp-noAB HNC vs. HC. (2) [b]Analytical design with "HPV status" and "Sample site" as fixed factors and "Antibiotic" as the nested random factor. Grp-All comparisons were as follows: HPV vs. HC (N = 23; N = 20), HPV+ vs. HC (N = 16; N = 20), HPV– vs. HC (N = 7; N = 20), and HPV+ vs. HPV– (N = 16; N = 7). Grp-noAB comparisons were as follows: HPV vs. HC (N = 15; N = 20), HPV+ vs. HC (N = 12; N = 20), HPV– vs. HC (N = 3; N = 20), and HPV+ vs. HPV– (N = 12; N = 3). Distinct species were determined using LEfSe and Mann–Whitney U-tests for Grp-All and Grp-noAB comparisons of HPV+ vs. HPV– for the sample site combination BPST. Significant LEfSe bacterial species for HPV+ group were investigated for their roles in periodontal disease using conventional methods (PubMed, Google Scholar, etc.). Significant LEfSe HPV+ species using the Mann–Whitney U-test were analyzed by using MedCalc ROC curve analysis. Multi-marker combinatorial analysis was completed using CombiROC online tool (http://combiroc.eu/) for the bacterial species identified with MedCalc log(RA + 1) ROC curve AUC of 0.75 or greater (n = 10 species). CombiROC utilized raw RA data and log(RA + 1) transformed data. Microbial Interaction Network Database (MIND) online tool was used to identify possible interactions between most significant bacterial species. Python$_{v3.6.2}$ code was used to extract PubMed abstracts matching key words to determine positive and negative training sets and were validated manually. Using NCBI LitSuggest online tool (https://www.ncbi.nlm.nih.gov/research/litsuggest/), 203 PubMed articles were classified to identify articles involving HPV infection in the oral microbiome.

five relevant comparisons (**Figure 1**). Species and genera RA data were square root transformed and converted into Bray–Curtis similarity matrices.

PERMANOVA analyses were performed using a mixed model with unrestricted permutation of raw data, 9,999 permutations, and type III partial sum of squares (Clarke and Gorley, 2006), as previously implemented (Mougeot et al., 2019, 2020). Fixed factors were "Diagnosis" (e.g., HNC vs. HC) and "Sample site" (B, P, S, and T). In this design, the "Antibiotic" treatment (yes or no) variable was used as a random factor nested into "Diagnosis" and "Sample site." Monte Carlo corrected p-values (α = 0.05) were determined, as appropriate for relatively small sample sizes.

Principal coordinate analysis (PCoA) was completed for the Grp-All: HNC vs. HC BPST sample site combination.

β Diversity Sub-Analyses

Sub-analyses were completed using subsets of Grp-All and Grp-noAB patients, based on n = 3 or 4 sample sites per patient (BPST, BST, and PST): HPV+ (N = 16) vs. HPV– (N = 7) and HPV+ (N = 12) vs. HPV– (N = 3), respectively. The sample site combinations BPST, BST, and PST and the previously mentioned data transformation were used for PERMANOVAs in PRIMER$_{v7}$ program. Fixed factors used were "HPV status" (positive and negative) and "Sample site" (B, P, S, and T). "Antibiotic" (yes or no) was used as a random factor and nested into "HPV status" and "Sample site." Monte Carlo corrected p-values (α = 0.05) were determined. PCoA was completed for the Grp-All: HPV+ vs. HPV– BPST sample site combination.

Linear Discriminant Analysis Effect Size

Taxonomy levels were added manually to ProbeSeq derived datasets for Grp-All (HPV+ vs. HPV–) and Grp-noAB (HPV+ vs. HPV–) subsets. The text files were then formatted for linear discriminant analysis (LDA) effect size (LEfSe) using the Galaxy$_{v1.0}$ online tool (Jalili et al., 2020). LEfSe data input consisted of "HPV status" as the option "Class" and "Patient ID" as the option "Subject" (Segata et al., 2011). Data were normalized. Using the "one-against-all" strategy for multi-class analysis, the factorial Kruskal–Wallis test and pairwise Wilcoxon signed-rank test were set at a Monte Carlo significance (α = 0.05) to calculate LDA scores. Log LDA scores were set at a threshold > 2.0. Histograms of the differential features (species) were generated, and each species was investigated for its role in periodontal disease.

Receiver-Operating Characteristic Curve Analyses

Conventional Receiver-Operating Characteristic Analysis

Mann–Whitney U-tests were completed for LEfSe differential features for HPV+ species probes from Grp-All and Grp-noAB groups. Significant species probes (α = 0.05) further underwent receiver-operating characteristic (ROC) curve analysis for Grp-All HPV+ and Grp-noAB HPV+ species probes using the BPST sample combination in MedCalc program (MedCalc Software Ltd, Ostend, Belgium).

RA data were log-transformed with the addition of a pseudo-count [i.e., log(RA + 1)]. Analysis was completed for Grp-All (HPV+; n = 64 samples and HPV–; n = 28 samples) and Grp-noAB (HPV+; n = 48 samples and HPV–; n = 12 samples) groups and for each non-zero RA probe in MedCalc program. The area under the curve (AUC) of each probe was calculated, and ROC curves were generated. Significance level was set at α = 0.05, and biomarker accuracy was calculated using methods described by Ray et al. (2010).

CombiROC Analysis

ROC curves from MedCalc that had an AUC greater than 0.75 were subjected to combinatorial analysis using CombiROC

online tool[2] (Mazzara et al., 2017) based on raw RA data and log(RA + 1) transformed RA data. Using CombiROC, marker profile plots were generated to confirm quality, and the detection threshold was set to 0.001. Using this threshold, combinational analysis was performed which calculated the sensitivity and specificity scores for each marker or combination of markers corresponding to the probability that the microbial data will be positive when HPV is present and the probability that microbial data will be negative when HPV is not present.

A minimum feature filter was set to include at least two markers. Based on a threshold of 10 for sensitivity and 50 for specificity, the best or "gold" combinations of markers were kept, thereby creating optimal multi-marker ROC curves and violin plots. Summary statistics were calculated and recorded for the top two AUC scores of the raw and log(RA + 1) transformed RA data.

Microbial Interaction Network Database Analysis

A microbial interaction network was created to illustrate possible interactions between *Haemophilus* spp. and *Leptotrichia* spp. with other bacterial genera or species, by using Microbial Interaction Network Database ($MIND_{v1.0}$) (Microbial Interaction Network Database, 2019). Default options were selected for human tissue sites, interaction weight, and health or disease conditions.

LitSuggest

An application programming interface was established using the National Center for Biotechnology Information guidelines (National Center for Biotechnology Information, 1988) and $Python_{v3.6.2}$ (van Rossum and Drake, 2009). $Python_{v3.6.2}$ was used to generate classifiers by extracting abstracts from PubMed (1997) through the keywords (i) "oral microbiome" and "HPV" to constitute a positive training set, (ii) "vaginal microbiome" and "HPV" keywords to constitute the negative training set, and (iii) "HPV" and "microbiome" to constitute the test set. Positive and negative training set abstracts were then manually validated. Using the NCBI LitSuggest[3] online tool; a total of 19 positively and 104 negatively classified articles were used to train the model (Allot et al., 2021). Test set classification was then completed using LitSuggest, and full articles were manually verified for relevancy.

RESULTS

The overall analytical strategy is presented in **Figure 1**. Demographics and clinical information of our HNC patient cohort (N = 30 patients, sub-cohort of OraRad study) are presented in **Table 1**. Clinical information, including caries and periodontal disease status for OraRad HNC patient cohort, associated with HPV status (N = 559 of 572 total patients) has been published elsewhere (Brennan et al., 2021). While no significant differences were noted in age and ethnicity, the male population was over-represented in the HNC patient set in OraRad and this study, as anticipated for oral SCC in general and for HPV-associated oropharyngeal cancers (Fakhry et al., 2018; Mahal et al., 2019). In our sub-cohort, most HPV+ HNC patients had oropharyngeal cancer (e.g., tonsil, base of tongue), whereas most of the HPV– HNC patients had cancer in other sites (**Table 1**).

Abundance, Species Detection, and α-Diversity

Probe count data are provided as **Supplementary Data Files** and can be downloaded from our lab's Github repository[4] (**Supplementary Data Files 1, 2**). Sequencing reads matched 737 total probes (117 genera and 620 species probes) for all samples from HNC and HC groups combined. Comparisons of species and genera detected for HNC vs. HC and HPV+ vs. HPV– are presented in **Supplementary Table 1**. Unmatched reads were removed from RA determinations. For all samples sequencing data, 442 of 620 species probes and 65 of 117 genus probes had at least one matched read. Significant α-diversity differences were identified for Grp-All and Grp-noAB for HNC vs. HC and Grp-noAB HNC vs. HC for the matched sample site combinations BPST, BST, and PST (**Table 2**). *Streptococcus* was the most abundant genus for HNC and HC groups, whereas *Rothia mucilaginosa* and *Haemophilus parainfluenzae* were the most abundant species detected in HNC patients and HC, respectively. Excluding HNC patients treated with antibiotics (Grp-noAB) did not affect these results (data not shown). Overall, the highest and lowest average number of taxa detected per sample were 96.08 and 117.66 for species probes and 24.75 and 26.83 for genus probes (**Supplementary Table 1**).

β-Diversity Analysis

PERMANOVA β-diversity analyses were performed for sample site combinations providing sufficient power based on available oral microbiome data (i.e., BPST, BST, and PST). Significance of β-diversity analyses is presented in **Figure 2**. For the Grp-All comparisons, including HNC HPV+ vs. HNC HPV– comparisons, all but one (i.e., HPV+ vs. HC, BST, pMonte Carlo = 0.261) were significant, regardless of the sample site combinations analyzed. All Grp-noAB comparisons were found significant for "HPV status" and "Sample site." Monte Carlo corrected p-values of all comparisons are presented in **Supplementary Table 2**. PCoA plots describing the variations explaining dissimilarity between groups (i.e., HNC vs. HC and HPV+ vs. HPV–) are presented in **Supplementary Figure 1**.

LEfSe Analysis

A total of 44 and 43 species were identified for Grp-All HPV– and HPV+, respectively. A histogram of the differential features is presented in **Figure 3A**. Species of the genera *Actinomyces* and *Leptotrichia* were the most representative of HPV– and HPV+ patient groups, respectively. A total of 52 and 38 species were identified for Grp-noAB HPV– and HPV+, respectively (**Figure 3B**). *Leptotrichia* spp. were the most represented taxa for

[2]http://combiroc.eu

[3]https://www.ncbi.nlm.nih.gov/research/litsuggest

[4]https://github.com/mbeckm01/HPV_HNC.git

TABLE 1 | Patient demographics and clinical characteristics.

	HNC[a]	HC[b]	Combined[c]	HNC HPV+[d]	HNC HPV−[e]	Combined[f]
Patient count (Male/Female)	30 (22/8)	20 (5/15)	50 (27/23)	16 (12/4)	7 (5/2)	23 (17/6)
Antibiotic treatment (Yes/No)	11/19	0/20	11/39	4/12	4/3	8/15
Primary cancer site						
Base of tongue	4		4	4	0	4
Nasopharynx	2		2	1	1	2
Oral cavity	1		1	0	1	1
Oropharynx	1		1	1	0	1
Supraglottis	1		1	0	1	1
Tongue	1		1	0	1	1
Tonsil	8		8	8	0	8
Unknown	5		5	2	3	5
Age:						
Median	55	55	55	54	61	54
Mean	54	52.7	54	54	51	53
Std Dev	12.02	15.29	12.93	6.47	20.29	11.93
Range	23–75	24–84	23–84	40–68	23–75	23–75
Ethnicity count						
M: Caucasian/African American	22/0	5/0	27/0	12/0	5/0	17/0
F: Caucasian/African American	7/1	13/2	20/3	3/1	2/0	5/1
Whole mouth average PD				2.28 (2.03–2.52)	2.09 (1.78–2.40)	2.26 (2.06–2.46)
Whole mouth average CAL				1.74 (1.42–2.07)	1.64 (1.02–2.26)	1.73 (1.46–2.01)
Sample combinations[g]						
BPST	120	80	200	64	28	92
BST	120	72	192	54	30	84
PST	105	69	174	54	21	75
BPS	93	63	156			

[a] *Head and neck cancer (HNC) patient group (primary cancer sites: base of tongue = 4; nasopharynx = 2; oral cavity = 1; oropharynx = 1; supraglottis = 1; tongue = 1; tonsil = 8; unknown = 5).*
[b] *Healthy control (HC) subject group.*
[c] *HNC and HC patient groups combined.*
[d] *HNC human papillomavirus positive (HPV+) patient group (primary cancer sites (N = 16); base of tongue = 4; nasopharynx = 1; oral cavity = 0; oropharynx = 1; supraglottis = 0; tongue = 0; tonsil = 8; unknown = 2).*
[e] *HNC human papillomavirus negative (HPV–) patient group (primary cancer sites (N = 7); base of tongue = 0; nasopharynx = 1; oral cavity = 1; oropharynx = 0; supraglottis = 1; tongue = 1; tonsil = 0; unknown = 3).*
[f] *HNC HPV+ and HPV– patient groups combined.*
[g] *Number of samples for site combinations including B (buccal), P (plaque), S (saliva), and/or T (tongue).*
PD, probing depth; CAL, clinical attachment loss; Std Dev, standard deviation. PD and CAL are shown as the average with 95% confidence intervals in parentheses. Mann–Whitney U-tests comparing whole mouth average PD and whole mouth average CAL separately for HPV+ vs. HPV– patient groups were not found to be significantly different ($p > 0.05$).

Grp-noAB HPV+ patients, and *Prevotella* spp. were the most represented ones for Grp-noAB HPV– patients. A total of 26 of 43 species in Grp-All HPV+ group (60.5%) and 24 of 38 species in Grp-noAB HPV+ group (63.2%) were recognized for their involvement in periodontal disease by performing manual searches in PubMed (**Supplementary Table 3**).

Receiver-Operating Characteristic Determination

Using the Mann–Whitney *U*-test, 31 of 43 bacterial species in Grp-All HPV+ and 29 of 38 bacterial species in Grp-noAB HPV+ LEfSe were significant ($p < 0.05$) (**Figures 3A,B**). Using MedCalc ROC curve analysis, one species (*Lachnoanaerobaculum sp083*) in Grp-All HPV+ was found not significant. All species in Grp-noAB HPV+ were, however, significant ($p < 0.01$). By minimizing zero inflation, we found 17 of the 31 Grp-All HPV+ species and 16 of the 29 Grp-noAB HPV+ species to be significant (MedCalc Software Ltd, Ostend, Belgium). Minimization of zero inflation is required to optimize ROC analysis for the bacterial species which are more consistently detected across subjects and to increase the "signal-to-noise" ratio for a panel of select candidate bacterial taxa biomarkers. Indeed, *Haemophilus pittmaniae*, *Rumonococcaceae G1* sp. *HOT 075*, and three *Leptotrichia* spp. were determined to be "Excellent" biomarkers in terms of sensitivity, specificity, and accuracy in the Grp-noAB using the log(RA + 1) transformed data with minimized zero inflation (**Supplementary Table 4**). Descriptive statistics of all the species with significant ROC curves are presented in **Supplementary Table 4**. Notably, *Leptotrichia* was the most represented significant genus for both Grp-All and Grp-noAB groups HPV+ vs. HPV– comparisons.

TABLE 2 | α-diversity comparisons: HNC vs. HC and HNC HPV+ vs. HPV–.

Variable[a]	Min[b]	Max[c]	Mean[d]	Std Dev[e]	*p*-value[f]
Grp-All HNC vs. HC					
BPST					
HNC	42	247	131.65	39.738	**0.045**
HC	53	281	143.5	42.448	
BST					
HNC	42	224	127.87	36.689	**0.004**
HC	72	281	145.96	41.343	
BPS					
HNC	45	247	138.28	42.348	0.378
HC	53	581	143.91	44.884	
PST					
HNC	42	267	135.09	42.93	**0.027**
HC	53	223	146.37	38.401	
Grp-noAB HNC vs. HC					
BPST					
HNC	45	247	131.17	38.434	0.056
HC	53	281	143.5	42448	
BST					
HNC	45	224	139.91	33.5	**0.017**
HC	72	281	145.96	41.343	
BPS					
HNC	45	247	139.55	41.855	0.54
HC	53	281	14.91	44.884	
PST					
HNC	69	267	136.13	42.264	**0.045**
HC	53	223	146.38	38.401	
Grp-All HPV+ vs. HPV–					
BPST					
Negative	42	218	127.25	37.99	0.413
Positive	45	247	133.47	39.39	
BST					
Negative	42	218	119.67	40.014	0.063
Positive	45	214	134.20	34.948	
PST					
Negative	42	218	130.52	40.964	0.483
Positive	69	267	140.30	43.242	
Grp-noAB HPV+ vs. HPV–					
BPST					
Negative	86	177	128.83	28.197	0.919
Positive	45	247	130.17	40.511	
BST					
Negative	86	174	128.08	28.273	0.787
Positive	45	214	130.71	34.437	
PST					
Negative	86	177	133.67	30.332	0.921
Positive	69	267	137.29	45.687	

[a] *Sample comparisons from head and neck cancer (HNC) patients with or without human papillomavirus (HPV) and healthy controls (HC) for sample sites buccal (B), plaque (P), saliva (S), and tongue (T) with and without (noAB) antibiotic treatment.*
[b] *Minimum number of species detected per sample.*
[c] *Maximum number of species detected per sample.*
[d] *Mean number of species detected per sample.*
[e] *Standard deviation of species detected per sample.*
[f] *Mann–Whitney U-test p-value.*
Significant values ($p > 0.05$) are shown in bold. Positive, HPV positive; Negative, HPV negative.

CombiROC and Microbial Interaction Network Database Investigation

From the ROC MedCalc analyses, 10 bacterial species from Grp-All HPV+ group had an AUC of at least 0.75, distinguishing HNC HPV+ from HNC HPV– group (**Supplementary Table 4**). Using RA data in CombiROC program (Mazzara et al., 2017), 24 "gold" combinations were generated out of 2,036 possible combinations containing at least two markers. The best two combinations (greatest AUC) were "Combo XXII" consisting of the microbial species probes *Ruminococcaceae sp075*, *H. parainfluenzae*, *H. pittmaniae*, *Leptotrichia sp212*, and *Leptotrichia sp417* and "Combo XV" consisting of *Ruminococcaceae sp075*, *H. pittmaniae*, *Leptotrichia sp212*, and *Leptotrichia sp417* (**Figure 4A**). "Combo XXII" and "Combo XV" had AUCs of 0.941 and 0.928, accuracies of 0.88 and 0.85, and positive predictive values of 0.69 and 0.65, respectively (**Figure 4A**). Using log(RA + 1) data, 46 "gold" combinations were created out of 1,013 possible combinations with the best two combinations of microbial probes being "Combo XLII" and "Combo XXXVI." "Combo XLII" contained a combination of *Gemella sanguinis*, *H. pittmaniae*, *Leptotrichia sp212*, and *Ruminococcaceae sp075*, while "Combo XXXVI" contained *TM7 G1 sp352*, *H. pittmaniae*, *Leptotrichia sp221*, *Leptotrichia sp417*, and *Ruminococcaceae sp075* (**Figure 4B**). "Combo XLII" and "Combo XXXVI" had AUCs of 0.943 and 0.938 and positive predictive values of 0.68 and 0.66, respectively. Both of these combinations from log(RA + 1) transformed data had an accuracy of 0.86 (**Figure 4B**). ROC curves, violin plots, and descriptive statistics of each data type are presented in **Figure 4**. Using MIND, *Haemophilus* and *Leptotrichia* were found to have many interactions in common (**Figure 5**).

LitSuggest Performance

From the $\text{Python}_{\text{v3.6.2}}$ data extraction code, 203 PubMed articles were identified for classification from the model matching the search terms "HPV" and "microbiome." From the classification set of these articles, 36 were determined as positively associated with the search terms "oral microbiome" and "HPV." LitSuggest program found 171 articles negatively classified. Manual validation of the 36 positively associated articles resulted in 21 articles being discarded. A total of 15 remaining articles were correctly determined as positively associated with HPV and the oral microbiome. Of these articles, three were reviews and 12 were research articles evaluating the HPV status in the context of oral tumor and microbiome relationship in SCC patients with oropharynx, including tonsil specifically, as the primary tumor site (**Table 3**).

DISCUSSION

This is the first study to evaluate the microbial differences in HNC HPV+ patients compared to those of healthy individuals and HNC HPV– patients by means of oral samples including saliva, buccal mucosa, supragingival plaque, and tongue swabs using multivariate analysis. We were able to identify 442

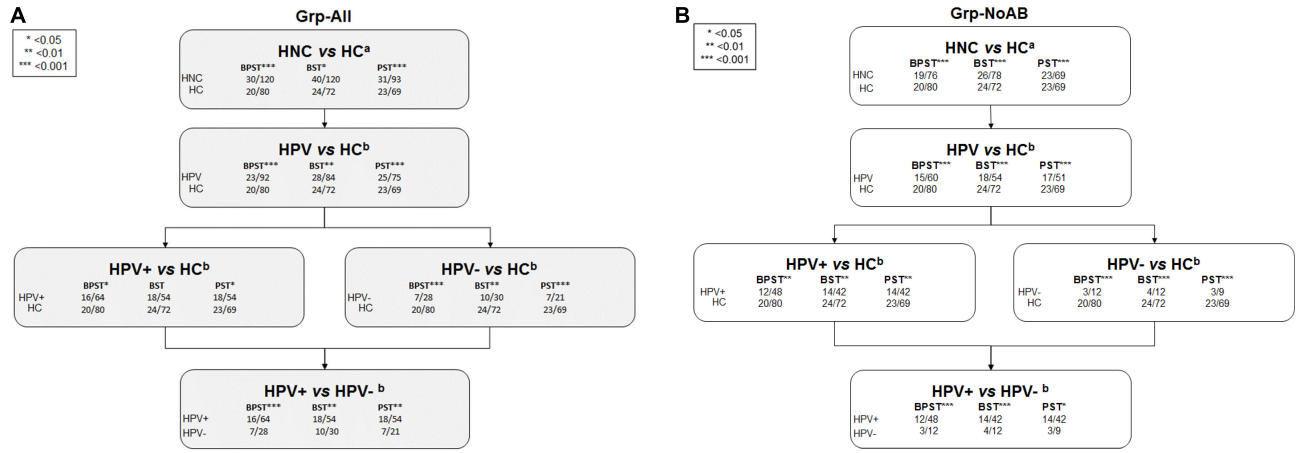


FIGURE 2 | PERMANOVA results comparisons flowchart. **(A)** Grp-All. **(B)** Grp-noAB. β-diversity analyses were performed using PERMANOVA in $PRIMER_{v7}$ software (PRIMER-E Ltd., IvyBridge, United Kingdom) to compare microbial profiles of head and neck cancer (HNC) patients to healthy controls (HC) and to compare microbial profiles of HNC HPV-positive (HPV+) patients to HNC HPV– patients. Sample sites consisted of three to four site combinations of buccal (B), plaque (P), saliva (S), and tongue (T). PERMANOVA analysis was completed using two different analytical designs based on Bray–Curtis similarity matrices determined from square root transformed relative abundance data of 737 probes (620 species and 117 genus probes). Sample site combinations consisted of BPST, BST, and PST for **(A)** Grp-All HNC vs. HC ($N = 30$; $N = 20$) and **(B)** Grp-no Antibiotics (Grp-noAB) HNC vs. HC ($N = 19$; $N = 20$). [a]For the Grp-All HNC vs. HC comparison, "Diagnosis" was the main fixed factor, and "Sample Site" was the secondary fixed factor. "Antibiotics" was nested into "Diagnosis" and "Sample site" factors as a random variable. Grp-noAB analytical design did not include antibiotics as a factor. [b]For the analytical design considering HPV, "HPV status" and "Sample site" were set as fixed factors and "Antibiotics" as nested as random factor. Grp-noAB analytical design did not include antibiotics as a factor. Level of significance is denoted using an asterisk (*): * < 0.05 = p-value less than 0.05; ** < 0.01 = p-value less than 0.01; *** < 0.001 = p-value less than 0.001.

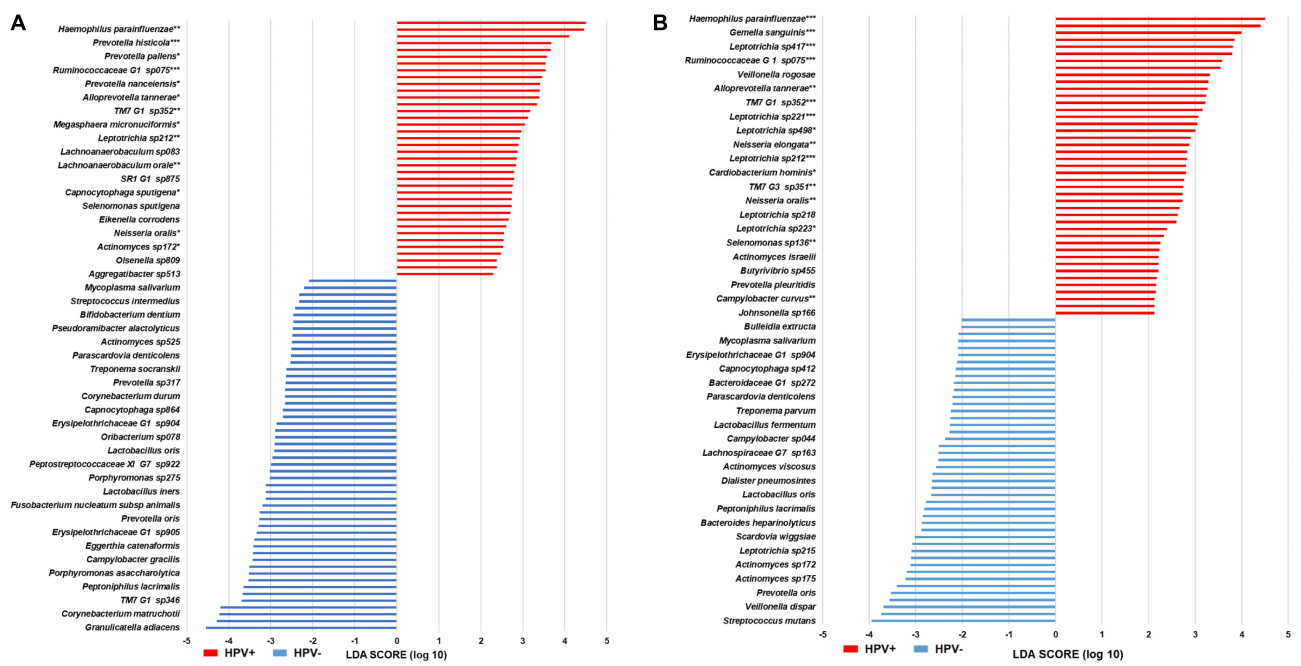
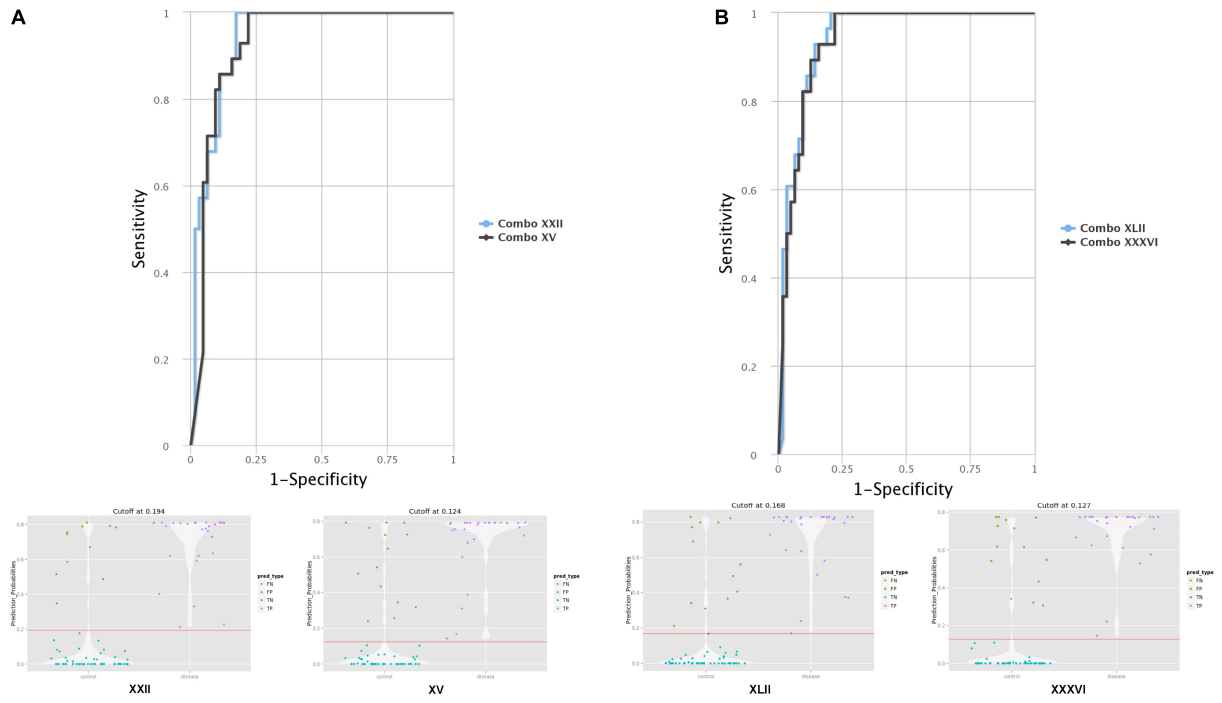


FIGURE 3 | LEfSe histograms of differential features in head and neck cancer patients. **(A)** Grp-All BPST. **(B)** Grp-noAB BPST. Linear discriminant analysis Effect Size (LEfSe) was performed to determine distinct microbiome features in oral samples [stimulated saliva (S) samples and swabs of buccal mucosa (B), supragingival plaque (P), and tongue (T)] of the following: **(A)** Grp-All patient cohort ($N = 23$) for the head and neck cancer (HNC) HPV-positive (HPV+; $N = 16$) vs. HPV-negative (HPV–; $N = 7$) comparisons. **(B)** Grp-noAB patient cohort ($N = 15$) for HNC HPV+ ($N = 12$) vs. HPV– ($N = 3$) that did not receive antibiotics within 2 weeks of sampling. Horizontal histograms depict the discriminant features, i.e., bacterial species, for Grp-All and Grp-noAB potential biomarkers for HPV+ (red) and HPV– (blue). Mann–Whitney U-tests were used to determine significance of HPV+ distinctive species. Level of significance is depicted by an asterisk (*): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



Combination	Species	AUC	SE	SP	ACC	Cutoff	PPV
Combo XXII	R. sp075, H. parainfluenzae, H. pittmaniae, L. sp212, L. sp417	0.941	1	0.828	0.88	0.194	0.69
Combo XV	R. sp075, H. pittmaniae, L. sp212, L. sp417	0.928	1	0.781	0.85	0.124	0.65

Combination	Species	AUC	SE	SP	ACC	Cutoff	PPV
Combo XLII	G. sanguinis, H. pittmaniae, L. sp212, L. sp417, R. sp075	0.943	1	0.797	0.86	0.168	0.68
Combo XXXVI	TM7 sp352, H. pittmaniae, L. sp221, L. sp417, R. sp075	0.938	1	0.781	0.86	0.127	0.66

FIGURE 4 | HNC HPV+ vs. HNC HPV– CombiROC analysis using RA and log(RA + 1) transformed abundance data. **(A)** CombiROC analysis for biomarkers with AUC > 0.75 on RA data. **(B)** CombiROC analysis for biomarkers with AUC > 0.75 on log(RA + 1) data. Significant ($\alpha = 0.05$) CombiROC program-generated receiver operator characteristic (ROC) curves for **(A)** RA data and **(B)** log(RA + 1) transformed abundance data of candidate microbial biomarkers (AUC > 0.75; $n = 10$ bacterial species) identified *via* MedCalc Software for Grp-All HPV+ vs. HPV– group are shown. **(A)** ROC curves, violin plots, and descriptive statistic summary table for the best two combinations from 10 potential markers using the RA are shown. "Combo XXII" consists of a combination of the markers *Ruminococcaceae sp075*, *Haemophilus parainfluenzae*, *Haemophilis pittmaniae*, *Leptotrichia sp212*, and *Leptotrichia sp417* (ROC curve blue line). "Combo XV" contains markers *Ruminococcaceae sp075*, *H. pittmaniae*, *Leptotrichia sp212*, and *Leptotrichia sp417* (ROC curve black line). **(B)** ROC curves, violin plots, and descriptive statistic summary table for the best two combinations from 10 potential markers using the log(RA + 1) transformed data are shown. "Combo XLII" contains potential markers *Gemella sanguinis*, *H. pittmaniae*, *Leptotrichia sp212*, and *Ruminococcaceae sp075* (ROC curve blue line). "Combo XXXVI" consists of species probes *TM7 G1 sp352*, *H. pittmaniae*, *Leptotrichia sp221*, *Leptotrichia sp417*, and *Ruminococcaceae sp075* (ROC curve black line). Cut-off refers to the optimal value or the highest true positive rate that has the lowest false positive rate. FN, false negative; FP, false positive; TN, true negative; TP, true positive; AUC, area under the curve; SE, sensitivity; SP, specificity; ACC, accuracy; PPV, positive predictive value.

species and 65 genera detected based on HOMI*NGS* sequencing data. We confirmed findings from multiple studies indicating that shifts in microbiome profiles which may be defined as "dysbiosis" occur in HNC patients compared to HC subjects (Guerrero-Preston et al., 2016, 2017; Tuominen et al., 2018).

Furthermore, we were able to establish that HNC HPV+ patients have significantly different microbiome than that of HNC HPV– patients (**Supplementary Table 2**). While α-diversity was not significantly different between HNC HPV+ and HNC HPV– patients, α-diversity differed between HNC patients and HC subjects (**Table 2**). Additionally, β-diversity differences were significant for all comparisons in this study except for one out of 30 comparisons (**Supplementary Table 2**). There was a clear separation between the Grp-All HNC patients and HC subjects (**Supplementary Figure 1A**). We were also able to determine that although antibiotic treatment within 2 weeks of sampling is a confounding variable, excluding antibiotic-treated HNC patients did not affect the main results, by comparing the microbiome data of HNC HPV+ patients with the data of HNC HPV– patients (i.e., GrpAll and GrpNoAB) (**Supplementary Table 1**). A visualization of the division of the Grp-All HNC HPV+ vs. HNC HPV– can be seen in **Supplementary Figure 1B**.

Regarding periodontal disease and dental caries status, our sub-cohort is similar to that of the larger OraRad cohort (**Table 1** and Brennan et al., 2021). A study identified by LitSuggest, reviewing findings pertaining to oral HPV infection in relation to periodontitis, suggests periodontal pockets may act as a reservoir for HPV and that oral HPV prevalence may be associated with periodontitis (Shigeishi et al., 2021a). Another recent study characterizing HPV16 DNA prevalence and periodontal disease inflammation in a population of older Japanese women identified an increase of *Prevotella intermedia* and *Porphyromonas* and

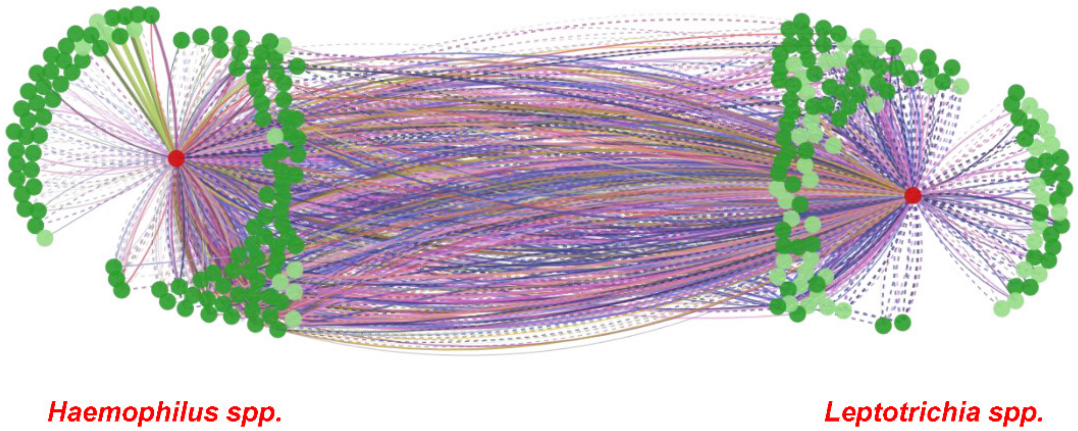


FIGURE 5 | MIND interaction network of *Haemophilus* and *Leptotrichia* spp. Microbial Interaction Network Database (MIND; http://www.microbialnet.org/mind_home.html) results, illustrating a network between *Haemophilus* spp. (left red circle) and *Leptotrichia* spp. (right red circle) by using MIND default options, are presented. Dark green circles depict genera that interact with *Haemophilus* spp./*Leptotrichia* spp., and light green circles depict species that interact with *Haemophilus* spp./*Leptotrichia* spp. The lines between *Haemophilus* spp. and *Leptotrichia* spp. show interactions in common with other genera and species. The different color lines depict the human tissue sites where interactions have been demonstrated in previous studies (MIND; http://www.microbialnet.org/mind_home.html).

a decrease of *Veillonella* and *Prevotella* to be associated with periodontal disease inflammation (Shigeishi et al., 2021b).

Furthermore, a study by Chowdhry et al. (2019) exploring deep-seated infected tissues removed during periodontal flap surgery in chronic periodontitis patients, observed an increased abundance of *Veillonella arula*, *Selenomonas noxia*, *Neisseria oralis*, *P. intermedia*, *Prevotella nigrescens*, and *Capnocytophaga ochracea* in HPV+ samples. Interestingly, in our study we found species of *Capnocytophaga*, *Neisseria*, *Prevotella*, *Selenomonas*, and *Veillonella* spp. to represent distinct taxa for HNC HPV+ patients through LEfSe analysis (**Figure 3**). Species from these genera were also found to be significant using Mann–Whitney *U*-test ($p < 0.05$) (**Figure 3**). While ROC curves were also significant ($p < 0.01$), we determined none of these species to be excellent biomarkers (**Supplementary Table 4**). We agree with Shigeishi et al. (2021b) suggesting that sampling methods of the oral microbiome should be carefully selected for periodontal tissue to ensure detection of HPV DNA directly along with the associated periodontal microbiome.

LEfSe analysis showed 43 bacterial species differentiating Grp-All HNC HPV+ from Grp-All HNC HPV– patients (**Figure 3**). Grp-noAB group analysis confirmed these findings since 24 bacterial species characterizing the HNC HPV+ patients were in common with the species distinctive of Grp-All HNC HPV+ patients (**Figure 3**). *Leptotrichia* spp. were the most prominent and significant species in comparisons performed for both Grp-All and Grp-noAB groups, precluding the possibility that antibiotics alone account for differences between HNC HPV+ and HPV– groups. In addition to LEfSe, Mann–Whitney *U*-tests comparing the RA of HNC HPV+ to HNC HPV– found 5/6 (83%) and 6/6 (100%) *Leptotrichia* spp. for Grp-All and Grp-noAB, respectively, to be significant ($p < 0.05$) (**Figure 3**). For these *Leptotrichia* spp., the RA was found to be greater in samples of HNC HPV+ compared to HNC HPV– patients for Grp-All and Grp-noAB (data not shown). ROC analysis on log(RA + 1) data further confirmed these findings with five Grp-All *Leptotrichia* spp. and six Grp-noAB *Leptotrichia* spp. to have an AUC significantly different from that of 0.5 ($p < 0.01$) (**Supplementary Table 4**). By minimizing zero inflation on log(RA + 1) transformed abundance data for the Grp-noAB group, we determined three *Leptotrichia* spp. (*Leptotrichia sp215*, *sp392*, and *sp417*) to be excellent biomarkers distinguishing HNC HPV+ from HNC HPV– patients, with a sensitivity >95%, a specificity = 100%, and an accuracy >95% (**Supplementary Table 4D**).

A previous study by Bahig et al. (2020) investigating the tumor microenvironment of HPV-associated SCC patients, determined an increased abundance of *Leptotrichia* genus in oral samples at baseline which declined over the course of radiation. This study was positively classified in our LitSuggest analysis (**Table 3**). A study by Oliva et al. (2021) found that *Leptotrichia hofstadii* was abundant in stage III oropharynx cancer, while Zakrzewski et al. (2021) determined *Leptotrichia* genus to be decreased in oropharynx HPV- tumor samples.

Surprisingly, other studies have found *Leptotrichia* spp. to be absent or less abundant at SCC primary tumor sites (Schmidt et al., 2014; Guerrero-Preston et al., 2016, 2017). *Leptotrichia* spp. have also been investigated for their role in periodontal disease (i.e., gingivitis and periodontitis) (**Supplementary Table 3**; Eribe and Olsen, 2017). A systematic review by Pérez-Chaparro et al. (2014) described a study by Griffen et al. (2012) that correlated an increased abundance of *Leptotrichia* genera, *Leptotrichia oral taxon 210*, *Leptotrichia EX103*, and *Leptotrichia IK040* to be associated with deep pockets of patients with periodontal disease. Accordingly, *Leptotrichia* species consist of non-motile facultative anaerobic and anaerobic species mostly present in the oral cavity (Eribe and Olsen, 2017).

We also observed that *H. pittmaniae* had a higher RA in HNC HPV+ than HNC HPV– patients in Grp-All and Grp-noAB (data not shown). This species was identified as a differential feature of HPV+ by LEfSe and was found to have a significant ROC curve using log(RA + 1) data with an AUC of 0.824 (**Figure 3** and **Supplementary Table 4**). Additionally, this species was determined to be an excellent biomarker in the Grp-All and Grp-noAB log(RA + 1) ROC curve analysis with zero inflation minimized as well as a good biomarker when zero inflation was not minimized (**Supplementary Table 3**). *H. pittmaniae* was also

TABLE 3 | LitSuggest positively classified articles (*n* = 15) involving HPV and the oral microbiome in HNC patients.

Year[a]	Author[b]	Purpose of study[c]	Findings[d]	PMID[e]
2021	Gougousis et al., 2021 (review)	Review the significance of biomarkers based on epigenetics and microbiome profile in the diagnosis of HPV-related OSCC.	*Streptococcus salivarius* (+), *Streptococcus gordonii* (+), *Gemella haemolysans*, *Gemella morbillorum* (+), *Johnsonella ignava* (+), and *Streptococcus parasanguinis* (+) highly associated with tumor site. *Gemella adiacens* (+) association with non-tumor site. HPV+ correlation between the genera *Haemophilus* and *Gemella* in oral cavity cancer. *Actinomyces* (+), *Parvimonas* (+), *Selenomonas* (+), and *Prevotella* (+) in OCC compared to OPC. *Corynebacterium* (+) and *Kingella* (+) are associated with decreased risk of oral cancer.	33521000
2021	De Keukeleire et al., 2021 (review)	Knowledge and biomarkers in HNC-SCC.	HPV is a biomarker of HNC-SCC; *Lactobacilli* (+); *Haemophilus* (–); *Neisseria* (–); *Gemellaceae* (–); *Aggregatibacter* (–); *Streptococci* (–); *Fusobacteria* (+); *Fusobacterium nucleatum* (+) associated with lower tumor stage	33916646
2021	De Martin et al., 2021	Characterize microbiome of human palatine tonsil crypts in patients with high-risk HPV-associated tonsil cancer compared to sleep apnea controls.	*Firmicutes* (+); *Actinobacteria* (+); *Veillonella* (+); *Streptococcus* (+); *Prevotella* (+); *Filifactor alocis* and *Prevotella melaninogenica* were distinct features of tonsil cancer	34367729
2021	Oliva et al., 2021	Characterize oral and gut microbiome of HPV+ OSCC patients before and after CRT.	*F. nucleatum* (+), *G. morbillorum* (+), *G. haemolysans* (+), *Leptotrichia hofstadii* (+), *Selenomonas sputigena* (+), and *Selenomonas infelix* (+) in stage III OSCC	33750907
2021	Rajasekaran et al., 2021	Characterize microbiome in patients with HPV-associated early tonsil SCC compared to benign tonsil specimens.	*Burkholderia pseudomallei* was unique to cancer specimens. *Fusobacteria* was identified in HPV-associated OSCC patients in tonsil and lymph node specimens. Negative nodes showed signatures for *Anaplasma phagocytophilum*, *Bacillus subtilis*, *Chlamydia trachomatis*, *Chlamydophila psittaci*, *Lactococcus lactis*, and *Proteus mirabilis*	33905914
2021	Shigeishi et al., 2021a	Characterize HPV16 DNA prevalence and PD inflammation in older Japanese women.	*Prevotella intermedia* (+), *Porphyromonas* (+), *Veillonella* (–), and *Prevotella* (–) in HPV+ periodontal inflammation	33456534
2021	Shigeishi et al., 2021b (review)	Review recent findings of oral HPV infection in relation to periodontitis.	HPV localizes to inflammatory periodontal tissue, and periodontal pockets may act as a reservoir for HPV. Smoking is associated with HPV and periodontitis. Carcinogenic HPV and periodontitis may lead to OCC, but HPV E6/E7 has not been fully investigated in patients with periodontitis. Oral HPV prevalence may be associated with periodontitis.	33728046
2021	Zakrzewski et al., 2021	Compare microbial composition, diversity, and specific bacterial phytotypes between HPV+ and HPV– oropharyngeal tumors using saliva, normal tissue, and tumor tissue.	*Treponema* (+) and *Spirochaetes* (+) were associated with normal tissues of HPV+ patients; *Neisseria*, *Veillonella*, *Fusobacterium*, *P. melaninogenica*, and *Porphyromonas* were associated with HPV status (not significant). *Fusobacteria* (–) in saliva samples (not significant); *Leptotrichia* (–) in HPV-; *Rothia* (–) in HPV+ tumor tissues; *Atopobium* (–) in normal tissue HPV+ patients.	34278648
2020	Bahig et al., 2020	Characterize tumor microenvironment of HPV-associated OSCC with RT +/– cisplatin-based chemotherapy using surface swab of tonsil, base of tongue, and buccal mucosa.	Decreased α-diversity over course of treatment. *Veillonella* (+) and *Leptotrichia* (+) at tumor site. *Actinomyces* (–) and *Leptotrichia* (–) over the course of radiation. *Gemella* (–) and *Streptococcus* (–) between baseline and 1 week and returned to baseline at week 5. *Veillonella* (+) and *Topobium* (+) at week 5.	33367119
2019	Chowdhry et al., 2019	Explore deep-seated infected granulation tissue removed during periodontal flap surgery procedures for residential bacterial species between HPV+ and HPV– chronic periodontitis patients.	Deep-seated granulation tissues showed *Firmicutes* (+), *Proteobacteria* (+), and *Bacteroidetes* (+). *Veillonella arula* (+), *Selenomonas noxia* (+), *Neisseria oralis* (+), *P. intermedia* (+), *Prevotella nigrescens* (+), *Capnocytophaga ochracea* (+) in HPV+ samples. *Prevotella* (+), *Macellibacteroides fermentans* (+), *Porphyromonas endodontalis* (+), *Campylobacter rectus* (+), *Treponema phagedenis* (+) in HPV– samples. *Pseudoxanthomas kaohsiungensis* (+) in females and *Desulfobulbus rhabdoformis* (+) in males.	31111067

(Continued)

TABLE 3 | (Continued)

Year[a]	Author[b]	Purpose of study[c]	Findings[d]	PMID[e]
2018	Lim et al., 2018	Characterize the oral microbiome fluctuation associated with OCC and OSCC compared to healthy controls using oral wash samples.	*Rothia* (–), *Haemophilus* (–), *Corynebacterium* (–), *Paludibacter* (–), *Porphyromonas* (–), *Capnocytophaga* (–) in OCC and OSCC. *Oribacterium* (+) in OCC and OSCC. *Actinomyces* (+), *Parvimonas* (+), *Selenomonas* (+), and *Prevotella* (+) in OCC compared to OSCC. *Haemophilus* (+), *Gemella* (+) with HPV+. *Actinomyces* (+), *Actinobacillus* (+), *Lautropia* (+), *Fusobacterium* (+), *Aggregatibacter* (+) in high-risk individuals. Panel of bacterial species *Rothia*, *Haemophilus*, *Corynebacterium*, *Paludibacter*, *Porphyromonas*, *Oribacterium*, and *Capynocytophaga* showed an area under curve of 0.98, sensitivity of 100%, and specificity of 90%	30123780
2018	Tuominen et al., 2018	Investigate the association between HPV infection and microbiome composition in the placenta, uterine cervix, and mouth in women	*Selenomonas* (+), *TM7* (+), *Megasphaera* (+) with HPV+ in oral samples. *Haemophilus* (+) with HPV– oral samples. Higher richness in HPV+ than HPV– samples.	29955075
2017	Guerrero-Preston et al., 2017	Characterize microbial species in the saliva microbiome and tumor characteristics in HNC-SCC patients.	*Veillonella dispar* (+) in all samples. *S. salivarius* (+), *Streptococcus vestibularis* (+) in HNC-SCC samples. *Lactobacillus* spp. (+), *Parvimonas micra* (+), *Streptococcus mutans* (+), and *F. nucleatum* (+) in salivary HNC-SCC samples. *Fusobacterium periodonticum* (–), *Leptotrichia trevisanii* (–), *L. hofstadii* (–), and *Leptotrichia* (–) in HNC-SCC compared to controls. Lower diversity in HNC-SCC than controls regardless of HPV status. No significant differences when comparing HPV+ to HPV– saliva HNC-SCC samples with control. *F. periodonticum* (+) in saliva from HNC-SCC patients. *Lactobacillus rhamnosus* (+), *Lactobacillus salivarius* (+), *Lactobacillus vaginalis* (+), *Lactobacillus reuteri* (+), *Lactobacillus fermentum* (+), *Lactobacillus johnsonii* (+), *Lactobacillus gasseri* (+) in subset of HNC-SCC samples from Johns Hopkins University. *Lactobacillus* was 710 time higher, and *L. vaginalis* was 52 times higher in HNC-SCC samples compared to controls.	29340028
2017	Wolf et al., 2017	Compare oral salivary microbiome samples of patients with OCC and OSCC vs. healthy controls.	Shannon index found higher diversity in tumor patients but was not significant. Highest LEfSe LDA was from *Proteobacteria*. *Prevotella* (+), *Haemophilus* (+), *Neisseria* (+), *Streptococcus* (+), and *Veillonella* (+) in healthy controls. *Actinomyces* (+), *Schwartzia* (+), *Treponema* (+), and *Selenomonas* (+) in HNC-SCC patients. HPV+ patients demonstrated normal microbiome compared to healthy controls.	28725009
2016	Guerrero-Preston et al., 2016	Compare saliva microbiome from HPV+ and HPV–, OCC, OSCC, and normal cavity epithelium.	*Firmicutes* (+), *Proteobacteria* (+), *Bacteroidetes* (+), *Actinobacteria* (–), and Fusobacteria (–) prior to surgery. At lower levels *Streptococcus* (+), *Prevotella* (+), *Haemophilus* (+), *Lactobacillus* (+), *Veillonella* (+), *Citrobacter* (–), *Kingella* (–) prior to surgery. HNC-SCC patients exhibited lower richness and diversity compared to controls. *Streptococcus*, *Dialister*, and *Veillonella* were able to discriminate tumor from control samples. *Neisseria* (–), *Aggregatibacter* (–), *Haemophilus* (–), and *Leptotrichia* (–) in tumor samples. *Enterobacteriaceae* and *Oribacterium* discriminate OCC from OSCC and normal samples. *Gemellaceae* (+) and *Leuconostoc* (+) only observed in HPV+ samples. α-diversity was reduced post-surgery.	27259999

Using Python$_{v3.6.2}$ program, 203 PubMed articles were retrieved for classification from the model that matched the search terms "HPV" and "microbiome." LitSuggest program determined 36 articles to be positively associated with "HPV" and "oral microbiome." Manual validation of the 36 positively classified articles resulted in 21 articles being discarded. The remaining 15 articles were manually validated as positively classified articles that relate to HNC, HPV, and the oral microbiome.

[a] *Year of publication.*

[b] *First listed author.*

[c] *Purpose or outcomes explored during the study.*

[d] *Findings/results of the study.*

[e] *PubMed ID.*

HPV, human papillomavirus; HNC, head and neck cancer; OSCC, oral squamous cell carcinoma; OCC, oral cavity cancer; OPC, oropharyngeal cancer; SCC, squamous cell carcinoma; CRT, chemoradiotherapy; PD, Parkinson's disease; LDA, linear discriminant analysis; RT, radiotherapy.

included in all four multi-marker ROC combinations including *Leptotrichia* species, suggesting it is a contributor to HPV+ SCC progression (**Figure 4**). *H. pittmaniae* has been suggested as a pathogen possibly responsible for respiratory tract infections in patients with lung diseases (Boucher et al., 2012) but has also been identified at significantly higher levels in male children with active caries (Ortiz et al., 2019). While little is known about *H. pittmaniae* and its role in periodontal disease, the

Haemophilus genus was identified in many positively classified studies per our LitSuggest text mining analysis. In a recent study by De Keukeleire et al. (2021) a decrease in *Haemophilus* was associated with HNC-SCC, confirming findings by Wolf et al. (2017) and Lim et al. (2018). However, studies by Guerrero-Preston et al. (2016) and Gougousis et al. (2021) found the opposite to be true. In our study, we were able to verify findings by Lim et al. (2018) and Tuominen et al. (2018) that an increase in abundance of *Haemophilus* in the oral cavity is associated with HNC-SCC HPV+ samples. Altogether, a microbiome metagenome/metatranscriptome survey focused on *Haemophilus* and *Leptotrichia* at the species and strain levels could provide reliable biomarker signatures with clinical implications for HNC HPV+ patients in the future.

MIND analysis found many microbial interactions between the genera *Leptotrichia* and *Haemophilus* connected through many species and genera (**Figure 5**). These genera have also been shown as two of the nine taxa that facilitate structures in oral plaque and intermingle at the micron scale (Mark Welch et al., 2016). The study by Mark Welch et al. (2016) also suggests that *Corynebacterium* forms long structures with *Streptococcus* and *Porphyromonas* in direct contact. *Streptococcus* creates an environment rich in CO_2, lactate, and acetate, facilitating the contact with *Haemophilus*, *Aggregatibacter*, and *Neisseriaceae* (Mark Welch et al., 2016). With the exception of *Neisseriaceae*, these species are likely essential to aerobic metabolism, allowing *Fusobacterium* and *Leptotrichia* to thrive as key participants in the metabolism of sugars, producing lactic acid (Mark Welch et al., 2016). It is suggested that this metabolism is involved in degradation of oral tissues, possibly leading to dental caries and/or periodontal disease (Eribe and Olsen, 2017).

Text mining used in this study suggests a lack of information involving HPV and the oral microbiome of HNC patients. Of 203 HPV microbiome articles, only 15 were verified as relevant to HPV and the oral microbiome in SCC. Only two studies identified by LitSuggest related oral SCC with HPV and periodontitis (Chowdhry et al., 2019; Shigeishi et al., 2021a). Furthermore, few studies investigated multiple primary tumor sites in SCC patients. Most identified studies in our analysis focused on investigating oropharyngeal SCC (Guerrero-Preston et al., 2016; Wolf et al., 2017; Lim et al., 2018; Bahig et al., 2020; Gougousis et al., 2021; Oliva et al., 2021; Zakrzewski et al., 2021). Only two positively classified articles were found to characterize the microbiome in the tonsil of SCC patients (**Table 3**; De Martin et al., 2021; Rajasekaran et al., 2021). In the future, more studies on HNC-SCC HPV+ patients using larger patient cohorts will be required to determine HNC risk in relation to the oral microbiome and HPV status.

Limitations

While this study was able to show the significance of microbial composition in HNC-SCC HPV+ patients compared to HNC-SCC HPV– patients, we were unable to account for the immune status of the patients. Furthermore, our patient cohort was relatively small, and our design was not optimally balanced due to the various primary cancer sites in our patient cohort. In addition, many factors not addressed in this study may affect HNC progression, such as genetics, oral hygiene practices, and periodontal treatment. However, our main conclusion remains pertinent, in that the species identified as multi-marker combinations, i.e., *H. pittmaniae* and *Leptotrichia* spp., increase in HNC-SCC HPV+ patients regardless of the primary cancer site.

DATA AVAILABILITY STATEMENT

The data presented in the study are provided as **Supplementary Material** and have been deposited in a public GitHub repository. The data can be found here: https://github.com/mbeckm01/HPV_HNC.git.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Atrium Health Institutional Review Board and University of Connecticut Health Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-LM and FB conceived this microbiome study. MTB and RL had previously established the cited clinical outcomes study "OraRad" and provided clinical insights for this study. J-LM directed the statistical analyses implemented and verified by MFB and HL. MFB, HL, J-LM, and FB contributed to the writing of the manuscript, the overall analysis, and biological interpretation. All authors participated in the revisions of the manuscript and interpretation of the results, gave their final approval, and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.794546/full#supplementary-material

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Periodontal and Peri-Implant Microbiome Dysbiosis Is Associated With Alterations in the Microbial Community Structure and Local Stability

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Periodontitis and peri-implantitis are common biofilm-mediated infectious diseases affecting teeth and dental implants and have been considered to be initiated with microbial dysbiosis. To further understand the essence of oral microbiome dysbiosis in terms of bacterial interactions, community structure, and microbial stability, we analyzed 64 plaque samples from 34 participants with teeth or implants under different health conditions using metagenomic sequencing. After taxonomical annotation, we computed the inter-species correlations, analyzed the bacterial community structure, and calculated the microbial stability in supra- and subgingival plaques from hosts with different health conditions. The results showed that when inflammation arose, the subgingival communities became less connective and competitive with fewer hub species. In contrast, the supragingival communities tended to be more connective and competitive with an increased number of hub species. Besides, periodontitis and peri-implantitis were associated with significantly increased microbial stability in subgingival microbiome. These findings indicated that the periodontal and peri-implant dysbiosis is associated with aberrant alterations in the bacterial correlations, community structures, and local stability. The highly connected hub species, as well as the major contributing species of negative correlations, should also be given more concern in future studies.

Keywords: periodontitis, peri-implantitis, microbiome, community structure, metagenomic sequencing, dysbiosis, local stability

INTRODUCTION

Periodontitis is a prevalent disease in the human oral cavity and the major cause of dentition defects (Albandar, 2005). It is a complex infectious disease resulting from infection-induced inflammation and hyperimmune response toward various microbial pathogens (Kajiya et al., 2010; Bueno et al., 2015). Previous studies have proved that periodontitis is initiated with microbial dysbiosis in the periodontium (Kinane et al., 2017). The prevalence of periodontitis is estimated from 4 to 76.0% in developed countries and from over 50% to almost 90% in developing ones (Jiao et al., 2021). Approximately over 700 million adults are suffering from periodontitis worldwide (Kassebaum et al., 2014), which has become a severe burden in the oral health of humankind (Marcenes et al., 2013).

Peri-implantitis has been described as a pathological condition around dental implants where inflammation continuously affects connective tissue and finally leads to the loss of the supporting bone matrix (Schwarz et al., 2018). Similar to periodontitis, peri-implantitis is also caused by the hyper-inflammation in peri-implant tissue and the aberrant change in the microbial community (Alcoforado et al., 1991; Leonhardt et al., 1999; Wang et al., 2016). A meta-analysis in 2017 indicated that the weighted mean prevalence of peri-implantitis was around 19.83% at patient level (Lee et al., 2017). As implant-supported prostheses are being more and more widely used to replace missing teeth (Buser et al., 2017), there will be an increasing number of patients suffering from peri-implantitis in the coming future.

Periodontitis and peri-implantitis share many clinical and etiological features, including biofilm-mediated infection, hyperinflammatory reaction, and progressive absorption of alveolar bone (Berglundh et al., 2011; Carcuac and Berglundh, 2014; Liu et al., 2020). Most importantly, the accumulation of dental plaque and the following microbial dysbiosis are considered to be the initiation of both diseases (Ng et al., 2021). Given the shared nature as infectious diseases between periodontitis and peri-implantitis, it is necessary to delve into the microbial communities around teeth and implants to understand the two diseases further.

The stability of commensal microbial communities in human bodies has been proved essential to human health (Relman, 2012). However, previous studies investigating oral microbiota using high-throughput sequencing approaches have mainly focused on the taxonomical profile or microbial functionalities (Dabdoub et al., 2016; Ai et al., 2017; Babaev et al., 2017; Belstrom et al., 2017; Ghensi et al., 2020; Komatsu et al., 2020; Ng et al., 2021). Yet, the community structure and the microbial stability have not been fully illustrated, especially when the complexity of numerous bacterial correlations cannot be fully identified by isolating pairwise interactions. To fill this insufficiency, we analyzed 64 microbial samples from plaque around teeth and implants in different health conditions using metagenomic shotgun sequencing. We annotated taxonomical information at the species level, visualized the bacterial co-occurrence network, analyzed the community structure, and calculated the microbial stability of our samples to further our understanding of periodontitis and peri-implantitis.

MATERIALS AND METHODS

Participant Recruitment

This study enrolled 34 participants, including 19 subjects for the healthy group and 15 subjects with periodontitis or peri-implantitis for the diseased group (See **Supplementary Tables 1, 2**). All participants were Chinese natives who sought care at the College of Stomatology, Xi'an Jiaotong University, and provided written consents. Natural teeth were considered periodontal health when there was no bleeding on probing (BOP), no clinical attachment loss (CAL), or radiographic bone loss (RBL) and the maximum probing depth (PD) was less than 3 mm. Periodontitis was diagnosed with an increased PD of more than 4 mm, examinable RBL, and interdental CAL, which corresponded with the latest diagnostic criteria for Stage II-IV periodontitis (Papapanou et al., 2018). As for implants, subjects were considered peri-implant health when peri-implant tissue showed no redness, suppuration, BOP, and no more than 1-mm marginal RBL beyond bone remodeling. Peri-implantitis was diagnosed when there was clinical inflammation, increased PD of more than 6 mm, and radiographic evidence of more than 3 mm RBL compared to baselines (Lindhe et al., 2008). Detailed inclusion and exclusion criteria are listed in **Table 1**.

Clinical Examination and Sample Collection

Before sampling, full-mouth examinations were conducted on all subjects by the same calibrated clinician to record clinical and demographic features, including sex, age, PD, BOP, and RBL. Especially for subjects with implants, we also recorded their implant type, location, and functional time (**Supplementary Tables 1, 2**).

The selection of sampling sites followed the criteria in our **Supplementary Information**. When sampling commenced, patients first gargled with distilled water for 1 min. Then, we used cotton rolls to isolate the selected sites and sampled the supragingival plaque using sterile curettes by a single horizontal stroke on each site. Bacteria were washed off from the curettes by rinsing in 1.5-ml microcentrifuge tubes containing phosphate-buffered saline (PBS). The remaining supragingival plaque was then removed. Afterward, we used sterile endodontic paper points for subgingival sampling (Jervoe-Storm et al., 2007), by inserting paper points as deep as possible into the periodontal or peri-implant sulcus and staying for 20 seconds. After taking out, paper points were transferred into 1.5-ml microcentrifuge tubes containing PBS. All samples were stored at −80°C and were then sent to BGI Institute (BGI Group, Shenzhen, China) for genomic DNA extraction, metagenomic libraries preparation, and sequencing.

DNA Extraction and Metagenomic Sequencing

Genomic DNA of the samples was isolated using QIAmp DNA Micro Kit (Qiagen, Valencia, CA) with "Protocol: Isolation of Genomic DNA from Tissues" according to the handbook. The sequencing libraries were then prepared following BGI's

TABLE 1 | Detailed inclusion and exclusion criteria for subject recruitment.

Type	Health condition	Inclusion criteria	Exclusion criteria
Teeth	Periodontal health	• Individual normal occlusion with no less than 28 teeth left in dentition; • No RBL or examinable CAL; • Maximum PD $\leq$ 3 mm; • No BOP or redness examined.	• Diabetes mellitus or other severe systemic diseases; • HIV infection or other severe immune diseases; • A history of tobacco smoking; • A history of immunosuppressant therapy; • A history of bisphosphonates, steroids, or other therapy influencing bone metabolism; • Antibiotic therapy, oral antiseptic therapy, or oral prophylactic treatment undergoing or in recent 3 months; • Having other dentures in any form besides the selected dental implant; • Pregnancy or lactation; • Over 60 years old or below 20 years old.
	Periodontitis	• Individual normal occlusion with no less than 20 teeth left in dentition; • Examinable interdental CAL $\geq$ 3 mm; • PD $\geq$ 4mm; • Examinable RBL; • Existing BOP and/or suppuration.	
Implants	Peri-implant health	• A single implant with a single cement-retained crown seated to replace the missing tooth; • Implant in function for over 2 years; • Radiographic MBL $\leq$ 1 mm; • No redness, suppuration, or BOP examined around the implant.	
	Peri-implantitis	• A single bone-level implant with a single cement-retained crown seated to replace the missing tooth; • Implant in function for over 2 years; Radiographic MBL $\geq$ 3 mm compared to baseline; • PD $\geq$ 6 mm around the implant.	

instruction (BGI Group, Shenzhen, China). The libraries were sequenced on the BGI SEQ-500 sequencing platform (BGI Group, Shenzhen, China). Raw reads generated from the sequencing platform were then filtered and cleaned before further analysis.

Metagenomic Analysis

To obtain high-quality data, we firstly filtered the raw reads when they contained more than 10 low-quality bases (< Q20) or 15 bases of adapter sequences with a self-constructed script. Using BWA software (version 0.7.17), we aligned the read data to the human genome (hg19) and filtered the reads when the alignment length exceeds 40% of the read length (Li and Durbin, 2009). After the removal of host mapped reads, the clean metagenomic data were applied for the following metagenomic analysis.

Using MetaPhlAn3 (Truong et al., 2015), we aligned the filtered reads to the microbial database of specific marker genes (mpa_v30_CHOCOPhlAn_201901) and obtained the taxonomical annotation results. Based on the microbial profiling, we calculated the relative abundances of bacteria at phylum, class, order, family, genus, and species levels, respectively (see **Supplementary Data Sheet 1**). After the taxonomical annotation, we performed permutational multivariate analysis of variance (PERMANOVA) to evaluate the impact of environmental factors on the microbiome (permutation number equals 9,999), calculated alpha diversity using the Chao1 and Shannon indexes, and detected the Spearman correlation coefficients among the species with relative abundance over 0.01%. We kept the relations with coefficients <-0.6 or > 0.6 (adjusted p-value < 0.05) to construct the bacterial interacting matrix (**Supplementary Data Sheets 2, 3**) and to plot the bacterial co-occurrence networks by applying Gephi (version 0.4.2) and Cytoscape (version 3.8.2) for further analysis (Shannon et al., 2003; Bastian et al., 2009; Otasek et al., 2019). Species with more than 25 correlations were defined as hub species, which indicated their pivotal places in the bacterial co-occurrence networks. We screened and compared these species between different microbiomes.

Local Stability Analysis

Local stability measures the tendency of a community to return to its equilibrium after perturbation. The community is stable if it can return to its equilibrium after perturbation. Following the work by May and Allesina (May, 1972, 1973; Allesina and Tang, 2012), we used the community matrix generated from our co-occurrence network (**Supplementary Data Sheets 2, 3**) to analyze the local stability of oral microbiome (**Figure 1A**). The community matrix incorporates several structural properties, including the number of interacting species, the connectance, the types and strength of interactions, and the degree distribution. Connectance was defined as the fraction of non-zero off-diagonal elements of the community matrix (May, 1972, 1973), or briefly as the ratio of actual bacterial correlations to all topologically possible correlations. The types of interactions were extracted from our co-occurrence networks illustrated above. The degree of a species referred to the count of its correlations with other species. The local stability theory indicates that a stable system requires that all eigenvalues of the community matrix should have negative real parts (**Figure 1B**), which means the real part of the rightmost eigenvalue in the complex plane can be used to measure the extent of stability. A more negative real part corresponds to a more stable community, which grants it more robustness when resisting perturbations that tend to alter the abundance of its members (**Figure 1C**). Based on experimental data, we performed a series of simulations to show the differences in stability among different groups (see also **Supplementary Information**).

Statistics

For the Chao1 and Shannon indexes calculated for different groups, we performed the Wilcoxon rank-sum test to check whether significant differences exist between groups. All the

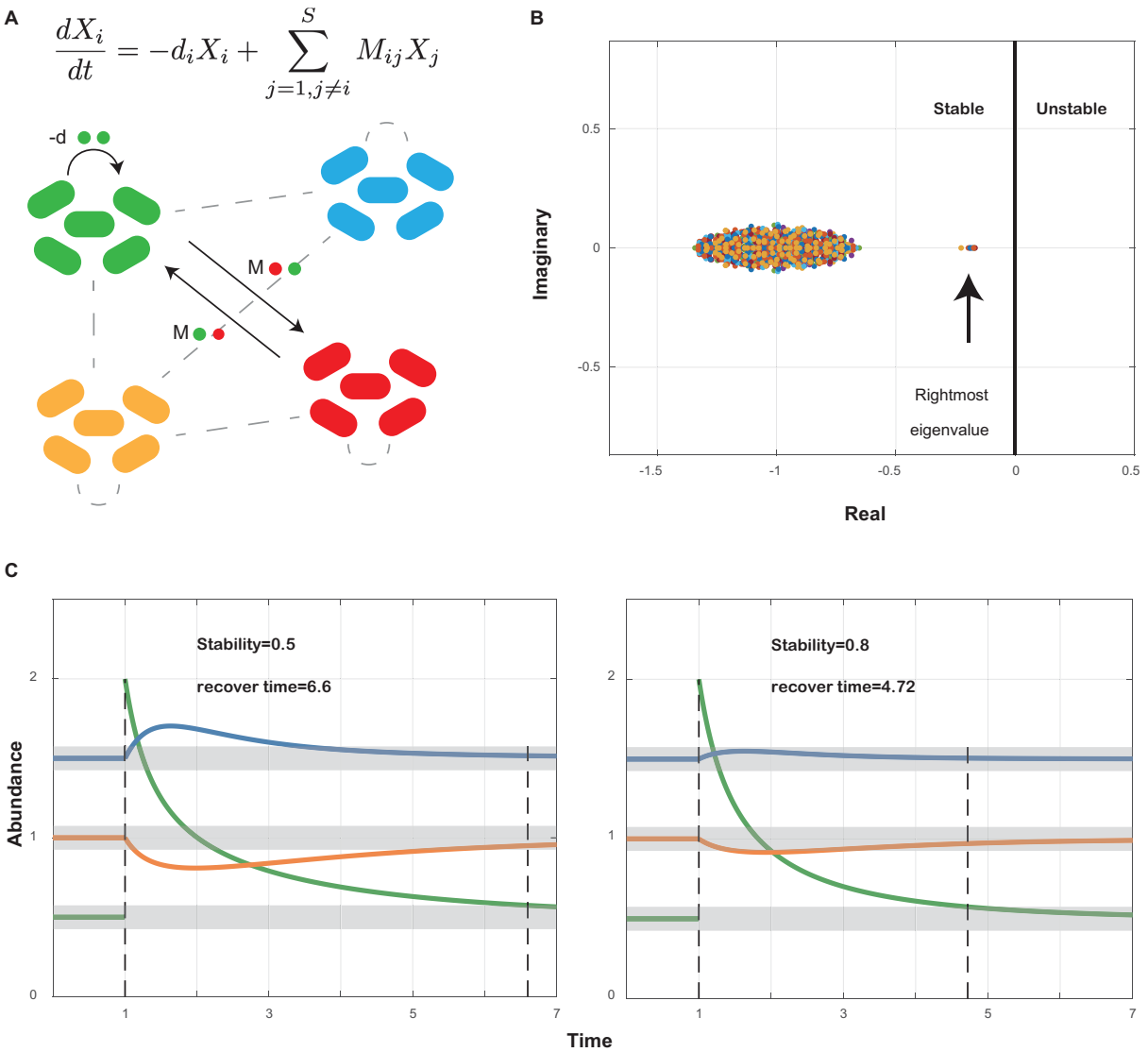

FIGURE 1 | Local stability theory. **(A)** A schematic diagram shows a small community with bacterial species interacting within themselves ($-d$) and with other species (M_{ij}). Ordinary differential equations measure the abundance change of species i after perturbation around the equilibrium point. X_i, abundance of species i; $-d_i$, self-regulating effect of species i; M_{ij}, effect of species j on species i. **(B)** All eigenvalues of community matrix M are shown in the complex plane. The community is stable if all eigenvalues have negative real parts. Therefore, the sign of the rightmost eigenvalue decides whether a community is stable or not, and the value of its real part decides how stable the community is: the more negative its real part, the more stable the community (see also **Supplementary Information**). **(C)** A community will return to its former equilibrium after perturbations if it is stable. A community with higher stability will recover faster than a less stable community.

Spearman correlation coefficients among the species were adjusted with Benjamini and Hochberg method (adjusted $p < 0.05$). As for the counts of negative and positive correlations, we applied the chi-square test for the detection of significant differences between the health and disease groups.

RESULTS

Taxonomical Annotation

After low-quality filtration and host-read removal, a total of 1,926,649,953 sequences were obtained from 64 samples, with an average of 30,103,906 sequences per sample (range from 1,004,522 to 77,090,552). Overall, 310 bacterial species have been identified (see **Supplementary Data Sheet 1**). The clinical and demographic characteristics of recruited subjects were summarized (**Supplementary Table 3**). There were no significant differences in mean age and sex distribution among all subjects, and functional time between healthy and diseased implants ($p > 0.05$).

PERMANOVA was performed to evaluate the differences in microbial communities contributed by several factors (**Supplementary Figure 1**). The results indicated a significant difference between the compositions of supra- and subgingival

communities (R^2 = 0.02631, p = 0.047). Based on this finding, we therefore analyze and discuss supragingival and subgingival communities separately in the following procedures.

Using the interacting matrix extracted from our taxonomical annotations (see Materials and Methods), we plotted co-occurrence networks in healthy and diseased sites (**Figure 2**). In our networks, positive and negative coefficients represented potentially cooperative and competitive interactions between bacterial species, respectively. Overall, subgingival microbiome from periodontitis and peri-implantitis patients exhibited less connected and competitive bacterial networks. On the contrary, supragingival microbiome from the diseased subjects showed more connected and competitive bacterial networks when compared with their healthy controls.

Structural Properties of Bacterial Co-occurrence Networks

Besides the proportions of negative and positive interactions, we visualized more structural properties including the numbers of interacting species, the connectance, and the degree distributions of the networks using bar charts (**Figures 3A–D**), to further

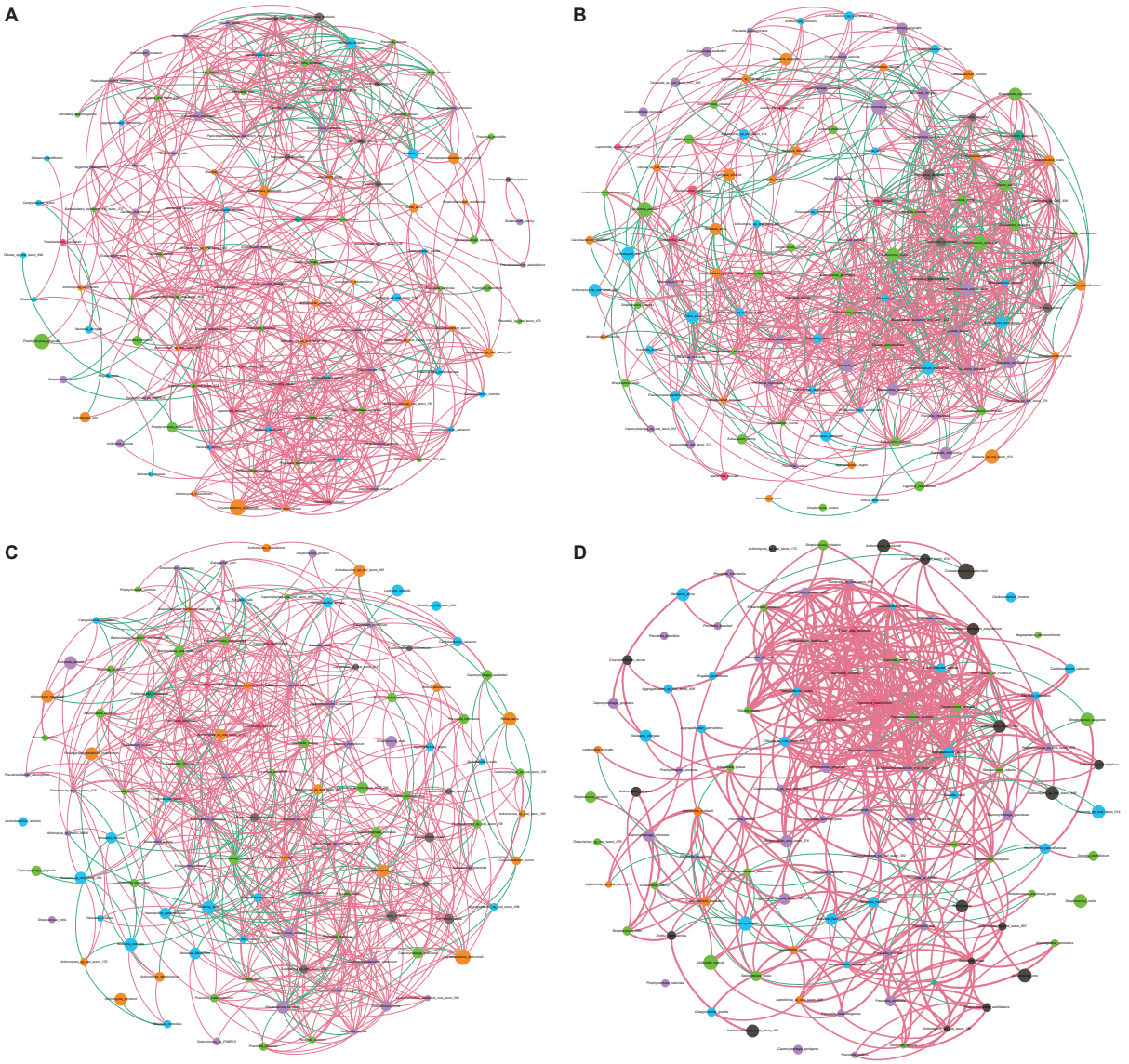

FIGURE 2 | Bacterial co-occurrence networks. **(A)** Network of diseased subgingival microbiome. **(B)** Network of healthy subgingival microbiome. **(C)** Network of diseased supragingival microbiome. **(D)** Network of healthy supragingival microbiome. Species from different phyla were marked in different colors. The larger nodes represented the higher mean relative abundance of the species. We selected those interactions with Spearman correlation coefficient <-0.6 or >0.6 (adjusted p < 0.05). Positive and negative correlations are shown in red and green lines, respectively. Thicker lines meant higher absolute values in Spearman coefficient. Generally, the healthy subgingival network was more complex than the diseased subgingival network, while the healthy supragingival network was less complex than the diseased supragingival network.

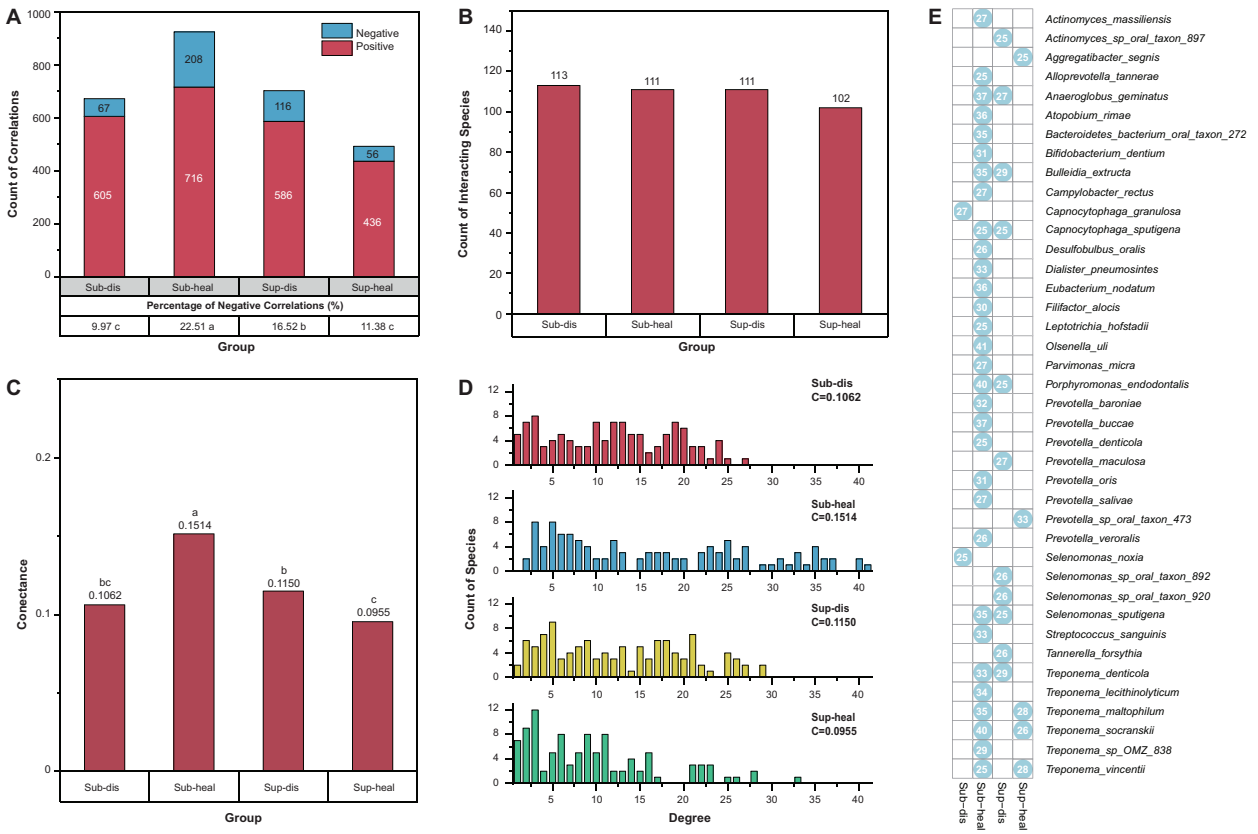

FIGURE 3 | Properties of the community structures in different microbiomes. **(A)** Positive and negative correlations were shown in red and blue, respectively. Positive correlations were predominant in all communities. The percentage of negative correlations in diseased subgingival communities was significantly lower than that in healthy subgingival communities. However, such difference was reversed between diseased and healthy supragingival communities ($p < 0.05$, Pearson chi-square). **(B)** All communities in our study had similar counts of interacting species ($p > 0.05$, Pearson chi-square). **(C)** When associated with periodontitis and peri-implantitis, the subgingival community exhibited a decrease in connectance while the supragingival community exhibited an increase in connectance. Significance of differences was marked in letters. **(D)** Degree distributions of the diseased subgingival, healthy subgingival, diseased supragingival, and healthy supragingival networks are shown in red, blue, yellow, and green bars, respectively. *C* stood for connectance. A conspicuous difference was observed in the degree distribution of healthy subgingival communities as there were significantly more high-degree (degree > 25) species ($p < 0.05$ Pearson chi-square). **(E)** Hub species in the diseased subgingival, healthy subgingival, diseased supragingival, and healthy supragingival microbiome are shown in the heatmap. A blue dot means the species had more than 25 interspecies correlations in the corresponding microbiome. Numbers within the dots showed the counts of correlations of the species.

dissect the community structure within these networks. In both supra- and subgingival samples, there are similar amounts of interacting species between healthy and diseased microbiome. However, in subgingival microbiome, healthy communities had higher connectance and more high-degree species than diseased communities ($p < 0.05$, Pearson chi-square and Fisher exact test). Besides, the healthy subgingival network had a larger proportion of negative correlations (22.51%, 208 of 924) than the diseased subgingival network (9.97%, 67 of 672) ($p < 0.05$, Pearson chi-square). As for supragingival microbiome, differences were reversed where healthy communities had lower connectance and exhibited a cluster in lower degrees when compared with diseased communities. Also, the healthy supragingival network showed a lower proportion of negative correlations (11.38%, 56 of 492) than the diseased supragingival network (16.52%, 116 of 702) ($p < 0.05$, Pearson chi-square).

Based on the degree distribution, we selected those hub species with more than 25 correlations (degree > 25) in each group. These hub species were the pivotal members in the co-occurrence networks which were highly connected with other species (**Figure 3E** and **Supplementary Table 5**). There were more hub species in the healthy subgingival microbiome than the diseased subgingival microbiome (31 in healthy microbiome and 2 in diseased microbiome). Such difference was again reversed in the supragingival group where diseased microbiome had more hub species (5 in healthy microbiome and 11 in diseased microbiome). The results above revealed distinct bacterial co-occurrence networks and community structures in different microbiomes and built the foundation for further stability analysis.

Alterations in Bacterial Interactions

Bacterial interactions are known to have an impact on oral health (Diaz and Valm, 2020), especially the competitive interactions which have been proved essential in preserving the fitness of microbial communities (Stacy et al., 2014). To evaluate how

inflammation around teeth and implants would alter such bacterial interactions, we extracted all negative correlations unique to different health conditions for further comparison (**Figure 4**). As expected, there was a great change in the bacterial competition with the shift from health to disease. Each group had its own distinctive set of unique correlations.

In subgingival microbiome (**Figure 4A**), *Streptococcus sanguinis* (l = 31, number of negative linkages equal 31 with R < -0.6 and adjusted p < 0.05), *Streptococcus oralis* (l = 17), *Haemophilus parainfluenzae* (l = 10), *Rothia aeria* (l = 12), *Corynebacterium matruchotii* (l = 18), *Leptotrichia hofstadii* (l = 11), *Actinomyces massiliensis* (l = 22), and *Capnocytophaga sputigena* (l = 14) participated in a large number of negative correlations in healthy communities. When inflammation arose, the negative correlations were significantly weakened and those interactions associated with the above species were altered, among which *Corynebacterium matruchotii*, *Leptotrichia hofstadii*, *Actinomyces massiliensis*, and *Capnocytophaga sputigena* lost all their negative correlations, while *Streptococcus sanguinis* (l = 10), *Streptococcus oralis* (l = 3), *Haemophilus parainfluenzae* (l = 5), and *Rothia aeria* (l = 2) established fewer new negative correlations with other species. Instead, in the diseased communities, *Lautropia mirabilis* (l = 15), *Actinomyces naeslundii* (l = 8), and *Capnocytophaga gingivalis* (l = 7) emerged to become the concentrated nodes of negative correlations.

Changes in supragingival microbiome were quite different (**Figure 4B**), where healthy communities had significantly fewer negative correlations than diseased communities. *Kingella oralis* (l = 3), *Lautropia mirabilis* (l = 9), *Prevotella multiformis* (l = 5), and *Actinomyces massiliensis* (l = 15) were the major contributors of negative correlations in healthy communities, while in diseased communities, there were complex sets of negative correlations coming from *Streptococcus sanguinis* (l = 14), *Neisseria sicca* (l = 10), and *Capnocytophaga sputigena* (l = 23).

In contrast with alterations of negative correlations, there were also some correlations shared by all communities despite health conditions or sampling sites (**Figure 4C**). This shared network was mainly constructed by species from phyla *Bacteroidetes*, *Firmicutes*, and *Spirochaetes*. Different from the unique negative correlations which defined the health status of the microbiome, these shared correlations seemed to be constant and might have formed a fundamental framework for periodontal and peri-implant microbiome.

Stability Analysis

To compare the stability among different microbial communities, the above structural properties were required for numerical simulations. The number of interacting species, the connectance, and the types of interactions could be drawn directly from our taxonomical annotation and the co-occurrence networks. However, acquiring the strength of interactions would usually require a time-sequence analysis from longitudinal samples according to previous studies (Schloissnig et al., 2013; Stein et al., 2013; Oh et al., 2016). This seemed inapplicable to studying diseased subjects due to ethical reasons, as clinicians were supposed to treat the periodontitis or peri-implantitis rather than observing the diseased status without interference. In this scenario, we introduced a strategy to analyze the stability

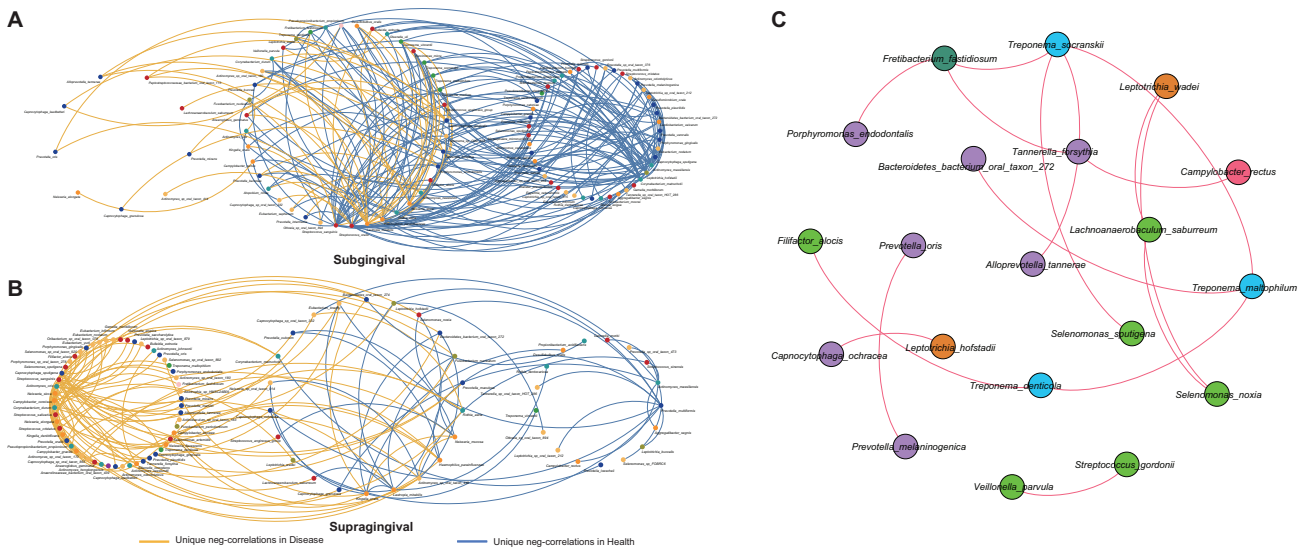

FIGURE 4 | Alterations in negative correlations from health to disease and the shared correlations. **(A)** Unique negative correlations of healthy and diseased subgingival communities. *Streptococcus sanguinis*, *Streptococcus oralis*, *Haemophilus parainfluenzae*, *Rothia aeria*, *Corynebacterium matruchotii*, *Leptotrichia hofstadii*, *Actinomyces massiliensis*, and *Capnocytophaga sputigena* were the concentrated nodes of negative correlations in health, while *Lautropia mirabilis*, *Actinomyces naeslundii*, and *Capnocytophaga gingivalis* were the concentrated nodes in disease. **(B)** Unique negative correlations of healthy and diseased supragingival communities. *Kingella oralis*, *Lautropia mirabilis*, *Prevotella multiformis*, and *Actinomyces massiliensis* were the concentrated nodes of negative correlations in health, while *Streptococcus sanguinis*, *Neisseria sicca*, and *Capnocytophaga sputigena* were the concentrated nodes of negative correlations in disease. **(C)** The shared correlations of all communities. All shared correlations were positive and were mainly constructed by phyla *Bacteroidetes*, *Firmicutes*, and *Spirochaetes*.

of microbial communities using cross-sectional samples based on Spearman coefficient (see Materials and Methods, see also **Supplementary Information**).

We assigned the strength of interactions following the assumptions by Allesina (Allesina and Tang, 2012) (see **Supplementary Information**) and mainly focused on comparing the stability among different communities rather than numerically calculating the absolute stability value of a specific community. Stability analysis showed that healthy subgingival communities had the worst stability among four groups while diseased subgingival communities possessed the highest stability (**Figure 5**). As for the supragingival group, the healthy and diseased supragingival communities showed similar stability in our analysis. We performed a series of simulations using different parameter sets and concluded the same result, which proved its robustness (**Figure 5**, see also **Supplementary Figure 3**).

To figure out why healthy subgingival microbiome was far less stable than the others, we generated unstructured ER (Erdős–Rényi) networks with the same amount of interacting species, connectance, and the positive–negative ratio of interactions as our original networks. Yet the sole different property was that these unstructured communities were distinguished from the original communities by having concentrated degree distributions (**Figure 6A**). Using the same method above, we compared the stability differences caused by distinct degree distributions between the original communities and the unstructured communities (**Figures 6B–E**). All original communities showed decreased stability when compared with their ER network counterparts in most parameter sets, while the healthy subgingival microbiome showed the largest extent of stability decrease. This indicated that the degree distributions of the original communities were somehow destabilizing,

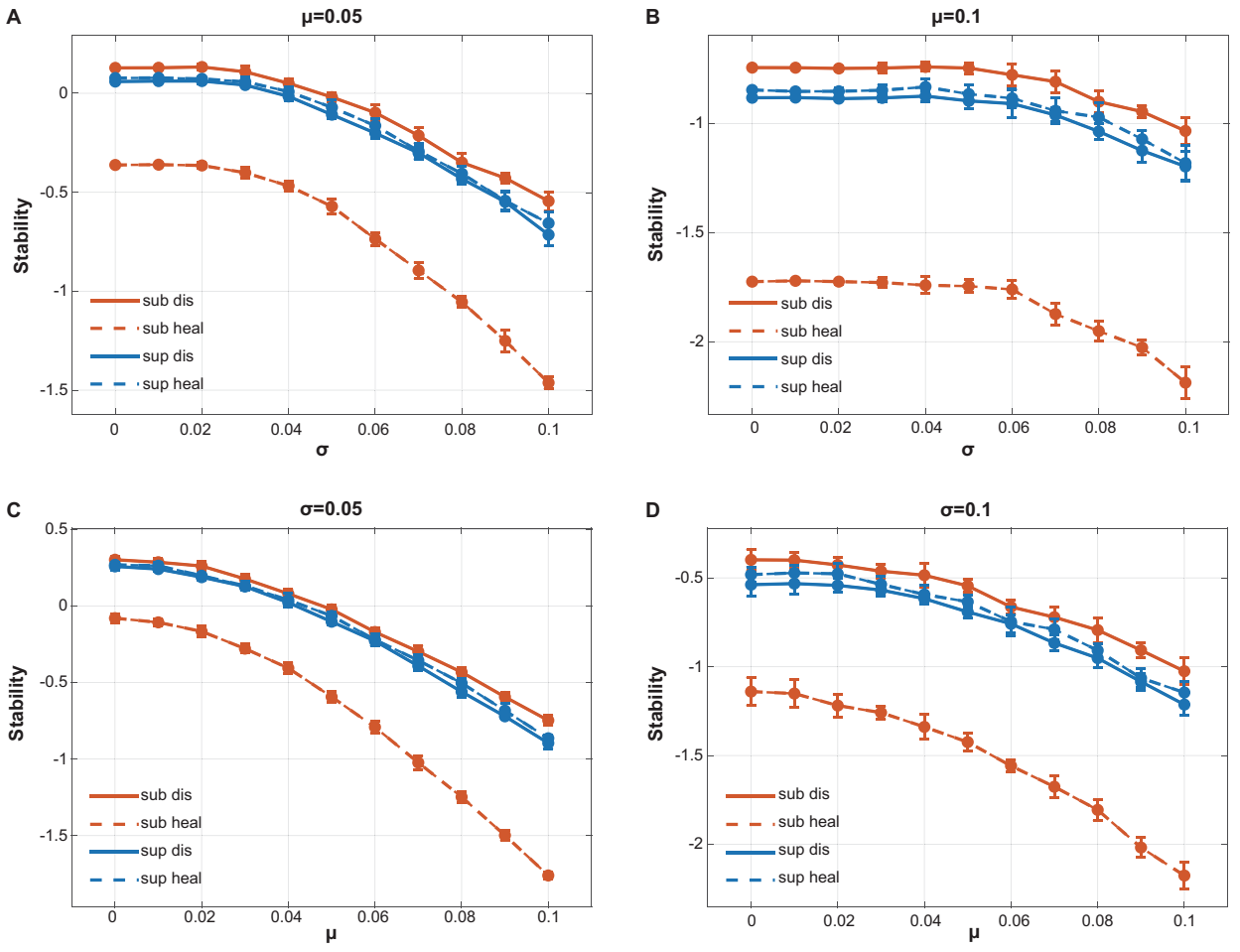

FIGURE 5 | Calculation of local stability. Red lines stood for supragingival communities while blue lines stood for subgingival communities. Healthy and diseased communities are shown in dotted and solid lines, respectively. Connectance, interacting species richness, and bacterial correlations were drawn directly from our interacting matrix. The strength of bacterial interactions was assigned to follow a normal distribution with mean μ and variance σ^2. By changing the value of μ and σ, we performed a series of calculations to compare the stability of our communities (see also **Supplementary Figure 3**). **(A)** μ = 0.05 with variable σ. **(B)** μ = 0.1 with variable σ. **(C)** σ = 0.05 with variable μ. **(D)** σ = 0.1 with variable μ. All calculations showed the same tendency that the healthy subgingival communities had the worst local stability while the diseased subgingival communities had the highest. However, the stability difference in supragingival communities was not as distinct as that in subgingival communities.

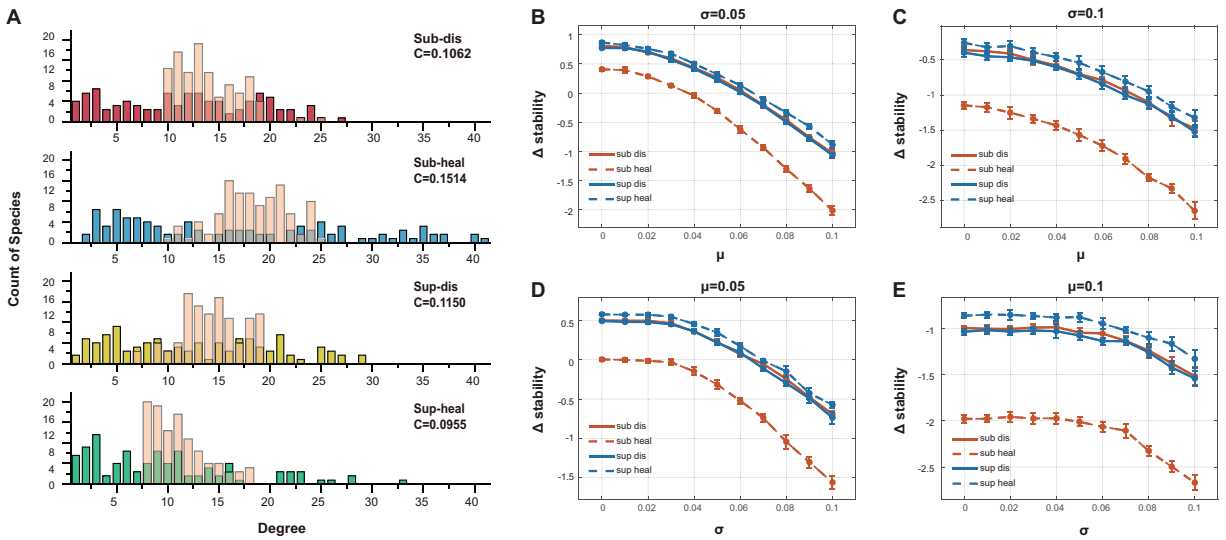

FIGURE 6 | Evaluation of the association between degree distribution and local stability. **(A)** The transparent pink bars showed the degree distribution of the ER networks while the opaque-colored bars showed the degree distribution of the original networks. The major difference was that the ER networks had concentrated degree distributions. **(B)–(E)** The vertical axis showed the stability change after ER randomization ($\Delta stability$). $\Delta stability = sta_{origin} - sta_{ER}$, in which sta_{origin} was the stability of the original communities, while sta_{ER} was the stability of the ER networks. It was clear that all original communities were less stable than their ER counterparts in all parameter sets (different μ and σ as in **Figure 5**), which indicated that their network structure tended to be destabilizing. The extent of stability decrease in the healthy subgingival group was much more than the other three groups, which meant that the network structure of the healthy subgingival community hampered stability the most.

among which the degree distribution of the healthy subgingival microbiome tended to hamper stability the most.

DISCUSSION

Distinct Structures Between Healthy and Diseased Communities

The oral microbiome is structurally and functionally organized, which means the properties of a microbial community are more than the sum of the components within it (Kuramitsu et al., 2007; Marsh and Zaura, 2017). To fully understand a microbial community, we are supposed to explore the whole structure and the aggregation of all interactions more than focusing on single or pairwise species. In this scenario, we investigated the bacterial co-occurrence networks and the community structures to explore the effect of periodontitis and peri-implantitis on the oral microbiome in a new perspective.

Our study revealed that when inflammation arose around teeth and implants, the subgingival bacterial networks tended to become less connected and less competitive. However, networks in supragingival communities seemed to shift in an opposite direction, with higher connectance and a larger proportion of competitive interactions in the diseased communities than their healthy counterparts.

Bacterial competition has been reported to be beneficial to both competitors involved and might even improve the fitness of the whole microbial community (Stacy et al., 2014), as they form a defensive mechanism in oral microbiome where the colonization of exogenous species was prevented (Marsh and Zaura, 2017). However, our results indicated that inflammation would alter the competition among species in periodontal and peri-implant microbiome. Such alterations could be observed in both supra- and subgingival microbiome and were not just in terms of number or proportion. In fact, the whole community seemed to reestablish a brand-new network with its own distinctive negative correlations and own centers for these correlations. These major changes in the community structure might lead to changes in the keystone compositions of the biofilm and come with the pathologic shift from health to disease (Marsh and Zaura, 2017).

The degree distribution of ecological networks is usually right-skewed with many low-degree vertices and only a small number of high-degree vertices (Girvan and Newman, 2002). Such was the case in our networks where the majority of the species were in low degrees. However, it was still clear that the degree distribution of the healthy subgingival microbiome distinguished itself among groups by having significantly more hub species, which also contributed to hampering the local stability of healthy subgingival community according to our further analysis.

The connectance was another important property of the community structure. Our result showed that when associated with periodontitis and peri-implantitis, the connectance of subgingival microbiome tended to decrease while the connectance of supragingival microbiome tended to increase. Previous studies proved that an ecosystem with higher connectance was more persistent when subjects to colonization–extinction dynamics (Gravel et al., 2011) and was less prone to losing hub species than systems with lower connectance (Kulkarni and De Laender, 2017). However, other studies on the dynamics of complex ecosystems showed that when connectance

rose beyond a certain threshold, the local stability of the community would decrease rapidly (Gardner and Ashby, 1970). The healthy subgingival microbiome in our study had a larger number of hub species, which were sensitive to selective loss accordingly. Nonetheless, the high connectance helped prevent these species from losing. As for whether the connectance of our communities had crossed the threshold where local stability began to drop, we suggested that more studies were needed to draw the conclusion. However, we were able to plot the overall outcome of these factors and to compare the stability differences between the healthy subgingival microbiome and the other three groups (see below).

All findings above showed that healthy and diseased oral microbiome had distinct community structures. We addressed that these aberrant changes in bacterial competition, connectance, and degree distribution were crucially associated with the onset and progression of periodontitis and peri-implantitis. Among all communities in our study, we found that the differences between healthy and diseased subgingival microbiomes were most striking and complicated. Future studies should pay more attention to the relationship between community structures and oral infectious diseases, especially the changes in the community structure of subgingival microbiome.

Association Between Ecological Stability and Health Conditions

Patterns of the bacterial networks in supra- and subgingival microbiome were associated with health and disease. Moreover, the multiple interactions gave the community resilience to environmental perturbations (Marsh and Zaura, 2017). As mentioned above, the stability of a community mainly depends on its community matrix, which incorporates structural properties such as interaction types, connectance, and degree distribution. According to previous studies, competitive interactions tend to increase stability by decreasing diversity within the influence range of the competitors (Coyte et al., 2015), while connectance that reaches beyond a critical level might rapidly destabilize a microbial community (Gardner and Ashby, 1970; May, 1972). Interestingly, in our study, those communities with larger proportions of competitive interactions turned out to have higher connectance too. These communities, or more specifically, healthy subgingival communities and diseased supragingival communities, received antagonistic effects from both stronger competition within species and higher connectance. To plot the outcome of various effects on the stability in our study, we performed a series of simulations following the work of Allesina to compare the stability differences among our communities.

The result showed that healthy subgingival microbiome had the worst local stability among four groups while diseased subgingival microbiome had the highest. This meant that the equilibrium of healthy subgingival microbiome was more delicate and more prone to perturbations. When perturbations reached beyond resilience, equilibrium may break down with changes in microbial composition and shift in the community structure. That could be where dysbiosis happened and be the essence of the initiation of periodontal and peri-implant diseases. On the other hand, the high local stability in diseased subgingival microbiome explained why, if without interventions, the periodontal and peri-implant microbiome could not spontaneously change back to health once infected by periodontitis or peri-implantitis as the diseased equilibrium was very robust.

By comparing the stability between randomly generated ER communities and our original communities, we revealed that the degree distribution of healthy subgingival microbiome tended to be most destabilizing. As healthy subgingival microbiome was characterized by having more hub species, we hereby hypothesized that hub species were in some way a weak point during the breakdown of the current equilibrium, for changes in these highly connected species could trigger a massive alteration in the whole network. This explained why the stability of healthy subgingival microbiome was far lower than other microbiomes. In this scenario, we suggested that more caution should be raised toward these hub species together with their roles during the shift from health to disease.

Relationship Between Hub Species and Health Conditions

Hub species were those with a large number of interspecies correlations. Whether abundant or not, hub species played roles as "traffic centers" in the bacterial network. In one respect, these species were spatially or functionally related with many others and therefore contributed to the integration of the community. In another respect, they might also be responsible for destabilizing the community as mentioned above. Our study showed that the healthy subgingival microbiome had the highest count of hub species, of which species from genus *Prevotella* and *Treponema* made up a major part. In the diseased subgingival microbiome, there were only two hub species, *Capnocytophaga granulosa* and *Selenomonas noxia*. As for the supragingival microbiome, differences between healthy and diseased networks were not as distinct as subgingival microbiome and seemed to change in an opposite direction where the diseased network had more hub species than the healthy one. The supragingival hub species came from various genus including *Actinomyces*, *Aggregatibacter*, *Anaeroglobus*, *Bulleidia*, *Capnocytophaga*, *Porphyromonas*, *Prevotella*, *Selenomonas*, *Tannerella*, and *Treponema*.

The microbial community is extremely complex and sophisticated which subjects to numerous influences ranging from microbial compositions to environmental and genetic factors. It is difficult to explicitly address the role of a specific species in the community. Although most of the hub species of communities in our study had been proven associated with periodontal and peri-implant destruction (Morita et al., 1991; Ellen and Galimanas, 2000; Takeuchi et al., 2001; Ohishi et al., 2005), we suggested that their pivotal roles in the bacterial network should be treated dialectically, the roles that on the one hand contributed to their pathogenicity, but on the other hand, were also essential in integrating the community network. Future studies should pay more attention to the important roles of these hub species and associate the pivotal places in the network with their pathogenicity.

Limitations of the Study

One major limitation in this study is that the sample size, although equivalent to other congener studies (Dabdoub et al., 2016; Belstrom et al., 2017; Komatsu et al., 2020), is relatively small to describe the oral microbiome of the whole human population. As the oral microbiome is very individualized (Belibasakis et al., 2019), we suggest that future studies with a larger sample size are needed to further generalize our findings.

The strategy provided in this study is sound and rigorous in theoretical aspect. However, these methods were mainly based on taxonomical annotations. They revealed the phenomena observed from the samples within this study yet could not validate the mechanisms behind the phenomena in biochemistry or molecular view. We appeal that further studies using either *in vitro* models or *in vivo* trials are needed to figure out the detailed mechanisms and provide more clinical implications.

Predicting the stability of microbial community usually requires a time-sequence analysis from longitudinal samples, as longitudinal studies offer control for confounding factors including age, gender, diet and so on. Although cross-sectional samples can also provide prediction on community stability following our strategy, it can be less powerful than longitudinal ones (Knight et al., 2018).

CONCLUSION

In conclusion, we revealed distinct community structures in healthy and diseased microbial communities around teeth and implants. By extracting the bacterial correlation networks, we found that the subgingival microbiome tended to become less connective and competitive when inflammation arises. In contrast, the supragingival microbiome tended to become more connective and competitive. We also observed a great change in competitive interspecies correlations between healthy and diseased microbiome. These alterations contributed crucially to the shift from health to disease and were highly associated with periodontal and peri-implant microbiome dysbiosis in the aspect of community structures. Besides, by applying dynamic models on these microbial communities, we concluded that the healthy subgingival community was far less stable than the inflamed subgingival community. We also managed to prove that it was those highly connected species in the network that contributed to destabilizing the biofilm. Our results suggested these hub species should also be given more concern in future studies. Preserving these species and maintaining their normal functionalities might be of much meaning in preventing periodontal and peri-implant diseases. Combining the above findings, we revealed that microbiome dysbiosis in the periodontium was not limited to the changes in bacterial compositions. With durative perturbations from microbial pathogens, the former equilibrium broke down and the microbiomes formed new bacterial networks with distinct interspecies correlations and community structures. During this progress, the subgingival biofilm established a more stable and stubborn community with even higher resilience.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of College of Stomatology, Xi'an Jiaotong University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YZ designed the details of the study, conducted the statistical analysis, interpreted the analysis results, and wrote this manuscript. YL performed the bioinformatics analyses, interpreted the analysis results, and revised the manuscript. YY conducted mathematical simulations and interpreted the results. YW helped perform statistical analysis and revised the manuscript. XC and YX helped with the collection of samples and the revision of the manuscript. YJ revised the manuscript and performed statistical analysis. QZ and SCL supervised the whole project and polished the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.785191/full#supplementary-material

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