Microbes and Infection 26 (2024) 105339

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

Oral microbiota signatures associated with viremia and CD4 recovery in treatment-naïve HIV-1-infected patients

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ARTICLE INFO

Article history:
Received 7 January 2024
Accepted 11 April 2024
Available online 16 April 2024

Keywords:
HIV
Oral microbiota
Immunological responders
Proteobacteria
Microbiota-immunity axis
Viremia

ABSTRACT

Purpose: Few reports focused on the role of oral microbiome diversity in HIV infection. We characterized the microbiota-immunity axis in a cohort of treatment-naïve HIV-1-infected patients undergoing antiretroviral therapy (ART) focusing on the oral microbiome (OM) and immunological responsivity.
Methods: The sequencing of 16S rRNA V3−V4 hypervariable region was performed on salivary samples of 15 healthy control (HC) and 12 HIV + patients before starting ART and after reaching virological suppression. Then, we correlated the OM composition with serum cytokines and the Short Chain Fatty acids (SCFAs).
Results: The comparison between HIV patients and HC oral microbiota showed differences in the bacterial α-diversity and richness. We documented a negative correlation between oral *Prevotella* and intestinal valeric acid at before starting ART and a positive correlation between oral *Veillonella* and gut acetic acid after reaching virological suppression. Finally, an increase in the phylum *Proteobacteria* was observed comparing saliva samples of immunological responders (IRs) patients against immunological non-responders (INRs).
Conclusions: For the first time, we described an increase in the oral pro-inflammatory *Proteobacteria* phylum in INRs compared to IRs. We provided more evidence that saliva could be a non-invasive and less expensive approach for research involving the oral cavity microbiome in HIV patients.

1. Introduction

Currently, the human immunodeficiency virus (HIV) infection is one of the main causes of morbidity and mortality worldwide [1], even if the widespread use of antiretroviral therapy (ART) has made this infection become a chronic manageable disease. After a transmission event, HIV spreads through mucosal tissues, and reaches the lymphoid organs, infecting primarily CD4+ T cells. According to epidemiological reports, oral symptoms are experienced by 30% of HIV patients first, and once their immunity has been compromised, oral and maxillofacial infections develop [2]. Often, oral lesions are the first symptom for identifying HIV infection, and pharyngitis is usually the first manifestation of oral inflammation [3]. Moreover, despite effective ART, several oral diseases, such as oropharyngeal candidiasis (OPC) [4] and periodontitis [5] are frequently reported in all stages of HIV infection. So, the oral HIV symptoms are currently given a great deal of focus by the medical community.

Many studies have investigated the role of gut microbiota (GM) and its metabolites in HIV infection. Of note, we recently explored the gut-immunity axis (comparing the fecal microbial composition, serum and fecal microbial metabolites, and serum cytokine profile) on treatment-naïve HIV patients before starting ART and after reaching virological suppression (HIV RNA < 50 copies/mL) [6]. This

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https://doi.org/10.1016/j.micinf.2024.105339

study showed that the microbiota remained unchanged in overall gut bacterial diversity before and after achieving virological suppression, while their systemic inflammatory status seemed not to be completely restored [6].

Recent reports suggested that oral microbiome diversity may also be critical in systemic inflammation in HIV-infected humans [7]. Moreover, despite the absence of oral lesions, in some patients, the persistent state of heightened immune activation and inflammation results in microbial translocations and oral mucosal alterations with a similar pattern of gastrointestinal disruption [8]. Finally, during disease progression and in the absence of ART, patients with a CD4 count less than 200 positive cells/μl are diagnosed with AIDS and acquire opportunistic infections, including oral ones such as candidiasis. Therefore, imbalance of the oral microbiota (OM) can contribute to oral manifestation in HIV patients [9]. A recent study [10] found that some oral species, including opportunistic pathogens, might diffuse from the oral cavity to the gut, which may directly cause intestinal inflammation.

Nowadays, the comparison between the OM composition of HIV patients and healthy controls (HC) showed conflicting results. Studies are reporting modest changes in the relative abundances of numerous bacterial taxa between HIV-infected patients and HC [11], while other ones document significant differences in the saliva bacterial communities [12]. Recently Gou et al. [13] performed for the first time a stage comparison of salivary microbial differences in HIV-infected patients, demonstrating that the OM changes in the saliva of untreated HIV-positive patients were characterized by greater alpha-diversity.

Moreover, the impairment of local immunity in HIV infection, including decreased salivary IgA, defensins, and cytokines, might convert commensal microorganisms to microorganisms with increased pathogenicity and lead to the OM dysbiosis, which could increase the risk of opportunistic infections [14]. Finally, the OM compositions in HIV-infected subjects with ART became more similar to those in HIV-uninfected controls; however, a difference remains between the groups [15].

Notably, the OM is complex, involving several niches in the oral cavity, including the saliva, tongue, supragingival and subgingival plaque, gingiva-crevicular fluid, buccal cavity, and soft (mucosal) and hard tissues. Saliva plays a significant role in determining the OM composition and activity as it delivers components of the adaptive and innate host defences. So, it represents the most suitable sampling to describe the complex relationship between the host and the resident microbiota [16].

Based on these premises, we integrated and deepened our previous results concerning the characterization of the microbiota-immunity axis in a cohort of treatment-naïve HIV-1-infected patients undergoing ART [6]. We aim first to assess differences in the OM structure between HIV patients and HC, considering the oral health conditions. In addition, we performed a longitudinal evaluation of the oral-gut microbiota-immunity axis from HIV-infected patients before and after ART beginning. Finally, we characterized the OM profile and its functionality related to the condition of the host immunological response to ART, and sub-grouped the patients in immunological responders *vs* immunological non-responders.

2. Methods

2.1. Study population

Treatment-naïve HIV-infected patients and healthy donors (HC) were recruited by the Department of Infective and Tropical Disease at University Hospital of Careggi (Florence, Italy). The study was approved (Rif CEAVC 15035) by the local Ethical Committee (Area Toscana Centro) and all patients signed written informed consent before participation. We excluded patients that had used antibiotics, probiotics/prebiotics, or had experienced diarrhoea or digestive symptoms within the previous 1 month. Plasma HIV-RNA was measured using Test v1.5 Roche COBAS AmpliPrep, Roche TaqMan HIV-1 Test v2.0 (Roche Diagnostics, Branchburg, NJ, United States) and Siemens Versant K PCR (Siemens Healthcare GmbH, Erlangen, Germany), with lower limits of detection of 20 copies/mL, respectively. At each time point, we collected salivary samples. After collection, saliva samples were immediately frozen and stored at −80 °C until DNA extraction, in order to assess the oral microbiota composition. Patients underwent medical visits at 0 and 24 weeks after the study enrolment. In addition, they underwent a comprehensive physical examination and medical history inquiry, urine toxicology panel testing, clinical laboratory tests including plasma HIV RNA, specimen collection, and detailed behavioural questionnaire survey.

2.2. Salivary microbiota characterization

Genomic DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) from frozen (−80 °C) saliva samples according to the manufacturer's instructions as reported in a previous study by our group [17].

2.3. Bioinformatic and statistical analysis of 16S rRNA

Demultiplexed sequence reads were processed using the bioinformatic tools in QIIME2 2022.8. In particular, the sequencing primers and the reads without primers were removed using Cutadapt tool. DADA2 was used to perform paired-end reads filtering, merging and chimeras removal steps after trimming low quality nucleotides from both forward and reverse reads (–p-trunclen-f 275 and –p-trunclen-r 168). Hence, ASVs (amplicon sequence variants) were generated and the taxonomic assignments were performed through Scikit-learn Bayesian Classificator retrained on V3−V4 16S region of SILVA 138.

The statistical analyses on bacterial communities were performed in R 4.2.1 with the help of the packages phyloseq 1.44.0, vegan 2.6−4, DESeq2 1.40.1 and other packages satisfying their dependencies. The packages ggplot2 3.4.2, ggh4x 0.2.2 and ggpubr 0.40 were used to plot data and results. A saturation analysis on ASVs was performed on every sample using the function rarecurve (step 100 reads), further processed to highlight saturated samples (arbitrarily defined as saturated samples with a final slope in the rarefaction curve with an increment in ASV number per reads < 1e-5). The observed richness, Shannon and Pielou's evenness indices were used to estimate the bacterial alpha-diversity in each sample using the function estimate_richness from phyloseq. The evenness index was calculated using the formula $E = S/\log(R)$, where S is the Shannon diversity index and R is the observed ASV richness in the sample. Differences in alpha-diversity indices were analyzed using the Mann−Whitney test when comparing different subjects and the Wilcoxon test when comparing the same patient along the time. PCoAs was performed using Hellinger distance computed on ASV counts. A PERMANOVA was used to test the statistical significance of the beta-diversity distances at ASV, genus and phylum level. At different taxonomic ranks, the differential analysis of the abundances has been computed through DESeq2 on raw count data, specifying a paired design when exploring the treatment effect on the patients. To control the false discovery rate, the Benjamini-Hochberg multiple test correction has been applied on DESeq2 results. In addition, differentially abundant taxa with a DESeq2 baseMean value less than 100 have been discarded from the displayed results, irrespective of their statistical significance, to limit noisy and possibly irrelevant information. A random forest

model has been trained to distinguish the microbiota of Healthy and HIV samples using only the bacterial families with an average relative abundance over 0.1% in all the datasets and a minimal abundance of 0.5% at least in two samples.

PICRUSt2 v2.5 with the SEPP algorithm was used to predict pathway's expressions based on the KO (KEGG ORTHOLOGY) database and then the significant differences between the groups have been explored using LEfSe (Linear discriminant analysis Effect Size) v1.1.2. Only the LEfSe results with a log10 LDA score over 2 have been considered significant. Finally, the monotonic relationships between the five most abundant genera and the FFA values in HIV patients have been explored at two time points, namely 0 weeks and 24 weeks, using Spearman's correlation. The resulting p-values have been adjusted according to the Benjamini-Hochberg method.

3. Results

3.1. Patients

Twelve treatment-naïve HIV-infected patients and fifteen healthy donors (HC) matched for sex and age were enrolled. Clinical information about the treatment-naïve HIV-infected patients is reported in Table 1.

3.2. Salivary microbiota differences between naïve HIV patients and healthy controls

We compared the salivary microbiota composition of naïve HIV patients and HC to detect differences due to the presence of the HIV infection. In detail, the alpha diversity analysis revealed significant differences according to the observed richness ($p = 0.0004$) and Shannon ($p = 0.0085$) indexes, while no differences were documented by Evenness index. This result suggests that the saliva samples from HIV-infected patients harbored significantly richer bacterial communities than those from HC (Fig. 1A). As expected, the PCoA evidenced that HIV patients and HC were grouped into two distinct clusters (Fig. 1B). Moreover, the taxonomic analysis of both HIV patients' group (at T0 and T24) and HC revealed that *Proteobacteria*, *Fusobacteriota*, *Actinobacteriota*, *Bacteroidota* and *Firmicutes* are the five most abundant microbial phyla while *Haemophilus, Prevotella, Streptococcus, Veillonella, Porphyronomas* are the five most abundant genera in saliva samples (SFig. 1). Furthermore, the differential abundance analysis between HC and treatment-naïve HIV infected patients highlighted that only two families significantly changed: Porphyromonadaceae and Spirochaetaceae were increased in HIV infected patients (Fig. 2).

Lastly, a random forest model has been built to distinguish the microbiota of HC and HIV patients. The computed model is characterized by good yet not perfect prediction accuracy ("out of bag" estimate of error rate: 18.52%). Despite this, we used its estimations to further augment the reliability of our conclusions by matching the most important families according to this random forest with the DESeq2 results. Accordingly, the abundance of Porphyromonadaceae family has been confirmed to be a relevant change between the two groups. Further details about this model are available in the R script (see Methods) provided as supplementary files.

3.3. Salivary microbiota differences in naïve HIV patients associated with the presence of oral infections

We then divided the naïve HIV patients into two groups, based on the presence/absence of oral infections at T0 (STable 1). Specifically, the alpha diversity analysis did not reveal significant differences (data not shown). The beta diversity analysis on transformed ASV, genus and phylum counts reported a significant p-value; however, this difference in the related PCoA plot does not appear as clear (Fig. 3A). Furthermore, the differential abundance analysis highlighted that only two families significantly change between HIV patients with and without oral infections. In detail, *Mycoplasmatales* and *Treponema* were decreased in HIV infected patients with oral infections (Fig. 3B).

Finally, regarding the oral health conditions, the HIV patients were also shifted into three additional groups, or smokers *vs* no smokers, presence *vs* absence of caries, and use of mouthwash.

Table 1
Patients and clinical data.

	Age	Sex	ART regimen	Timepoints (wk)	Viral load (copies/mL)	CD4+ cells/mm3	CD8+ cells/mm3	CD4/CD8 ratio
1	37	Male	3TC/ABC/DTG	T0	597,463	110	420	0.3
				T24	<20	520	832	0.6
2	38	Male	FTC/TDF/EVG/c	T0	4489	630	670	0.9
				T24	TND	831	740	1.1
3	34	Male	FTC/TDF/EVG/c	T0	165,516	253	725	0.3
				T24	TND	504	363	1.4
4	39	Male	FTC/TDF/EVG/c	T0	859,883	360	974	0.4
				T24	33	781	986	0.8
5	38	Male	3TC/ABC/DTG	T0	4860	1341	928	1.4
				T24	TND	1881	988	1.9
6	41	Male	FTC/TDF/RPV	T0	213	814	690	1.2
				T24	TND	845	519	1.6
7	25	Male	3TC/ABC/DTG	T0	23,098	516	1149	0.4
				T24	<20	942	1019	0.9
8	22	Male	FTC/TAF/EVG/c	T0	12,188	654	1055	0.6
				T24	TND	668	733	0.9
9	48	Male	3TC/ABC/DTG	T0	175	833	1520	0.5
				T24	TND	941	1258	0.7
10	53	Male	3TC/ABC/DTG	T0	40,545	863	1196	0.7
				T24	TND	612	515	1.2
11	40	Male	3TC/ABC/DTG	T0	859,000	399	980	0.4
				T24	39	648	652	1
12	51	Male	FTC/TDF DTG	T0	4410	884	1066	0.8
				T24	<20	1130	1261	0.9

ART: Antiretroviral therapy; 3 TC: Lamivudine; ABC: Abacavir; DTG: Dolutegravir; FTC: Emtricitabine; TDF: Tenofovir disoproxil; EVG/c: Elvitegravir/cobi; RPV: Rilpivirine.

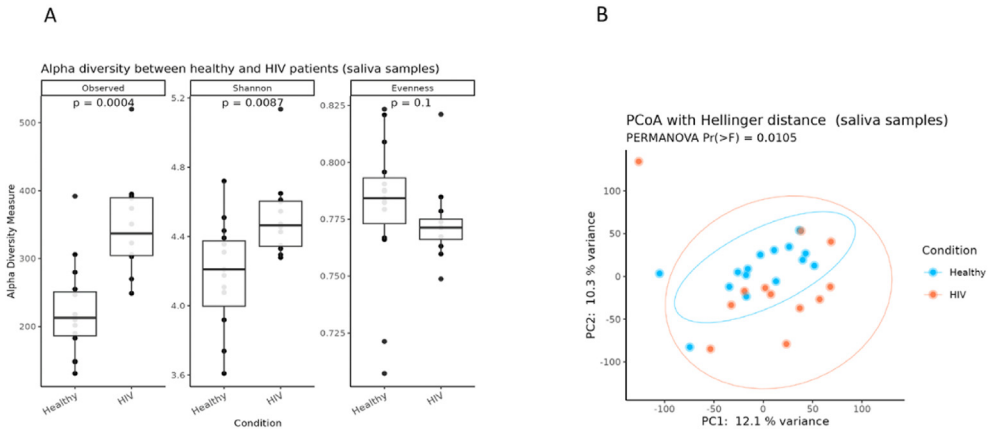

Fig. 1. (A) Box-plots showing alpha diversity indices (Observed richness index, Shannon index, Evenness index) in salivary samples of HIV patients and HC. The p-values of the difference between the groups for each index are reported on the top of the bar plot. P-values less than 0.05 were considered statistically significant; (B) Principal coordinate analysis (PCoA) conducted with Hellinger distance on ASVs in salivary samples of HIV patients and HC. The reported PERMANOVA p-value is computed at ASVs level.

However, no significant differences were observed in alpha diversity, PCoA and differential abundance analysis in all the analysed groups (data not shown).

3.4. Oral microbiota profile associated with HIV viremia

Then, we compared the OM before and after ART, in order to examine potential changes resulting from HIV infection and ART. We analysed the longitudinal variation of OM composition in patients at two different time points: T0, corresponding to "high viremia" condition (HIV- RNA > 50 copies/mL) and T24, corresponding to "viral suppression" condition (HIV-RNA ≤ 50 copies/mL). The alpha diversity of saliva samples did not display significant differences for any index (SFig. 2A).

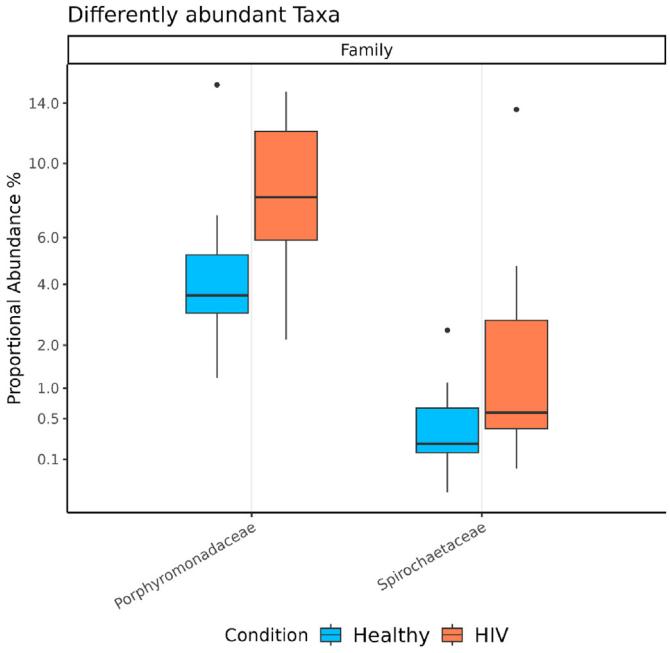

Fig. 2. Boxplot showing the results of taxa differential abundance analysis in the salivary samples from HIV patients and HC at T0. All results have an adjusted p-value < 0.05.

In order to explore similarity of patients' OM abundance profiles a beta diversity analysis on ASV, genus and phylum transformed counts was performed. This analysis did not reveal a significant difference in the OM at T0 and T24 for each patient, suggesting that the 24 weeks' ART therapy does not induce substantial alterations in the OM profile of the single patient (SFig. 2B).

However, the paired comparison of the abundance of single microbial ranks in saliva samples showed a significant decrease for the *Absconditabacteriales* family (SFig. 3).

Moreover, as we reported slight differences of the OM architecture between T0 and T24 weeks, we did not perform the comparison analysis between the healthy and HIV OM compositions at T24 weeks.

3.5. Correlations along the oral-gut microbiota axis associated with HIV viremia

The cytokines' serum signature and the intestinal microbiota of the same enrolled patients have already been characterized in our previous report [6]. As fecal, serum and saliva samples have been collected at the same time points as our previous study (T0 and T24 weeks), we wondered if there might be some microbial correlation between the samples along the oral-gut microbiome axis, with a different modulation induced by ART.

So, we first correlated the five most abundant genera detected in saliva samples (*Haemophilus, Prevotella, Streptococcus, Veillonella, Porphyromonas*) with serum cytokines' profile [6] (at T0 and T24 weeks). In detail the immunological panel included macrophage inflammatory protein-1α (MIP-1α), interleukin (IL)-27, IL-1β, IL-2, IL-4, interferon gamma-induced protein 10 (IP-10), IL-8, IL-10, IL-17A, interferon (IFN)-γ, IFN-α, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein 1(MCP-1), IL-9, IL-1α, IL-18, IL-21, IL-22. However, no significant correlations have been detected at both times (data not shown).

We further tested correlations between the five most abundant genera in saliva samples with the intestinal microbial metabolites (Short Chain Fatty acids-SCFAs) previously measured (at T0 and T24) [6], in detail acetic, propionic, butyric, isobutyric, isovaleric, 2-methylbutyric, valeric, and hexanoic acids. Data analysis revealed a negative correlation between oral *Prevotella* and intestinal valeric acid at T0 (Fig. 4A). Moreover, we observed a positive correlation between oral *Veillonella* and intestinal acetic acid at T24 weeks (Fig. 4B).

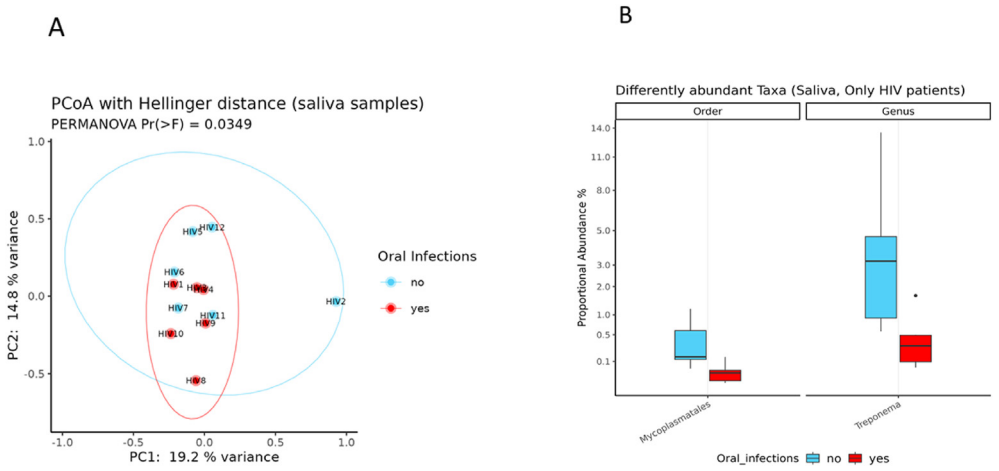

Fig. 3. (A) Principal coordinate analysis using Hellinger distance on ASVs in HIV patients with and without oral infections at T0. The reported PERMANOVA p-value is computed at the ASVs level; (B) Boxplot showing the results of taxa differential abundance analysis in the salivary samples from HIV patients with and without oral infections at T0. All results have an adjusted p-value < 0.05.

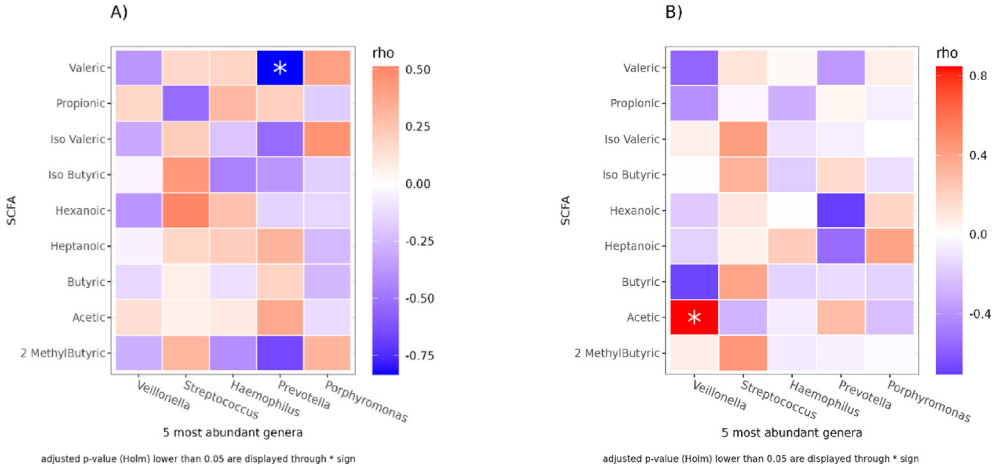

Fig. 4. Heatmaps of Spearman correlation values between intestinal SCFAs (rows) and the five most abundant genera in saliva samples (columns) at T0 (A) and T24 weeks (B). Comparisons with adjusted p-values lower than 0.05 are marked with an asterisk.

3.6. Microbiota differences at phylum level associated with CD4+ T-cell counts

In the last part of this study, we divided the patients into two groups: immunological responders (IRs) and immunological non-responders (INRs), based on the CD4/CD8 ratio > 1 or < 1, respectively. As regard the alpha diversity of salivary samples, we documented a significant difference for the Observed richness index ($P = 0.041$) but not for Evenness and Shannon indices (SFig. 4A). Beta diversity analysis did not reveal a marked sample separation between IR and INR (SFig. 4B). However, comparing the abundance of single microbial taxa revealed some significant differences at phylum level between the two sample groups. In detail, an increase of *Proteobacteria* and a decrease of *Campilobacterota* were observed in IRs saliva samples (Fig. 5).

3.7. Functional OM profiles associated with CD4+ T-cell counts

As we documented important differences at phylum level, the functional metagenomics contents using PICRUSt2 were inferred to investigate better how the salivary bacterial functional profiles may differ between IRs and INRs. In detail, we performed functional prediction through PICRUSt2 and then observed various genetic pathways that may be differently expressed between the two clinical conditions (SFig. 5). In particular, the IRs were positively associated with glycolysis III (from glucose), superpathway of hexuronide and hexuronate degradation, superpathway of beta-D-glucuronide and D-glucuronate degradation, glycogen degradation I (bacterial), L-leucine degradation I, pentose phosphate pathway (non-oxidative branch), L-lysine biosynthesis III, catechol degradation I (meta-cleavage pathway), GDP-mannose biosynthesis, UMP biosynthesis, 5-aminoimidazole ribonucleotide biosynthesis I, 5-aminoimidazole ribonucleotide biosynthesis II, superpathway of 5-aminoimidazole ribonucleotide biosynthesis, CMP-legionaminate biosynthesis I, protein N-glycosylation (bacterial), adenosine ribonucleotides de novo biosynthesis, D-fructuronate degradation, superpathway of demethylmenaquinol-6 biosynthesis II, NAD salvage pathway I, superpathway of L-threonine biosynthesis.

On the other hand, INRs were positively associated with biotin biosynthesis I, heme biosynthesis II (anaerobic), superpathway of (R,R)-butanediol biosynthesis, ubiquinol-7 biosynthesis (prokaryotic), ubiquinol-9 biosynthesis (prokaryotic), ubiquinol-10 biosynthesis (prokaryotic), ubiquinol-8 biosynthesis (prokaryotic), superpathway of L-alanine biosynthesis, superpathway of heme

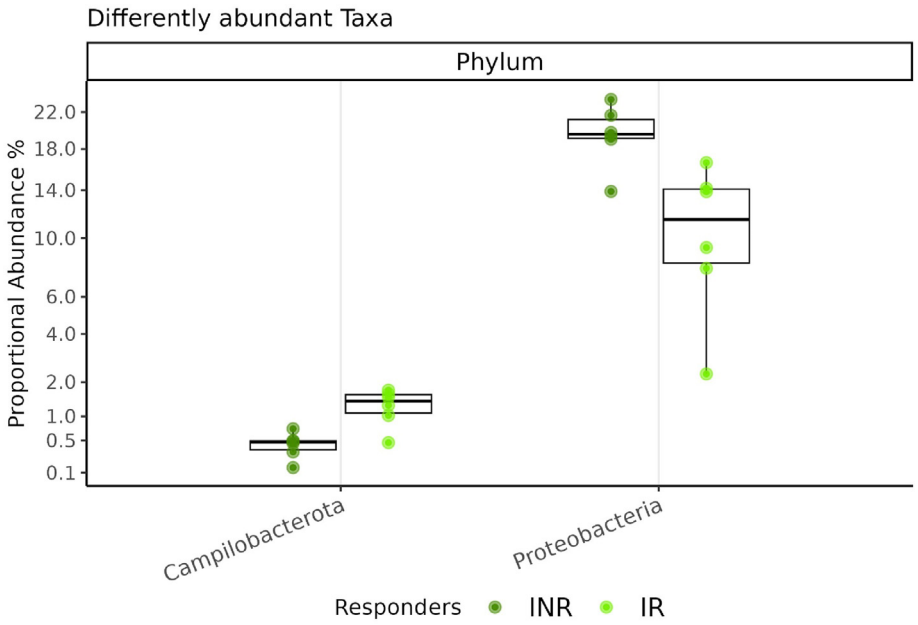

Fig. 5. Boxplot showing the significantly abundant taxa between the salivary samples of HIV IRs and INRs patients. All results have an adjusted p-value < 0.05.

biosynthesis from uroporphyrinogen-III, tRNA processing, superpathway of ubiquinol-8 biosynthesis (prokaryotic).

4. Discussion

Currently, numerous reports describe the intestinal microbial dysbiosis (MD) in HIV patients [18]. However, despite oral disease manifestations are often the first clinical expression of HIV infection, its alterations still need to be elucidated. HIV infection appears to affect immunity both locally and systemically, resulting in oral opportunistic infections. Finally, the high frequency of opportunistic oral infections in HIV patients and their correlation with CD4 T cells' count suggest that oral microbiota may be modulated also by various immunological responses to ART [19].

Nowadays, the comparison between the OM of HIV patients and HC shows conflicting results. Some studies reported unassuming changes in the relative abundances of numerous bacterial taxa between HIV-infected patients and controls [11], while other ones highlighted significant differences in the saliva bacterial communities [12]. Possible explanations might include the different enrolled patients, samples' collecting and sizes, and other factors not involved in the analysis.

In agreement with previous reports, we observed differences in the bacterial α-diversity and richness, which supports HIV-associated salivary dysbiosis [20]. We reported that the saliva samples from HIV patients harboured significantly richer bacterial communities than HC ones.

Interestingly, the setting of increased OM diversity and richness has been associated with periodontal disease [21] and according to the literature, we detected an increased abundance of *Porphyromonadaceae* [14], and Spirochaetaceae [20] family in HIV patients. However, according to our random forest model, the difference of Porphyromonadaceae abundance is more “important” than Spirochaetaceae *one*. In detail, the Porphyromonadaceae family has been associated with the normal (healthy) human oral microbial ecosystem, and harbours taxa that can act asperiodontal pathogens like *Porphyromona gingivalis* and *Tannerella forsythia* [22]. Intriguingly, *in vitro* model, HIV and *Porphyromonas gingivalis* were able to co-infect mucosal epithelial cells [23], suggesting that these OM bacteria may promote HIV acquisition on oral mucosal sites.

Vice versa, the Spirochaetaceae are prominent in the polymicrobial infections causing periodontal diseases; it is reported that they can activate immune responses, leading to tissue injury, but impair some crucial innate responses, including neutrophil function and TLR activation, thus preventing their eradication [24].

So, based on our preliminary results, we may speculate that the disruption of oral mucosal immunity in HIV infection might adversely affect the colonization of commensal bacteria in the oral cavity leading to an increase in microbial diversity, promoting an increased risk of HIV-associated oral diseases. Indeed, compared with HC, the HIV-positive patients show an increased risk for dental caries and periodontal diseases [25]. In addition, the impairment of systemic defence mechanisms, by reduction of CD4 T cells and decreased salivary IgA, defensins and cytokines, below protective levels and impairment of local immunity in the saliva might lead to the conversion of commensal bacteria, as Porphyromonadaceae and Spirochaetaceae, to microorganisms with increased pathogenicity, causing an imbalance in the OM composition and hence an increased risk for additional opportunistic local and systemic infections.

Unexpectedly, we also reported a significant decrease in *Mycoplasmatales* and *Treponema* in salivary samples of HIV patients with oral infections. *Mycoplasmatales* are commensal pathogen that usually colonize the throat, bacterial biofilm, or tartar found in the oral cavity and certain species such as *Mycoplasma salivarium* were reported to cause serious infections in HIV-positive patients [26]. In addition, this taxon is associated with infectious processes and is also considered a stimulating factor for chronic inflammatory conditions [27].

On the other hand, the oral *Treponema* belonging to *Spirochaetes* phylum exists as part of a polymicrobial biofilm accreted to the tooth surface in the gingival crevice [28]. *Treponema* plays a role in the etiology of several chronic diseases of humans including syphilis and yaws (*Treponema pallidum*), periodontal diseases, including chronic periodontitis and acute necrotizing ulcerative gingivitis (*Treponema denticola, Treponema lecithinolyticum, Treponema socranskii*, and others), and finally endodontic infections and

some acute dental abscesses [29]. Probably, the presence of other oral infections leads to a stimulation of the immune system. We may speculate that this boosting condition could keep under control the presence of other pathogens, such as *Mycoplasmatales* and *Treponema*, in HIV patients with oral infections.

Regarding the treatment effect, few studies have addressed the OM interaction with different immune responses to ART on HIV-infected patients [30]. It has been reported that the dysbiotic OM was not fully restored after effective ART, although some other local microbiota were restored [15]. In our study, we did not report huge changes in the OM composition before and after ART, we detected only a decrease in *Absconditabacteriales* family at T24 weeks. Indeed, the ART treatment inhibited HIV-1 viral replication effectively but did not heavily affect the oral microenvironment's overall bacterial composition. This result aligns with previous recent investigations documenting that ART, especially non-reverse transcriptase inhibitors (NRTIs), have considerably more impact on microbiota composition and diversity in the gut, leading to dysbiosis, than in the oral cavity [31]. The modest diversity we observed between the two sample groups might be associated with lowering viremia. It is relevant that ART, viral load, and CD4+ T cell count differentially contribute to salivary dysbiosis resulting in a decrease or increased different bacterial species [11]. Therefore, our study design makes it difficult to discriminate between various factors without longitudinal studies pre-infection, pre-ART, the type of ART regimens, and well-matched HIV-infected untreated controls sampling.

Moreover, we tackled the potential bacterial exchange between the salivary and gut microbiota due to the anatomical and physiological link between saliva and the gastrointestinal tract. In detail, we wondered if there might be some correlation between the oral-gut microbiome axis, and potential modulation before and after ART. We correlated the oral genera significantly changed before and after ART with the intestinal microbial products (SCFAs) previously analysed in the same patients' cohort [6]. We reported a negative correlation between oral *Prevotella* and intestinal valeric acid at T0 and a positive correlation between oral *Veillonella* and intestinal acetic acid at T24. Notably, *Prevotella* is recognized as one of the core anaerobic genera in the oral microbiota. Oral *Prevotella* species get constant access to the gastrointestinal tract via saliva swallowing [32]. Oral bacteria surviving to pass acidic stomach environment get access to the small intestine and colon, where they can interfere with gut bacteria [33]. Intriguingly, valeric acid is formerly known to have anti-inflammatory properties while *Prevotella* exhibit increased inflammatory properties, as demonstrated by increased release of inflammatory mediators from immune cells and various stromal cells [34].

In addition, *Veillonella* is a frequent oral-gut transmitter and its genus members are no-motile, gram-negative diplococci, usually present in normal mouth flora but often associated with oral infections. They require lactate for growth but are unable to metabolize normal dietary carbohydrates. Thus, they convert lactate, produced by other microorganisms, into a range of weaker and probably less cariogenic organic acids. *Veillonella* can produce propionate, acetate, and CO2 as the major end products of glucose and glycerol fermentation. Our results are in line with the metabolic activity of *Prevotella* and *Veillonella*, however we are not able to finely estimate its contribution on the fecal SCFAs' amounts.

Interestingly, up to 20% of HIV-infected patients fail to restore CD4+ T cell counts to the levels similar to HC ones. Poor CD4+ T cell reconstitution mechanisms on suppressive ART are not fully understood. In addition, the contribution of the microbiota, as a counterpart of the immune response, needs to be further explored. As previously mentioned, OM may be affected by various immunological responses to ART as there is high prevalence of opportunistic oral infections in HIV patients correlated with the low CD4+T cell count [19]. Conversely, recent studies have reported that CD4+ T cell counts and immune status do not affect the oral, airway or palatine tonsil microbiota in HIV + patients [35]. A previous, comparing the community composition of the salivary microbiota in HIV-infected IR and NIR [36], observed a higher alpha diversity (Simpson's index of diversity and Shannon index) of the salivary microbiota in IR patients. In agreement with these data, we reported a significantly higher value for the Observed richness index in IR patients.

Moreover, for the first time, INRs showed a high abundance of *Proteobacteria* phylum in salivary samples when compared to IRs. *Proteobacteria*, a phylum of gram-negative bacteria, is often overrepresented in several intestinal and extraintestinal diseases [37] and it tends to increase in the oral cavity with age and is frequently associated with inflammatory diseases and some non-infectious disorders [37]. In addition, due to its correlation with the inflammatory phenotype, it has been proposed as a “microbial signature” of disease [37]. However, since the *Proteobacteria* is the second most abundant phylum in the mouth, their relevance in the OM is largely underexplored and few data are available regarding its diversity and ecology [37,38]. This main phylum can include genera whose members have high potential to acquire virulence and antibiotic resistance genes [39]. The *Proteobacteria* overgrowths in the oral microbiome have been associated with oral health problems, including periodontal disease.

Regarding all the salivary samples evaluated, we detected a prevalence of the *Haemophilus* genus among *Proteobacteria* components. This genus consists of diverse species and includes pleomorphic, gram-negative coccobacilli, implicated in various opportunistic infections.

A previous study reported that *Haemophilus parainfluenzae* was significantly associated with HIV + patients, and it was positively correlated with CD4+ T cell counts within the HIV-positive group [40]. On the contrary, a recent report found that the genus *Haemophilus* was correlated negatively with CD4+ T cell count [41]. Interestingly, the LPS of *Proteobacteria*, can further affect T cells by promoting regulatory T cell phenotypes with suppressive properties or by impairing the antigen-presenting capacity of antigen presenting cells (APCs) [42]. In addition, bacterial fragments or whole bacteria can appear in the blood through translocation, due to injury to the buccal mucosa, and influence host immune system. Recently it is explored the translocated microbiota in INRs and analyzed its effects on CD4+ T cells' recovery from ART [43]. Quantitative and qualitative plasma microbial translocation differed in INRs compared to IRs and healthy controls. Further, INR-enriched microbial LPS induced proinflammatory responses, CD4+ T cell apoptosis and dysfunction; whereas IR-enriched microbial LPS did not exhibit such pathogenic activity [43]. So, the plasma of INRs is enriched with proinflammatory bacterial strains, whereas the IRs shows plasmatic non-inflammatory bacterial strains. In this scenario, we may hypothesize that the increased *Proteobacteria* in the salivary microbiome of INRs patients may contribute to the level of blood LPS from proinflammatory bacterial strains, thus affecting chronic inflammation and immune reconstitution failure in HIV patients after ART. Of course, our result needs to be further explored with further *in vivo* and *in vitro* experiments.

Furthermore, the enrolled IR patients showed an increase in salivary *Campilobacterota* phylum and previous reports have documented the association between oral *Campylobacter* infections and increased risk of oral cancer [44].

In addition, increased levels of *Campylobacterot*a have been reported in oral leukoplakia (OL) [45] and the microbiome of proliferative verrucous leukoplakia (PVL) patients is significantly enriched in *Campylobacter jejuni* [46]. Both IL and PVL are oral

potentially malignant disorders (OPMDs), reported also as HIV comorbidities, and defined as any oral mucosal abnormality that is associated with an increased risk of occurrence of oral cancer.

Finally, the INRs displayed a functional metagenomic association with the prokaryotic ubiquinone pathway as ubiquinol-7 biosynthesis, ubiquinol-9 biosynthesis, ubiquinol-10 biosynthesis, ubiquinol-8 biosynthesis, superpathway of ubiquinol-8 biosynthesis. In detail, ubiquinones (also known as coenzyme Q) is an isoprenoid quinone that functions as an electron carrier in membranes. CoQ10 has shown a hematological activity for humans and an influence on host immunity. Indeed, it increases the activity of macrophages, as well as the proliferation of granulocytes [47]. This data may indicate a particular selection in saliva, albeit purely theoretical, of ubiquinone producing bacteria in INRs that needs further exploration.

Therefore, our explorative study shows some limitations. First, we investigated some factors of the oral-gut microbiota axis potentially involved in the modulation of viremia and CD4 recovery, but being the patients' number low, our results need to be confirmed in a larger cohort. Second, the functional analysis performed is based on inferred pathways and not actual measurements. Finally, concerning the longitudinal design, we only considered two time points (0-24-weeks ART) with no further follow up. Future studies, considering also more than two time points, could explore the oral microbial alterations and the restoration of immune function after long-term effective ART.

5. Conclusions

Although previous works investigated the dysbiosis of the tongue [48] and periodontal tissues in HIV-infected patients [49], our results further support that saliva provides a non-invasive, less expensive and informative approach for oral cavity microbiota-related analysis. In addition, this study advances our knowledge of the oral-gut microbiota axis and its mutual interplay with the host immunity in HIV patients at different clinical conditions. In detail, we reported a description of a dysbiotic oral microbiome in HIV patients, due to the increase of specific bacteria, as Porphyromonadaceae and Spirochaetaceae, that can convert into pathogenic, when the immune system is compromised, favouring an increasing risk for additional opportunistic infections. Curiously, among the Spirochaetaceae, we detected a decrease of *Treponema* (widely-considered to play important roles in periodontal disease), in naïve patients with oral infections. Additionally, we reported a potential communication between the dysbiotic oral and intestinal bacterial flora in HIV patients along the oral-gut microbiota immunity axis, differentially modulated before and after ART. Moreover, for the first time, we described an interesting increase of the oral pro-inflammatory *Proteobacteria* phylum in INRs compared to IRs. This taxon is able to support oral opportunistic infections and may contribute to increase the LPS blood level affecting the CD4 T cell reconstitution failure in HIV patients after ART. Finally, this oral *Proteobacteria* signature could be further explored as a non-invasive bacterial biomarker of immunological response to ART.

It is evident that the oral microbiota plays a relevant role in the pathogenesis of HIV-correlated disease, a better understanding of its dynamics might improve the mouth health in HIV-infected patients. So, additional investigations are needed to evaluate the impact of the potential interventions on oral microbiome in HIV infection, which is groundwork for the task of developing new approaches for the prevention and treatment of HIV/AIDS-associated diseases.

Future therapeutic strategies targeting anti-bacterial peptides [50], focused on bacterial strain LPS-mediated pathogenesis, and the current ART could improve the CD4+ T cell recovery balancing so the chronic immune activation and inflammation and decreasing the morbidity/mortality in human HIV infection.

Funding

This study was supported by University of Florence, No. 35° PhD Program and the European Union—NextGenerationEU—National Recovery and Resilience Plan, Mission 4 Component 2—Investment 1.5—THE—Tuscany Health Ecosystem—ECS00000017—CUP B83C22003920001.

Data availability

Further details about the reads processing and the statistical analysis are available at github.com/LeandroD94/Papers/tree/main/2023_Saliva_HIV_paper_Nannini.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Area Toscana Centro (Rif CEAVC 15035).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

CRediT authorship contribution statement

Giulia Nannini: Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Investigation. **Leandro Di Gloria:** Data curation, Formal analysis, Software. **Edda Russo:** Conceptualization, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Gaetana Sterrantino:** Conceptualization, Resources, Supervision, Validation. **Seble Tekle Kiros:** Data curation, Formal analysis. **Marco Coppi:** Formal analysis, Investigation. **Elena Niccolai:** Formal analysis, Investigation, Supervision, Validation. **Simone Baldi:** Formal analysis, Investigation. **Matteo Ramazzotti:** Data curation, Formal analysis, Methodology, Supervision, Validation, Writing – original draft. **Vincenzo Di Pilato:** Formal analysis, Investigation, Methodology, Supervision. **Filippo Lagi:** Data curation, Formal analysis, Investigation, Supervision. **Gianluca Bartolucci:** Formal analysis, Investigation. **Gian Maria Rossolini:** Conceptualization, Methodology, Supervision, Validation, Visualization. **Alessandro Bartoloni:** Conceptualization, Methodology, Supervision, Validation, Visualization. **Amedeo Amedei:** Conceptualization, Data curation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105339.

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