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Alterations in the oral microbiome in HIV-infected participants after ART administration are influenced by immune status

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Abstract

Objective: To characterize the oral bacterial microbiome in HIV-infected participants at baseline and after 24 weeks of EFV/FTC/TDF.

Design: Thirty-five participants co-enrolled in two AIDS Clinical Trials Group (ACTG) studies, A5272 and A5280, with paired saliva samples and complete data sets were assessed.

Methods: Paired saliva samples from were evaluated for bacterial microbiome using 16S rDNA PCR followed by Illumina sequencing. Diversity and differential abundance was compared between groups. A random forest classification scheme was used to determine the contribution of parameters in classifying participants' CD4 count.

Results: Bacterial communities demonstrated considerable variability both within participants and between timepoints, although they became more similar after 24 weeks of ART. At baseline, both the number of taxa detected and the average alpha diversity were variable between participants, but did not differ significantly based on CD4, VL or other factors. After 24 weeks of ART samples obtained from participants with persistently low CD4 counts had significantly higher bacterial richness and diversity. Several differentially abundant taxa, including *Porphyromonas* species associated with periodontal disease, were identified which discriminated between baseline and post-treatment samples. Analysis demonstrated that while inflammatory markers are important in untreated disease, the salivary microbiome may play an important role in CD4 count recovery after ART.

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Conclusions: Shifts in the oral microbiome after ART initiation are complex, and may play an important role in immune function and inflammatory disease.

Keywords

HIV; human microbiome; saliva; anti-HIV agents

Background

Opportunistic oral infections are common among persons living with HIV (PLWH), and have been linked to deteriorating immune function, weight loss, and poor nutrition [1–3]. Even in the current antiretroviral therapy (ART) era, oral candidiasis, HIV salivary disease, human papilloma virus (HPV)-associated oral lesions, xerostomia and ulcerations are frequently reported [4]. Periodontal disease, also commonly seen in PLWH, contributes to cardiometabolic diseases[5][6]. In a prospective cohort of PLWH, an increase in periodontal disease was associated with increases in carotid artery intima media thickness, a surrogate measure of atherosclerosis [7]. This highlights the need to characterize the oral microbiome in the setting of HIV infection and ART initiation.

The microbiome of the oral cavity is distinct from that found in the nares, skin, and gastrointestinal tract [8]. Notably, the oral cavity presents a niche with high alpha diversity but the low beta diversity, i.e. the oral cavity is a very diverse environment in terms of absolute bacterial diversity but that these communities are quite similar between individuals, remaining stable in both short and long term longitudinal assessments [9, 10]. Furthermore, in longitudinal analyses, when variability exists in the microbial phyla that are present in the oral cavity, active metabolic pathways remain conserved indicating functional stability in the oral microbiome [8].

The oral microbiome has been primarily studied in periodontal disease and its link to systemic diseases such as cardiovascular disease (CVD) and arthritis [11]. In periodontal disease, overgrowth of pathogenic bacteria (e.g. *Fusobacteria, Prevotella, Porphyromonas spp.*) has been hypothesized to induce endotoxemia and resultant systemic inflammation [12][13]. Recurrent exposure to endotoxin in animal models results in increased mortality and cardiac fibrosis [14]. In the FINRISK cohort, an HIV-negative cohort, high antibody response to periodontal pathogens predicted CVD events [15]. These data suggest a link between oral bacterial communities and systemic disease, however a full understanding has yet to be defined, particularly in PLWH.

Previous studies have indicated that the oral microbiome may be altered by HIV infection or ART, although results are highly variable, with different species associated with HIV infection, including increases in *Streptococcus* species in some studies and decreased in others [16–21]. Similarly, ART has been associated with variable changes [22, 23]. Other components of the microbiome, including the mycobiome and virome have been less well characterized. Fungal colonization is also altered demonstrating increased *Candida* with HIV [17, 22, 24], and HPV DNA was increased after 24 weeks of ART [25]. While intriguing, these studies are limited by small sample size, variations in technique, and their mostly cross-sectional nature. The enteric microbiome demonstrates significant inter- and intraindividual variability [26], as well as significant variability associated with genetics, diet, sexual activity and season [27–29][30]. Because of this, longitudinal studies of the changes in the microbiome associated with changes in health in an individual may provide better information than cross sectional studies comparing PLWH to unrelated, uninfected controls. The objective of our study was to explore changes in the oral microbiome data from 35 HIV-infected persons over a 24-week period following initiation of therapy with EFV/FTC/TDF.

Methods:

Study cohort:

Samples were collected from participants co-enrolled in two AIDS Clinical Trials Group (ACTG) studies, A5272 (NCT01029249) and A5280 (NCT01403051) [25, 31]. A5280 was a randomized-placebo controlled study in which ART naïve participants initiated ART with EFV/FTC/TDF. A5272 was a prospective, observational study of HIV-1 infected ART-naïve adults (≥18 years old) designed to collect throat wash and saliva specimens and evaluate changes in HPV oral shedding and the incidence of oral warts following ART initiation. For the present analysis, we obtained samples from all participants who had available paired samples from both visits as well as complete biomarker datasets. Saliva samples (70 samples from 35 participants) met these criteria and were used for this study. The protocol was approved by the institutional review boards of all participating sites. All participants provided written informed consent.

Biomarker Assays:

HIV-1 RNA and CD4 counts were performed at local CLIA certified laboratories. Serum samples were stored at −70°C until batched analysis at the Irving Institute Biomarkers Core at Columbia University Medical Center (New York, NY) as part of the A5280 study [31]. We measured IL-6 (ELISA; R&D Systems, Minneapolis, MN); soluble receptors of TNFα (sTNFr-I and –II; ELISA; R&D Systems, Minneapolis, MN), and soluble CD14 (sCD14, ELISA; R&D Systems, Minneapolis, MN).

16S rRNA gene sequencing and analyses:

Bar-coded PCR primers directed at the V4 region of the bacterial 16S rRNA gene were used to generate amplicons from nucleic acid isolated from saliva samples [32]. Multiplex sequencing of amplicons with sample-specific barcodes was performed using an Illumina MiSeq instrument (paired end 2×250 nt reads).

Demultiplexing of 16S rRNA gene sequences was performed in QIIME (Quantitative Insights Into Microbial Ecology, v.1.8.0) [33] (Full commands in Supplementary Information). Read quality control and the resolution of amplicon sequence variants (ASV) were completed using the dada2 R package (v.1.5.2) [34]. Ten low quality 5’ bases of the forward read and 50 3’ bases of the reverse read were removed following inspection of QC plots. All sequences with any Ns, reads mapping to phiX, and reads with a maximum expected error of > 2 following truncation were also removed. Error estimation was calculated on all samples (pooled) following dereplication. Following chimera removal (consensus method), taxonomy was assigned independently to the RDP v.14 16S rRNA gene sequence database [35]. Additional species level assignment to the RDP species training sets was accomplished using the *addSpecies* function of dada2. Blastn against the NCBI 16S reference database was used to further define the taxonomy of selected ASV. ASV were aligned using the R MSA package [36] and arranged into a maximum likelihood phylogeny (GTR model with optimization of the proportion of invariable sites and the gamma rate parameter) using the phangorn package [37]. The resulting phylogenetic tree was combined with the table of ASV and merged with sample data for loading into the PhyloSeq package [38]. Full details of the dada2 workflow are available at https://github.com/shandley/HIV-Oral in the file HIV_oral_16S_preprocessing.Rmd.

Samples with fewer than 50 ASV were removed. ASV not assigned to the kingdom bacteria or assigned to the mitochondrial family or chloroplast class were also removed. PhyloSeq was used to calculate alpha diversity measures of richness and Shannon Diversity and beta diversity using weighted UniFrac on log transformed counts [39]. Permutational multivariate analysis of variance of the distance matrices between groups were compared using ADONIS from the R package vegan [40]. Differential abundance testing of taxa between groups was completed using the DeSeq2 algorithm using a beta prior and Wald's significance test with local fitting of dispersion [41]. Random forest classification was implemented using the random Forest R package (citation: https://cran.r-project.org/web/packages/randomForest/citation.html). Taxa specific plots were generated using rlog normalized data. Intra- and inter- sample distance comparisons were calculated using Hellinger transformed data on Euclidian distances using the vegdist function in the R package vegan [40]. Full details of the dada2 workflow are available at https://github.com/shandley/HIV-Oral in the file HIV_oral_analysis.Rmd

Statistical Analysis:

Descriptive data characteristics were used to summarize the cohort. Continuous variables were summarized using median and IQR; categorical variables were summarized using frequency and percent (%). Spearman's rank correlations were used to examine bivariate associations between study variables. Fisher's exact and chi-square tests were used to compare categorical variables between the study groups. Mann-Whitney test and Kruskal Wallis test (indicated by p value in text) with Dunn's post hoc analyses (p values in figures) were used for comparing continuous variables. No correction for multiple comparisons was performed unless otherwise stated. Statistical analyses and graphing were performed in R and Prism version 6.05 for Windows (GraphPad Software, http://www.graphpad.com). All p values with $p < 0.05$ were considered significant.

Results:

Cohort Characteristics

The study participants were predominantly male (88.6%); 45.7% white, 31.4% African American, and 20% Hispanic, with a median age of 36 years (range: 19–61). The median $CD4^+$ T-cell count was 326 c/ml (range: 7–804), with 5 participants having entry $CD4^+$ T-cell counts <200 c/ml (herein CD4 low vs. CD4 high if CD4 T-cell counts ≥ 200 c/ml) (Table 1). None had opportunistic infections within the past 30 days, and none were coinfected with tuberculosis or HCV. Although $CD4^+$ T-cell counts increased in all participants, three participants still had $CD4^+$ T-cell counts <200 c/ml at the week 24 time point (Figure S1A). The median viral load was 30,136 cp/mL (range: 1,091–1,614,781 cp/mL); 9 participants had baseline viral load >100,000 cp/mL (herein viral load high vs. viral load if < 100,000 cp/ml) (Figure S1B). All participants achieved virologic suppression by week 12, although one participant rebounded to 9,100 cp/mL at week 24.

Effects of ART on the salivary bacterial microbiome

The bacterial microbiome was characterized using 16S rRNA gene profiling. Sequencing resulted in an average of 4,070 ASV per sample (SD: 4,901.82) (Figure S2). Similar to previous reports, at baseline the most abundantly detected bacterial phyla in the salivary microbiome were *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, with lower relative abundance of *Fusobacteria*, *Spirochaetes, Actinobacteria*, and *Tenericutes* (Figure 1A). Following 24 weeks of ART, the dominant phyla represented in the saliva remained similar to that found at baseline (Figure 1B). There was no significant difference in beta diversity when comparing baseline samples to those collected following 24 weeks of ART (Figure 1C, ADONIS test p-value: 0.744). However, the communities within each participant did change over time (Figure 1C). Similarly, alpha diversity (richness and Shannon diversity) was not significantly different between samples collected at baseline and samples collected following 24 weeks of ART (Figure S3A&B). However, there was a large range of alpha diversity values across all samples at both time points (Richness min: 15, max: 169, median: 91, SD: 40.007, Shannon Diversity min: 2.115, max: 4.252, median: 3.362, SD: 0.461) suggesting high person-to-person variability in the salivary bacterial microbiome within this cohort at both baseline and after 24 weeks of ART. Bacterial communities within the same person (intrasample) were just as similar to each other as they were to those in other people (intersample) (Figure 1D). On average, the bacterial communities became more similar across all participants following 24 weeks of ART (Figure 1D).

Detection of differentially abundant ASV between baseline samples and samples collected following 24 weeks of ART yielded only two discriminatory sequences (Figure 2A and Table S1). A sequence with only 91% identity to *Muribaculum intestinale* ($\log_2$fold change = −4.193) was more frequently detected (Fisher's exact test p-value = 0.0005) in baseline samples, although the average abundance was not significantly different (Figure 2B). *Treponema lecithinolyticum* ($\log_2$fold change = 2.848) was detected at similar frequencies, but was on average more abundant in week 24 samples (Figure 2C). Of note, the 91% sequence similarity to *M. intestinale* suggests that this is a novel representative of the phylum Bacteroidetes, family Porphyromonadaceae which is not currently in sequence databases.

Effects of CD4 count and HIV viral load on the salivary microbiome

To determine the effect of $CD4^+$ T-cell counts on the salivary bacterial microbiome, we compared these measurements to the alpha and beta diversity as well as determined differentially abundant taxa between participants with CD4 low (< 200 c/ml) vs. CD4 high (≥ 200 c/ml) at both sampling time points. At baseline, both the number of bacterial taxa detected and the average alpha diversity of samples at baseline were not significantly

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different (Figure S6A&C). However, following 24 weeks of ART, samples obtained from the 3 participants who had persistently low CD4 counts had significantly higher bacterial richness (p=0.0007) and Shannon diversity (p=0.0053) when compared to those with CD4 counts that remained or recovered to greater than 200 c/ml (Figure S6B&D). Bacterial alpha diversity did not significantly change in CD4 low or CD4 high samples when corresponding samples were compared at baseline and following 24 weeks of ART (richness and diversity baseline vs. week 24 CD4 low: p-value = 0.500 and 0.2500, CD4 high p-value = 0.2492 and 0.8314). Beta diversity was not significantly different between CD4 low and CD4 high samples as determined by the ADONIS permutational multivariate analysis of variance test (Baseline: p-value = 0.7130, Week 24: p-value = 0.0740).

A single ASV with 100% identity to *Haemophilus parainfluenza* was statistically associated with CD4 group (Figure 3A and Table S2). This ASV was more abundant in baseline CD4 high samples ($\log_2$fold change = 3.149). After further investigation this sequence was found to increase in abundance following 24 weeks of ART specifically in the CD4 low participant samples (Figure 3B). The abundance was significantly higher in CD4 high samples than CD4 low samples at baseline, and switched to be more abundant in CD4 low samples than CD4 high samples following 24 weeks of ART.

In participants with viral load (VL) < 100,000 cp/ml at baseline, the numbers of detectable bacterial taxa following 24 weeks of ART treatment were significantly reduced when compared to their paired baseline samples, although the bacterial diversity was similar in both groups (Figure S5). No statistically significant differences were detected in alpha diversity measures at baseline or following 24 weeks of ART when comparing participants with VL< 100,000 cp/ml to those with VL≥ 100,000 cp/ml (Figure S4). There were no significant differences in between VL high and VL low groups at baseline as determined by the ADONIS test (p-value = 0.632). It was not possible to calculate significance in VL group differences in samples collected following 24 weeks of ART as no participants had a high VL at that time point. There were no discriminatory differentially abundant taxa identified when comparing viral load groups at baseline or following 24 weeks of ART.

Effect of demographic data on the salivary bacterial microbiome

In order to determine if demographic parameters had an effect on the bacterial microbiome, we tested for significant group differences in bacterial communities between groups using the ADONIS test. Testing for significance between sex, ethnicity and race failed to identify any significant differences (ADONIS p-value at baseline: race = 0.492, sex = 0.405, ethnicity = 0.351. Similar results were seen following 24 weeks of ART: race = 0.701, sex = 0.114, ethnicity = 0.750).

The salivary bacterial microbiome as a discriminating feature of ART and CD4 count

Inflammatory biomarkers changed variably with ART (Figure S7). We implemented a random forest classification scheme to determine the contribution of inflammatory biomarkers (IL-6, sTNFr-I, sTNFr-II, sCD14), virologic (HIV-1 viral load), and bacterial microbiome (richness and diversity) parameters in classifying participants' CD4 category (CD4 low vs CD4 high) both pre- and post-ART. Prior to ART, the most important factor for categorizing CD4 category was sTNFr-II followed by viral load (Figure 4A). However, following 24 weeks of ART the most important factors for CD4 category classification became the alpha diversity of the salivary microbiome (Figure 4B).

Discussion:

We report on the effect of 24 weeks of ART with EFV/FTC/TDF on the oral bacterial microbiome in HIV-infected participants. Key strengths of the current study include prospective data collection, uniform ART therapy given to all participants, and longitudinal sample collection from baseline and following 24 weeks of ART using individuals as their own controls. Our study is limited by a lack of HIV-negative controls. Given significant individual variability, the best control samples would be taken from participants prior to acquisition of HIV. Because all participants are HIV-infected, our study evaluates the effect of both HIV-control and ART on the salivary microbiome.

Most prior studies of the oral microbiome were designed as cross-sectional studies comparing HIV-infected participants with HIV-negative controls [16–24]. Only two studies evaluated longitudinal changes after ART administration, with conflicting findings as one demonstrated and increase and the other a decrease in the prevalence of *Aggregatibacter* after ART administration [22][23]. These studies differed in methodology from our study, in that the microbiome was assessed using culture techniques. Regardless, these studies suggest that HIV and ART drive changes in specific bacterial taxa in the oral microbiome. Many of these are associated with periodontal disease and should be considered in the pursuit of developing treatments to minimize HIV-associated periodontal disease.

We did not observe a characteristic shift in diversity after ART initiation, but did see substantial change in individual participant salivary bacterial community structures between the baseline and after treatment. When comparing participants with high baseline CD4 or low baseline VL we demonstrate a relative decrease in richness after 24 weeks of ART, similar to prior reports [22]. In participants with low CD4, we demonstrate an increase in bacterial alpha diversity associated with the elimination of viremia. Taken together, these data suggest that alpha diversity is associated with both low CD4 counts and low HIV load, with immune reconstitution and viral suppression correlated with decreases in bacterial diversity. The variability of these individual responses may reflect changes induced by (1) immune reconstitution, (2) a direct influence of the ART used (EFV/FTC/TDF) or (3) a shift in the microbiome reflective of the individual prior to HIV infection. It is difficult to differentiate between these possibilities without an uninfected control group, pre-infection samples, samples from participants treated with other ART regimens, or a well-matched untreated control group. We identified two differentially abundant ASV between baseline and week 24 samples. One sequence was more frequently detected in baseline samples, and is most closely related to an enteric bacterium of mice, *M. intestinale*. The low percent identity (91%) demonstrates that this ASV is distinct from *M. intestinale* but likely belongs to a member of the *Porphyromonadaceae*. *Porphyromonadaceae* have been associated with the human oral cavity, and are important periodontal pathogens [8]. Without specific taxonomic assignment it is unclear why this bacteria is specifically associated with oral microbiomes of PLWH prior to ART, however, it is a specific bacterial marker as it is almost entirely absent following 24 weeks of ART. Our finding is similar to prior findings that among participants with untreated HIV, there is an overgrowth of *Porphyromonas* [12][13]. The second ASV was associated with the salivary bacterial microbiome in samples collected after 24 weeks of ART. This sequence has 100% identity to *T. lecithinolyticum*, a Spirochaete also implicated causing periodontal disease [42]. These findings could partially explain the ongoing incidence of periodontal disease in treated PLWH [43].

At baseline, an ASV with 100% identity to *H. parainfluenzae* presence is noted to discriminate between high and low CD4 groups. *H. parainfluenzae* was ubiquitous in both CD4 high and low groups. Its abundance was not significantly changed in CD4 high samples, but increased in CD4 low samples following 24 weeks of ART, suggesting that immunodeficiency is associated with low abundance of *H. parainfluenzae* which is then restored to normal or higher levels following ART.

Further analysis demonstrated that the CD4 count classification of participants prior to ART initiation is most related to viral load and inflammatory cytokines. After suppression of viremia, features of the microbiome, including richness and diversity, play a more prominent role in CD4 count. These limited data raise the intriguing possibility that persistent changes in the microbiome may play a role in CD4 count recovery, and suggest that measurement of salivary bacterial diversity could serve as a biomarker of immune reconstitution following ART.

In summary, these data suggest that the oral microbiome is highly variable from person to person, with some shifts toward more similarity after 24 weeks of ART with virologic suppression. This may be due to a variety of factors, including antibiotic administration, immune compromise, oral pathology or differences in oral care. The expression of these bacterial communities may also lead to further immune activation, as microbial translocation from the oral cavity may contribute to microbial translocation and systemic immune activation, similar to what has been postulated in the lower GI tract. Future studies should address how the microbiome shifts during the complex time period after ART initiation. These studies should incorporate data about immune function and inflammation, as well as control for ART regimen, the passage of time, and the extensive variety of factors which are known to influence the microbiome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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RMP and ETO were the lead investigators, developed the protocol, interpreted the data and wrote the manuscript. SH performed the statistical analysis and wrote the manuscript. LD performed the sequencing and contributed to the statistical analysis. All the other authors participated in protocol development, study accrual, and reviewed, revised and approved the final manuscript.

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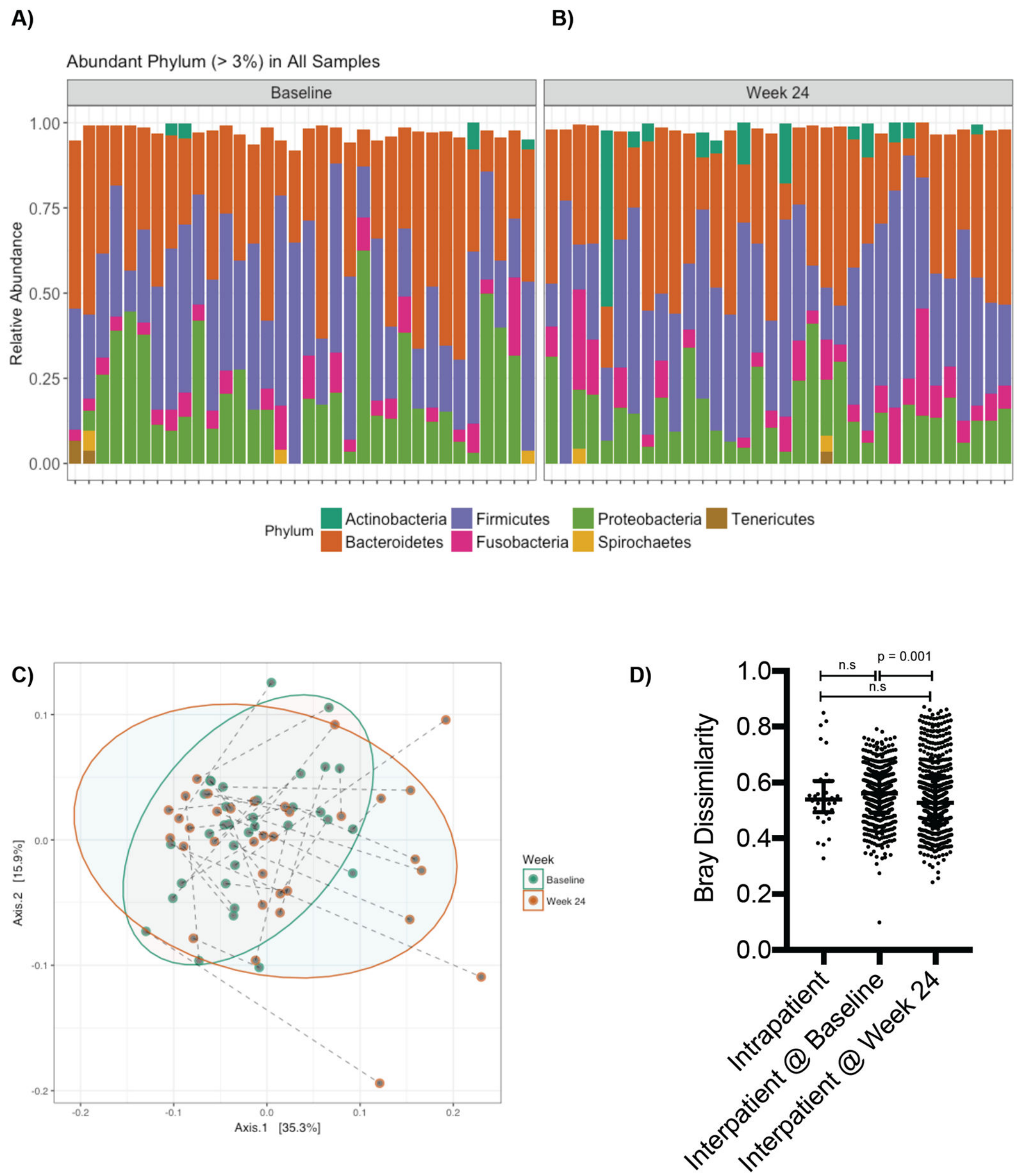

Figure 1. Effect of ART on the salivary bacterial microbiome.

Bacterial phyla (present at > 3% of total relative abundance) are in (A) samples collected at baseline and (B) samples collected following 24 weeks of ART. Principle coordinate analysis of weighted UniFrac distances of samples collected at baseline and following 24 weeks of ART (C). Dashed lines connect samples from the same patient. Bray-Curtis dissimilarity of intra- and inter-patient distances (D). Differences between groups were considered statistically significant if $p < 0.05$ using the nonparametric analysis of variance correcting for multiple comparisons using the method of Benjamini, Krieger and Yekutieli. The median for each group is indicated with a horizontal line, and error bars indicate the interquartile range. n.s. indicates not significant.

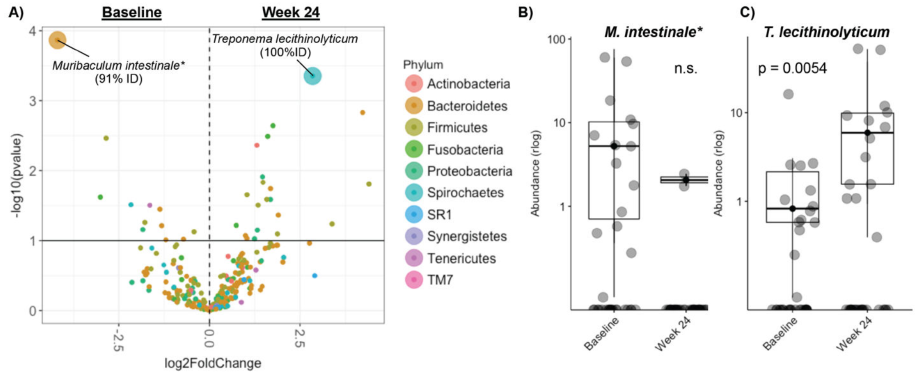

Figure 2. Bacterial taxa differentially abundant in saliva prior to and following 24 weeks of ART.
(A) Volcano plots of the $\log_2$fold changes and –log10 p-values when comparing taxa abundances between baseline and week 24 samples. Large circles indicate taxa with FDR significance < 0.10. (B) Comparison of ASV with 91% identity to *Muribaculum intestinale* and (C) 100% ID to *Treponema lecithinolyticum* identified with differential abundance testing. Differences in abundance between groups were considered statistically significant if p < 0.05 using the nonparametric Wilcoxon signed-rank test. The median for each group is indicated with a horizontal line, and error bars indicate the interquartile range. n.s. indicates not significant, FDR indicates false discovery rate *The 91% sequence similarity to *Muribaculum intestinale* suggests that this is a novel representative of the family Porphyromonadaceae.

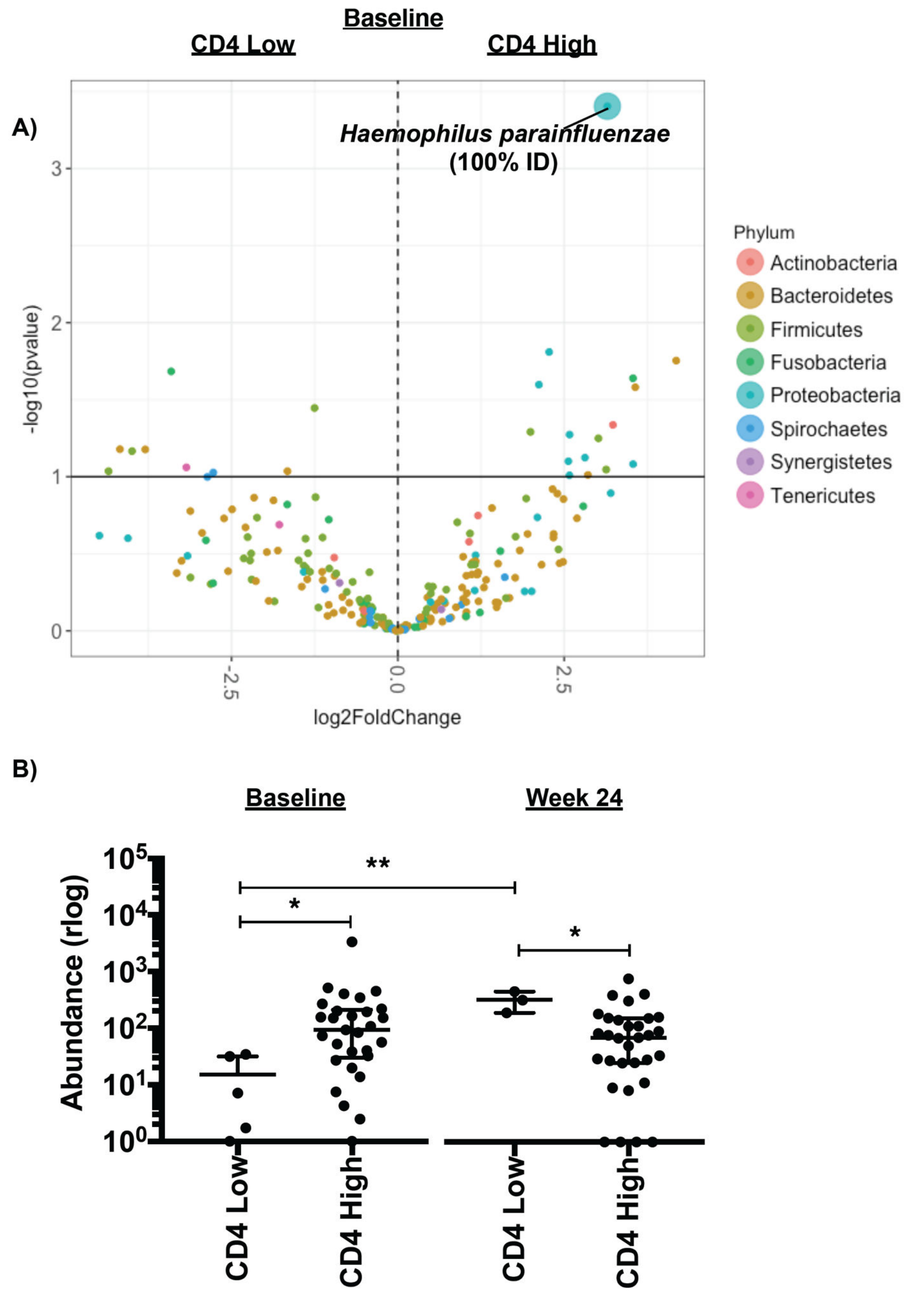

Figure 3. Bacterial taxa differentially abundant in CD4 low or CD4 high patient saliva.

(A) Volcano plots of the $\log_2$fold changes and −log10 p-values when comparing taxa abundances between CD4 low (< 200 c/ml) and CD4 high (CD4 > 200 c/ml) samples. Large circles indicate taxa with FDR significance < 0.10. (B) Comparison of ASV with 100% identity to *Haemophilus parainfluenzae* identified with differential abundance testing. Differences between groups were considered statistically significant if p < 0.05 using the nonparametric Kruskal-Wallis Test correcting for multiple comparisons using the method of Benjamini, Krieger and Yekutieli. *p ≤ 0.05, **p ≤ 0.01. FDR indicates false discovery rate.

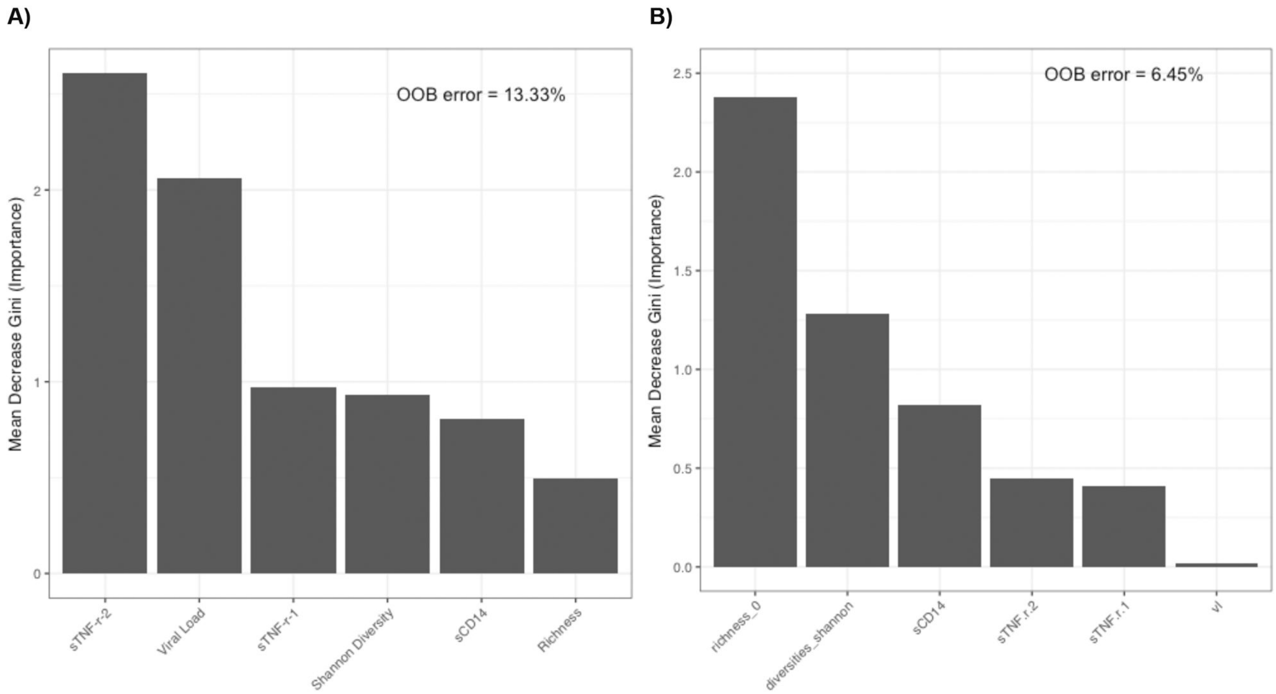

Figure 4. Random forest classification of salivary samples from patients into CD4 category.
Ranked importance of factors importance for classifying samples in CD4 category (High or Low) in (A) baseline of (B) samples collected following 24 weeks of ART. OOB = Out-Of-Box error rate.

Table 1:

Cohort description with demographic data

Characteristics		**Number (%)/Median (range)**
Sex	Men	31 (88.6)
	Women	4 (11.4)
Race	White	16 (45.7)
	Black	11 (31.4)
	Hispanic	7 (20)
	Other	1 (2.9)
IVDU	Never	33 (94.3)
	Previously	2 (5.7)
Age at entry		36 years (19–61)
CD4+ at entry		326 c/ml (7–804)
CD4+ <200 c/ml		5 (14.2)
Baseline HIV VL		118,465 cp/mL (1091–1,614,781)
VL>100,000 cp/ml		9 (25.7)
Baseline BMI		25.66 kg/m^2 (17.4–37.45)