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Oral Microbiome in HIV-Infected Women: Shifts in the Abundance of Pathogenic and Beneficial Bacteria Are Associated with Aging, HIV Load, CD4 Count, and Antiretroviral Therapy

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Abstract

Human immunodeficiency virus (HIV)-associated nonacquired immunodeficiency syndrome (AIDS) conditions, such as cardiovascular disease, diabetes, osteoporosis, and dementia are more prevalent in older than in young adult HIV-infected subjects. Although the oral microbiome has been studied as a window into pathogenesis in aging populations, its relationship to HIV disease progression, opportunistic infections, and HIV-associated non-AIDS conditions is not well understood. We utilized 16S rDNA-based pyrosequencing to compare the salivary microbiome in three groups: (1) Chronically HIV-infected women >50 years of age (aging); (2) HIV-infected women <35 years of age (young adult); and (3) HIV-uninfected age-matched women. We also examined correlations between salivary dysbiosis, plasma HIV RNA, $CD4^+$ T cell depletion, and opportunistic oral infections. In both aging and young adult women, HIV infection was associated with salivary dysbiosis characterized by increased abundance of *Prevotella melaninogenica* and *Rothia mucilaginosa*. Aging was associated with increased bacterial diversity in both uninfected and HIV-infected women. In HIV-infected women with oral coinfections, aging was also associated with reduced abundance of the common commensal *Veillonella parvula*. Patients taking antiretroviral therapy showed increased numbers of *Neisseria* and *Haemophilus*. High plasma HIV RNA levels correlated positively with the presence of *Prevotella* and *Veillonella*, and negatively with the abundance of potentially beneficial *Streptococcus* and *Lactobacillus*. Circulating $CD4^+$ T cell numbers correlated positively with the abundance of *Streptococcus* and *Lactobacillus*. Our findings extend previous studies of the role of the microbiome in HIV pathogenesis, providing new evidence that HIV infection is associated with a shift toward an increased pathogenic footprint of the salivary microbiome. Taken together, the data suggest a complex relationship, worthy of additional study, between chronic dysbiosis in the oral cavity, aging, viral burden, $CD4^+$ T cell depletion, and long-term antiretroviral therapy.

Keywords: HIV, oral microbiome, saliva, opportunistic infection, aging, disease progression

Introduction

Although human immunodeficiency virus (HIV) remains a leading cause of death worldwide, the development of antiretroviral therapy (ART) has dramatically increased life expectancy of individuals who have reliable access to treatment. As a result, the number of aging individuals living with HIV/acquired immunodeficiency syndrome (AIDS) is rapidly growing. According to the Centers for Disease Control and Prevention (CDC) estimates, more than half of HIV-infected individuals in the United States are now over the age of 50.[1] Moreover, due to the upsurge in

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aging Americans caused by emergence of the ''baby boomers,'' older individuals have also become the fastest growing population with new HIV infections.

Evidence has accumulated in recent years that HIV-associated non-AIDS conditions occur more prevalently in older populations than in young or uninfected adults. These include, but are not limited to; cardiovascular disease,[2–5] lung disease,[6] infection-related and noninfection-related cancers,[7,8] HIV-associated neurocognitive disorders,[9,10] osteoporosis,[11,12] cirrhosis,[13] and renal disease.[14] It is commonly believed that the development of non-AIDS events is driven by relentless systemic inflammation associated with chronic HIV infection. However, the picture remains less clear regarding the breadth of physiological events that actually fuel the inflammatory responses.

In recent years, much attention has been focused on characterizing the impact of HIV infection and ART on host–microbe interactions in the gut, and the potential role of gastrointestinal dysbiosis in immune activation[15–17] and microbial translocation.[18–20] In light of the valuable insights on HIV pathogenesis provided by these studies, we were surprised to find a comparative paucity of studies focused on the role of the oral microbiome in HIV pathogenesis and susceptibility to opportunistic oral disease. This discrepancy is magnified by our understanding that oral mycoses and other opportunistic oral infections remain a significant problem during HIV infection despite the success of ART.[21]

We attempt to address this knowledge gap in the current study by exploring the relationship between oral dysbiosis, aging, and HIV infection. In addition, our investigation focuses on another critical disparity in HIV research, underrepresentation of women. We have utilized next-generation sequencing (NGS) of the bacterial 16s ribosomal subunit to characterize the salivary microbiome of a subset of women enrolled in the Women's Interagency HIV Study (WIHS), a multicenter, prospective study of HIV-1 infection in women.[22,23] Our cohort spans a spectrum of ages, HIV burden, $CD4^+$ T cell depletion, and oral health status. The salivary microbiome was characterized and compared between aging (over 50 years of age) and young adults (under 35 years of age) in the presence and absence of HIV infection.

Methods

Study subjects and saliva sample collection

We studied 28 participants in the Bronx WIHS site. The WIHS cohort comprises both HIV-infected and demographically comparable uninfected women who were interviewed, examined, and had laboratory evaluations semiannually. Between 1995 and 2004, a subset of subjects participated in the Oral Sub-study, which encompassed a comprehensive examination and evaluation of oral health and the collection of stimulated and unstimulated saliva. The Institutional Review Board at each site approved the investigation and each woman provided informed consent.

Seeking subjects with a spectrum of ages, plasma HIV RNA levels, $CD4^+$ T cell depletion, and oral disease, we selected women from the WIHS Oral Sub-study with the following characteristics: (1) Chronically HIV-infected women who, at the time of sample collection, were >50 years of age (aging); (2) HIV-infected women <35 years of age (young adult); and (3) HIV-uninfected age-matched women (Supplementary Table S1). Additional HIV-infected women who displayed distinctive oral opportunistic infections were also studied. Clinical data (peripheral $CD4^+$ and $CD8^+$ T cell numbers and plasma HIV RNA counts) collected for each patient on the date the saliva samples were collected was provided through WIHS database archives.

Saliva samples

Unstimulated saliva was collected by expectoration. During routine visits to the WIHS, stimulated saliva ($\sim$1–3 mL) was collected in 50-mL conical tubes, divided into 1 mL aliquots, and archived at −80°C at the WIHS repositories in the Bronx, NY, and a single 1 mL aliquot of each sample was assigned for use in the current study. To perform 16S phylogenetic analysis and quantitative polymerase chain reaction (PCR) assays, DNA was extracted as needed from 250 μL aliquots of saliva.

DNA extraction, 16S library construction and sequencing

Total genomic DNA was extracted from saliva samples. Samples were incubated in an enzymatic cocktail containing lysozyme (0.2 mg/mL) and proteinase K (0.4 mg/mL) followed by bead beating using a Biospec Products Mini-BeadBeater-8™ (BioSpec Products, Inc.). DNA was then extracted from cell lysates using the PowerSoil Kit (MO BIO Laboratories, Inc.) according to manufacturer-supplied protocols. The extracted DNA was amplified by PCR enrichment of 16S ribosomal RNA encoding sequences using primers 515F and 806R (V3-V4 hypervariable region) as previously described.[24] To facilitate assemblies and longer accurate reads, paired-end (PE) libraries were constructed. The PE amplicons were modified by adding a unique set of eight oligonucleotide barcodes for purposes of multiplexing. PE amplicons were purified and quantified on an Invitrogen Qubit system, and sequenced using an Illumina MiSeq system.

Phylogenetic analysis

Sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) open-source computational package[25] (version 1.8.0). Data were quality filtered and clustered into operational taxonomic units (OTUs) as previously described.[26] Clustered OTUs were then aligned against the Greengenes database,[27] version 2011 to provide taxonomic assignments. For final graphical output, additional filtering was performed to display only taxa that comprised at least 0.5% of the total salivary community in any sample.

Statistical analysis

Alpha diversity scores for experimental groups were calculated in Explicet open source software[28] (version 2.9.4). Statistical significance of differences between experimental groupings was determined by Student's t-tests and Wilcoxon rank-sum analysis. Correlations between changes in bacterial phylogenetic proportions, age, HIV and oral coinfection status, and smoking and drinking habits were evaluated by Spearman's rank correlation coefficient test (GraphPad Software, Inc.).

Principal coordinates analysis (PCoA) was performed based on the taYC index in Mothur[29] and plots were visualized using

rgl package in R (www.r- project.org). Analysis of molecular variance[29] was used for statistical analysis between groups compared in each PCoA plot. Linear discriminant analysis effect size[30] (LEfSe) was utilized to identify statistically different bacterial taxa in their relative abundance between HIV-positive young adults and HIV-positive aging adults with coinfection. Kruskal–Wallis test was used to process the dataset with LEfSe alpha values set at 0.05 and logarithmic linear discriminant analysis score >2 for discriminative feature filtering.

Analysis of Epstein–Barr virus and Candida albicans *by quantitative PCR*

PCR-based assays were utilized to determine copy numbers of Epstein–Barr virus (EBV)[31] and *C. albicans*[32] in each saliva sample. Primer and probe sequences for EBV were generated for the BALF5 gene according to previous studies.[33] The primer and probe sequences for *C. albicans* were designed for the internal transcribed spacer region of the rRNA gene.[34] On both probes, a 6-carboxy-fluorescein (FAM) reporter was covalently attached to the 5′ end, and a 6-carboxy-tetramethyl-rhodamine (TAMRA) quencher was covalently attached to the 3′ end. Fluorescence signal was detected with an ABI Prism 7700 sequence detector (PE Applied Biosystems) and data captured and analyzed with Sequence Detector Software (SDS). Standard curves for EBV were generated as previously described.[35] *C. albicans* standard curves were constructed using a serial dilution of ATCC culture, strain CBS 562.

Results

Study population

To investigate the relationship between age, oral dysbiosis, $CD4^+$ T cell depletion, and HIV burden, in women, we analyzed the salivary microbiome of 28 women exhibiting a spectrum of ages, HIV disease progression, and oral health status. Virologic and immunologic characteristics and the age ranges of the study groups are summarized in Supplementary Table S1. We compared aging (>50 years of age), young adult (<35 years of age), chronically HIV-infected patients, and age-matched HIV-uninfected women. To further explore the relationship between HIV infection and oral disease, we characterized the microbiome of women with specific HIV-associated opportunistic oral infections diagnosed at the time of sample collection. These included individuals with hairy leukoplakia, oral candidiasis, and lesions resulting from Kaposi's sarcoma virus (KSV) and human papilloma virus (HPV). Finally, we also included a subset of women who reported treatment with ART for more than 1 year ($n=3$; ages; 33, 49, and 52 years of age; no diagnosed coinfections).

Despite the small number of women in the study, all HIV-infected groups displayed significantly reduced $CD4^+$ T cell counts in peripheral blood as compared with those of HIV-uninfected women (Fig. 1A). Notably, HIV-uninfected aging women also showed reduced $CD4^+$ T cell levels nearing statistical significance ($p=.055$) when compared with HIV-uninfected young adults, supporting previous reports that the process of aging can impact T cell repertoires.[36] Young adult HIV-infected women had the highest mean peripheral viral loads, but the difference was not statistically significant and all other HIV patient groups had similar plasma HIV RNA copy numbers (Fig. 1B).

Impact of HIV infection on the salivary microbiome

We utilized the Illumina MiSeq NGS platform to perform phylogenetic profiling of the salivary microbiome. Our findings indicate that chronic HIV infection was associated with alterations in community structure that were common to both young adult and aging patients. Evaluation of alpha diversity within the four overarching subgroups showed that, in general, HIV infection was associated with a marked

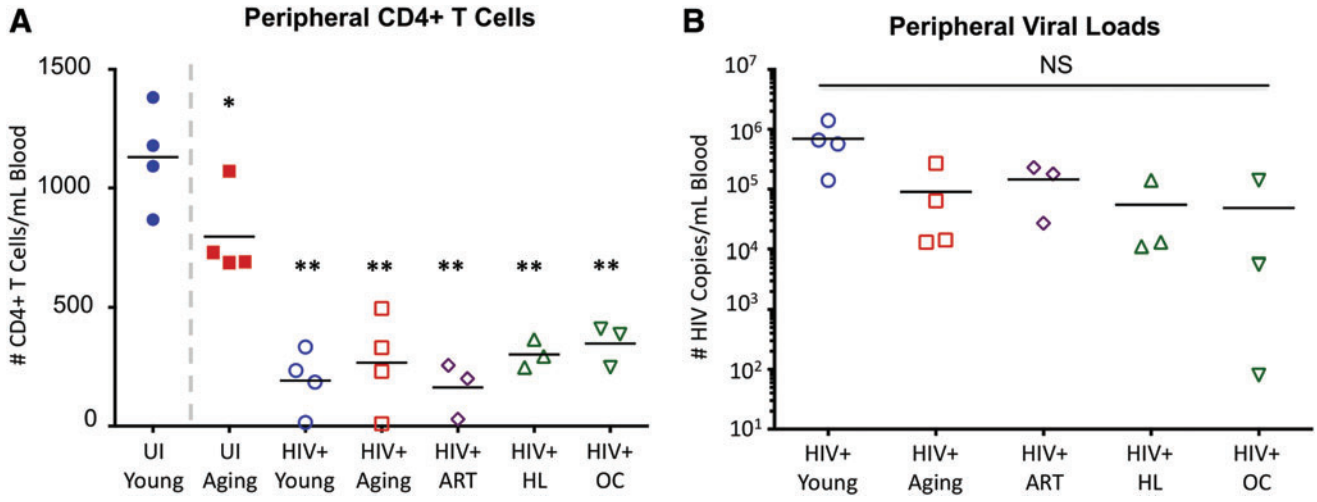

FIG. 1. Immunologic and virologic characteristics of HIV patient groups and HIV-uninfected women. **(A)** Comparison of absolute peripheral $CD4^+$ T cell numbers in HIV patient groups and HIV-uninfected women quantitated by flow cytometry indicated a statistically significant depletion in each infected group. *p*-Values were calculated by Student's *t*-test. $*p<.05$, $**p<.01$. **(B)** Although young adult HIV-infected women displayed peripheral viral loads that were about one log higher than other infected groups, the difference was not statistically significant. The remaining HIV patient groups all displayed similar levels of plasma HIV RNA. UI, uninfected; HL, hairy leukoplakia; OC, oral candidiasis; HIV, human immunodeficiency virus; NS, not significant. Color images are available online.

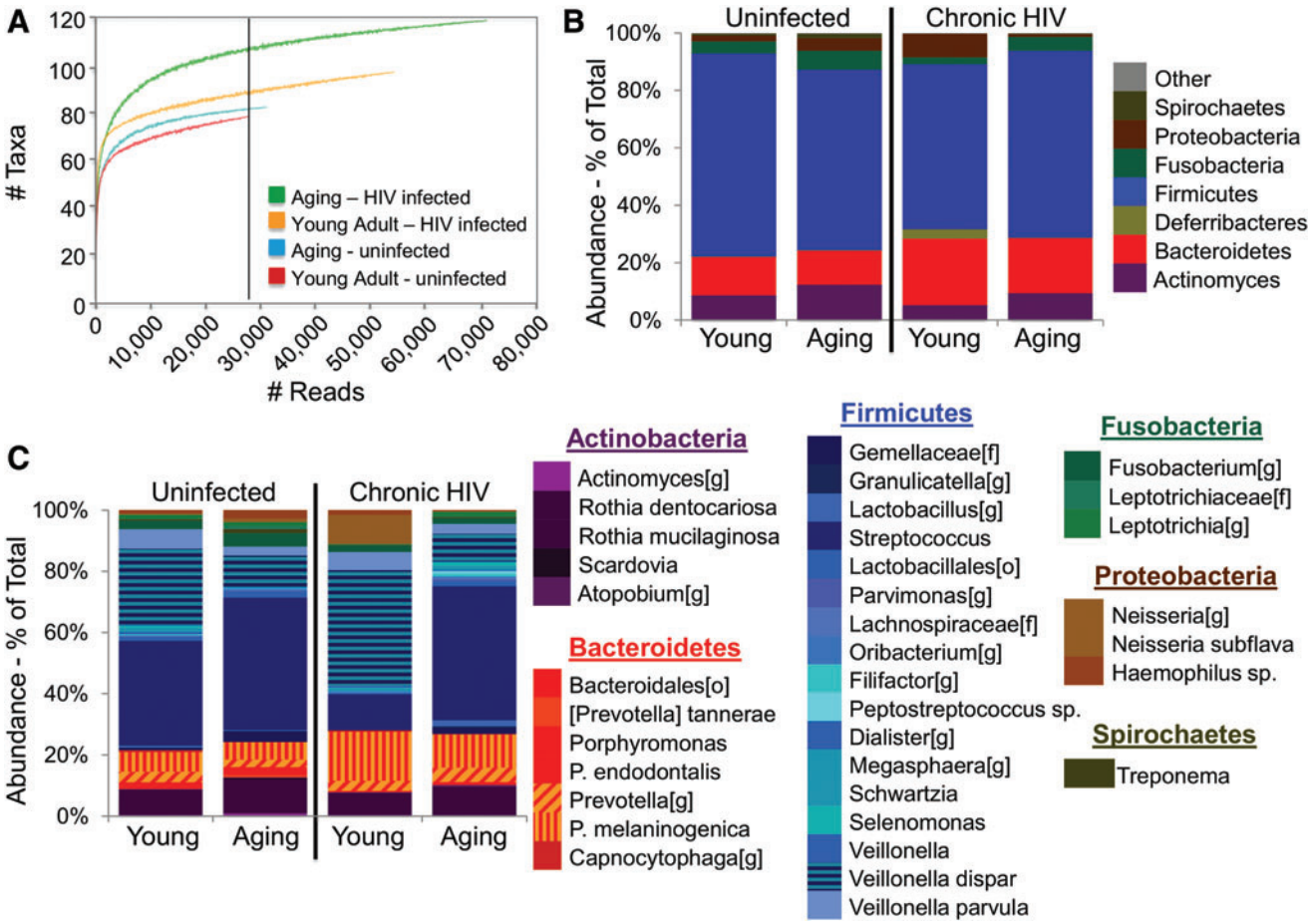

FIG. 2. Phylogenetic analysis of the impact of aging and HIV infection on the salivary microbiome. The richness and abundance of bacterial taxa in the salivary microbiome was evaluated and compared through alpha (Shannon index) diversity scores **(A)**. HIV-infected young adult (*orange line*) and aging (*top*, *green line*) groups harbored significantly more diverse communities than their age-matched control counterparts (*red* and *blue lines*, respectively). **(B)** Mean phylogenetic distribution at the phylum level taxonomic resolution of bacteria comprising at least 0.5% of the total bacterial community in the saliva of young adult (<35) and aging (>50) untreated HIV-infected women and age- and gender-matched HIV-uninfected women. **(C)** Mean distribution of highest taxonomic levels detected within each phylum. Color images are available online.

increase in the number of different bacterial taxa when compared with HIV-uninfected controls (Fig. 2A).

At phylum-level taxonomy, an average but insignificant increase in colonization of *Bacteroides*, and reduction in *Actinobacteria* was detected in both young adult ($p = .185$) and aging ($p = .133$) HIV-infected groups compared with age-matched HIV-uninfected controls (Fig. 2B). Closer examination of the highest taxonomic levels revealed that the *Bacteroidetes* increase reflected consistently higher proportions of the *Prevotella* species, including *Prevotella melaninogenica* (Fig. 2C; highlighted by slanted and vertical red bars, respectively). While several commensal *Prevotella* are commonly found in oral flora, it is worth mentioning that *P. melaninogenica* has been linked with increasing subgingival plaque in older adults[37] and reported to have potential links to oral cancer[38,39] and rheumatoid arthritis.[40]

Impact of aging

While both young adult and aging HIV-infected women displayed higher bacterial diversity in the salivary microbiome than their HIV-uninfected counterparts (Fig. 2A), we found that the highest alpha diversity measured was associated with the aging HIV-infected group. At phylum-level taxonomy, both age groups of HIV-uninfected controls displayed relatively similar taxonomic profiles (Fig. 2B), with the exception of higher levels of *Proteobacteria* in the aging group that approached statistical significance ($p = .059$). During HIV infection, however, that profile was reversed, with the young adult group trending toward higher proportions of *Proteobacteria* than the aging group ($p = .106$). It may be noteworthy, given the small number of patients that the decline in *Proteobacteria* in the aging group was statistically significant ($p = .021$).

In addition, higher proportions of *Actinobacteria* and *Fusobacteria* were observed in the saliva of aging HIV-infected and aging HIV-uninfected women compared with their young adult counterparts, but this discrepancy also did not reach statistical significance (Fig. 2B). Similarly, young adult HIV-infected women displayed tended to show higher proportions of *Bacteroidetes* and *Proteobacteria* than aging HIV-infected women.

Despite an overall reduction of *Firmicutes* in young adult HIV-infected compared with their HIV-uninfected counterparts, we found that HIV infection was associated with higher proportions ($p = .076$) of *Veillonella dispar*, (Fig. 2C; highlighted by horizontal bars). *V. dispar* is a commensal anaerobic lactate fermenter believed to strongly influence the diversity of the oral microbial community. Additional examination of species-level taxonomic hits revealed that the detected increase in *Actinobacteria* in aging HIV-uninfected and HIV-infected groups was representative of higher proportions of another common oral commensal *Rothia mucilaginosa.* In light of the increased prevalence of oral cancer in aging populations and the current study's focus on aging, it may be worth noting that acetaldehyde production by *R. mucilaginosa* has been implicated as a potential contributor to oral carcinogenesis.[41]

The salivary microbiome profiles of each individual HIV-infected patient and HIV-uninfected control sample are presented at phylum level in Supplementary Figure S1, and at the highest taxonomic levels detected in Supplementary Figure S2. A summary of the differences in abundance of bacterial genera that reached or approached statistical significance between young and aging HIV-uninfected and HIV-infected groups is provided in Supplementary Figure S3. In general, we found that statistically significant differences were limited due to small patient groups and the high overall variability in microbiome profiles.

Impact of ART

Three patients in the study were taking ART at the time of saliva sample collection, and this group represented a mixture of young adult and aging HIV-infected women. The bacterial community structure of this group appeared similar to that of untreated HIV-infected individuals, but showed even higher levels of *Prevotella* (Fig. 3) that approached statistical significance ($p = .059$) when compared with all HIV-uninfected controls. We also noted that one HIV-infected patient on ART showed markedly high proportions of *Neisseria* and *Haemophilus spp.* (Supplementary Fig. S2). Of additional note perhaps (see Discussion) in women receiving ART, was the detection of statistically significant increases in the abundance of two low-level genera, *Oribacterium* and *Moryella*, as compared with HIV-uninfected patients (Supplementary Fig. S3).

Impact of opportunistic oral infections

In contrast to young adult and aging HIV-infected women without coinfection, patients with KSV and HPV infection at the time of sample collection showed reduced abundance of *Prevotella* compared with HIV-uninfected women (Fig. 3). HIV-infected women with oral candidiasis displayed increased growth of both *Prevotella* and *Porphyromonas*. We were intrigued to find that, although representative of only a single patient, oral HPV coinfection was associated with a striking reduction of bacterial diversity. In this case, the community was dominated by outgrowth of *Streptococcus spp.* that encompassed more than 80% of the total population.

Further evaluation by PCoA revealed no significant taxonomic clustering when considering HIV status (Fig. 4A), aging (Fig. 4B, C), or opportunistic oral infections (Fig. 4D). However, when evaluating multiple variables, we detected a statistically significant difference between the microbiome of

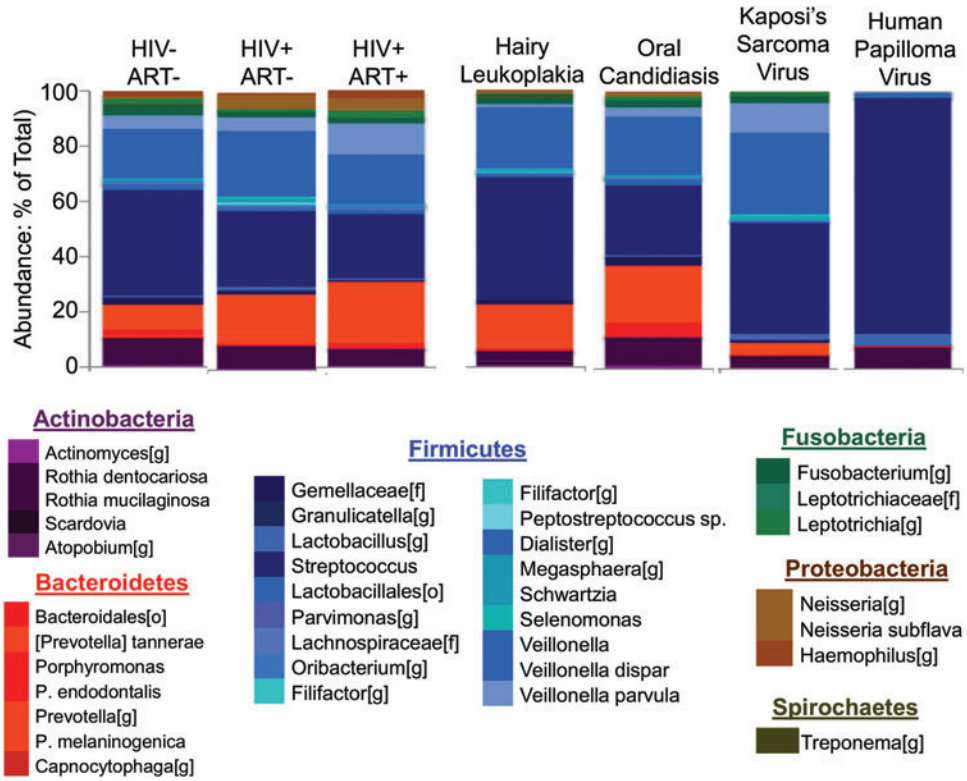

FIG. 3. Impact of ART and opportunistic oral infections on the salivary microbiome. Bar graphical representation of the mean phylogenetic distribution of bacteria comprising at least 0.5% of the total bacterial community in the saliva of untreated HIV-infected women, HIV-infected women on long-term ART, and HIV-infected women with diagnosed oral opportunistic infections. ART, antiretroviral therapy. Color images are available online.

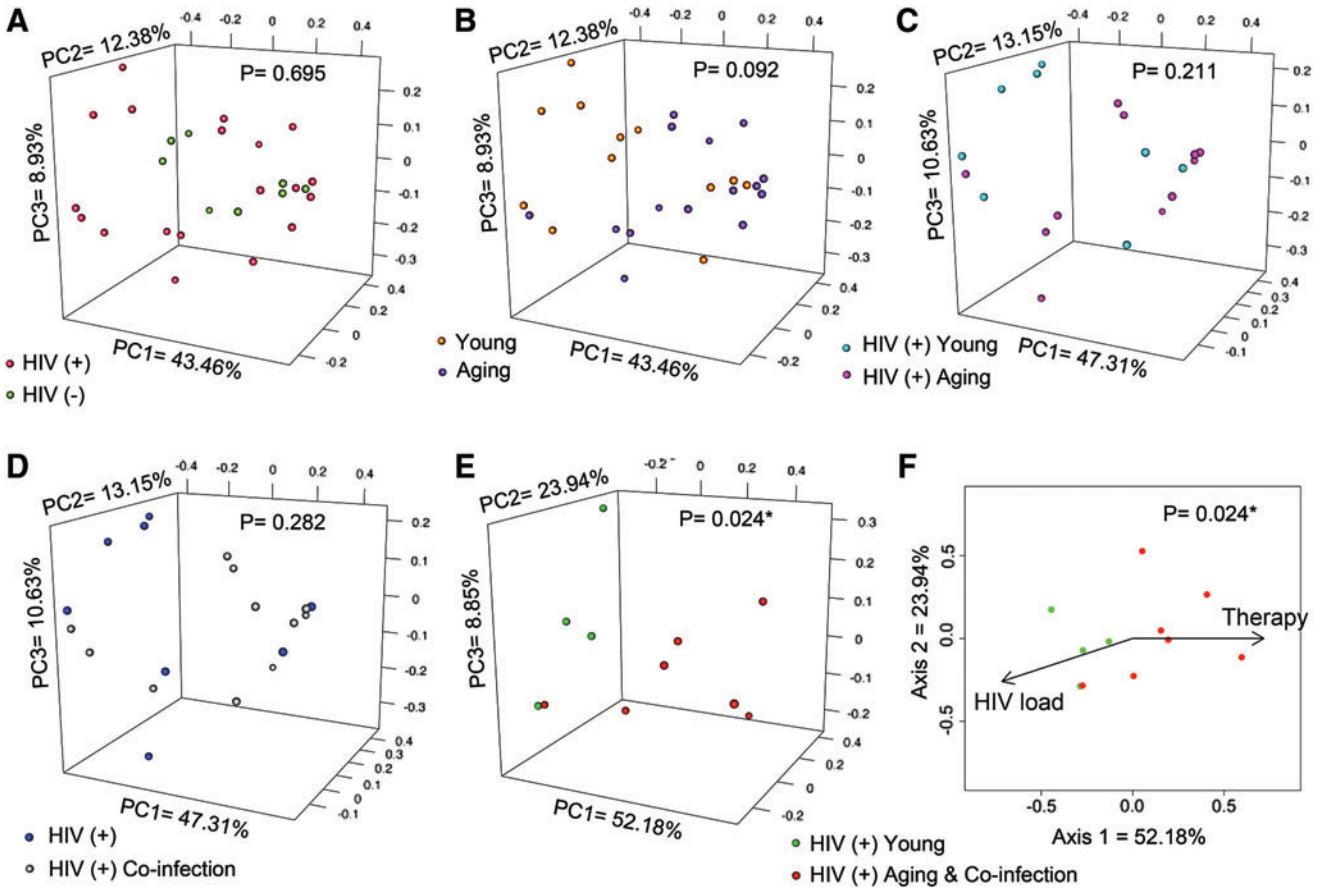

FIG. 4. Principal coordinates analysis (PCoA). PCoA was utilized to evaluate differences in bacterial community structure. Microbiome structure was not significantly different based on HIV incidence, age (in both HIV-infected and HIV-uninfected groups), or by coinfection **(A–D)**. However, significant clustering ($p = .024$) was observed among HIV-infected subjects when age and coinfection status were considered collectively **(E)**. Additionally among these two groups, HIV RNA loads significantly correlated **(F)** with young adult HIV-infected women ($p = .013$), whereas therapy status correlated significantly with HIV-positive aging subjects with coinfection ($p = .012$). Color images are available online.

aging HIV-infected women with oral coinfections versus young adult HIV-infected women without coinfection (Fig. 4E). This discordance was also observed when HIV RNA loads ($p = .013$) and therapy status ($p = .012$) were considered collectively (Fig. 4F). While the relevance of taxonomic differences between these particular subgroups is unclear within the scope of this study, it may hint of a broader pattern of oral dysbiosis in HIV-infected patients that are susceptible to opportunistic oral disease.

Utilizing linear discriminant analysis, we found a significantly higher abundance of *Veillonella parvula* and *Bulleidia moorei* (a.k.a. *Solobacterium moorei*) in the young HIV-infected group without coinfection (Fig. 5), whereas two gut-associated species, *Mucispirillum schaedleri* and *Lactobacillus reuteri*, were more prevalent in aging HIV-infected women with coinfections. Although the relevance of these differences could not be ascertained in the scope of the present study, it is interesting to note that *V. parvula* has been identified as a member of the ''purple complex'' of species (*Actinomyces odontolyticus and V. parvula*) associated with oral health.[42] Thus, it may be warranted in future investigations to determine whether a loss of salivary *V. parvula* is consistently linked with aging and oral coinfections in HIV-infected patients.

To provide further insights into the influence of HIV infection, aging, and opportunistic oral infections on viral and fungal components of the oral microbiome, we analyzed levels of EBV and *C. albicans*, respectively, in saliva samples by quantitative PCR. Our findings suggest that HIV infection was linked to significant increases in the level of EBV in young adult women, and a similar elevation in a smaller subsection of older women (Fig. 6A). In contrast, the abundance of salivary *C. albicans* was not significantly different between aging and young adult HIV-infected and HIV-uninfected women, including the two HIV-infected patients with diagnosed *Candida* infections (Fig. 6B). Our inability to detect a higher abundance of *C. albicans* in these two patients may reflect limitations of the assay itself, or could be potentially due to discordance between the species or strain actually causing the infection and the reference strain that was chosen.

Cumulative impact of aging, HIV infection, and lifestyle choices

In addition to the effects of aging, HIV infection, and the presence or absence of opportunistic oral infection, we were

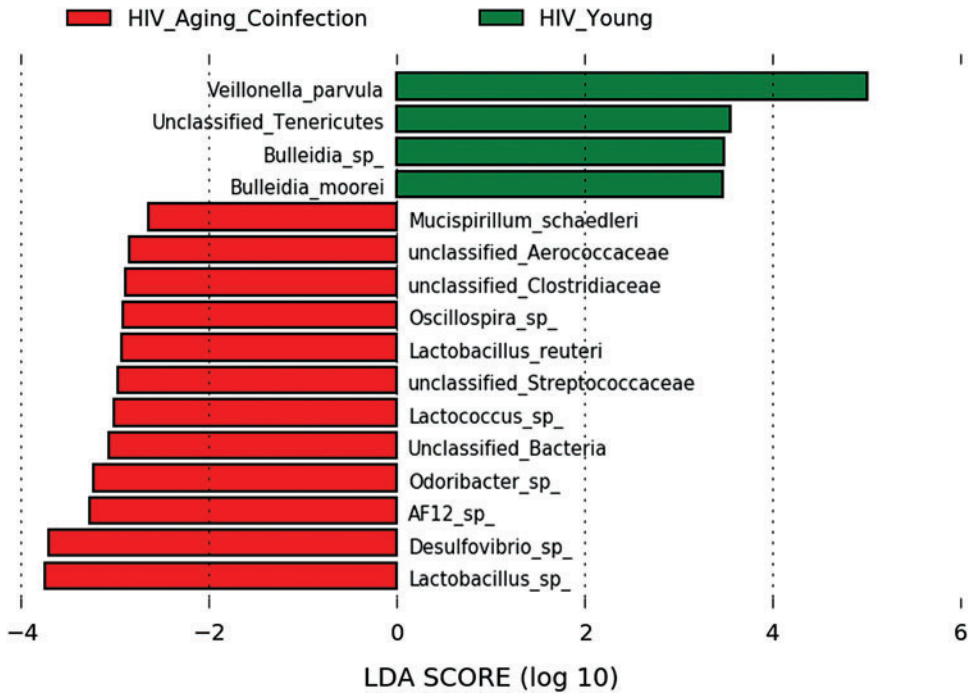

FIG. 5. Linear discriminant analysis effect size (LEfSe). LEfSe was performed to identify statistically different bacterial taxa in their relative abundance between young adult HIV-infected women versus aging HIV-infected women with coinfection. Kruskal–Wallis test was used to process dataset while LEfSe alpha values were set at 0.05 and logarithmic linear discriminant analysis score >2 for discriminative feature filtering. Color images are available online.

interested in evaluating the potential influence of smoking and drinking on the oral microbiome of experimental and control groups. Details on the amount of smoking and alcohol consumption were provided for a subsection of our WIHS cohort, and we comprehensively evaluated correlations between these metadata; patient age, $CD4^+$ and $CD8^+$ T cell counts, HIV, EBV, and *Candida* burden, and modulations in the salivary microbiome, including alpha diversity calculations. A cumulative summary of these data is shown in a heat map format in Figure 7.

Our findings indicate that increased aging was associated with increasing abundance of salivary *Oribacterium* and *Moryella* in HIV-uninfected study participants ($p = .04$, .024, respectively). A negative correlation was also detected in the same group between age and *Lautropia* counts ($p = .023$). We also noted that *Lautropia* prevalence and age were negatively correlated in HIV-infected women ($p = .038$), in addition to *Veillonella* ($p = .005$).

Focusing on clinical parameters, we found that peripheral $CD4^+$ T cell counts correlated positively with abundance profiles of *Streptococcus* and *Lactobacillus* in saliva (Fig. 7) across all study participants ($p = .04$), and with *Lactobacillus* alone when considering only HIV-infected patients ($p = .02$). In HIV-uninfected controls (both age groups), increasing

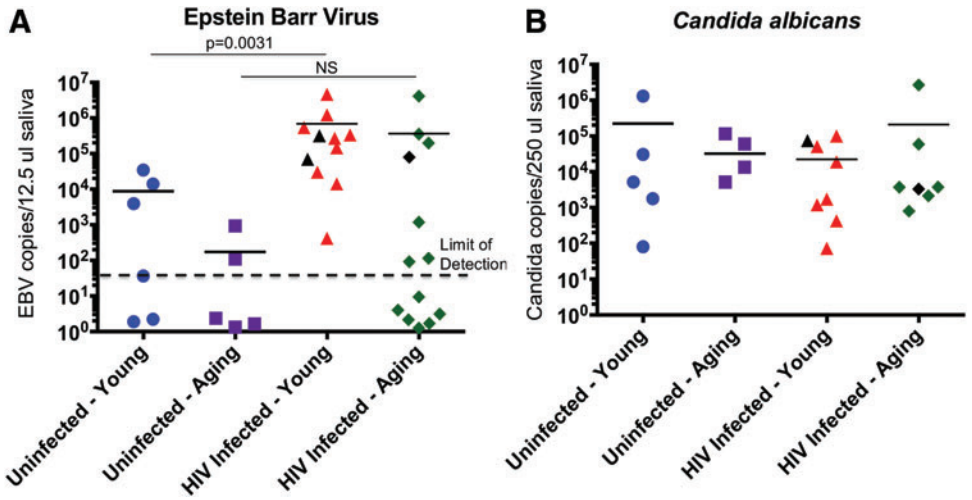

FIG. 6. Comparison of salivary EBV and *Candida albicans* levels in aging and young adult HIV-infected individuals and HIV-uninfected women. The quantities (DNA copy numbers) of **(A)** EBV and **(B)** *C. albicans* in saliva were determined by quantitative polymerase chain reaction. A statistically significant increase in EBV was observed in the HIV-infected young adult group in comparison to age-matched HIV-uninfected women. No significant change in C. albicans was detected between experimental and control groups. EBV, Epstein–Barr virus. Color images are available online.

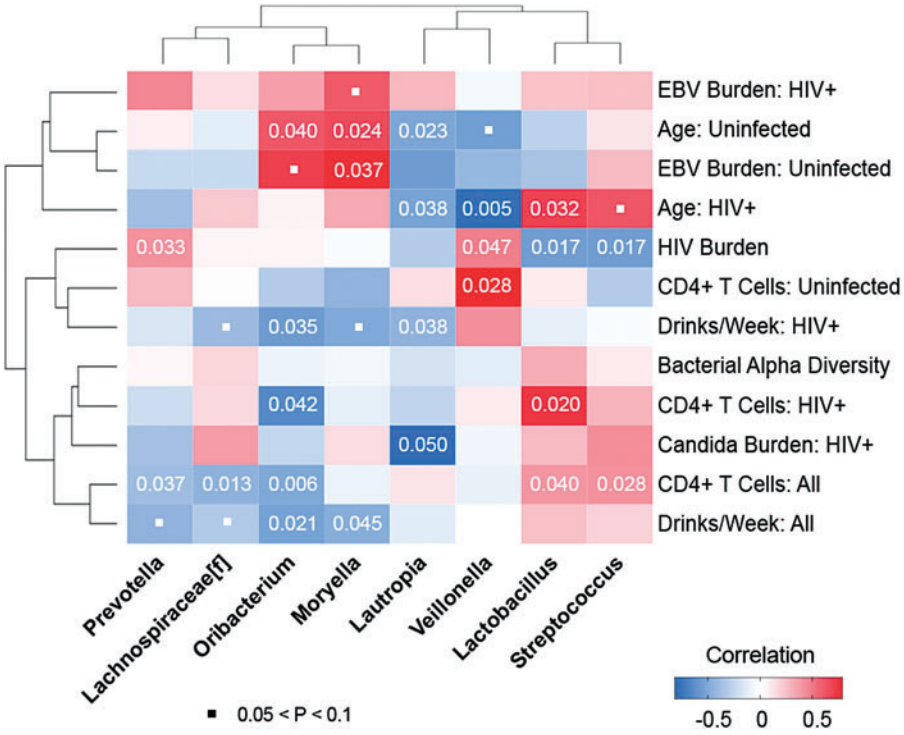

FIG. 7. Correlation matrix of relationships between salivary dysbiosis, age, clinical status, and alcohol consumption. Correlative relationships were determined between age, plasma HIV RNA, peripheral CD4$^+$ T cell depletion, salivary EBV and *C. albicans* copy numbers, alcohol consumption, and the predominant genus-level changes in the salivary microbiome of HIV-infected patients. Positive correlations are shown in *red* and negative correlations in *blue* with increasing color intensity corresponding with decreasing *p*-values. All of the bacterial genera (X-axis) showing statistically significant correlations to one or more of the study parameters (Y-axis) described above are depicted. Correlations were determined by Spearman's rank correlation coefficient test. Statistically significant (<0.05) *p*-values are shown. <.01. *White squares* are used to highlight trends toward correlation, where .05 < *p-value* < .2. Color images are available online.

CD4$^+$ T cell numbers were associated with higher proportions of *Veillonella* ($p = .028$). In contrast, peripheral CD4$^+$ T cells were linked negatively across all study participants to decreasing abundance of *Oribacterium* ($p = .006$), *Prevotella* ($p = .037$), and *Lachnospiraceae* ($p = .013$).

We also detected potential relationships between salivary microbiome profiles and plasma HIV RNA levels (Fig. 7). *Lactobacillus* and *Streptococcus* abundance correlated negatively with plasma HIV RNA (Fig. 7; $p = .017$, in both cases). Intriguingly, this pattern directly contrasted the positive correlation observed between the same genera and circulating CD4$^+$ T cell counts. On the other hand, increasing amounts of HIV RNA in blood correlated with higher proportions of salivary *Veillonella* ($p = .047$) and *Prevotella* ($p = .033$).

In our previous comparisons of the differences in the mean proportions of salivary *Prevotella spp*. between HIV-infected and HIV-uninfected groups, we detected consistent increases associated with infection that did not reach statistical significance thresholds (Supplementary Fig. S3). Because the inclusion of correlative analysis appeared to shine additional light on such differences, we decided to examine correlations between the prevalence of Prevotella and other bacterial genera in the salivary community. Notably, these data showed that across all patient samples, higher levels of *Prevotella* were associated with increased percentages of *Veillonella*, *Moryella*, and *Oribacterium* (Supplementary Fig. S4A–C). In contrast to this potential costimulatory profile, *Prevotella* abundance correlated strongly ($p = .0004$) with decreasing prevalence of oral *Streptococcus spp.* (Supplementary Fig. S4D).

Discussion

Our findings of HIV-associated salivary dysbiosis in the current study extend to previous investigations of the tongue,[43] periodontal,[44,45] and salivary[23,46] microbiome in HIV-infected individuals. We note that saliva expectoration not only provides a complete view of the oral cavity microbiome, something more frequently employed tongue and buccal swabs do not, but it is also less expensive and invasive than stool or biopsy sampling. Because of this, it is an advantageous method for serialized sampling and long-term studies. In the current investigation, we examine and compare HIV-related changes in aging and young adult female cohorts, helping to fill an important gender gap that prevails in much of the history of HIV research. In addition, our study bridges correlations between virologic, immunologic, and oral microbiome parameters that provide a more comprehensive and holistic view of patient condition.

It is noteworthy that the increased abundance of salivary *Prevotella* we observed in young and aging HIV patient groups and individuals taking ART is in accordance with some previous findings from both the gut[47] and oral cavity.[48,49] Although not yet investigated vigorously for oral species, increased growth of *Prevotella* species in the gut has also been linked to increased systemic inflammation in rheumatoid arthritis.[50] Further large-scale microbiome sequencing studies will help to determine if these conditions are also consistently associated with outgrowth of oral *Prevotella spp.* in HIV infection, and whether aging patients may be intrinsically more susceptible. Such studies are becoming more economically feasible due to rapidly decreasing sequencing costs, and are further advantaged through the ease by which the oral cavity can be sampled noninvasively multiple times per day.

It is also notable that, despite small group numbers, we detected statistically significant increases in *Moryella* and *Oribacterium* in aging HIV-uninfected women as well as both young and aging HIV-infected patients compared with

young adult HIV-uninfected patients. While very little is known about these oral bacterial genera, they both are considered opportunistic pathogens and have been linked to dysbiotic profiles observed in tuberculosis patients.[51] Because oral tumors, especially those that are glycolytic or hypoxic, are known to consume acetate at a rapid rate, it is also interesting to note that both *Moryella* and *Oribacterium* ferment glucose and produce acetate as a metabolic byproduct. Further studies may be warranted to determine if increased acetate production resulting from higher abundance by specific *Moryella* and *Oribacterium* species plays a significant role in the increased manifestation of oral cancers in immunosuppressed HIV-infected individuals.

Interestingly, like older HIV-infected patients, patients who reported taking ART showed increased proportions of *Moryella* and *Oribacterium* as well as higher abundances of *Prevotella* and *Lautropia* in the salivary microbiome. Our findings of oral dysbiosis in treated patients are in agreement with previous reports indicating that, although ART decreases viral titers and increases CD4 T cell counts, it does not lead to restoration of a healthy microbiome.[52–54] Li *et al.*, recently reported positive correlations between $CD4^+$ T cell counts and levels of oral *Streptococcus*, and showed that the prevalence of several potentially pathogenic *Prevotella*, *Campylobacter*, *Fusobacterium*, *Capnocytophaga*, *Actinomyces*, and *Atopobium*, was increased 6 months after initiation of ART.[48] Collectively, the observations of dysbiosis in patients with a successful clinical response to ART suggest that a novel, more holistic and systemic therapeutic strategy may provide substantial added benefit, especially for the growing population of aging HIV-infected individuals. One attractive and relatively inexpensive approach could involve combining an aggressive ART regimen with administration of pre and probiotic formulations[55] validated to restore and maintain healthy colonization of the oral and gut microbiome.

In summary, the patterns of dysbiosis we have observed in the salivary microbiome of women enrolled in the WIHS cohort reveal an increased abundance of potentially pathogenic bacteria associated with HIV infection and plasma HIV RNA, aging, ART administration, and occurrence of opportunistic oral infections. Our findings are consistent with previous microbiome studies of HIV-infected patients and support the prevailing hypothesis that chronic dysbiosis may contribute to local and systemic inflammation. Additional studies are required to determine the influence of oral dysbiosis on HIV disease progression and the development of associated non-AIDS conditions. Ultimately, large-scale longitudinal metagenomic investigations will be necessary to fully understand the complex relationship between aging and HIV infection on phylogenetic and functional changes in the oral microbiome.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Table S1

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