The oral microbiome in human immunodeficiency virus (HIV)-positive individuals

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Human immunodeficiency virus (HIV) infection is associated with a range of oral conditions, and increased numbers of disease-associated microbial species have previously been found in HIV-positive subjects. The aim of this study was to use next-generation sequencing to compare the composition of the oral microbiome in HIV-positive and -negative individuals. Plaque and saliva were collected from 37 HIV-positive individuals and 37 HIV-negative individuals, and their bacterial composition determined by pyrosequencing of partial 16S rRNA genes. A total of 855,222 sequences were analysed. The number of species-level operational taxonomic units (OTUs) detected was significantly lower in the saliva of HIV-positive individuals (mean 5303.3) than in that of HIV-negative individuals (mean 5365.5) (P < 0.0003). Principal coordinates analysis (PCoA) based on community membership (Jaccard index) and structure (Yue and Clayton measure of dissimilarity) showed significant separation of plaque and saliva samples [analysis of molecular variance (AMOVA), P < 0.001]. PCoA plots did not show any clear separation based on HIV status. However, AMOVA indicated that there was a significant difference in the community membership of saliva between HIV-positive and -negative groups (P = 0.001). Linear discriminant analysis effect size revealed an OTU identified as \( \text{Haemophilus parainfluenzae} \) to be significantly associated with HIV-positive individuals, whilst \( \text{Streptococcus mitis/HOT473} \) was most significantly associated with HIV-negative individuals. In conclusion, this study has confirmed that the microbial composition of saliva and plaque is different. The oral microbiomes of HIV-positive and -negative individuals were found to be similar overall, although there were minor but significant differences in the composition of the salivary microbiota of the two groups.

INTRODUCTION

There were an estimated 35 million people infected with the human immunodeficiency virus (HIV) at the end of 2013 and AIDS-related illnesses were responsible for 1.5 million deaths in a single year (Joint United Nations Programme on HIV/AIDS, 2014). Wider and improved access to highly active antiretroviral therapy (HAART) in recent years has led to an increase in life expectancy of HIV-infected individuals (World Health Organization, 2013).

HIV infection is associated with a number of oral conditions linked to lowered CD4+ T-cell counts. These include opportunistic infections by \( \text{Candida} \) spp. (candidiasis) and by viruses such as herpes simplex virus, varicella-zoster virus, Epstein–Barr virus and human papilloma viruses (Reznik, 2005). Oral candidiasis is a
particularly common problem in HIV-infected individuals, although the introduction of HAART has led to a decline in its incidence (Hodgson et al., 2006). Infection with HIV is also associated with the periodontal diseases: linear gingival erythema, necrotizing ulcerative gingivitis (NUG) and acute necrotizing periodontitis (ANP) (Mataftsi et al., 2011). NUG and ANP typically have a sudden onset and can cause severe pain. ANP is characterized by a rapid destruction of periodontal tissue, which may result in tooth loss. Whilst a number of studies have investigated the bacteria associated with these forms of periodontal disease (Aas et al., 2007; Murray et al., 1989; Paster et al., 2002; Ramos et al., 2012; Rams et al., 1991), relatively few studies have compared the normal oral microbiome in HIV-infected and uninfected individuals. One study used the Human Oral Microbe Identification Microarray (HOMIM) to compare the proportions of approximately 300 of the most prevalent oral species on the dorsal surfaces of the tongues of antiretroviral-treated and untreated HIV-infected individuals, and an uninfected control group (Dang et al., 2012). The authors detected significantly increased proportions of pathogenic/disease-associated species, including Campylobacter concisus, Campylobacter rectus, Prevotella pallens and Megasphaera micronuciformis, in the untreated HIV-infected group relative to the uninfected control group (Dang et al., 2012). Another study using HOMIM, denaturing gradient gel electrophoresis and culture compared the salivary microbiota in HIV-positive and HIV-negative individuals, and monitored the changes in microbial diversity after HAART in the HIV-positive group (Li et al., 2014). The authors found that the microbial diversity of saliva was lower in the HIV-positive group than in the HIV-negative group, and that diversity was reduced following HAART treatment.

A recent study used an open-ended deep sequencing approach to characterize the composition of both the oral mycobiome and bacteriome in 12 HIV-infected and 12 uninfected individuals (Mukherjee et al., 2014). The authors reported no significant differences in the bacterial community composition of oral rinse samples from these groups. However, they revealed differences in the fungal communities (mycobiome). In particular, Pichia species were associated with uninfected individuals and had a negative correlation to the presence of Candida species. The authors also showed that Pichia spp. could inhibit Candida growth in vitro. Whilst the authors reported that the bacteriomes of HIV-infected and uninfected individuals were similar, it is possible that differences in specific habitats such as tooth surfaces were overlooked, as a single oral rinse sample was used for each individual. In addition, the number of participants in each group was relatively low.

The aim of this study was to use next-generation sequencing to compare the composition of oral bacterial communities present in plaque and saliva of HIV-positive and -negative individuals.

**METHODS**

**Study groups.** Ethical approval for this study was granted by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand, and written informed consent was obtained for all of the participants. A group of HIV-positive patients was recruited for cross-sectional comparison to a group of HIV-negative subjects (Tables 1 and S1, available in the online Supplementary Material). The groups were matched for age and gender and all of the subjects were non-smokers who had not used antibiotics during the 6 months prior to sampling. All of the individuals were Thai who lived in Bangkok and visited Chulalongkorn University dental clinics for dental care. The subjects did not have any recent history of oral lesions (6 months prior to sampling), oral mucositis, active caries involvement or dental implants. HIV-positive subjects had been infected for at least 10 years prior to sampling and had been undergoing antiretroviral therapy for at least 5 years. The HIV viral load of the HIV-seropositive patients was determined using quantitative real-time PCR (Roche Molecular Systems) and expressed as the number of viral copies ml⁻¹. The number of CD4⁺ cells of the HIV-positive subjects was determined by flow cytometry with a Becton Dickinson instrument and expressed as cells mm⁻³.

**Sample collection.** Dental plaque and saliva were collected from both groups. Probing for pocket depth and bleeding was performed after saliva collection, but before plaque collection, during the same visit. Plaque samples were taken using a sterile curette from the buccal surfaces of three teeth (one molar, one premolar and one incisor) in the quadrant containing the sampling tooth/site, the adjacent tooth was used. There was no evidence of bleeding on probing at the sites used for sampling plaque. Two millilitres of saliva was collected by expectoration into 10 ml sterile tubes. Plaque and saliva samples were stored at −70 °C and processed within 24 h of collection.

**DNA extraction.** DNA was extracted from saliva and plaque using the GenElute Bacterial DNA Extraction kit (Sigma-Aldrich), with an additional lysis step to increase the recovery of Gram-positive bacteria. The samples were incubated with 45 mg lysozyme ml⁻¹ solution at 37 °C for 30 min.

**16S rRNA gene PCR and 454 pyrosequencing.** The protocol used as described previously (Kistler et al., 2013) with some minor modifications. PCR amplification of a fragment of the 16S rRNA gene, approximately 500 bp in length covering the V1-V3 hypervariable regions, was performed using composite fusion primers. The fusion primers comprised the broad-range 16S rRNA gene primers 27F and 518R. The protocol used as described previously (Kistler et al., 2013) with some minor modifications. PCR amplification of a fragment of the 16S rRNA gene, approximately 500 bp in length covering the V1-V3 hypervariable regions, was performed using composite fusion primers. The fusion primers comprised the broad-range 16S rRNA gene primers 27F and 518R.

<table>
<thead>
<tr>
<th>Table 1. Summary of the study groups</th>
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<tr>
<td><strong>No. of subjects</strong></td>
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<tr>
<td><strong>HIV-positive</strong></td>
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<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Mean (±SD) age (years)</td>
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<tr>
<td>44 (±10)</td>
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<tr>
<td>Male : female</td>
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<tr>
<td>18:19</td>
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<tr>
<td>Mean CD4⁺ T-cell count (±SD)</td>
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<tr>
<td>(cells mm⁻³)</td>
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<td>510 (±232)</td>
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NA, Not applicable.
FYM (Frank et al., 2008) and 519 R (Lane et al., 1985) along with Roche GS-FLX Titanium Series adaptor sequences (A and B) for 454 pyrosequencing using the Lib-L emulsion-PCR method. Previously described 12-base error-correcting ‘Golay’ barcode sequences (Fierer et al., 2008) were incorporated into the forward primers to enable multiplexing of samples in the same sequencing run. The appropriate barcoded forward primer and reverse primer were used in PCRs along with Extensor High-Fidelity PCR Mastermix (Thermo-Scientific). The PCR conditions were as follows: 5 min initial denaturation step at 95 °C, followed by 25 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s and a final extension of 72 °C for 5 min. PCR amplicons were then purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions. The size and purity of the amplicons were checked using the Agilent DNA 1000 kit and the Agilent 2100 Bioanalyzer. Quantification of the amplicons was performed by means of a fluorometric assay using the Quant-IT Picogreen fluorescent nucleic acid stain (Invitrogen). The amplicons were then pooled together at equimolar concentrations (1 × 10⁹ molecules µl⁻¹). Emulsion-PCR and unidirectional sequencing of the samples were performed using the Lib-L kit and the Roche 454 GS-FLX+ Titanium series sequencer by the Department of Biochemistry, Cambridge University, Cambridge, UK.

Sequence analysis. Sequence analysis was performed using the ‘mothur’ software suite version 1.33 (Schloss et al., 2009), following the 454 standard operating procedure (Schloss & Westcott, 2011) on http://www.mothur.org. The sequences were denoised using the AmpliconNoise algorithm, as implemented by mothur. Sequences that were less than 440 bases in length and/or had one of the following: w 2 mismatches to the primer, w 1 mismatch to the barcode regions, and homopolymers of w 6 bases in length, were discarded. The remaining sequences were trimmed to remove primers and barcodes and aligned to the SILVA 16S rRNA gene reference alignment. The UChime algorithm was used to identify chimeric sequences, which were then removed from the dataset. Sequences were clustered into operational taxonomic units (OTUs) at a genetic distance of 0.015 using the average neighbour algorithm and identified using a naive Bayesian classifier with the Human Oral Microbiome Database (HOMD) reference set (version 13). A distance of 0.015 was chosen because many named oral bacterial species have high sequence identity in their 16S rRNA genes, particularly among the streptococci, Actinomyces and Neisseria. Therefore, a number of different species are likely to be combined into the same OTU when applying the more commonly used distance of 0.03. In addition, species-level phylogenotypes in the HOMD have been defined based on a 98.5 % 16S rRNA gene sequence identity cut-off (Dewhirst et al., 2010). For those OTUs that could not be identified using the Bayesian classifier, representative sequences were obtained in mothur (the sequence with the smallest distance to all other sequences in that OTU) and identified using BLAST against the HOMD reference set. The possible alternatives for the species identification were then provided. In order to perform OTU-based comparisons of bacterial diversity, the number of sequences in each sample was normalized by subsampling down to the number of sequences equal to that of the sample with the lowest number. The diversity of the communities was estimated and compared in mothur using Simpson’s inverse diversity index (Simpson, 1949). Good’s non-parametric coverage estimator was used to determine how well sampled the communities were (Good, 1953). The beta-diversity of the samples was analysed using distance matrices generated using the Jaccard index for community membership and the Yue and Clayton measure of dissimilarity (thetaYC calculator) for community structure (Yue & Clayton, 2005). The distance matrices were visualized using dendrograms and principal coordinates analysis (PCoA) plots. Three-dimensional PCoA plots were generated in R (http://www.r-project.org) using the ‘rgl’ package.

Statistical analysis. Analysis of molecular variance (AMOVA) (Excoffier et al., 1992), as implemented by mothur, was used to determine if there were any statistically significant differences between groups and sample types in the distance matrices based on the Jaccard and thetaYC indices. Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was used to identify OTUs that were significantly differentially abundant between saliva and plaque and between HIV-positive and -negative groups. The alpha values for the factorial Kruskal–Wallis and pairwise Wilcoxon tests were set to 0.05 and the LDA score threshold for discriminative features was set to 3.5. Two sample t-tests were performed in R to determine if there were any significant differences in OTU richness and diversity between HIV-positive and -negative groups. Correlations between the relative abundances of OTUs and metadata for the HIV-positive group, including CD4⁺ cell counts and subject age, were determined using Pearson’s rank correlation coefficient as implemented by mothur in the ‘otu.association’ command; OTUs that were detected in less than 50 % of the saliva or plaque samples were not included in the analysis.

RESULTS

Pyrosequencing

A total of 855 222 sequences were included in the final analysis after filtering and removal of chimeric sequences. This resulted in a mean of 5980 per sample (range: 3583–9464). Five samples were not included in the pyrosequencing run due to poor PCR amplification. This resulted in a final total of 36 plaque and 36 saliva samples in the HIV-positive group and 35 plaque and 36 saliva samples in the HIV-negative group for comparison. The mean numbers of quality-filtered sequences per sample were 6355 (range: 3587–9245) and 5599 (range: 3583–9464) in the HIV-positive and -negative groups respectively.

Composition of plaque and saliva samples

The sequences were assigned to a total of 12 phyla. The predominant phyla in both plaque and saliva were Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. Other phyla detected, but not in every sample, included Chloroflexi, GN02, SR1, Spirochaetes, Synergistetes, TM7 and Tenericutes. The relative abundances of the predominant phyla in plaque and saliva are shown in Fig. S1. A total of 168 different genera were detected among all of the samples. The predominant genera in plaque (constituting > 5 % mean relative abundance) were Streptococcus, Corynebacterium, Prevotella, Capnocytophaga, Actinomyces, Veillonella and Selenomonas, whilst in saliva the predominant genera were Streptococcus, Prevotella, Veillonella and Rothia. The bacterial composition of all the samples (classified to species level where possible) is shown in Dataset S1.

Comparisons of OTU richness and diversity

Table S2 shows the OTU richness and diversity (Simpson’s inverse diversity index) of each of the plaque and saliva samples included in the analysis (when subsampled to 3583 sequences per sample). The mean number of OTUs
detected in the saliva of the HIV-positive individuals was significantly lower than in the HIV-negative individuals (two sample \( t \)-test, \( P<0.0003 \)) (Fig. 1a). Two saliva samples from HIV-positive individuals had a particularly reduced richness with 180 and 184 OTUs detected in each sample. Interestingly, the same individuals had the lowest richness in their plaque samples (141 and 177 OTUs). There was, however, no significant difference \( (P=0.058) \) between the mean number of OTUs detected in the plaque samples of the HIV-positive and -negative groups (Fig. 1a). In addition, there were no significant differences in the Simpson's inverse diversity index of saliva or plaque samples of the two groups. The mean diversity of plaque samples was higher than that of saliva samples (Fig. 1b).

**Comparisons of bacterial community membership and structure**

The community membership and structure of plaque and saliva samples from HIV-positive and -negative groups was compared using PCoA plots. Comparisons of samples from both HIV-positive and negative groups in PCoA plots revealed clear separation of plaque and saliva (Fig. 2). AMOVA tests confirmed that saliva and plaque were highly significantly different \( (P<0.001 \) for both membership and structure). The plots did not show a clear separation/difference between the HIV-positive and -negative groups for either the plaque or saliva samples (Fig. 3). AMOVA confirmed that there was no significant difference in community structure between the groups. However, AMOVA indicated that there was a significant difference between the community membership of saliva, but not plaque, of HIV-negative and -positive subjects \( (P=0.001) \) (Fig. 4).

Comparison using LEfSe revealed 121 OTUs that were significantly differentially abundant between saliva and plaque samples. The differentially abundant OTUs with LDA scores of \( \geq 3.5 \) are shown in Fig. 5. The OTU most significantly associated with saliva was identified as *Streptococcus salivarius/Streptococcus vestibularis*, whilst a *Corynebacterium matruchotii* OTU was most significantly associated with plaque. Comparison of the saliva of HIV-positive subjects to that of HIV-negative subjects revealed 116 OTUs that were significantly differentially abundant. However, only three OTUs had LDA scores of \( \geq 3.5 \) and many of the associated OTUs were detected at low relative abundances. Of the OTUs with LDA scores of \( \geq 3.5 \), a *Haemophilus parainfluenzae* OTU was associated with HIV-positive individuals, whilst a *Streptococcus mitis/Streptococcus pneumoniae/HOT473* OTU and a *Streptococcus mitis* bv.2 OTU were associated with HIV-negative individuals. LEfSe comparison of plaque from HIV-positive and -negative individuals was not performed, as there was no significant difference in community membership or structure by AMOVA.

**Correlation of OTUs with CD4\(^+\) cell counts and subject age**

The OTUs that showed a statistically significant correlation (Pearson's rank correlation coefficient) with CD4\(^+\) T-cell counts and with subject age in the HIV-positive group are shown in Tables S3 and S4. For both plaque and saliva samples, stronger correlations were obtained with age than with the CD4\(^+\) cell counts. The OTUs that showed the strongest negative correlations with CD4\(^+\) cell counts were OTU 30 *Alloprevotella tannerei* \( (\rho=0.42, P<0.009) \) and OTU 373 *Eubacterium yurii* \( (\rho=0.45, P<0.004) \) in saliva and plaque, respectively. The OTUs

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Fig. 1. Bar charts comparing the mean species-level OTU richness (a) and Simpson's inverse diversity index (b) in plaque and saliva samples of the HIV-positive and -negative groups. The saliva of HIV-positive individuals had a significantly lower richness than the saliva of HIV-negative individuals (indicated by the asterisk) using a two-sample \( t \)-test. There was no significant difference in Simpson's diversity index between groups. The error bars indicate SEM.
**Fig. 2.** PCoA plots comparing plaque and saliva from HIV-positive and -negative individuals based on their community membership (Jaccard index) (a) and structure (thetaYC calculator) (b). Plaque samples are shown in red and saliva samples are in blue. Plaque and saliva had a highly significantly different community membership and structure by AMOVA ($P<0.001$ for both). The proportion of variance shown for (a) and (b), respectively, was: PC1 = 6.1 % and 18.4 %, PC2 = 2.4 % and 7.3 %, PC3 = 1.5 % and 5.5 %.

**Fig. 3.** PCoA plots comparing plaque and saliva from HIV-positive and -negative individuals based on their community membership (Jaccard index) (a) and structure (thetaYC calculator) (b). Red, saliva from HIV-positive subjects; blue, saliva from HIV-negative subjects; purple, plaque from HIV-positive subjects; green, plaque from HIV-negative subjects. There was a significant difference between the community membership, but not structure, of saliva between groups using AMOVA ($P=0.001$). No other significant differences were detected. The proportion of variance shown for (a) and (b), respectively, was: PC1 = 6.1 % and 18.4 %, PC2 = 2.4 % and 7.3 %, PC3 = 1.5 % and 5.5 %.
DNA–DNA hybridization, Mager et al. (2003) showed that the distribution of 40 selected bacterial species in saliva was most similar to that found on the tongue surfaces, suggesting that saliva predominantly comprises species that are shed from tongue biofilms. In that study, saliva and tongue communities were characterized by increased numbers of Prevotella melaninogenica and Veillonella parvula relative to other oral sites/samples. Next-generation sequencing data from the Human Microbiome Project also showed that saliva samples were highly similar in composition to samples from the tongue dorsum (Segata et al., 2012). Supra- and subgingival plaque had a distinct composition from samples from other oral sites and was characterized by having significantly higher proportions of members of the orders Burkholderiales, Cardiobacteriales, Flavobacteriales, Fusobacteriales, Neisseriales and Pasteurellales. However, the sequences were not classified to the species level.

Comparisons of the community composition of plaque and saliva of HIV-positive and HIV-negative individuals in PCoA plots showed that their bacterial communities were largely similar, supporting the findings of a recent deep-sequencing study (Mukherjee et al., 2014). The plaque samples in the present study were, however, only taken from periodontally healthy sites in both groups. It is therefore possible that there are significant differences in plaque composition between HIV-infected and uninfected individuals at inflamed/diseased sites. Differences in OTU community membership between groups were detected in saliva by AMOVA and a number of species-level OTUs were identified as being significantly differentially abundant. In addition, the saliva of the HIV-positive individuals had a significantly lower OTU richness than that of the HIV-negative individuals. This is in agreement with the findings of a previous study that compared the salivary microbiota of HIV-positive and -negative individuals using culture and other culture-independent methods (Li et al., 2014). The authors reported a decreased microbial diversity (Shannon diversity index) in the saliva of HIV-positive subjects compared with that of the negative control group and also showed that the microbial diversity within the HIV-positive group was reduced following HAART. There is evidence that HAART can reduce salivary flow (Navazesh et al., 2009) and this in turn might affect the richness and composition of the salivary microbiota. A previous study has shown that individuals with reduced salivary secretion have increased numbers of lactobacilli, which are caries-associated, in their saliva compared with a control group (Almstål & Wikström, 1999). All of the HIV-positive patients in the present study were undergoing HAART. The importance of the OTUs that were associated with HIV-positive individuals is unclear as they were found to be relatively common oral commensals species. An H. parainfluenzae OTU was most significantly associated with the HIV-positive group. This commensal species is a member of the HACEK group of Gram-negative bacilli that have been implicated in various opportunistic infections.

**DISCUSSION**

This study has comprehensively characterized the bacterial composition of dental plaque and saliva of HIV-positive individuals, at the species level, and compared it with that of HIV-negative individuals. The results confirmed the findings of previous studies, which showed that saliva and plaque have a different bacterial composition (Segata et al., 2012; Simón-Soro et al., 2013). In addition, the results showed that plaque had a higher bacterial diversity (Simpson’s inverse diversity index) than saliva. Species that were most strongly associated with saliva included salivarius- and mitis-group streptococci, Rothia mucilaginosa and Prevotella melaninogenica whilst species most strongly associated with plaque included Corynebacterium matruchotii, Streptococcus sp. HOT058 and Capnocytophaga granulosa. The hard, non-shedding tooth surfaces on which plaque forms provide a distinct microbial habitat to the shedding mucosal surfaces of the mouth and is thus associated with a differing microbial community structure (Mager et al., 2003; Segata et al., 2012). Using chequerboard analysis, the differences in the composition of plaque and saliva between HIV-positive and HIV-negative individuals were quantified and the results showed that plaque had a lower bacterial diversity than saliva. The authors reported a decreased microbial diversity (Shannon diversity index) in the saliva of HIV-positive subjects compared with that of the negative control group and also showed that the microbial diversity within the HIV-positive group was reduced following HAART.

Fig. 4. PCoA plot comparing saliva from HIV-positive and -negative individuals based on their community membership (Jaccard index). Red, saliva from HIV-positive subjects; blue, saliva from HIV-negative subjects. There was a significant difference in community membership between the two groups by AMOVA ($P=0.001$). The proportion of variance shown is: PC1 = 6.1 %, PC2 = 2.4 %, PC3 = 1.5 %.

most strongly positively correlated with CD4+ T-cell counts were OTU 623 H. parainfluenzae (0.46, $P<0.004$) and OTU 269 Actinomyces sp. (0.50, $P<0.002$) in saliva and plaque, respectively.
infections including endocardial infections, upper respiratory tract infections, brain abscesses and joint infections (Janda, 2013). Whilst *H. parainfluenzae* was associated with the HIV-positive individuals, within the HIV-positive group it was positively correlated with CD4+ cell counts. This suggests that *H. parainfluenzae* does not increase in proportion as the disease progresses and CD4+ cell numbers decline.

In conclusion, this study found that the oral microbiomes of HIV-positive and -negative individuals were similar overall, although minor but significant differences in the composition of the salivary microbiota were detected. The results also confirmed that plaque and saliva have a distinct microbial composition.

**ACKNOWLEDGEMENTS**

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