

Composition of gut microbiota of children and adolescents with perinatal HIV infection taking antiretroviral therapy in Zimbabwe

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Summary: HIV infected African children and adolescents have altered gut microbiota compared to HIV uninfected. ART was significantly associated with a higher alpha diversity, and prolonged ART may restore richness of the microbiota closer to that of HIV uninfected children.

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Abstract

Background: HIV infection causes impairment of the gastrointestinal barrier, with substantial depletion of CD4⁺ T-cells in the gut. Antiretroviral therapy (ART) restores CD4⁺ counts and may have beneficial effects on gut microbiota in adults. Little is known about effect of long-term ART on gut microbiome in HIV infected children. We investigated composition of gut microbiota in HIV infected and uninfected children and assessed associations between gut microbiota and patient characteristics.

Methods: In a cross-sectional study, rectal swabs were collected from 177 HIV infected and 103 HIV uninfected controls. Gut microbial composition was explored using 16S rRNA sequencing (Illumina Miseq).

Results: HIV infected children had significantly lower alpha-diversity and higher beta-diversity compared to HIV uninfected. No association was observed between microbiome diversity and CD4⁺ T-cell count, HIV viral load or HIV-associated chronic lung disease. We found enriched levels of *Corynebacterium* ($p < 0.01$), *Finegoldia* ($p < 0.01$) and *Anaerococcus* ($p < 0.01$) in HIV infected, and enrichment of *Enterobacteriaceae* ($p = 0.02$) in participants with low CD4⁺ counts (< 400 cells/mm³). Prolonged ART-treatment (≥ 10 years) was significantly associated with a richer gut microbiota by alpha diversity.

Conclusion: HIV infected children have altered gut microbiota. Prolonged ART may restore the richness of the microbiota closer to that of HIV-uninfected children.

Keywords: HIV infection; antiretroviral therapy; gut microbiota; children; adolescents; Africa

Introduction

The gastrointestinal (GI) tract plays an important role in the pathogenesis of human immunodeficiency virus (HIV) infection, with the majority of CD4⁺ T-cells residing in the GI-tract and associated lymphatic tissue [1]. HIV-induced depletion of CD4⁺ T-cells causes structural impairment of the GI epithelial barrier, systemic microbial translocation and ultimately alteration of the gut microbial community composition [2].

Recent evidence indicates that HIV-associated gut dysbiosis is characterized by decreased abundance of commensal (protective) bacteria and enrichment of potentially pathogenic taxa [3]. For example, the genera *Pseudomonas*, *Enterobacteriaceae*, *Acinetobacter* and *Campylobacter* are thought to have infectious and inflammatory properties and are enriched in adults with HIV [3, 4].

Studies show altered gut microbiota is associated with elevated circulating inflammatory markers such as C-reactive protein and interleukin-6 [5-8], as well as markers of microbial translocation such as lipopolysaccharide and lipopolysaccharide binding protein [9, 10]. Further, studies suggest ART may only partially restore the gut microbiota towards levels observed in HIV uninfected populations, and patients continue to suffer from dysbiosis even when HIV infection is controlled [1, 11, 12].

Moreover, gut dysbiosis, and associated microbial translocation may drive systemic chronic inflammation which increases the risk of chronic non-infectious HIV complications, such as cardiovascular disease and lung complications [13, 14] 15, 16].

Few studies have investigated the gut microbiome in sub-Saharan African children and its relation to the development of HIV-associated chronic complications. Most studies to date have been performed in adult populations and potentially confounded by sexual preference, and are therefore not directly comparable to our study. The overall aim of our study was to investigate the gut microbiota in HIV infected and HIV uninfected children in Harare (Zimbabwe), and to evaluate the association between gut microbial composition and clinical and laboratory parameters (chronic lung disease, CD4⁺ T-cell count, viral load (VL)).

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Materials and methods

Study population

This study investigated bacterial profiles of rectal swabs collected from participants of the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial [17] (clinicaltrials.gov identifier NCT02426112). Chronic lung disease was defined as forced expiratory volume in one second (FEV₁) z-score less than -1.0 with no reversibility (<12% improvement in FEV₁ after 200 ug of salbutamol inhaled using a spacer). The detailed study protocol has been described previously [17]. For the present sub study only participants enrolled in Harare (Zimbabwe) were included. HIV infected children aged 6-16 years without chronic lung disease, meaning no prior history of heart/lung diseases, tuberculosis (TB), no chronic cough, reported chest pain or shortness of breath during exercise, and HIV uninfected participants were recruited at the same outpatient clinic. These were recruited as comparison groups and not randomized into the trial. The route of HIV transmission was likely perinatal for most of the HIV infected participants. HIV-infected participants had to be stable on ART for at least 6 months, in order to meet eligibility criteria. All study participants completed a detailed questionnaire regarding demographic, socio-economic characteristics and clinical history.

The study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee; Harare Central Hospital Ethics Committee; Medical Research Council of Zimbabwe; The Regional Committee for Medical and Health Research Ethics REC North 2015/1650; and University of Cape Town Human Research Ethics Committee. All

participants and/or legal guardians gave written informed consent to participate in the study.

Sample collection

Rectal swabs were collected from all participants at enrolment into the trial by study nurses. Swabs were immediately preserved in 1,5 ml of transport medium PrimeStore® MTM (Longhorn diagnostics, Maryland, USA), directly stored on ice for maximum one hour, and then frozen at -80 °C before shipment on dry ice to the laboratory at the University of Cape Town (UCT).

DNA extraction

The Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep kit (Zymo Research, California, USA) was used for DNA extractions. DNA was extracted according to the manufacturer's description, with modifications. Briefly, a 400 µl aliquot of each sample was mixed with 400 µl BashingBead™ Buffer in a ZR BashingBead™ Lysis Tube. Mechanical lysis (bead beating) was performed using the TissueLyser LT™ (QIAGEN, Hilden, Germany) set to 50 Hz for 5 minutes. 500 µl of supernatant was transferred to a Zymo-Spin™ III-F Filter (Zymo Research, California, USA) and centrifuged at 8000 x g for 1 minute. Chemical lysis was done by adding Genomic Lysis Buffer. All other procedures were done according to manufacturers protocol.

16S library preparation and gene sequencing

In order to assess DNA quality and total bacterial load, a real-time quantitative polymerase chain reaction (qPCR) was performed as previously described [18].

Subsequently, two PCR sets targeting the V4 hypervariable region of the 16S ribosomal ribonucleic acid (rRNA) gene were performed according to previously described protocols [19, 20] (Supplementary file).

Samples were sequenced on an Illumina Miseq® instrument using the Miseq® Reagent v3 kit, (600 cycles) (Illumina, California, USA). The final library was diluted to a 6 pM concentration, and a 25% PhiX library spike-in was added as internal control [21]. The pre-processing of sequence reads was done using the H3ABioNet 16S rDNA diversity analysis package (<https://github.com/h3abionet/h3abionet16S>) [20], with the exception that taxonomy of representative reads was assigned using the SILVA version 132. Raw sequence files have been submitted to the European Nucleotide Archive (ENA), accession number PRJEB32077.

Data analysis

Statistical analyses were performed in STATA 14 (StataCorp LLC, Texas USA) and R Statistical software (<http://www.r-project.org/>). Characteristics between study groups were compared using Fisher's exact test (for categorical parameters) and Kruskal-Wallis or Wilcoxon rank sum test (for continuous parameters).

Richness of bacterial taxa within a single sample was represented by the number of operational taxonomic units (OTUs) and Chao1 index [22]. Chao1 index uses mark-release-recapture-like ratio to estimate richness by adding a correction factor to the observed number of species. Richness and evenness (relative abundances of the different

species) were characterized by Shannon's index [23]. Alpha diversity measures were calculated at sample depth 4000 reads to include 95% of samples.

Interindividual differences, beta diversity, were determined using Bray-Curtis dissimilarity index [24] with sample depth set at 2000 reads to include 99% of samples. Beta diversity comparisons were explored using Principal Coordinate plots generated by the *stats* package in R. Comparisons were made using Wilcoxon rank sum test where not specified otherwise. We also used Kruskal-Wallis test in cases with more than two groups. The same groups were compared using permutational multivariate analysis of variance (PERMANOVA) in QIIME2 (version 2018.4) [25], with number of permutations set to 999. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [26].

Relative abundance

To assess relative abundance, a linear discriminant analysis was performed using linear discriminant analysis effect size [27] with default settings (alpha values for the statistical test 0.05). To reduce the number of markers, the effect size threshold was set to 1.0 for the plots. Relative abundance comparison plots were generated using the MicrobiomeAnalyst web-based software tool with standard feature filtering [28]. Heatmaps were generated using only the taxa significantly different using linear discriminant analysis effect size comparison (Supplementary figure 1-3). The average fraction of each taxa was calculated from all samples within each group. The data was transformed to fractional abundance (Phyloseq) before performing the linear discriminant

analysis effect size analysis. All p-values reported are corrected for multiple testing using false discovery rate (FDR).

Alpha diversity indices between study groups were compared using Wilcoxon rank-sum test. P-values were corrected for multiple testing using FDR. Spearman's rank correlation with Bonferroni correction was used to assess the association between alpha diversity indices and continuous parameters. We fitted a linear regression model in order to estimate the association between HIV status and alpha diversity indices. BMI, age, and sex were adjusted for a priori. An interaction term between HIV status and antibiotics the three previous months (co-trimoxazole prophylaxis for HIV-infected) was included into the regression model to determine whether antibiotics modify the effect of HIV status on alpha diversity estimates. The association between other participant characteristics and alpha diversity indices was further evaluated in regression analysis stratified by HIV status and adjusted for BMI, age and sex. A two-tailed significance level of 0.05 was used.

Results

Study population

In total 149 HIV infected participants with chronic lung disease, 28 HIV infected participants without chronic lung disease, and 103 HIV uninfected participants were enrolled. All HIV infected participants were on ART, for a median of 6.6 years for those with chronic lung disease and 8.0 for those without. 89% of HIV infected participants were taking co-trimoxazole prophylaxis as per WHO guidelines [29]. No HIV uninfected participants were taking co-trimoxazole. The study group characteristics are presented in Table 1.

HIV infected participants were older compared to the HIV uninfected participants ((15.6 years (IQR 12.8-17.7) vs 9.9 (IQR 7.4-12.7), $p<0.001$)) and were more likely to be stunted and underweight compared to HIV uninfected participants (stunted: 41% vs 5%, $p<0.001$; underweight: 45% vs 5%, $p<0.001$). The proportion of participants who experienced diarrhoeal episodes during the last three months prior to enrolment was also higher in HIV infected than in HIV uninfected group (11% vs 3%, $p=0.03$).

Alpha diversity

Species richness (OTUs, Chao1) was significantly higher in HIV uninfected compared to HIV infected participants. There was no difference in Shannon index between these two study groups (Figure 1, Supplementary table 1). After adjustment for BMI, age and sex using linear regression analysis the negative association between richness indices and HIV status remained significant ($p=0.02$ for OTUs, $p=0.001$ for Chao1 index). The use of antibiotics during the three previous months did not change the significant effect of

positive HIV status for the Chao1 index (Supplementary table 2). HIV infected participants with suppressed VL had borderline higher OTUs (Median (IQR): 192.5 (145.5-228.5) vs 176 (138-220), $p=0.18$) and higher Chao1 index (259.3 (201.2-302.1) vs 233.2 (175-276), $p=0.05$) compared to non-suppressed participants in regression analysis adjusted for BMI, age and sex (Supplementary table 3, 4).

We stratified HIV infected participants based on their time spent on ART (ART $\leq$ 5 years; $n=53$, ART 5-10 years; $n=100$, ART $\geq$ 10 years; $n=23$). When comparing HIV infected participants based on these subgroups, we found that participants who had been on ART $\geq$ 10 years had an alpha diversity similar to the HIV uninfected study group (Table 2).

There was no difference in alpha diversity indices between HIV infected participants with and without chronic lung disease (Supplementary table 5). The same was observed after adjusting for BMI, age and sex using regression analysis. The associations between participant characteristics and alpha diversity indices in HIV infected participants is presented in Supplementary table 4.

Prolonged ART treatment was the only parameter significantly associated with richer gut microbiota after adjustment for age and sex, suggesting a positive effect of prolonged ART. No parameters were found to be significantly associated with alpha diversity estimates in HIV uninfected group (Supplementary table 6).

Beta diversity

We found significantly higher beta diversity amongst HIV infected, compared to HIV uninfected participants ($p < 0.01$) (Figure 2a). ART duration had no impact on beta diversity when stratified by years spent on ART. There was no association between beta diversity and VL suppression, type of ART regimen, time on ART or prior TB in HIV infected participants (Supplementary table 7).

HIV infected participants with chronic lung disease had higher beta diversity compared to both HIV uninfected ($p < 0.01$) and HIV infected participants without chronic lung disease ($p = 0.03$). There was no significant difference between HIV infected participants without chronic lung disease and HIV uninfected ($p = 0.74$) (Figure 2b). Unweighted UniFrac analysis showed similar results.

Relative abundance

We identified 26 different phyla in the rectal swabs from all participants. Only five phyla contributed more than 1% of the total sequences of the entire dataset. Firmicutes (43.9%), Bacteroidetes (33.9%) and Epsilonbacteraeota (9%) (previously within the phylum Proteobacteria), Actinobacteria (5.3%) and Proteobacteria (7.7%), accounted for 99.8 % of the bacteria present.

HIV infected versus HIV uninfected participants

At phylum level HIV infected participants had significantly lower abundance of Epsilonbacteraeota (7%) ($p<0.01$) and Bacteroidetes (32%) ($p<0.01$) compared to HIV uninfected participants (with 13% and 38% respectively) (Supplementary figure 4).

At genus level HIV infected participants had enriched *Corynebacterium* ($p<0.01$), *Lawsonella* ($p<0.01$) and *Collinsella* ($p=0.04$), belonging to the Actinobacteria phylum; while in the Firmicutes phylum, *Finegoldia* ($p<0.01$), *Anaerococcus* ($p<0.01$), *Erysipelotrichaceae* ($p=0.02$) and *Lachnospirillum* ($p=0.04$) were enriched when compared to HIV uninfected.

HIV uninfected, compared to HIV infected participants, were enriched in *Campylobacter* ($p<0.01$), phylum Epsilonbacteraeota; *Porphyromonas* ($p<0.01$) and *Prevotella* ($p=0.03$), phylum Bacteroidetes; *Eubacterium coprostanoligenes_group* ($p<0.01$), *Ruminococcaceae* ($p<0.01$), *Fastidiosipila* ($p<0.01$), *Fournierella* ($p<0.01$), W5053 ($p<0.01$), *Coprococcus* ($p=0.02$) and *Murdochella* ($p<0.01$), phylum Firmicutes (Figure 3).

HIV infected with chronic lung disease had higher abundance of the genus *Faecalibacterium* ($p=0.05$), phylum Firmicutes, compared to participants without chronic lung disease. Participants without chronic lung disease had higher abundance of genus W5053 ($p<0.01$), phylum Firmicutes and *Prevotella* ($p=0.05$), phylum Bacteroidetes, compared to participants with chronic lung disease.

Characteristics of HIV infected participants and gut microbiota

When we stratified HIV infected participants based on CD4 count ($CD4 \leq 400$ cells/mm³ vs >400 cells/mm³), we found no statistically significant differences at genus level. However, we found higher proportions at family level of Enterobacteriaceae ($p=0.02$) and Burkholderiaceae ($p=0.04$) in those with CD4 counts ≤ 400 cells/mm³, whereas Succinivibrionaceae ($p=0.04$) was higher in those with CD4 counts > 400 cells/mm³. No differences in relative abundance were found at any taxonomic level between virally suppressed and non-suppressed participants (<1000 copies/ml vs ≥ 1000 copies/ml).

We compared HIV infected participants based on ART duration sub groups to HIV uninfected using linear discriminant analysis effect size analysis. Genera such as *Bacteroides*, *Prevotella*, *Porphyromonas*, *Blautia* and *Roseburia* were similarly abundant in HIV uninfected and HIV infected participants who have been on ART ≥ 10 years (Supplementary figure 1-3, 5-8). This finding may suggest prolonged ART helps shift microbial composition towards that of HIV uninfected, but needs further investigation. We found no differences in relative abundance when comparing HIV infected participants on ART < 5 years to those on ART for 5-10 years or for ≥ 10 years.

Discussion

Our study showed that gut microbiota in HIV infected, ART-treated children was less diverse than in HIV uninfected children. Children who had been taking ART for 10 years or more, had a more diverse microbiota resembling that of HIV uninfected children. Our results suggest that prolonged ART may minimize differences in gut microbiota between HIV infected and uninfected children.

Impact of HIV on gut microbiota

A number of studies in adults demonstrated that untreated HIV infection is associated with intestinal dysbiosis, reduced alpha diversity and increased beta diversity [9, 30, 31]. These changes may persist despite ART (5, 6, 10, 38, 39). Our results of overall lower alpha diversity and higher beta diversity in HIV infected, ART-treated children support these findings.

Published data are less consistent with regards to relative abundance of specific taxa in HIV infected individuals. Types of specimens used, study populations, geographical area, sequencing method and false discovery may explain these conflicting results. For example, rectal swab analysis from HIV infected, ART treated adults in Nigeria found higher abundance of *Finnegoldia* and *Anaerococcus* in HIV infected individuals [32], which is consistent with our findings. However, in the same study *Campylobacter* was significantly enriched in HIV infected participants, whereas we found enriched *Campylobacter* in the HIV uninfected group.

Several studies showed enriched levels of Proteobacteria in HIV infected, ART naive individuals [6, 7, 9], only one study showed similar findings in ART treated individuals [33]. We found enrichment of Proteobacteria in HIV infected individuals, but this was not statistically significant.

Impact of ART on gut microbiota

At least two studies have found a negative impact of ART on gut microbiota diversity [9, 32]. In a longitudinal study, Nowak P. *et al.* found significant decrease in number of observed species and Shannon index after ART introduction. However, Nowak and colleagues investigated the effect of ART initiation, with a relatively short follow up of 10 months [9]. In our population, we had no ART-naive participants, and minimum duration of ART was 1 year. We observed lower alpha diversity in those on ART < 10 years compared to HIV uninfected participants.

Previous studies that investigated the gut microbiome in individuals on long-term ART reported similar alpha diversity profiles in HIV infected, ART-treated and HIV uninfected individuals [30, 33]. For example, Dinh *et al.* found no significant difference in alpha diversity measures between HIV infected participants on ART for a median of 13.3 years and HIV uninfected controls [33]. This is similar to our findings for participants who received ART for 10 or more years. The impact of ART duration on gut microbiota was also noted by Lozupone *et al.* who found that individuals with longer ART duration showed closer resemblance to HIV uninfected individuals than to subjects with untreated HIV infection [34]. These studies together with our findings support the ability that long-term ART may restore HIV-associated dysbiotic gut microbiota.

We did not observe an association between immunological or virological markers (VL and CD4 count) and gut microbiome diversity measures. In contrast, other studies showed significantly lower microbiome diversity in those with more severe HIV status [9, 35, 36]. Findings of previous studies may have been affected by sample size and ART duration. A longitudinal study with repeated measurements of VL, CD4 and microbiome profiles is needed to uncover the relationship between these parameters.

We found enriched levels of Enterobacteriaceae in HIV infected participants with low CD4⁺ T-cell counts (≤ 400 cells/mm³). Enterobacteriaceae may cause gastrointestinal and urinary tract infections in HIV infected children [36], however the clinical significance is unclear, as Enterobacteriaceae are found as part of the normal intestinal flora. Burkholderiaceae, also enriched in those with low CD4⁺ T-cell counts (≤ 400 cells/mm³), includes species known to cause severe lung infections in patients with cystic fibrosis [37].

Gut-lung axis

Recent evidence suggests that gut microbiome is involved in maintaining lung health, and an altered gut microbiome composition is often observed in patients with lung diseases [15, 16]. For example, low gut microbiome diversity during infancy has been linked to asthma at school age [38]. In our study we did not observe any difference in alpha diversity estimates between participants with and without HIV-associated chronic lung disease, but there were some significant differences in relative abundance of specific taxa. For example, the genus *Faecalibacterium* was enriched in HIV infected individuals with

chronic lung disease, while *Prevotella* was enriched in HIV infected individuals without chronic lung disease.

Faecalibacterium have previously been regarded as a protective commensal, and is associated with a healthy gut. Depletion of this genus have been linked to the development of inflammatory bowel disease and asthma, and low levels have been shown in patients with cystic fibrosis [16, 40]. Some studies have challenged this, showing increased levels of the species *Faecalibacterium prausnitzii* in gut microbiome of paediatric patients with untreated Crohn's disease at the time of diagnosis [41]. Interestingly, a recent study also showed increased levels of *Faecalibacterium* in the gut microbiome of patients with active TB [42].

Co-trimoxazole prophylaxis

Since the majority (89%) of HIV infected participants in our study received co-trimoxazole prophylaxis, it is not possible to completely tease apart the effect of HIV from that of cotrimoxazole. Though it is known antibiotics cause substantial changes in the gut microbiota, data regarding the impact of co-trimoxazole prophylaxis on gut microbiota in HIV infected, ART-treated individuals are limited. However, recent evidence suggests co-trimoxazole does not affect global gut microbial composition, but rather specific inflammatory pathways in HIV infected [43]. In our study, the negative impact of positive HIV status on richness estimates remained significant after accounting for co-trimoxazole prophylaxis. Also, no effect of co-trimoxazole administration on alpha diversity in HIV infected participants was observed. Our results are in line with several

other studies where no significant difference in alpha diversity was observed in HIV infected individuals who took co-trimoxazole and those who did not [44-46].

Study strengths and limitations

Our study is one of few to assess the gut microbiome composition in children and adolescents with perinatally acquired HIV infection. Relatively large sample size and detailed characteristics of study participants allowed us to perform extensive statistical analysis. All participants in our study were from the same region - thus increasing the internal validity of our data.

Our study was cross-sectional and is therefore unable to directly assess relationships over time. The group of HIV infected participants without chronic lung disease was small and therefore gave limited power to detect differences. Further, we did not assess diet, social factors such as housing or level of education, which may have impact on gut microbiota. Age imbalance between HIV infected and uninfected participants is also a limitation of this study.

Conclusion

Our study is among the first to assess gut microbial composition of HIV infected children and adolescents in a very high HIV burden setting. Our results indicate that gut microbiota is altered in HIV infected children, though diversity improves with increasing duration of ART. Further studies, where the gut microbiota, markers of microbial translocation and immunological markers are measured are warranted to provide better insight to the pathogenesis of HIV and its related complications.

Footnote page.

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Contributions: MPN, TF, JPC, SCW, ES and TTF conceived and designed the study, participated in data analysis and revision of the manuscript. BREATHE study team were responsible sample collection and management. TTF performed the laboratory experiments, analyzed the data and wrote the first draft of the manuscript. SCW performed the laboratory experiments, participated in data analysis and reviewing the final manuscript. ES analyzed the data and wrote the first draft of the manuscript. EH and

KSM analysed the data. JOO, RAF, GM, MPN, JPC and TF revised the manuscript. All authors approved the final version.

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Figure 1. Alpha diversity indices in HIV infected and HIV uninfected participants.

Mid line showing median and error bars showing the IQR.

Figure 2. Beta-diversity comparison between study groups

Principal coordinate analysis-plot showing beta-diversity by Bray-Curtis dissimilarity comparing (a) HIV infected (red) and HIV uninfected (blue) participants ($p < 0.01$) and (b) HIV infected with chronic lung disease (red), HIV infected without chronic lung disease (green) and HIV uninfected (blue) participants. P-value obtained using wilcoxon rank sum test.

Figure 3. Linear discriminant analysis effect size (LEfSe)-plot

This plot shows enriched taxa that are significantly different between HIV infected (blue) and HIV uninfected (red) participants. Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant after adjustment for FDR.

Tables and figures

Table 1. Characteristics of study participants.

Parameter	HIV- (N=103)	HIV+ chronic lung disease + (N = 149)	HIV+ chronic lung disease - (N = 28)
Age, Median (IQR)	9.9 (7.4-12.7)	15.5 (12.8-17.7)	16.7 (11.7-18.1)
Male, N (%)	53 (52)	84 (56)	8 (29)
BMI-for-age z score, Median (IQR) ¹	-0.24 (-0.69 to 0.35)	-1.19 (-1.80 to -0.62)	-0.11 (-0.73 to 0.61)
Stunted (height-for-age z-score <-2), N (%) ¹	5 (5)	66 (44)	7 (25)
Underweight (weight-for-age z-score <-2), N (%) ¹	5 (5)	78 (52)	2 (7)
Took antibiotics the three previous months for HIV uninfected group or Co-trimoxazole prophylaxis for HIV infected group, N (%)	2 (2)	133 (89)	25 (89)
Episodes of diarrhea during the last three months, N (%) ²	3 (3)	11(13)	1 (4)
Residential area, N (%) ³			
High density	107 (95)	83 (98)	24 (86)
Medium density	4 (4)	1 (1)	3 (11)

Low density	2 (2)	1 (1)	1 (4)
HIV-related parameters			
ART regimen, N (%)			
NNRTI-based regimen	-	93 (62)	24 (86)
PI-based regimen	-	56 (38)	4 (14)
CD4 count ≤ 400 cells/mm, N (%) ⁴	-	40 (27)	9 (32)
VL suppression (VL<1000 copies/ml), N (%)	-	87 (58)	17 (61)
Age at ART initiation, Median (IQR) ⁴	-	8.2 (5.2-11.4)	8.6 (5.0-9.9)
Years spent on ART, Median (IQR) ⁴	-	6.6 (4.4-8.4)	8.0 (5.0-9.1)
ART duration categories, N (%) ⁴			
<5 years	-	46 (31)	7 (25)
5-10 years	-	83 (56)	17 (61)
≥ 10 years	-	19 (13)	4 (14)
Previously treated for TB, N (%)	-	54 (36)	2 (7)

¹ Parameters were calculated using British 1990 Growth Reference Curves

² Data on episodes of diarrhea during the last three months was missing for 64 participants

³ Data on residential area was missing for 64 participants

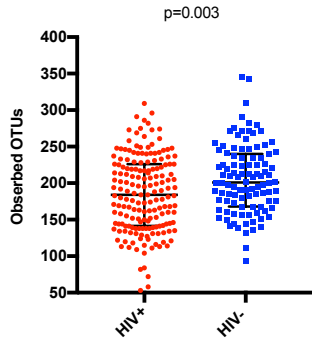
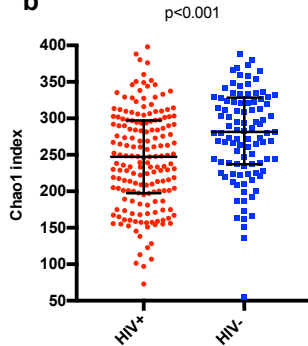
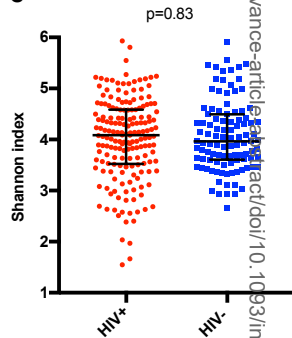
⁴ Data was missing for one participant

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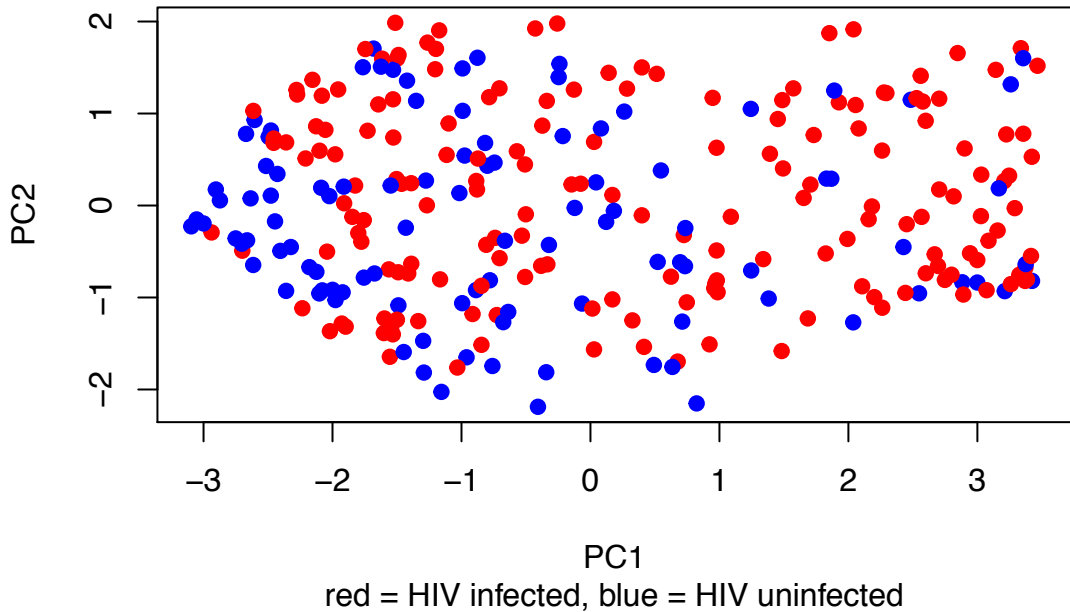
Table 2. Alpha diversity in HIV infected participants stratified by years on ART and in HIV uninfected participants.

	HIV+, <5 years on ART (N=53)	HIV+, 5-10 years on ART (N=100)	HIV+, ≥10 years on ART (N=23)	HIV- group, (N=103)	HIV+, <5 years on ART vs HIV-	HIV+, 5-10 years on ART vs HIV-	HIV+, ≥10 years on ART vs HIV-
	Median, IQR				p values *		
Observed OTUs	176 (138-214)	186.5 (143-223.5)	204 (162-242)	201 (168-240)	0.001	0.10	0.28
Chao1	229.4 (175.0-277.9)	249.6 (200.2-299.6)	268.9 (224.4-306)	281.3 (237.2-328.4)	<0.001	0.02	0.08
Shannon index	4.03 (3.48-4.39)	4.12 (3.52-4.58)	4.23 (3.82-4.84)	4.0 (3.6-4.5)	0.20	0.75	0.86

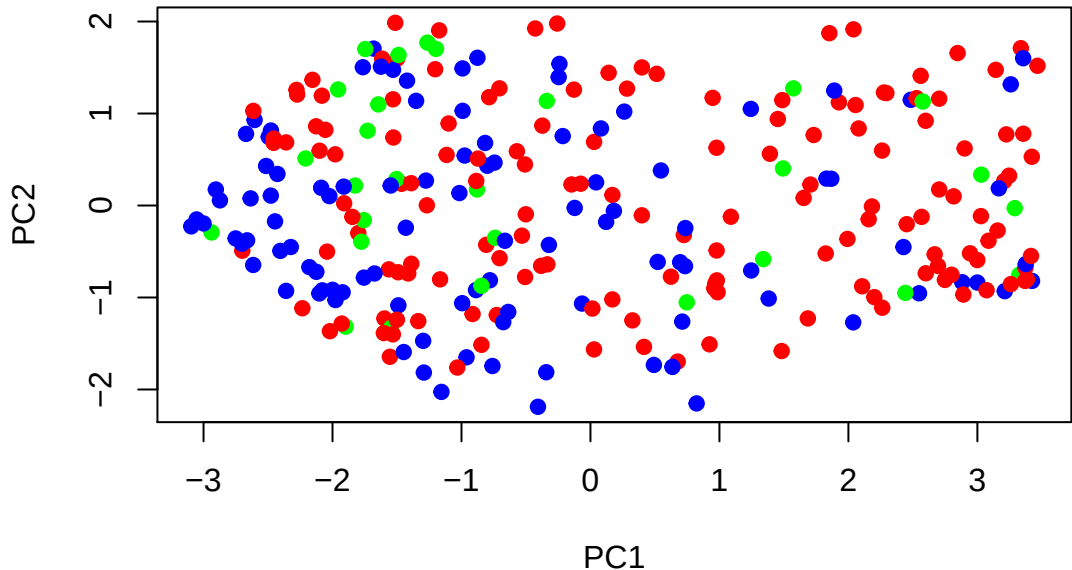
*presented p values were obtained from regression model adjusted for BMI, age and sex where HIV status with years of ART was introduced as an independent variable and alpha diversity estimates as a dependent (outcome) variable.

a**b****c**

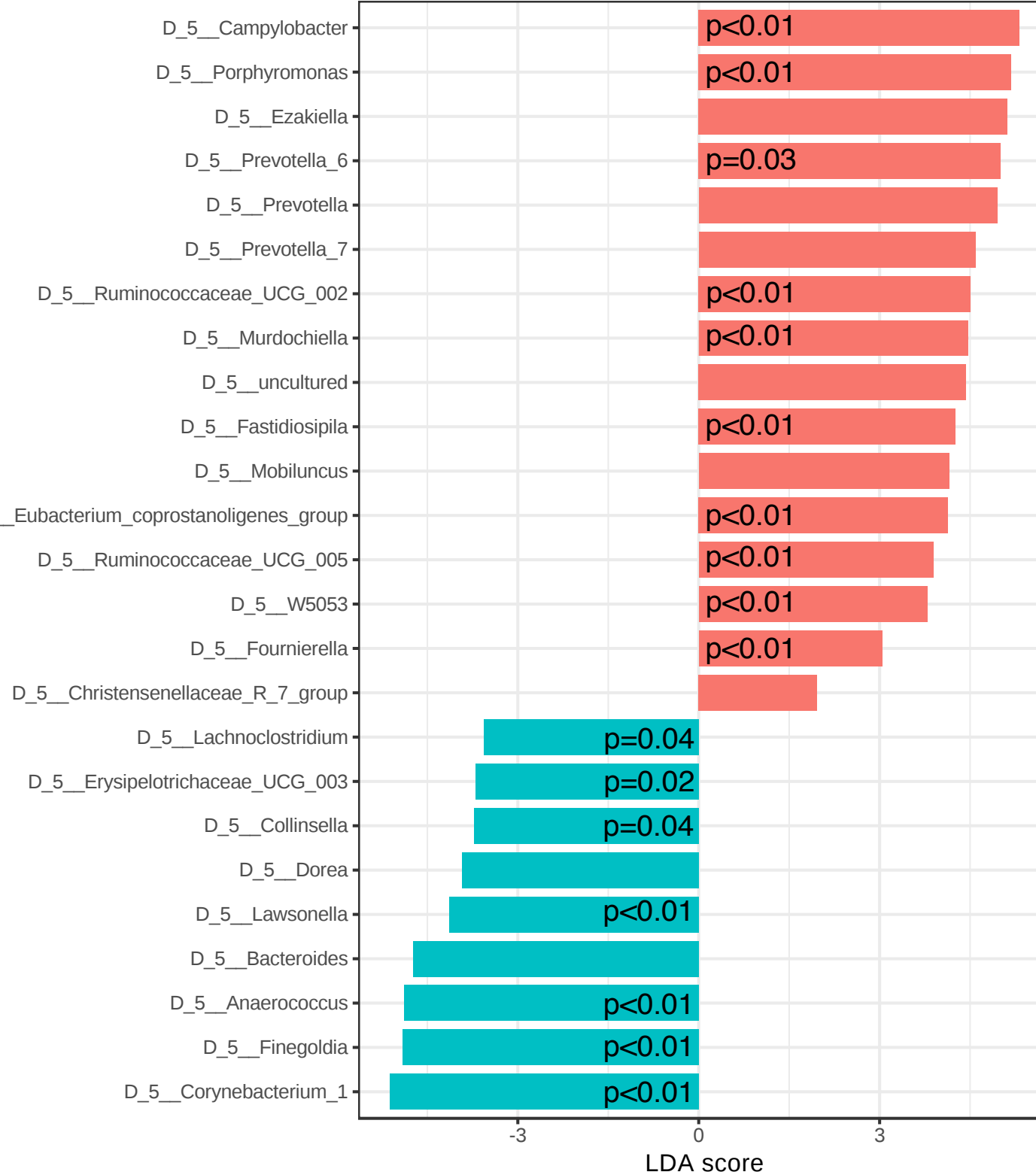
Main study group



Study sub groups



Features



Genus

HIV-
HIV+