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ORIGINAL ARTICLE

Gut Microbiota Diversity in HIV-Infected Patients on Successful Antiretroviral Treatment is Linked to Sexual Preferences but not CD4 Nadir

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Abstract
The effects of HIV infection and antiretroviral therapy (ART) on the gut microbiome are poorly understood and the literature data are inconsistent. The aim of this study was to assess the alpha and beta diversity of the fecal microbiota in HIV-infected patients on successful antiretroviral therapy with regard to sexual preferences and CD4 nadir. Thirty-six HIV-infected ART-treated patients with HIV viremia below 20 copies/ml and CD4 > 500 cells/µl were divided into two subgroups based on CD4 nadir. The composition of the intestinal microbiota was assessed by 16SrRNA sequencing (MiSeq Illumina). The alpha and beta diversity were analyzed according to CD4 nadir count and sexual preference. Several alpha diversity indexes were significantly higher in the MSM group than in heterosexual patients. The alpha diversity did not differ significantly between patients with CD4 nadir > 500 cells/µl and CD4 nadir < 200 cells/µl. Beta diversity was also associated with sexual preference. A significant difference in Weighted Unifrac was observed between all MSM and all non-MSM participants ($p = 0.001$). The MSM group was more diverse and demonstrated greater distances in Weighted Unifrac than the non-MSM group. The relative abundance of the Prevotella enterotype was higher in the MSM than the non-MSM group. Sexual preferences demonstrated a stronger influence on alpha and beta diversity in HIV-infected patients following successful antiretroviral treatment than HIV infection itself. The observed lack of association between CD4 nadir and alpha and beta diversity may be caused by the restoration of the faecal microbiota following antiretroviral treatment.

Keywords Gut microbiota · HIV · CD4 nadir · MSM

Introduction

During the early stage of HIV infection, a huge depletion of Th17 T cells in the gut-associated lymphoid tissue causes an impairment of mucosal immunity, resulting in the gut microbiota undergoing compositional changes and the subsequent translocation of microbial products into the blood. Microbial translocation from the gut to systemic circulation causes an increase in the production of pro-inflammatory cytokines. The resulting immunoactivation and exhaustion of the immune system contribute to HIV-associated immune dysfunction (Brenchley et al. 2004, 2006; Elhed and Unutmaz 2010; Liang et al. 2006; Liu et al. 2017; Paiardini et al. 2008). This immunoactivation is associated with the premature incidence of such age-related diseases as cardiovascular disease (Das 2010; Klein et al. 2003). It is well known that antiretroviral treatment prolongs life expectancy in HIV-infected patients by reducing the incidence of opportunistic infections and lowering immune activation, thus decreasing the risk of the development of non-HIV-associated disease (Kuller et al. 2008; Palella et al. 1998; Strategies for Management of Antiretroviral Therapy Study Group 2006).

During antiretroviral treatment, HIV-infected patients typically experience changes in the composition of the intestinal microbiota following CD4 T cell immune recovery and the restoration of the intestinal mucosa barrier. However, the degree to which HIV infection alters the gut microbiome, and the extent to which antiretroviral treatment reverses these processes, is not clear. These questions are

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complicated by the myriad of factors influencing the intestinal microbiota (Dong and Gupta 2019), which may not be revealed by studies on small samples of the impact of HIV infection itself or antiretroviral therapy (ART) on the gut microbiome. Nevertheless, some studies suggest that MSM (men who have sex with men), the group currently demonstrating the highest prevalence of HIV infection in Poland, may also possess a distinct microbiota composition (Noguera-Julian et al. 2016). If the composition of microbiota is affected by the type of sexuality, it is necessary to take this factor into account when assessing the impact of immune reconstitution on gut microbiome. Moreover, geographical conditions and diet variations (eating habits) in different populations can further influence the composition of the gut microbiome.

In Poland, there have been no studies analyzing the intestinal microbiota in patients infected with HIV to date. In our study, we particularly focused on the assessment of the gut microbiome in MSM, as this is currently the predominant group among the HIV-infected individuals.

The aim of this paper is to describe the composition of the intestinal microbiota with regard to CD4 nadir count and sexual preference in HIV-infected patients following successful antiretroviral treatment and who demonstrate last great CD4 recovery.

Materials and Methods

Study Group

The study group was comprised of patients registered at the Outpatient Clinic for Immune Deficiency, Lodz, Poland. All patients had received successful ART with CD4 > 500 cells/µl and viremia < 20 copies/ml in the last two samples prior to the study. The patients were divided into two subgroups based on CD4 nadir: one with a CD4 nadir above 500 cells/µl (good immunity group) and another with a CD4 nadir below 200 cells/µl (good response to treatment group). They were further divided into subgroups according to sexual preferences: MSM and non-MSM subgroups. The dominant group of HIV-positive patients in our study were MSM who have become a major population of HIV-infected patients in Poland in recent years. The non-MSM group was comprised of heterosexual patients and injecting drug users.

The study was approved by the ethical committee of the Faculty of Medicine, Medical University of Lodz. Informed written consent was obtained from all participants prior to enrollment. The control group consisted of age-, sex- and sexual preference-matched volunteers without HIV infection. $CD4^+$ and $CD8^+$ T cell counts and HIV viremia tests were performed by flow cytometry and Cobas Amplicor (Roche Molecular System Inc.) during routine clinical measurements.

All participants in this cross-sectional study met all the inclusion criteria and provided written informed consent before taking part.

The inclusion criteria were as follows: (1) age older than 18 years; (2) minimum 2 years of ART; (3) CD4 > 500 cells/µl; (4) CD4 nadir < 200 cells/µl or > 500 cells/ µl; (5) regular, non-specific diet; (6) informed written consent to take part in the study.

The exclusion criteria were as follows: (1) acute retroviral syndrome; (2) pregnancy; (3) treatment with antibiotics or probiotics in the previous 3 months; (4) symptoms of sexually transmitted diseases; (5) obesity or underweight; (6) diabetes; (7) diagnosis of inflammatory bowel disease; (8) symptomatic liver diseases; (9) hepatitis C virus (HCV) or hepatitis B virus (HBV) coinfections; (10) current alcohol or substance abuse; (11) vegan or vegetarian diet.

HIV-negative controls were recruited at our Outpatient Clinic. They were HIV, HCV, HBV negative sexually active male and female subjects, who come for HIV testing. The majority of subjects from the control group were MSM, as this group most frequently seeks access to HIV testing in Poland.

The subjects from the control group declared that they had not been diagnosed with chronic diseases and, like patients from the study group, had not been not on treatment with antibiotics or probiotics in the previous 3 months and did not follow a vegan or vegetarian diet.

Microbiome Analysis

Fresh stool specimens were collected into 50 ml sterile urine and stool specimen bottles. The samples were immediately frozen at − 80 °C after acquisition and stored under these conditions until DNA was isolated. After thawing, 100 mg of feces was transferred to sterile 2 ml microcentrifuge tubes and washed with 1 ml phosphate-buffered saline, 1X, without calcium and magnesium, pH 7.4 ± 0.1 (Corning, USA) and vortexed (3000 rpm, 30 s). After homogenization, 1 ml of the sample was transferred to the new 2 ml microcentrifuge tubes and the DNA was isolated using the QIAamp DNA Microbiome kit (Qiagen, Germany) and eluted in 50 µl of elution buffer. After isolation, DNA concentration was measured using the NanoVue®Plus spectrophotometer (GE Healthcare, UK). Each sample was diluted to a working concentration of 5 ng/µl. DNA libraries were prepared according to the 16S Metagenomics Sequencing Library Preparation protocol. The gene-specific sequences used in this protocol targeted 16S V3–V4 regions have been described previously (Klindworth et al. 2013). Each sample was dual indexed using the Nextera XT v2 Index Kit (Illumina, USA). DNA libraries were normalized and pooled. All samples were

sequenced using 2 × 250 bp paired-end reads with 100,000 reads per sample coverage on a MiSeq system (Illumina, USA) using v2 reagents (500 cycles).

Data Analysis After Sequencing

Output reads were analyzed using the Qiime2 platform. Each sample was represented by two files: R1 for forward reads and R2 for reverse reads. For each sample dataset, the same quality check and selection criteria were applied. Briefly, read quality was checked using FastQC software and statistics were performed in Qiime2. Any amplicon errors were corrected and chimeric sequences and ambiguities removed by denoising using the dada2 package. Reads were also trimmed at the eighth nucleotide position from the 5′ end in both read sets, and at the 247th and 240th nucleotide positions from the 3′ end in the respective forward and reverse reads. Subsequently, the sequences were clustered into operational taxonomic units (OTU) based on sequence similarity within the reads. Taxonomic identities were also assigned using the Green Genes classifier and Green Genes reference database; the OTU sequences were aligned and an OTU table was constructed representing the abundance of each OTU in each microbial sample. Based on the outcome of the denoised OTU, the threshold was set to 400 OTU appearing in a single sample.

All analyses were performed using the Qiime2 platform. Bacterial alpha diversity was determined by OTU analysis and presented as a box-and-whisker plot for the Shannon index, Simpson index and Chao1 index. Beta diversity was measured by Weighted Unifrac and principal coordinate analysis; these were calculated to display microbiome distances between the samples. The results of the bacterial taxonomic analyses are presented at the genus level as a heatmap.

The Mann–Whitney *U* test was used to evaluate the differences between continuous variables, such as age of the HIV-positive group vs age of the HIV-negative group. To compare categorical variables, Chi-square distribution or Fisher's exact test was used depending on the size of the studied group. To test the differences between more than two groups, we used the Kruskal–Wallis test with post hoc comparisons with the Conover–Iman test.

Results

The study group consisted of 32 males and 4 females. The median age of the HIV-infected patients was 42 years. Dominant route of HIV transmission was homosexual contacts (23 patients), non-MSM intercourse was the causes of HIV infection in 13 patients. The median CD4 nadir count was 174 cells/μL (488–104). The median duration of ART was 6 years (2–10).

The control group consisted of 23 healthy volunteers age, sex and sexual orientation matched. The characteristics of the patients are summarized in Table 1.

Alpha Diversity

HIV-Positive and HIV-Negative Patients Demonstrate the Same Alpha Diversity Values

No significant differences were found between the HIV-infected patients and control group with regard to the Chao1, Simpson or Shannon–Weaver Diversity Indexes ($p > 0.05$). The results are presented as graphs (Fig. 1).

Alpha Diversity Depends on the Routes of HIV Transmission

The highest Chao1 index was observed in the MSM HIV-negative group (MSM control), while the lowest was observed in the non-MSM–good response to treatment

Table 1 The characteristics of the patients

	HIV-positive patients	Control group (HIV-negative)	*p*
Sex (F/M)	4/32	3/20	> 0.05
Age (years) median (LQ-UQ)	42 (37–47.5)	37 (33–47)	> 0.05
Caucasian Ethnicity	100%	100%	
CD4 nadir median (LQ-UQ)	174 (488–104)		
CD4/CD8 nadir	0.17 (0.13–0.34)		
Present CD4 cells/μl median (LQ-UQ)	729.5 (590.5–867)		
Duration of ART (years) median (LQ-UQ)	6 (2–10)		
Risk group			
Non-MSM	13	13	> 0.05
MSM	23	10	

MSM men who have sex with men

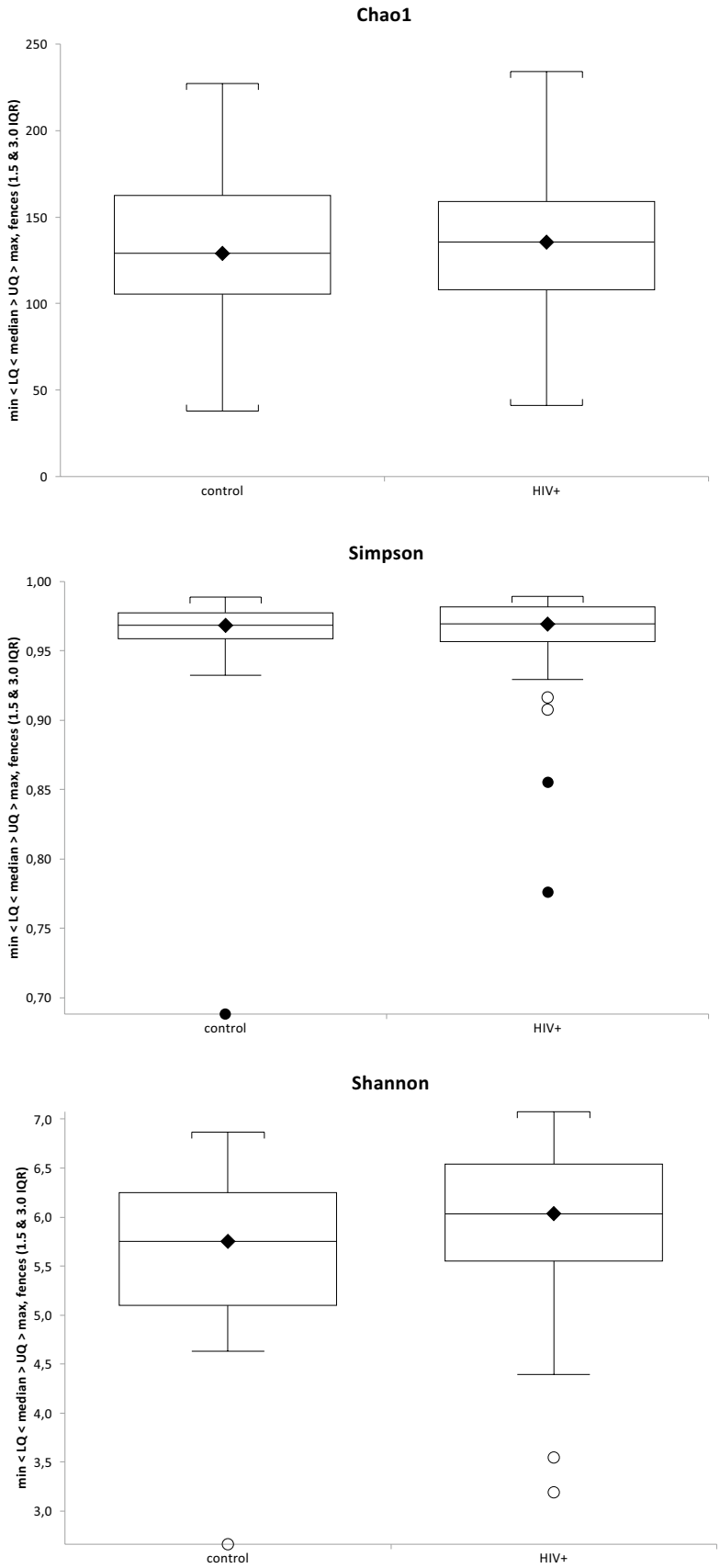


Fig. 1 Chao1, Simpson and Shannon–Weaver Diversity Indexes in all HIV-infected patients and control group

group. In the non-MSM control group, the Chao1 index was significantly lower than the MSM control ($p=0.0005$), as well as the MSM–good immunity ($p=0.0016$) and the MSM–good response to treatment subgroups ($p=0.0373$). The Chao1 index was significantly lower in the non-MSM–good response to treatment group than in the MSM control and MSM–good immunity groups ($p=0.0004$ and $p=0.0013$, respectively).

The lowest Simpson index was reported in the non-MSM–good response to treatment group and the non-MSM controls. The Simpson index was significantly lower in the non-MSM control group than the MSM–good response to treatment group ($p=0.0462$). The Simpson index was significantly lower in the non-MSM–good response to treatment subgroup than the MSM–good immunity ($p=0.0022$) and MSM–good response to treatment subgroups ($p=0.0053$).

The lowest Shannon index was found in the non-MSM–good response to treatment group. The Shannon index in MSM–good response to treatment group was significantly higher than in the non-MSM–control and non-MSM–good response to treatment groups ($p=0.0274$ and $p=0.0029$, respectively). The Shannon index was significantly higher in the MSM–good immunity group than the non-MSM–control group.

The Chao1, Simpson and Shannon–Weaver Diversity Indexes in the HIV-infected patients and controls are presented according to sexual preference and CD4 nadir in Fig. 2.

Beta Diversity-Weighted Unifrac Depends on the Route of HIV Transmission

No significant difference was observed between HIV-infected patients and HIV-negative patients with regard to the distance measured with Weighted Unifrac. A significant difference in Weighted Unifrac was observed between all MSM and all non-MSM participants ($p=0.001$).

Similarly, significant differences in Weighted Unifrac distance were found between the MSM control group and the non-MSM controls ($p=0.001$) and the non-MSM–good response to treatment group ($p=0.031$). Significant differences in Weighted Unifrac distance were also observed between the MSM–good response to treatment group and the non-MSM controls ($p=0.049$) and non-MSM–good response to treatment group ($p=0.005$). Weighted Unifrac in study groups are shown in Fig. 3.

The most common genera in each study group are presented in Fig. 4. The most prominent bacteria in study group were *Blautia* spp., *Coprococcus* spp., *Gemmiger* spp. and *Docea* spp. Some patients in the HIV^+ group exhibited significant amounts of *Clostridium* spp., *Peptostreptococcus* spp., *Sedimentibacter* spp. and *Methanosaeta* spp. The greatest difference in group composition was marked by the sexual preference (Fig. 5).

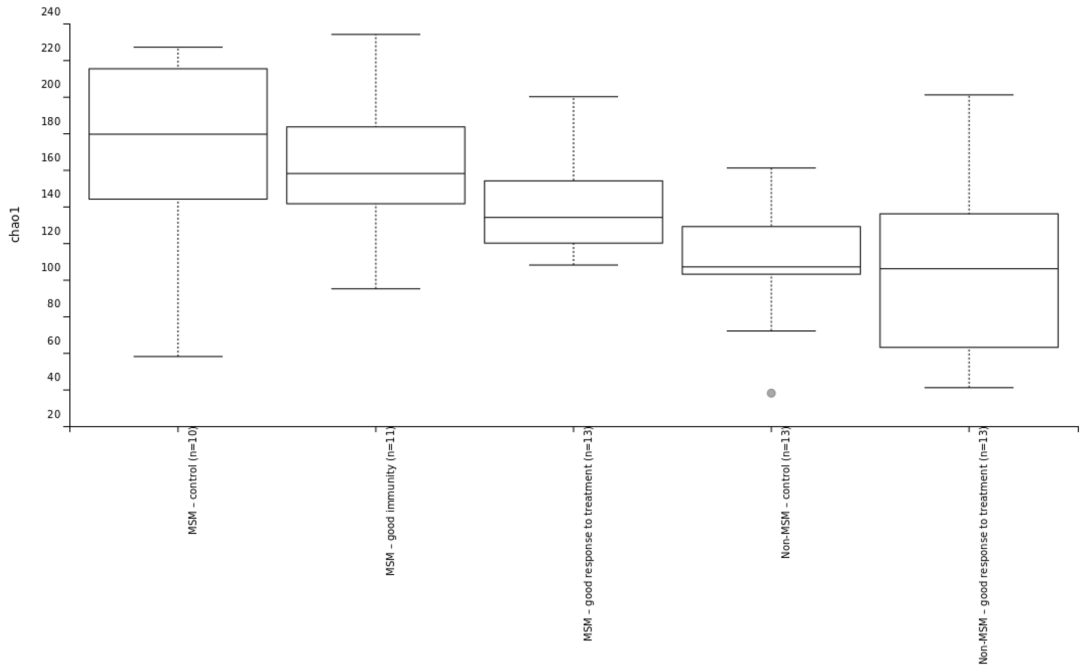
Composition of *Prevotella* spp. and *Bacteroides* spp.

Studies indicate that the *Prevotella*, and *Bacteroides* genera are of particular importance. In this study, the MSM group demonstrated higher relative abundance of *Prevotella* spp. than the non-MSM group (Fig. 6). Both non-MSM groups showed an overwhelming amount of *Bacteroides* spp. (Table 2).

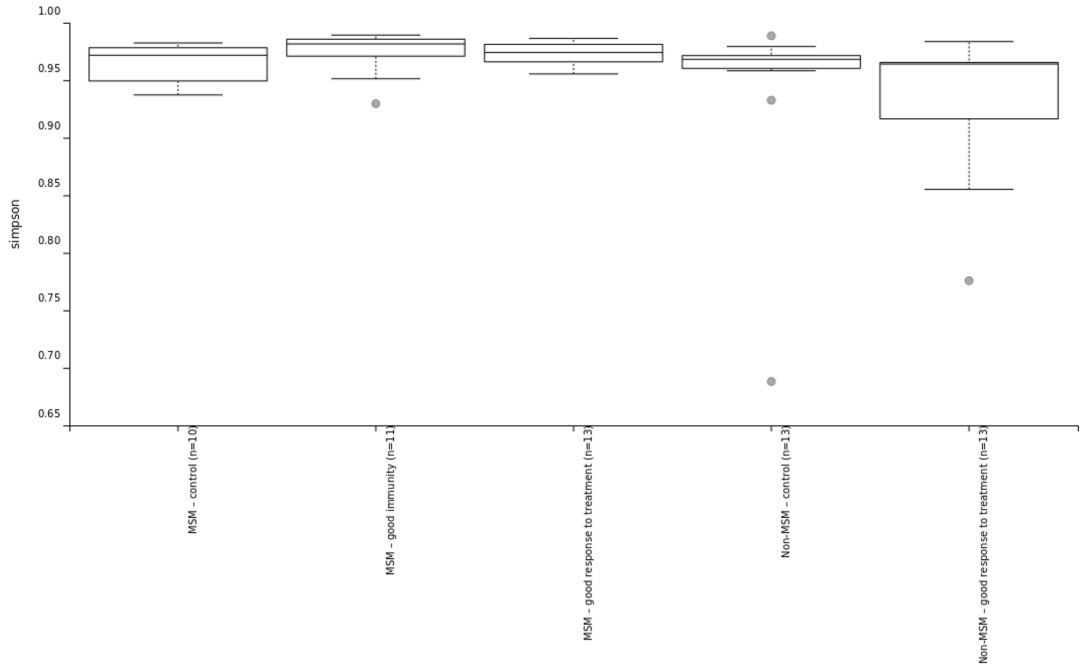
Discussion

The gut microbiota of HIV-infected patients differs from that of HIV-negative subjects (Dinh et al. 2015; Klindworth et al. 2013; McHardy et al. 2013; Mutlu et al. 2014; Nowak et al. 2015). In a study by Tuddenham et al. (2020), it has been observed that HIV status was associated with a decrease in measures of alpha diversity but only among men who have sex with women and women but not in men who have sex with men (MSM). The diversity and abundance of the gut microbiome are well known to be associated with the degree of immunodeficiency in HIV-positive patients who have not received ART. Nowak et al. (2015) report that HIV-infected patients present a smaller diversity of gut bacterial species before the introduction of ART compared to those who are HIV-negative. The number of confirmed bacterial species has been found to correlate positively with CD4 cell count and negatively with markers of microbial translocation and monocyte activation (Nowak et al. 2015). Furthermore, some authors report differences in taxa composition between HIV-positive and HIV-negative patients. The gut microbiome in elite controllers has also been found to be more diverse and abundant than in antiretroviral-naive non-elite controllers (Vesterbacka et al. 2017).

Our present findings do not indicate any difference in alpha or beta diversity between HIV-infected patients on successful ARV treatment (with CD4 > 500 cells/μl and HIV virema < 20 copies/ml) and healthy controls, irrespective of CD4 nadir count. Our observations are in accordance with results obtained by Wang et al. (2020) who also reported that bacterial community alpha diversity indices (Shannon index, Chao1 index, and Simpson's index) were not significantly different between groups with and without HIV. However, our research showed that the MSM and non-MSM groups displayed significant differences in the alpha and beta diversity of gut microbiota, with the MSM group demonstrating significantly higher alpha and beta diversity than the non-MSM group, irrespective of HIV serology status or CD4 nadir count. Recent evidence suggests also that status as MSM impacts gut microbiome measures, perhaps relating

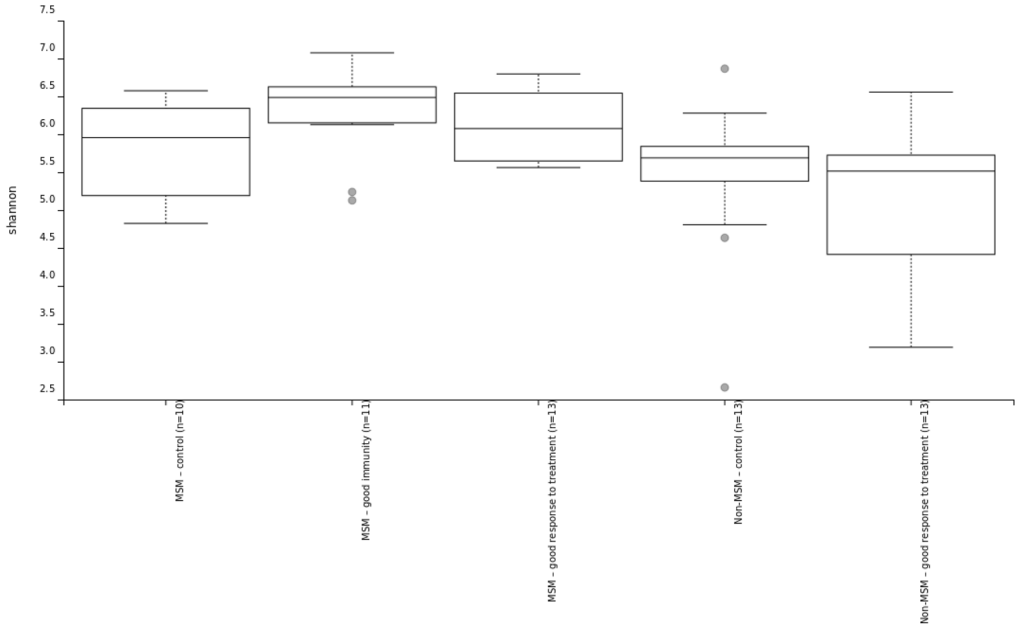


MSM positive control vs Non-MSM control p=0.0005
MSM positive good immunity vs Non-MSM control p=0.0016
MSM positive good response to treatment vs Non- MSM control p=0.0373
MSM positive good immunity vs Non MSM good response to treatment p=0.0013
MSM positive control vs Non-MSM good response to treatment p=0.0004



MSM positive good response to treatment vs Non-MSM good response to treatment p=0.0053
MSM positive good immunity vs Non-MSM control p=0.0462
MSM positive good immunity vs Non-MSM good response to treatment p=0.0022

Fig. 2 Chao1, Simpson and Shannon–Weaver Diversity Indexes in HIV-infected patients and control group depending on sexual preferences and CD4 nadir



MSM positive good response to treatment vs Non-MSM control p=0.0274
MSM positive good response to treatment vs Non-MSM good response to treatment p=0.0029
MSM positive good immunity vs Non-MSM control p=0.0085
MSM positive good immunity vs Non-MSM good response to treatment p=0.0008

good immunity group – HIV-infected patients with CD4 nadir above 500 cells/µl

good response to treatment group - HIV-infected patients with CD4 nadir below 200 cells/µl on successful ART with present CD4> above 500 cells/µl

MSM - men who have sex with men

Fig. 2 (continued)

to receptive anal intercourse or other behaviors (Noguera-Julian et al. 2016).

The differences in alpha and beta diversity observed between HIV-infected patients and HIV-negative subjects reported in prior studies may be associated with routes of HIV transmission and sexual preference. Interestingly, a study conducted on MSM group receiving ART for longer than 2 years by Lu et al. (2018) found no difference in gut microbiota alpha diversity between patients with CD4 ≥ 350 cells/µl and subjects with lower CD4 count, and no significant difference in alpha diversity was found between HIV-negative men and either subgroup of HIV-infected patients on ART.

Previous studies have identified two enterotypes of bacterial composition in humans: one dominated by *Bacteroides* spp. and a second one by *Prevotella* spp. Many studies report changes of relative abundance of *Prevotella* spp. and *Bacteroides* spp. in favor of *Prevotella* spp. in HIV-infected patients (Dillon et al. 2014; Lozupone et al. 2013; Noguera-Julian et al. 2016; Vazquez-Castellanos et al. 2015). However, no data have been obtained on the influence of sexual preferences. Lu et al. (2018) report a higher proportion of *Prevotella* spp. and fewer *Bacteroides* spp. in the stools of MSM HIV-positive men than HIV-negative individuals; however, the control group itself was significantly younger and the authors did not report whether it included MSM. Based on our present findings, future studies comparing microbiota between HIV-positive and HIV-negative patients should include sexual preference. In addition, it is possible that sexual preferences may have a key influence on the abundance of microbiota and its alpha and beta diversity.

Moreover, our findings indicate that the *Prevotella* spp. enterotype is more prevalent in the MSM group than the non-MSM group. In addition, the HIV-positive MSM group with low CD4 nadir demonstrated significantly higher relative abundance of *Prevotella* spp. than the non-MSM group

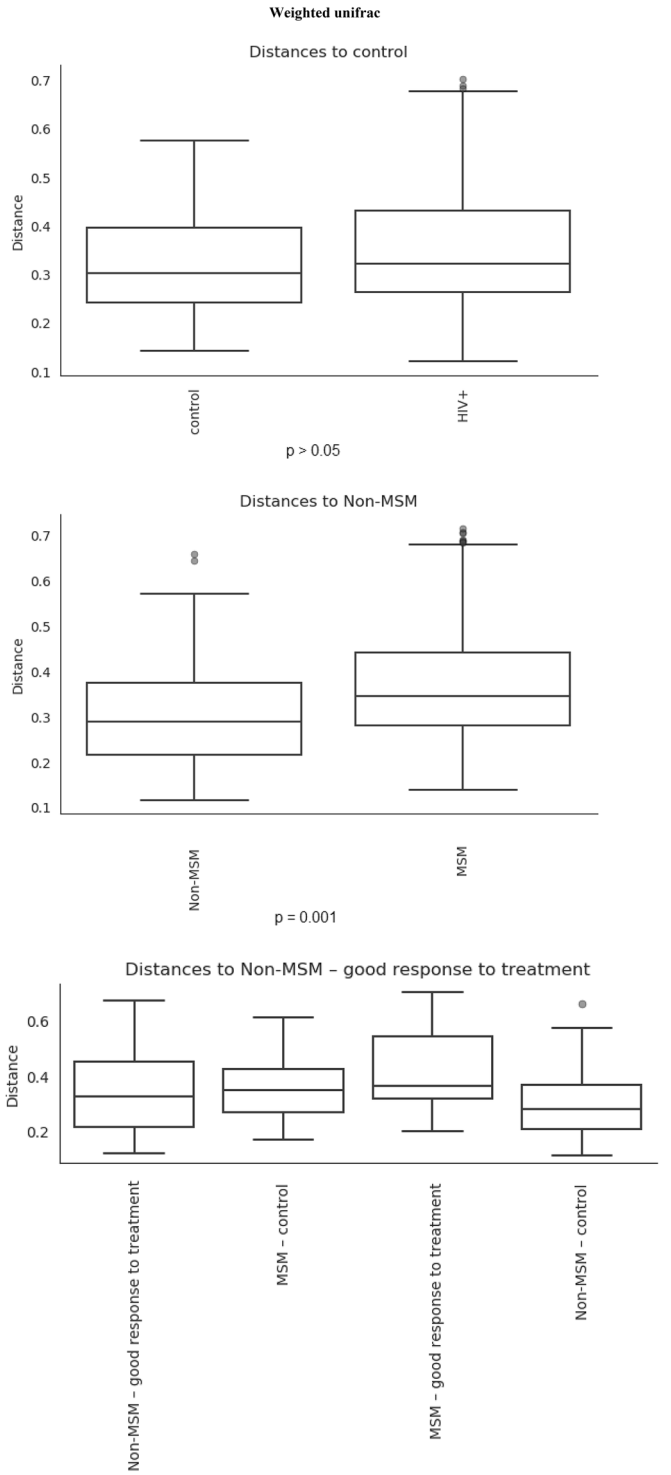


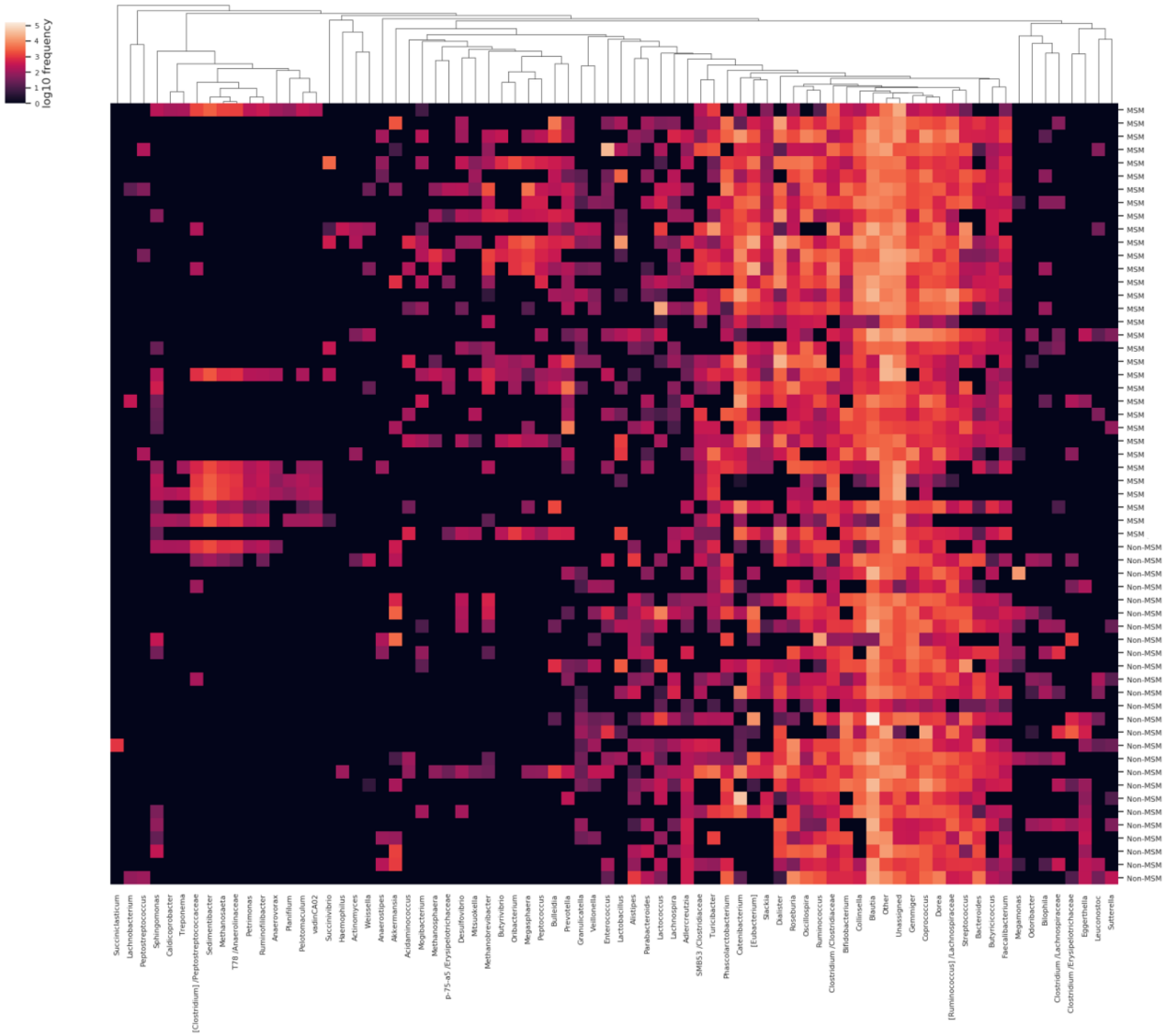
Fig. 3 Weighted Unifrac in study groups

MSM positive control vs Non-MSM control p=0.001
MSM positive control vs Non-MSM good response to treatment p=0.031
MSM positive good response to treatment vs Non-MSM good response to treatment p=0.005
MSM positive good response to treatment vs Non-MSM control p=0.049

good immunity group - HIV-infected patients with CD4 nadir above 500 cells/µl

good response to treatment group - HIV-infected patients with CD4 nadir below 200 cells/µl on successful ART with present CD4 above 500 cells/µl

MSM - men who have sex with men



good immunity group - HIV-infected patients with CD4 nadir above 500 cells/µl

good response to treatment group - HIV-infected patients with CD4 nadir below 200 cells/µl on successful ART with present CD4 above 500 cells/µl

MSM - men who have sex with men

Fig. 4 The most common genera observed in study groups

with a low CD4 nadir count. Our results are in accordance with those of Noguera-Julian et al. (2016), who also report that MSM subjects are more likely to display a *Prevotella*-rich enterotype than non-MSM subjects. However, they also note reduced bacterial richness among HIV-positive patients after stratification for sex orientation (Noguera-Julian et al. 2016). No such association was found in the present study, based on study group composed of patients with CD4 > 500 cells/µl.

As this is a cross-sectional study, the question arises as how ART influences the microbiota in patients with HIV infection, and whether patients with a low CD4 nadir demonstrate lower richness than those with a higher CD4 nadir levels before introduction of ART. Such a situation would mean that successful ART results in the return of microbial diversity to levels reported in healthy subjects. However, the data describing the impact of ART on faecal microbiota are particularly sparse. Nowak et al. (2015) report a decreased

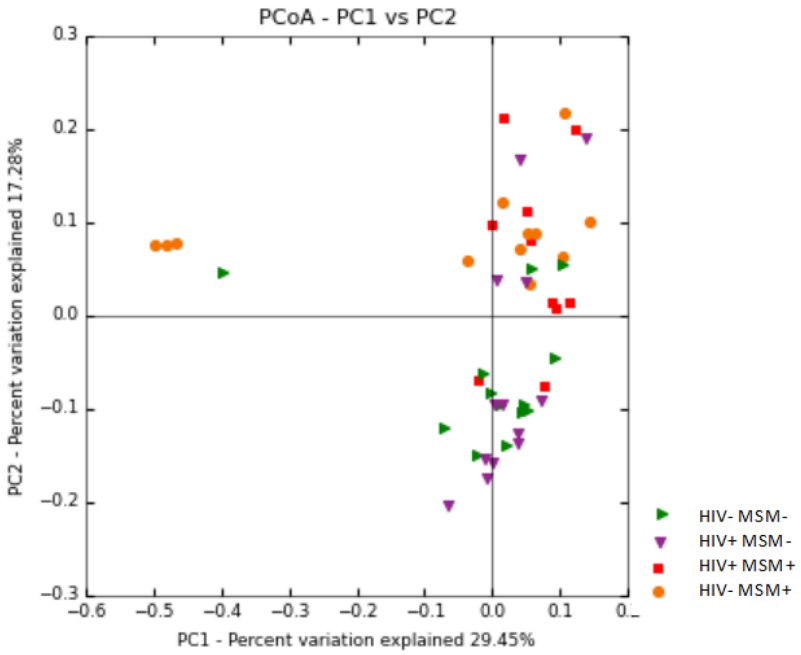
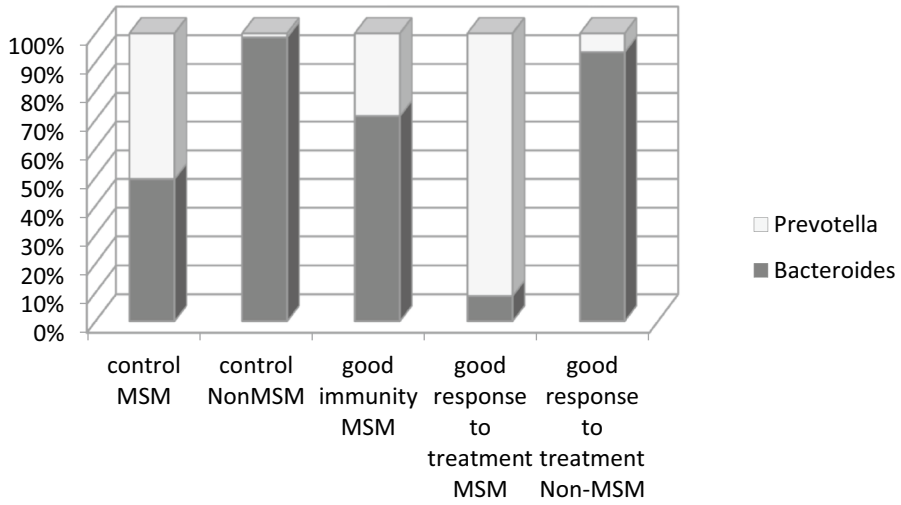


Fig. 5 Principal coordinate analysis (PCoA) graph showing similarities and differences between groups of patients based on Weighted Unifrac. MSM-negative patients (red and orange) form a separate group to MSM-positive (green and purple) patients

MSM - men who have sex with men



good immunity group - patients with CD4 nadir above 500 cells/µl

good response to treatment group - patients with CD4 nadir below 200 cells/µl on successful ART with present CD4 500 cells/µl

MSM - men who have sex with men

Fig. 6 Relative abundance of *Prevotella* spp. and *Bacteroides* spp. in all groups

number of bacterial species and Shannon index, as well as an increase in beta diversity after short-term ART (10 months).

Patients treated with integrase inhibitors have been found to demonstrate lower serum lipopolysaccharide and sCD14 levels and higher alpha diversity compared to those on protease inhibitors or NNRTI (Villanueva-Millan et al. 2017). These results suggest that integrase inhibitors have a beneficial effect on intestinal inflammation and restore

Table 2 Relative ratio in percentage between *Prevotella* spp. and *Bacteroides* spp. in study group

	Bacteroides	*Prevotella*
HIV-positive group		
MSM-positive	32%	68%
MSM-negative	97%	3%
Control group		
MSM-positive	49%	51%
MSM-negative	99%	1%
HIV-positive patients on ART		
Good immunity MSM-positive	71%	29%
Good response to treatment MSM-positive	9%	91%
Good response to treatment MSM-negative	93%	7%

Good immunity group: patients with CD4 nadir above 500 cells/µl; good response to treatment group: patients with CD4 nadir below 200 cells/µl on present successful ART with present CD4 above 500 cells/µl; MSM: men who have sex with men

gut microbiota diversity, which cannot be said about other classes of antiretroviral drugs; however, the impact of route of HIV transmission on the gut microbiota was not analyzed in the study.

Finally, Guillen et al. (2019) report that although a wide range of factors influence gut microbiota following ART, HIV infection itself does not appear to be a key determinant of gut microbiota composition. In fact, the observed aberrances in gut microbiota richness and composition in HIV-infected patients seem to be rather more subtle than had been supposed, and may not affect all HIV-positive patients (Guillen et al. 2019).

In conclusion, our findings suggest that sexual preference has an influence on the alpha and beta diversity of faecal microbiota. The influence of sexual preferences on the above-mentioned indexes was stronger than HIV infection itself. Future studies are intended to assess gut microbiota in MSM late presenters and non-late presenters following successful antiretroviral treatment. It is possible that HIV infection may be associated with changes in bacteria species, but this may be not apparent at higher taxonomic levels, such as at the phylum, as suggested by some authors; however, frequent not included other important factors influencing microbiome.

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