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Metagenomic analysis reveals rectal microbiota features associated with HIV and behavioral factors in Nigerian men who have sex with men

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Emerging data suggest unique features characterize the rectal microbiota of men who have sex with men (MSM) and people living with HIV (PLWH). The microbiota may have important health implications in these groups, but most studies have been conducted in the United States or Europe. This study leveraged metagenomic sequencing to evaluate relationships between rectal microbiota composition and clinical, behavioral and demographic characteristics in a cohort of Nigerian MSM. PLWH with suppressed viral load had lower α-diversity (richness) compared to people without HIV (PWoH), with similar trends for PLWH with an unsuppressed viral load. Lower α-diversity (Shannon) was associated with use of petroleum jelly lubricant for anal sex. Lower relative abundance of the genus *Prevotella* was seen in PLWH with a suppressed viral load versus PWoH. There were differences in abundance of the top 20 taxa associated with age, HIV status (enhanced in virally suppressed PLWH versus PWoH), lubricant use, receptive anal intercourse, and condom use, suggesting multiple clinical and behavioral factors impact the rectal microbiota. Future characterization of health outcomes associated with the rectal

or gut microbiota in MSM and PLWH as well as potential interventional insights will necessitate larger, dedicated studies across diverse geographic locations.

INTRODUCTION

Recent studies highlight unique features, including *Prevotella* predominance, which characterize the rectal and intestinal microbiota of men who have sex with men (MSM) in the US and Europe(1). Gut dysbiosis in people without HIV (PWoH) is hypothesized to drive local and systemic inflammation contributing to a wide diversity of diseases as well as increased risk of HIV acquisition via receptive anal intercourse (RAI)(1-3). Gut dysbiosis in people living with HIV (PLWH) might increase risk of inflammation-related chronic diseases such as cardiovascular disease and stroke(1-3). Given the high burden of rectal sexually transmitted infections (STIs) among MSM, there is speculation surrounding the potential relationship of the rectal microbiota to rectal STI susceptibility(1). Disparate studies have suggested factors such as HIV status, antiretroviral therapy (ART),

RAI, rectal lubricant use, number of anal sex partners and rectal STIs such as gonorrhea (NG) or chlamydia (CT) may influence the rectal or gut microbiota in MSM(4-12). Yet, results are inconsistent, and most studies have relied on 16S rRNA amplicon gene sequencing rather than metagenomic techniques that more reliably identify bacterial taxa down to the species level. Data on what determines and defines gut microbiota composition of MSM outside of the U.S. and Europe remain sparse; primarily limited to two small studies from Nigeria, one small study from Kenya, and one from China (6, 13-15), although MSM across the globe, including in sub-Saharan Africa, are disproportionately affected by HIV and other STIs.

Advanced metagenomics techniques are critical for characterizing the rectal microbiome and its association with HIV, ART, and sexual behaviors. These insights will inform future studies examining the role of the rectal microbiota in STI and HIV acquisition, and long-term health outcomes in MSM living with HIV. It was previously demonstrated, using 16S rRNA gene amplicon sequencing of rectal specimens from a cohort of MSM in Nigeria, that the microbiota may reflect ART use in HIV and link to rectal human papillomavirus (HPV) status(6, 15). Building on these findings, our study employs metagenomic sequencing to investigate associations between rectal microbiota features and clinical, demographic, and behavioral factors in MSM with and without HIV from Nigeria. The genus *Prevotella,* including *P. copri*, has been variably associated with MSM and HIV status in prior studies(9, 16-18). Thus, we conducted additional analyses on these taxa.

METHODS

Parent Cohort

This is a secondary analysis of rectal samples collected from the TRUST/RV368 cohort. This cohort has been described previously(19-21) (**Suppl. Methods**). In brief, TRUST and its sister study, RV368, enrolled a total of 2652 males who reported receptive or insertive anal sex with a male partner in the last 12 months in Abuja and Lagos, Nigeria from 2013-2020(22, 23). Participants were followed every 3 months up to 18 months. Each visit included behavioral interviews, NG/CT testing, and HIV testing if appropriate; PLWH were offered ART. Remnant rectal specimens from NG/CT testing were retained. Only rectal samples that tested negative for NG and CT were included in this cross-sectional analysis. All participants provided written informed consent prior to any procedures in TRUST/RV368. The study was approved by the University of Maryland Institutional Review Board (IRB) Baltimore, MD, USA; the Federal Capital Territory Health Research Ethics Committee, Abuja, Nigeria; Walter Reed Army Institute of Research IRB; Ministry of Defense Health Research Ethics Committee, Abuja, Nigeria; and approved or acknowledged at all collaborating institutions including the IRB at Johns Hopkins (IRB00271521). All methods were performed in accordance with the relevant guidelines and regulations.

Demographic, clinical and behavioral data

Data were obtained from questionnaires, diagnostic testing, clinical evaluations and pharmacy dispensation forms. Variables included age, education, HIV status, CD4 count, viral load (VL), RAI, condom use with anal sex, antibiotic use either

current or in the last 6 months, lubricant use with anal sex with men (insertive or receptive not specified)(24), and number of receptive anal sex partners (meaning the participant was the receiving partner) in the 12 months prior to enrollment. When CD4 count or VL were not available, the value was taken from a visit within 6 months for CD4 and within 4 months for VL.

Sample collection and metagenomic sequencing

In the parent cohort, physicians or participants inserted a rectal swab into the anorectum. The swab was gently rotated, then placed in Aptima media for NG/CT testing. Remnant samples were aliquoted to have at least 0.5mls per aliquot and stored at -80° Celsius, then shipped on dry ice from Nigeria to the University of Maryland. A 250µl aliquot was placed in Qiagen 96-well bead plates. Positive (Zymo) and negative controls were included at the genomic DNA extraction step. No contaminants were observed. Libraries were prepared with KAPA EvoPlus (Kapa Biosystems) according to manufacturer's specifications with dual unique indexing automated on a liquid handler. Samples were sequenced to target depth of 40M read pairs on Illumina Novaseq 6000 using S4 flow cells.

Bioinformatics analysis

Quality control of each metagenome was performed using tools from the BBMap software package(25) and the bioBakery3 suite2(26). Identical duplicated sequence reads were removed using the Clumpify tool in "dedupe" mode allowing 0 substitutions. PhiX spike-in sequence reads were removed using BBDuK. Kneaddata (v0.10.0) was used to trim low-quality bases, remove adapters, discard short reads, and filter human reads. Taxonomic profiles were estimated by

mapping reads to clade-specific marker genes using Metaphlan (version 4.0.6) with database version 3.1. A taxonomic table was generated with relative abundance of detected bacterial taxa for each sample.

Statistical analysis

One sample per participant was analyzed (**Suppl. Materials** for additional selection details). Kruskal-Wallis Rank Sum Test or Fisher's Exact tests were used to evaluate continuous and categorical variables across groups; for variables with significant heterogeneity, pairwise tests were conducted. The distribution of *Prevotella copri* was explored by RAI or HIV status using bar charts, medians, and interquartile ranges. Associations between participant characteristics and α-diversity (Shannon, inverse Simpson, Richness) were evaluated with linear regression. Multivariable linear regression models including different combinations of participant characteristics statistically significant in simple linear regression ($\alpha<0.05$) or in previous literature were built (**Table 1**). α-diversity indices were natural log-transformed prior to model fitting. Associations between participant characteristics and species β-diversity were assessed using constrained principal coordinates analysis (PCoA) of Bray-Curtis dissimilarities(27). Statistical significance was tested by permutational analysis of variance using distance matrices with 1000 permutations(28).

Table 1. Description of Models Included in Analysis

Model	Participants included	Factors included
Models to assess associations with α-diversity		
1	All	Age (in years), HIV status/viral load (PLWH with VL <200 copies/mL, PLWH with VL ≥200 copies/mL compared to HIV Negative) and Ever RAI compared to never
2	All	Age (in years), HIV status/viral load (PLWH with VL <200 copies/mL, PLWH with VL ≥200 copies/mL compared to HIV

		Negative), always condom use with RAI, not always condom use with RAI compared to Never RAI, type of lubricant used with anal sex with men compared to no lubricant use*
3	RAI only	Age (in years), HIV status/viral load (PLWH with VL <200 copies/mL, PLWH with VL ≥200 copies/mL compared to HIV Negative), and condom use (always compared to not always)*
Models to assess associations with top 20 most abundant taxa		
1	All	Age (in years), HIV status/viral load (PLWH with VL <200 copies/mL, PLWH with VL ≥200 copies/mL compared to HIV Negative) and Ever RAI compared to never
2	All	Age (in years), HIV status/viral load (PLWH with VL <200 copies/mL, PLWH with VL ≥200 copies/mL compared to HIV Negative), always condom use with RAI, not always condom use with RAI compared to Never RAI, type of lubricant used with anal sex with men compared to no lubricant use
3	RAI only	Age (in years), HIV status/viral load (PLWH with VL <200 copies/mL, PLWH with VL ≥200 copies/mL compared tor HIV Negative), and condom use (always compared to not always)
4	All	Model 2 with addition of antibiotic use in the past 6 months (yes compared to no) and number of anal sex partners (1, ≥2, compared to none).
5	RAI only	Model 3 with addition of antibiotic use in the past 6 months (yes compared to no) and number of anal sex partners (1, ≥2, compared to none)

*Number of anal sex partners was added in sensitivity analysis
RAI=receptive anal intercourse; PLWH=people living with HIV; VL=viral load; HIV=human immunodeficiency virus

Multivariable zero-inflated beta regression was used to determine differences in relative abundance of the 20 most abundant taxa by participant characteristics(29). Generalized additive models for location, scale, and shape (GAMLSS) were used to fit zero-inflated beta regression models. Presence or absence was modelled using a logit link, while for the continuous non-zero part a log link was used for location and scale parameters. Separately, for each taxon, we fitted three models which included the same covariates specified for our analyses of α-diversity; in sensitivity analysis we also added antibiotic use prior to the sample and number of receptive anal sex partners reported at the start of the study. The scale parameter was modelled as a function of sequencing depth. P-values were adjusted for multiple testing using the Holm method(30). To guide interpretation, model results are presented as the $\log_2$ fold change in the mean

relative abundance between groups for categorical variables, or per one unit increase in exposure for continuous variables. Mean relative abundances were estimated from fitted models(31). We repeated these analyses for the genus *Prevotella***.** Species detected in <5% of specimens were excluded from all analyses. Analyses were conducted in R version 4.2.2. PCoA and adonis2 were performed using the *vegan* package(32). Heatmaps were generated with *heatmap3*. Zero-inflated beta regression used the *gamlss* package(33).

RESULTS

Of the 425 participants sampled, 308 (72%) were living with HIV, of those 58% were virally suppressed (VL<200 copies/mL), with the majority (93%) having a CD4 count >200 cells/mm^3; HIV status was unknown for 3 participants; they were excluded from analyses with HIV status. Viral suppression was used as a biologic marker of ART adherence. Virally suppressed PLWH were older than PWoH and PLWH with unsuppressed VL (**Table 2**). PWoH were more likely to report never having RAI as compared with PLWH. More virally suppressed PLWH had used antibiotics and “always” used condoms with receptive anal sex compared with other groups. A higher proportion of PLWH with unsuppressed VL endorsed ≥2 receptive anal sex partners in the 12 months prior to enrollment than virally suppressed PLWH; PWoH endorsed the fewest partners. Those endorsing RAI were younger, more likely to be PLWH, and less likely to have attended higher than senior secondary school than those who did not (**Suppl. Table 1**).

Table 2. Demographics and behavioral factors by HIV status

Variable	All#	PWoH	PLWH: suppressed VL < 200 copies/mL	PLWH: unsuppressed VL ≥ 200 copies/mL	p-value
N	422	116	180	126	
Age (yrs) median [Q1,Q3]					
	25.00[22.00,28.00]	24.00[22.00,28.00]	26.00[23.00,29.00],A	24.00[21.25,27.00],A	**0.007**
CD4 Count cells/mm^3 (PLWH)					
< 200	21 (6.9%)	N/A	10 (5.6%)	11 (8.7%)	0.135
≥ 200	285 (93.1%)	N/A	170 (94.4%)	115 (91.3%)	
Receptive Anal Intercourse					
Never RAI	37 (8.8%)	25 (21.6%),A,B	8 (4.4%),A	4 (3.2%),B	**<0.001**
Ever RAI	385 (91.2%)	91 (78.4%),A,B	172 (95.6%),A	122 (96.8%),B	
Sexual orientation					
Bisexual	246 (58.3%)	79 (68.1%),A	101 (56.1%)	66 (52.4%),A	**0.040**
Homosexual	176 (41.7%)	37 (31.9%),A	79 (43.9%)	60 (47.6%),A	
Education					
No school	2 (0.5%)	0 (0.0%)	1 (0.6%)	1 (0.8%)	0.545
Quranic only	3 (0.7%)	1 (0.9%)	2 (1.1%)	0 (0.0%)	
Primary	8 (1.9%)	3 (2.6%)	3 (1.7%)	2 (1.6%)	
Junior Secondary	14 (3.3%)	5 (4.3%)	2 (1.1%)	7 (5.6%)	
Senior Secondary/SSS	223 (52.8%)	59 (50.9%)	96 (53.3%)	68 (54.0%)	
Higher than SSS	170 (40.3%)	48 (41.4%)	75 (41.7%)	47 (37.3%)	
Missing	2		1	1	
Current Antibiotic Use					
No	406 (96.2%)	115 (99.1%)	169 (93.9%)	122 (96.8%)	0.055
Yes	16 (3.8%)	1 (0.9%)	11 (6.1%)	4 (3.2%)	
Antibiotic Use in last 6 months					
No	325 (77.0%)	107 (92.2%),A	114 (63.3%),A,B	104 (82.5%),B	**<0.001**
Yes	97 (23.0%)	9 (7.8%),A	66 (36.7%),A,B	22 (17.5%),B	
Number of receptive anal sex partners 12 months prior to enrollment					
0	67 (15.9%)	36 (31.0%),A,B	22 (12.2%),A	9 (7.1%),B	**<0.001**
1	60 (14.2%)	16 (13.8%),A,B	28 (15.6%),A	16 (12.7%),B	
≥ 2	284 (67.3%)	62 (53.4%),A,B	124 (68.9%),A	98 (77.8%),B	
Missing	11	2	6	3	
Lubricant use with anal sex with men (insertive or receptive not specified)					
No	11 (2.6%)	3 (2.6%)	3 (1.7%)	5 (4.0%)	0.490
Yes	382 (90.5%)	100 (86.2%)	167 (92.8%)	115 (91.3%)	
Missing	29	13	10	6	
Ever lubricant use during sex (insertive or receptive not specified)					
No	33 (7.8%)	13 (11.2%)	13 (7.2%)	7 (5.6%)	0.243
Yes, condom (N)	27 (6.4%)	8 (6.9%)	8 (4.4%)	11 (8.7%)	
Yes, condom (Y)	191 (45.3%)	54 (46.6%)	87 (48.3%)	50 (39.7%)	
Sometimes, condom (N/Y)	171 (40.5%)	41 (35.3%)	72 (40.0%)	58 (46.0%)	
Type of lubricant use among those endorsing anal sex with men (insertive or receptive not specified)					
None	11 (2.6%)	3 (2.6%)	3 (1.7%)	5 (4.0%)	0.060
Petroleum Jelly/Vaseline	39 (9.2%)	11 (9.5%)	13 (7.2%)	15 (11.9%)	
Body creams/fatty creams	36 (8.5%)	11 (9.5%)	18 (10.0%)	7 (5.6%)	
Water-based	285 (67.5%)	68 (58.6%)	129 (71.7%)	88 (69.8%)	
Other/DK/Missing	51 (12.1%)##	23 (19.8%)	17 (9.4%)	11 (8.7%)	
Condom use with RAI					
N/A (Never RAI)	37 (8.8%)	25 (21.6%),A,B	8 (4.4%),A,C	4 (3.2%),B,C	**<0.001**
Not Always	158 (37.4%)	39 (33.6%),A,B	50 (27.8%),A,C	69 (54.8%),B,C	
Always	227 (53.8%)	52 (44.8%),A,B	122 (67.8%),A,C	53 (42.1%),B,C	

Fisher's Exact test for categorical and Kruskal-Wallis Rank sum test for continuous. If the global test across all three groups was significant ($p<0.05$), pairwise Kruskal-Wallis or Fisher's Exact tests were performed for continuous and categorical variables, respectively, with adjustment for multiple testing using the Bonferroni method. Rows with the same letter (A, B, C) indicate pairwise statistical significance at $p<0.05$. # HIV status unknown for 3 participants. ##N=19 DK: (Don't know), N=4 Other, N=28 Missing. VL=Viral load, RAI=receptive anal intercourse, PLWH=people living with HIV, PWoH=people without HIV

Rectal microbiota features

The most abundant species across all samples was *Prevotella copri* (**Figure 1)**. While median relative abundance of *P. copri* was 5%, a few individuals had a very high relative abundance of *P. copri* (>40%). There were no significant differences in the distribution of *P. copri* by RAI, HIV status, or VL (**Suppl. Figure 1A-C)**.

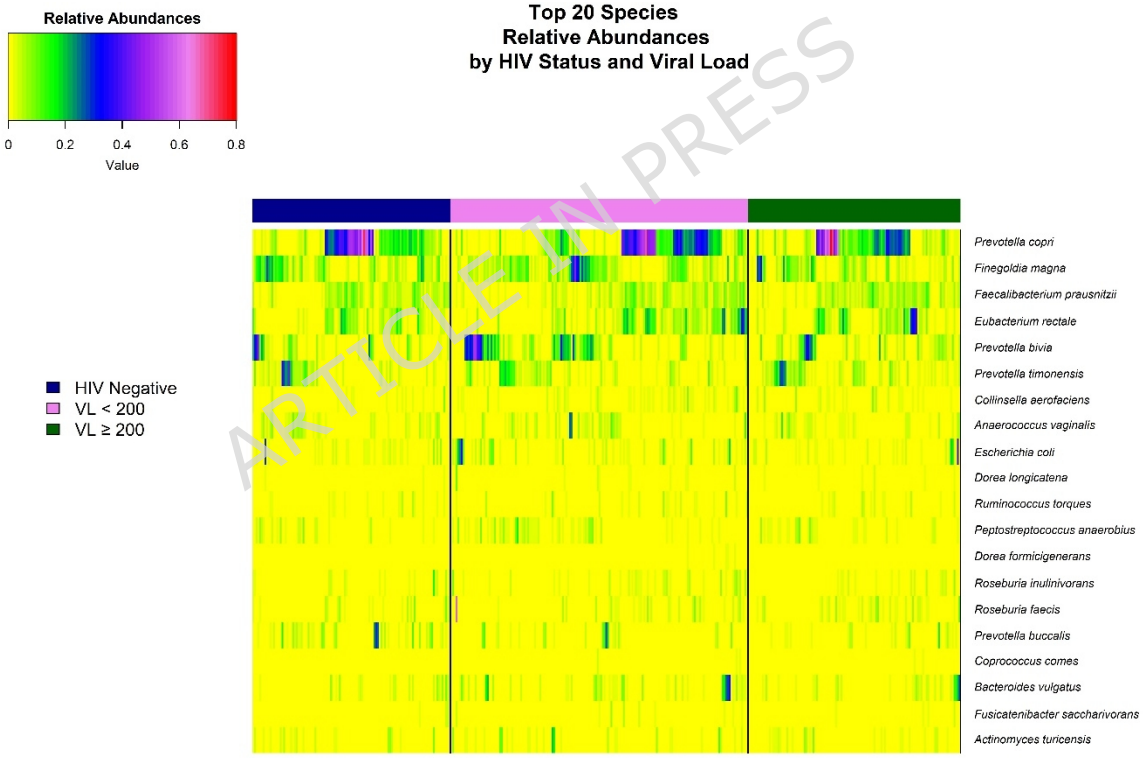


Figure 1. Heatmap showing 20 most abundant species identified over all rectal samples

Associations of participant factors with rectal microbiota diversity (alpha-diversity)

Measures of α-diversity (Shannon, inverse Simpson, and richness) were calculated. Several factors were associated with α-diversity in bivariable analysis (**Suppl.**

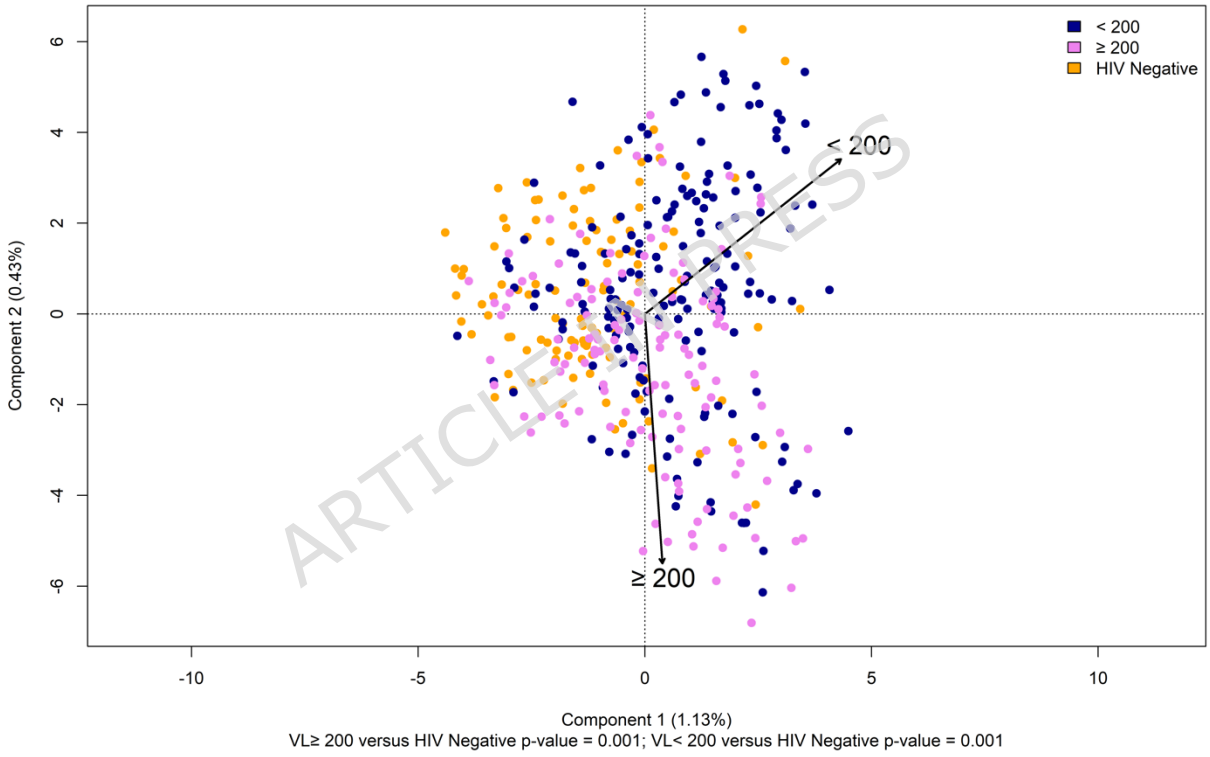
Tables 2A-C). Because age has been associated with diversity in previous literature, it was included in multivariable models. CD4 count lacked sufficient heterogeneity, was co-linear with VL and was not included. Sexual identity was collinear with RAI and not included. Due to limited sample size, as well as correlation of RAI with HIV, we built 3 multivariable models (**Table 1**). In adjusted analysis, use of petroleum jelly or vaseline as a lubricant for anal sex with men was associated with lower Shannon diversity in all participants and amongst those endorsing RAI. There was lower richness amongst PLWH with VL<200 and VL≥200 as compared to PWoH in Model 1 with similar trends in Model 2 and 3. In Model 1, there was lower richness in those endorsing RAI as compared to those who said they had never had RAI, but this was not statistically significant (p=0.08). In sensitivity analysis, adding number of RAI partners in the last 12 months to Models 2 and 3 did not change results; number of RAI partners was not statistically significant.

Comparison of overall rectal microbiota composition (beta diversity) between groups

In principle coordinates analysis supplemented by PERMANOVA, there were statistically significant differences between PWoH, PLWH with VL suppression and PLWH without VL suppression (**Figure 2**). No significant differences in beta diversity were seen by number of RAI partners, RAI, or condom use with RAI (**Supp. Figure 2A**). Results were unchanged when restricted to individuals endorsing RAI. Amongst PWoH, there were significant differences by age (**Suppl.**

Figure 2B) but not by RAI (**Suppl. Figure 2C**), or by antibiotic use in the past 6 months.

Figure 2. Comparing Beta Diversity-Principal Coordinates Analysis constrained by people living with HIV with HIV viral load<200 versus viral load ≥200 vs people without HIV with Visual plot and PERMANOVA



Associations between participant factors and top 20 most abundant taxa

We examined associations between the same factors assessed in the α-diversity analysis and relative abundance of the top 20 most abundant species (**Table 1:** models). In sensitivity analysis we added number of RAI partners and antibiotic to

Models 2 and 3, as antibiotic use and number of partners were associated with higher or lower relative abundance of specific species in univariate models. Descriptions of associations with specific taxa follow (**Figure 3, Suppl. Figure 3, Table 3, Suppl. Tables 3-5**). No significant associations were seen with antibiotic use after adjustment for multiple comparisons (**Suppl. Figure 3A, Suppl. Table 4E, 5E).**

HIV:

Based on Model 1, PLWH with VL<200 had a significantly higher relative abundance of *Bacteroides vulgatus, Colinsella aerofaciens* and *Escherichia coli* and a significantly lower abundance of *Coprococcus comes, Fusicatenibacter saccharivorans, Roseburia faecis,* and *Ruminococcus torques* compared to PWoH. Similar results were seen across the other models; additionally Models 2, 3, and 5 showed significantly lower *Eubacterium rectale*, Models 3 and 5 lower *Prevotella buccalis*, and Model 2 higher *Roseburia inulinivorans* in PLWH with VL<200 as compared with PWoH

Based on Model 1, PWH with a VL≥200 had higher relative abundances of *Bacteroides vulgatus* and lower *Prevotella buccalis.* Similar trends were seen across most other models. Models 2, 3, and 4 showed higher *Faecalibacterium prausnitzii*, and Models 3 and 5 showed higher *F. saccharivorans* and *E. rectale*, and decreased *E. coli* in PWH with a VL ≥200 as compared to PWoH (**Figure 3A**, **Suppl. Tables 3, 4, 5**). **Table 3** summarizes taxa that remained significant across the examined models after multivariable analysis with Holm method adjustment for multiple comparisons.

RAI and condom use with RAI:

Based on Model 1 (**Figure 3B, Suppl. Table 3**) before adjustment, there was lower relative abundance of *Prevotella buccalis, C. aerofaciens, Bacteroides vulgatus, Anaerococcus vaginalis* and higher *Actinomyces turcensis* in those endorsing RAI versus those who never endorsed RAI. However, there were no statistically significant associations after adjustment for multiple comparisons. Similar trends in *P. buccalis, B. vulgatus*, and *A. vaginalis* were seen across Models 2 and 4 in those endorsing condom use with RAI versus no RAI. Based on Model 2, after adjustment for multiple comparisons there was significantly lower relative abundance of *R. inulovorans, Prevotella timonensis* and *F. prauznitzii* in those who endorsed always condom use with RAI versus no RAI. There was lower relative abundance of *R. inulovorans, B. vulgatus* and *A. vaginalis* in those not always using condoms with RAI versus no RAI. Similar trends were seen in Model 4. However, when restricting to those endorsing RAI to try to isolate out condom use effects (Model 3, 5), the only significant association was lower relative abundance of *E. rectale* in those always versus not always using condoms (**Figure 3C, Suppl. Table 4, 5**)

Number of receptive anal sex partners:

In sensitivity analyses (Model 4), *P. buccalis* was higher in those endorsing 1 versus no RAI partners, and *P. buccalis* and *F. saccharivorans* were higher in those endorsing ≥2 partners vs. none. Similar results were seen in Model 5; additionally, *B. vulgatus* was higher amongst those endorsing ≥2 partners versus none in this model. *P. copri* was higher in those endorsing ≥2 partners versus none in both

models, but this was not statistically significant after adjustment for multiple comparisons (**Figure 3D, Suppl. Tables 4F, 5F**).

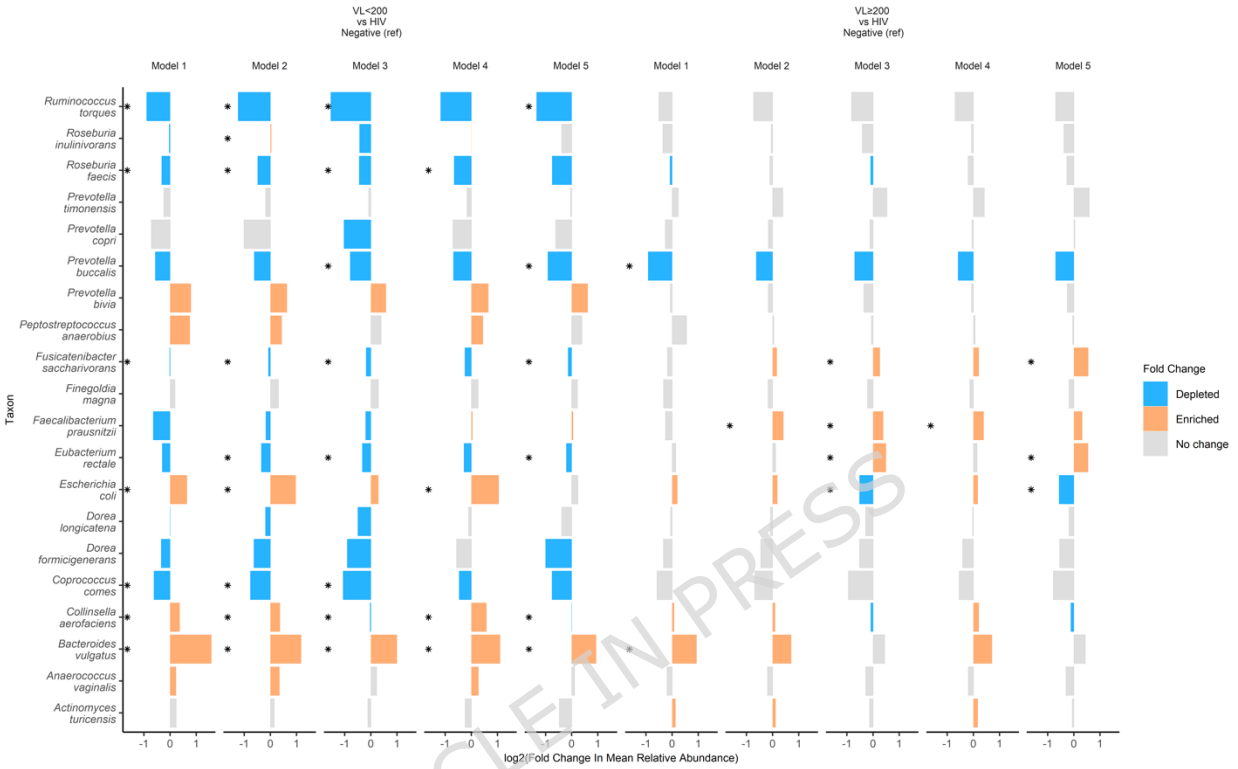
Lubricant used with anal sex:

Based on Model 2, all groups using lubricants had higher relative abundance of *A. turicensis* as compared to those without lubricants. All groups using lubricants except the other/"don't know"/missing group had higher *R. inulinivorans*, and all except the petroleum jelly and other/ "don't know"/missing groups had lower *E. coli*. Broadly similar results were seen across other models. However, in Models 3 and 5 (amongst those endorsing RAI) there was higher *F. saccharivorans* in all lubricant user groups compared to none, and lower *E. coli* in all lubricant user groups (including Petroleum jelly) except the Other/DK/Missing group. In Model 3, water-based creams associated with increased *E. rectale* versus none (**Figure 3E**, **Suppl Table 4, 5**).

Age:

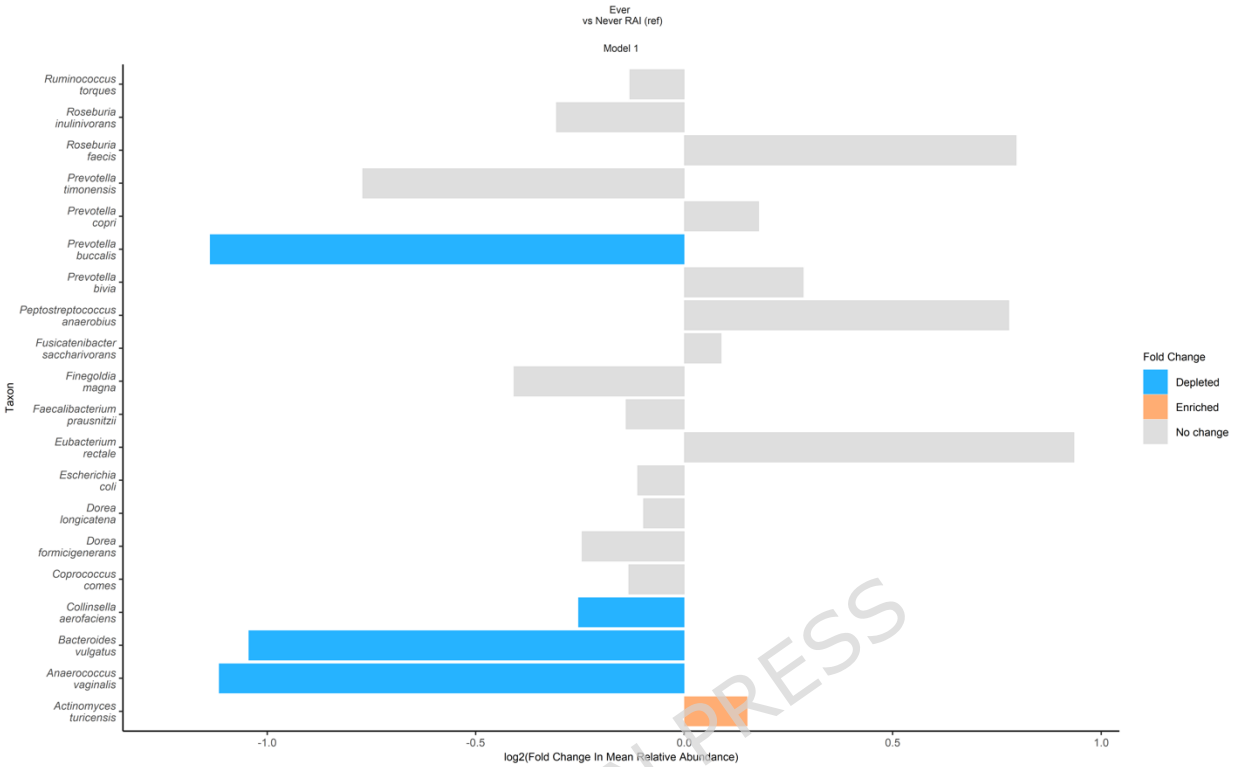
Based on Model 1 (**Suppl. Figure 3B**, **Suppl. Table 3A-C**)), there was significantly higher relative abundance of *E. rectale, F. prausnitzii, R. faecis, R. inulovorans*, and *R. torques* with older age. Similar trends were seen across Models 2 through 5 (**Suppl. Figure 3B, Suppl. Tables 4A-F**, **5A-F**), although based on Model 2 there was also a negative association of *C. aerofaciens* with older age; associations with *F. prauznitzii* were inconsistent and not statistically significant across Models 3, 4 and 5.

Figure 3A. Associations between relative abundance of top 20 most abundant species and viral load in people with HIV compared to people without HIV in Models 1 to 5.



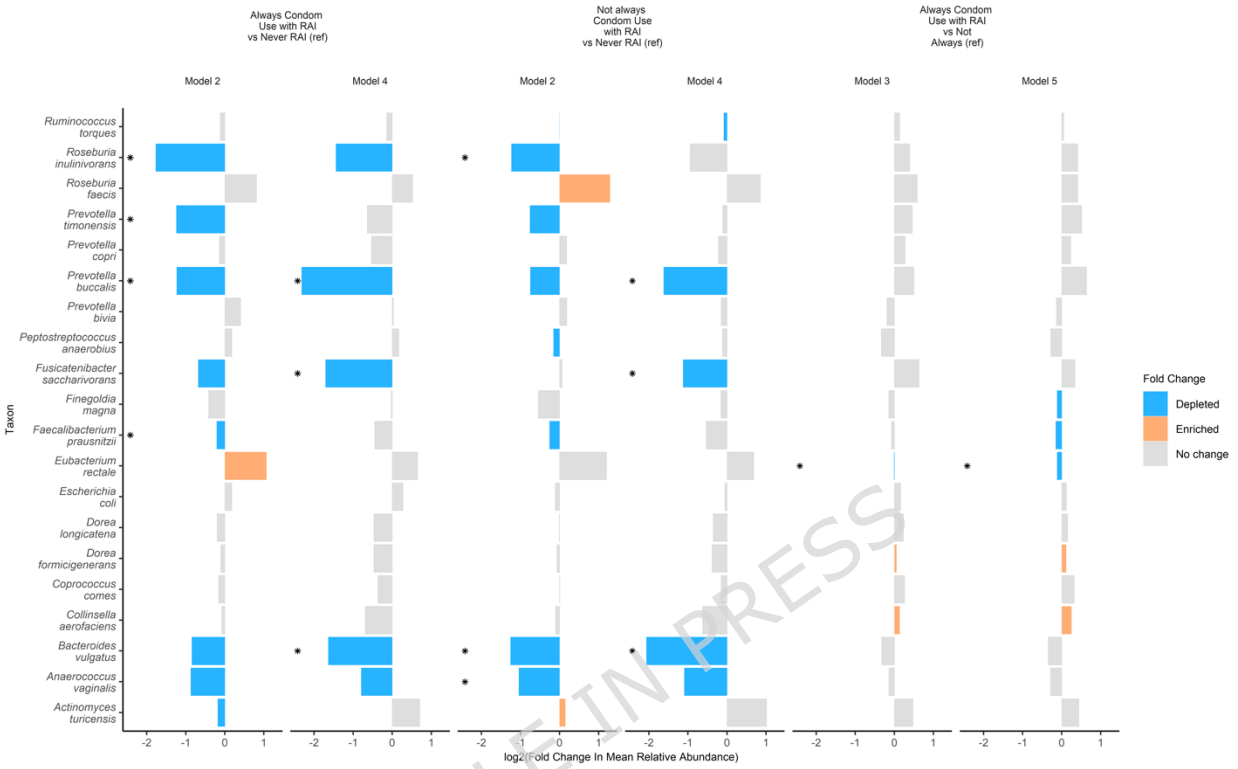
Associations which were statistically significant before adjustment for multiple comparisons are denoted in blue and orange. Associations statistically significant after adjustment by the Holm method are denoted with a *. RAI=receptive anal intercourse, VL=viral load, HIV=Human Immunodeficiency Virus. Model 1: age, HIV status, and RAI; Model 2: age, HIV status, condom use with RAI, and type of lubricant; Model 3: within those endorsing RAI only-age, HIV status, condom use, and type of lubricant, Model 4: age, HIV status, condom use with RAI, and type of lubricant use, antibiotic use in last 6 months, number of anal sex partners, Model 5: within those endorsing RAI only-age, HIV status, condom use, type of lubricant use, antibiotic use in the past 6 months and number of anal sex partners in the past 12 months.

Figure 3B. Associations between relative abundance of top 20 most abundant species and Ever RAI compared to Never RAI in Model 1.



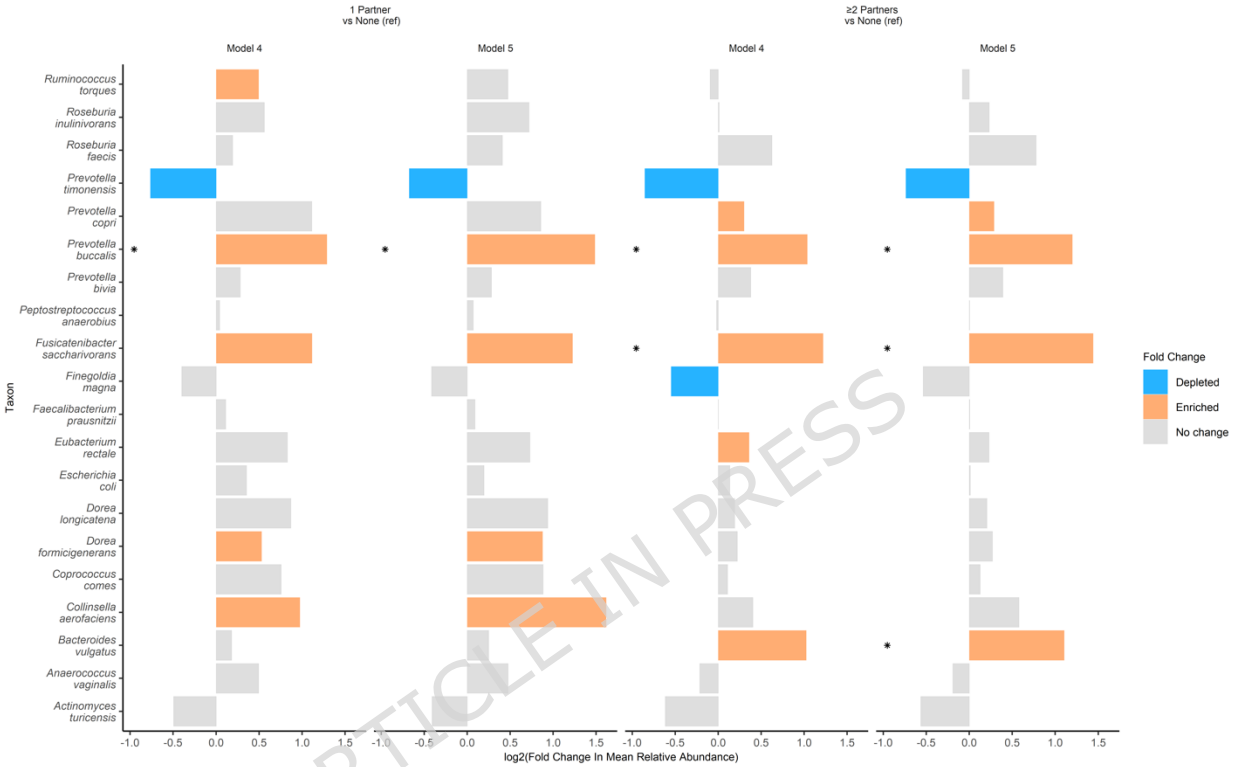
Associations which were statistically significant before adjustment for multiple comparisons are denoted in blue and orange. No associations statistically significant after adjustment by the Holm method. RAI=receptive anal intercourse. Model 1: age, HIV status, and RAI.

Figure 3C. Associations between relative abundance of top 20 most abundant species and frequency of condom use with receptive anal intercourse in Models 2 to 5.



Associations which were statistically significant before adjustment for multiple comparisons are denoted in blue and orange. Associations statistically significant after adjustment by the Holm method are denoted with a *. RAI=receptive anal intercourse, VL=viral load, HIV=Human Immunodeficiency Virus. Model 2: age, HIV status, condom use with RAI, and type of lubricant; Model 3: within those endorsing RAI only-age, HIV status, condom use, and type of lubricant, Model 4: age, HIV status, condom use with RAI, and type of lubricant use, antibiotic use in last 6 months, number of anal sex partners, Model 5: within those endorsing RAI only-age, HIV status, condom use, type of lubricant use, antibiotic use in the past 6 months and number of anal sex partners in the past 12 months.

Figure 3D. Associations between relative abundance of top 20 most abundant species and number of male anal sex partners compared to none in Models 2 and 5.



Associations which were statistically significant before adjustment for multiple comparisons are denoted in blue and orange. Associations statistically significant after adjustment by the Holm method are denoted with a *. RAI=receptive anal intercourse, VL=viral load, HIV=Human Immunodeficiency Virus. Model 4: age, HIV status, condom use with RAI, and type of lubricant use, antibiotic use in last 6 months, number of anal sex partners, Model 5: within those endorsing RAI only-age, HIV status, condom use, type of lubricant use, antibiotic use in the past 6 months and number of anal sex partners in the past 12 months.

Figure 3E. Associations between relative abundance of top 20 most abundant species and lubricant use

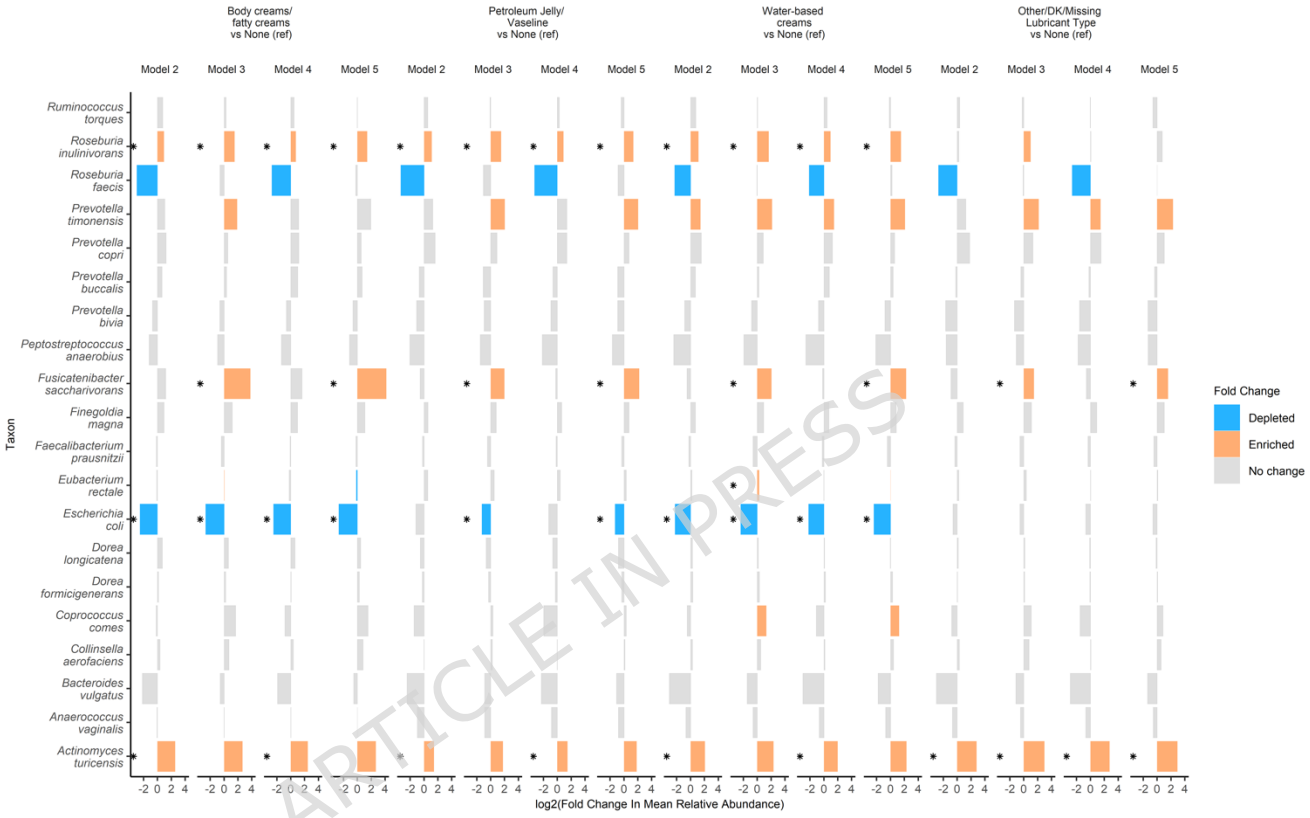


Table 3. Summary of Trends Across Models: Top 20 taxa*

Taxon	Older Age	RAI vs. no RAI	Condom use (Among RAI)	1 anal sex partner vs. none	≥2 anal sex partners vs. none	Lubricant use	Lubricant use Among RAI	HIV VL<200 vs. HIV neg	HIV VL ≥200 vs. HIV neg
Ruminococcus torques	Higher							Lower	
Roseburia inulinivorans	Higher	Lower				Higher	Higher	Higher (only in Model 2)	
Roseburia faecis	Higher							Lower	
Prevotella timonensis		Lower							
Prevotella copri									
Prevotella buccalis		Lower		Higher	Higher			Lower	Lower
Prevotella bivia									
Peptostreptococcus anaerobius									
Fusicatenibacter saccharivorans		Lower			Higher		Higher	Lower	Higher
Finegoldia magna						Lower (except Petroleum jelly)	Lower		
Faecalibacterium prausnitzii	Mixed trends; higher in Model 1, 2	Lower							Higher
Eubacterium rectale	Higher		Lower					Lower	Mixed (Higher in RAI only)
Escherichia coli								Higher	Mixed (Lower in RAI only)
Dorea longicatena									
Dorea formicigenerans									
Coprococcus comes								Lower	

Collinsella aerofaciens	Mixed trends: lower Model2							Higher	
Bacteroides vulgatus		Lower			Higher			Higher	Higher
Anaerococcus vaginalis		Lower							
Actinomyces turicensis							Higher		

*Association had to be statistically significant after adjustment in at least one model to be noted in the table in the colored boxes (orange for higher, blue for lower). Where no associations were statistically significant, boxes are colorless.

Prevotella genus specific analyses:

Based on Model 1 (**Suppl. Table 6A**), we found lower relative abundance of *Prevotella* in PLWH with VL<200 compared to PWoH. This remained consistent in Models 2 and 4 (**Suppl. Table 6B**). In Model 4 there was lower *Prevotella* in those endorsing 1 versus no RAI partners. These associations were no longer statistically significant when restricted to those endorsing RAI (Models 3, 5 **Suppl. Table 6C**).

DISCUSSION

In this cohort of Nigerian MSM, we found PLWH with VL<200 had less diverse rectal microbiota [richness (numbers of different species)] compared to PWoH, with similar trends for PLWH with VL ≥200. We found lower α-diversity with use of petroleum jelly lubricant for anal sex. Lower relative abundance of the *Prevotella* genus (although not *P. copri,* the most abundant taxa) was seen in PLWH with VL<200 versus PWoH. When examining relative abundance of the top 20 taxa, we found differences associated with age, HIV status (with more differences in those virally suppressed on ART when compared with PWoH than in those not virally

suppressed compared to PWoH), lubricant use, RAI, and condom use, suggesting multiple clinical and behavioral factors impact the rectal microbiota.

Early studies utilizing 16S rRNA gene amplicon sequencing had described a characteristic increase in abundance of the genus *Prevotella* in the gut or rectal microbiota of PLWH as compared to PWoH(1). However a study from Spain(17), now corroborated by several studies from the US and Europe and one from urban China(9, 14, 16, 18, 34-38), utilizing stool(5, 9, 14, 16, 18, 35, 37, 38) and rectal or anal samples(36, 38) suggested these findings were due to confounding, and that increases in *Prevotella* were associated with MSM rather than HIV status(1). Since then, in studies controlled for MSM status, a clear pattern of HIV-associated gut or rectal microbiota dysbiosis which remains consistent across populations and geography has not emerged(1). Adding complexity, associations with HIV may be different in MSM as compared to women or men who only have sex with women(7, 8). Furthermore, ART use may impact the microbiota (whether because of direct effects on the bacteria, indirect effects through immune reconstitution, or other, as yet poorly elucidated mechanisms). Several studies from the US or Europe have found HIV PrEP use associates with changes in specific rectal taxa(39-41); others have found differences in fecal(16) or rectal(42) microbiota of PLWH on ART compared to HIV negative individuals or PLWH not on ART. Finally, geography, whether due to differences in diet or other unmeasured factors, may impact these associations. Increases in *Prevotella* and decreases in *Bacteroides* in gut microbiota have been described in PWoH living in rural settings in Africa and the Amazon who consume higher fiber diets as compared with “Westernized” urban US/Europeans(1). A recent study(7) found not only increased *Prevotella* in the fecal

microbiota of PWoH from Botswana and Uganda as compared with Boston, but entirely different patterns of taxa differentiating PLWH from PWoH across populations in these different geographic locales. Collectively, these data imply gut or rectal microbiota associations with HIV status may be quite local and population specific - an implication which our findings support.

Below, we situate our major findings with respect to alpha diversity, *Prevotella* predominance, and the relationship of behavioral factors to the rectal microbiota in MSM in the context of existing literature.

Alpha diversity and HIV status: Our finding of decreased rectal microbiota richness (although not Shannon and Simpson diversity) in PLWH with suppressed VL (on ART) versus PWoH, suggests presence but not abundance of specific taxa differs among PLWH on ART versus PWoH. In US and European populations one study that included MSM found a similar decrease in gut α-diversity richness(9). However, one US study among MSM found an increase in rectal α-diversity richness in PLWH on ART compared to PWoH(43), others that included MSM in the US or Europe, and one in Malaysia found no difference based on gut(7, 16) or rectal(44, 45) samples. Some(46, 47), but not all(48), studies in African populations in non-MSM have found decreases in gut α-diversity in PLWH on ART. One found decreases in gut α-diversity in non-MSM with HIV on ART as compared with PWoH in Uganda but not in Botswana(7). Besides our group, very few studies have compared rectal microbiota in PLWH and PWoH in MSM outside of the US and Europe. The one study conducted in MSM in Africa (in Kenya) which examined 46

PWoH, 24 PLWH on ART, and 10 PLWH untreated, also found decreased rectal α-diversity in PLWH on ART as compared to PWoH, similar to our current study(13). A previous study using 16S rRNA gene amplicon sequencing of rectal microbiota in a smaller group from the same cohort as our study found no differences by HIV status in Shannon diversity, but richness was not calculated(6).

Prevotella predominance and HIV status: In our study, lower relative abundance of the Prevotella genus (although not P. copri, the most abundant species) was seen in PLWH with VL<200 versus PWoH. Most US or European studies that properly controlled for MSM status have not found higher *Prevotella* in PLWH based on fecal(9, 37, 47, 49) or rectal(41, 43, 45, 50) samples. In fact, one study from the Netherlands which controlled for MSM status found a decrease in fecal *Prevotella* in PLWH(9). A study from Boston found increased gut *Prevotella* spp. in PLWH compared with PWoH amongst MSM but not in non-MSM. Notably, in this same study, in a non-MSM cohort from Uganda, there were decreases in multiple *Prevotella* spp. in PLWH on ART as compared to PWoH; no such differences were observed in a Botswana cohort(7). In a Ghanaian (non-MSM) cohort, a decrease in gut *Prevotella* was observed in PLWH compared to PWoH(47). In the study of rectal microbiota in MSM in Kenya, there was a non statistically significant association between PLWH on ART being less likely to cluster in the high *Prevotella* group than PWoH(13). We had also seen decreases in *Prevotella* in PLWH on ART vs. PWoH in previous work in the same cohort using 16S rRNA gene amplicon sequencing on rectal samples(6). There is growing interest in whether *Prevotella* spp. are pro-inflammatory(34, 51) and contribute to dysregulated immune responses in PLWH. *P. copri*, in particular, has been linked to rheumatoid

arthritis(1) and, along with *P. stercorea,* has shown strong associations with MSM status in the few studies that have analyzed fecal(16, 52-54) microbiota at the species level. *P. copri* was the most prevalent taxa across all groups on our study, (similar to findings utilizing stool among MSM in Boston and non-MSM in Uganda and Botswana(7)). However, we did not observe any significant associations with *P. copri* by HIV status, RAI or any other factor. Additional work has suggested many different clades of *P. copri* (or *Segatella copri*) may figure in the fecal microbiota of MSM(34), may differ across geography, and that some of these may occupy important and possibly beneficial niches(55). Finally, for other individual taxa, there was not much overlap between our results and that of the only other group that published research on rectal microbiota MSM conducted in Africa (Kenya(13)), but comparing results is difficult because our study utilized metagenomics whereas theirs utilized 16S rRNA gene amplicon sequencing.

Behavioral factors and associations with the rectal microbiota: Although RAI is hypothesized to be a factor linked to key features of the rectal or gut microbiota in MSM, including increased *Prevotella*(1), we found no significant associations either with the *Prevotella* genus or with *P. copri* in multivariable analysis comparing those who endorsed RAI vs. no RAI. The Kenya MSM study similarly did not find significant associations between *Prevotella* predominance and sexual behaviors(13). In the US and Europe, some studies have described increased *Prevotella* and decreased *Bacteroidaceae* in fecal(9) or rectal(36, 38) microbiota in those endorsing RAI, but others using stool(5) or rectal(56) samples have not. An increased number of sexual partners correlated with abundance of rectal *Prevotella* in one US study(41), and >3 partners (insertive or receptive anal sex

was not defined) was associated with increased *Prevotellaceae* and *Segatella*/*Prevotella* clades in the fecal microbiota of MSM from Europe(5). However, we did not observe similar trends; in fact, in one analysis we found lower *Prevotella* in those endorsing 1 versus no anal sex partners. Some have speculated rectal semen exposure might affect the microbiota of MSM(1, 13) . In the Kenya study(13), reporting “not always” using condoms with regular partners was associated with lower rectal α-diversity; however we did not observe this. We found only one taxa level change in those engaging in RAI using condoms vs. not using condoms, though we did observe decreases in a few taxa associated with RAI with or without condom use as compared to no RAI. One study conducted in Europe(5) found “never” condom users had increased fecal Shannon diversity and richness versus “always” and “sometimes”, respectively, whereas a study from the Netherlands found no significant difference in fecal community composition(9). Another factor speculated to affect the microbiota is lubricant use with RAI. One small study in the US in which MSM without HIV (n=20) used hyperosmolar water based rectal lubricant daily for 7 days, (n=19) or oral TDF/FTC (n=21), or both found a non-significant increase in the genus *Prevotella* in rectal samples after lubricant use(10). We found no associations between lubricant use and *Prevotella*, but did find changes in other taxa with lubricant use; using petroleum jelly as a lubricant associated with decreased α-diversity.

The strengths of our study include the sample size. This is the largest study to evaluate the rectal microbiota of MSM in any country, and certainly in Africa, where only one other group (Kenya(13)) has examined the rectal microbiota of MSM. Also, we used metagenomic sequencing, which allowed us to better identify

taxa down to the species level which few other studies(34) (none in Africa) have done in MSM. Finally, we were able to control for multiple clinical and behavioral factors to best assess the relative contribution of these factors to the microbiota. There are also important limitations. This was a cross-sectional study, so longitudinal assessments of the microbiota and relative contributions of each studied factor were not assessed. While substance use, receptive oral sex, and diet have all been linked to the microbiota, we could not assess these factors. All behaviors were by self-report, which could have been subject to bias, it is also possible that there were unmeasured differences between groups. We could not assess number of anal sex partners longitudinally and had to utilize the number reported at the beginning of the study rather than necessarily when each sample was collected. We were unable to differentiate whether lubricant use with anal sex with men was insertive versus receptive. Rectal STIs may impact the rectal microbiota(4), however, all participants had tested negative for rectal gonorrhea and chlamydia. Some data suggests that there may be regional differences in the microbiota in the colon as well as between colonic, fecal and rectal or anal microbiota(38, 58, 59), although other data suggests that anal or rectal samples may be a reasonable surrogate for either colonic or fecal samples(60-64). Thus, it is not certain whether our rectal microbiota results would be generalizable to the colonic or fecal microbiota. Finally, we only assessed associations between factors of interest and the top 20 taxa identified to the species level; we could have missed important associations with less prevalent taxa.

There is interest in whether modifying the gut or rectal microbiota in MSM could decrease acquisition of HIV or other STIs, and whether novel treatments to modify

the gut microbiota in PLWH could decrease inflammation-related chronic disease(1). However accumulating evidence from our study and others suggests many different clinical and behavioral factors may shape the rectal (and likely colonic) microbiota of MSM and that these factors may differ by geography. MSM in countries across sub-Saharan Africa, in particular, are understudied though they remain burdened with high rates of HIV and other STIs. We provide important foundational data showing complex relationships between HIV, ART, and clinical and behavioral factors and the rectal microbiome among MSM in Nigeria. Future characterization of health outcomes associated with the rectal or gut microbiota in MSM and PLWH more broadly as well as potential interventional insights will necessitate larger, dedicated studies across diverse geographic locations.

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