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Characteristic Shifts of the Rectal Mucosal Microbiota associated with Condomless Receptive Anal Intercourse among HIV-Negative MSM

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Abstract

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By Nicole Pescatore, MPH Candidate

Men who have sex with men (MSM) continue to be disproportionately affected by HIV, accounting for 67% of new diagnoses of HIV in the US in 2015. The majority of HIV infections among MSM occur after exposure to the rectal mucosa during anal intercourse. Therefore, a better description of the rectal mucosal immune environment of MSM engaging in condomless receptive anal intercourse (CRAI) is necessary to understand rectal HIV transmission. In this analysis, the microbial composition of the rectal mucosa of HIV-negative MSM who engage in CRAI will be compared to men who have never engaged in anal intercourse. Several measures of microbiota diversity were assessed, and the most abundant genera were identified and compared for both study groups. Furthermore, to determine whether specific patterns of the microbiota composition were consistent between groups, correlations between genera were calculated. Our results were consistent with previous studies in showing the microbiota of MSM to be enriched with the *Prevotella* genus, while non-MSM were enriched with the *Bacteroides* genus. Interestingly, *Prevotella* was found to be inversely correlated with multiple genera associated with pathogenic bacteria in MSM despite being more prevalent in relation to men who have never engaged in CRAI. There were no significant differences in genus diversity and richness between MSM engaging in CRAI and men who have never engaged in CRAI. These results further characterize the microbial alterations associated with CRAI, provides insight on the role of *Prevotella* in the rectal mucosa, and will be useful in the design of future HIV prevention methods where the composition of the microbiota may play a role in efficacy (e.g. vaccine response).

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CHAPTER I: LITERATURE REVIEW

Human Immunodeficiency Virus:

Human immunodeficiency virus (HIV) is a lentivirus that can lead to acquired immunodeficiency syndrome (AIDS) if it is not treated (1). The virus infects CD4+ T cells, a type of helper T-cell, which are white blood cells that send signals to other immune cells responsible for protecting the body from foreign microorganisms. HIV leads to a depletion of CD4+ T cells and, consequentially, leaves the body susceptible to a multitude of opportunistic infections or infection-related cancers that it would normally be able to combat (1). The virus originated from simian immunodeficiency virus (SIV) found in chimpanzees in Central Africa (1) and it is commonly believed that SIV mutated into HIV when it was transmitted from chimpanzees to humans as early as in the late 1800's (1).

There are three stages of HIV that may occur if a person does not receive adequate treatment: 1) *Acute HIV infection*, 2) *Clinical latency*, 3) *Acquired immunodeficiency syndrome* (1). Patients are considered highly contagious during the first stage of HIV, which happens two to four weeks after infection. Indications of acute HIV infection include flu-like symptoms; however, some may not present with any symptoms at all (1). During the second stage of HIV, the virus replicates at lower levels. In patients who have untreated HIV, clinical latency can last over ten years and eventually advance to stage three of HIV infection (1). The last stage of infection leads to the progression of AIDS. Those with AIDS are extremely immunocompromised and have an expected survival of approximately three years if left untreated (1). AIDS is diagnosed

as having a CD4+ T cell count of less than 200 cells per cubic millimeter of blood or having manifested an opportunistic illness (1).

There is currently no cure for HIV, but the virus can be kept under control with antiretroviral therapy (ART) (1). This treatment can not only greatly improve patients' quality and longevity of life, but it can also reduce their likelihood of transmitting the virus to others (1). The Centers for Disease Control and Prevention (CDC) recommends a wide variety of prevention methods to reduce one's risk of transmitting and acquiring HIV. Condom use, reducing the number of sexual partners, getting tested for HIV and other STDs, pre-exposure prophylaxis (PrEP), and post-exposure prophylaxis (PEP) are among the most common and effective methods of HIV prevention (2). The CDC guidelines strongly recommend individuals between the ages of 13 and 64 receive HIV testing as part of their routine healthcare visits (3).

Approximately 1.2 million people in the United States have HIV, and more than 10% of those people are unaware of it (4). Homosexual and bisexual men are the highest risk group for HIV, accounting for 82% of HIV diagnoses among males in 2015 (4). Black/African American gay and bisexual men are at higher risk of infection compared to White and Hispanic gay and bisexual men in the United States primarily due to social determinants of health (e.g. higher rates of poverty, lower levels of educational attainment, access to healthcare, etc.), insular sexual networks with high HIV prevalence, and not knowing their HIV status (4).

HIV Transmission:

HIV is spread when certain bodily fluids from an HIV infected person come in contact with a mucous membrane or site of damaged tissue of another person (5). Mucosal membranes are found in the rectum, vagina, penis, and mouth. Fluids that may contain the virus include blood, semen, pre-seminal fluids, rectal fluids, vaginal fluids, and breast-milk (5). People are more likely to be exposed to or become infected with HIV by participating in certain risk behaviors. The most common HIV risk behaviors in the United States are needle-sharing during injection drug use and condomless receptive anal or vaginal intercourse (6).

Although HIV can be spread through several modes of sexual activities, anal intercourse is the riskiest sexual behavior for both contracting and transmitting HIV for men and women (7). During anal intercourse, the partner who inserts the penis into the anus is the insertive partner, and the partner receiving the penis is the receptive partner (7). The receptive partner is far more likely to contract the virus than the insertive partner. Previous studies have shown that HIV transmission risk is 12-fold higher with receptive anal intercourse among men who have sex with men (MSM) compared with other routes of sexual transmission (8). This is due to the composition of the lining of the rectum, or rectal mucosa, which is more susceptible to tissue damage, may allow the virus to enter the body (7). The human gastrointestinal tract, which includes the rectal mucosa, is the primary target for HIV infection and replication, because it is a reservoir of CD4+ T cells (9). The insertive partner is also at risk of contracting HIV through the urethra or damage to the penile tissue (5). Risk of infection may be reduced when condoms are used; however, they are not 100% effective (5).

Anal intercourse can also lead to other infections such as sexually transmitted diseases (STD) (6). HIV-negative individuals with an STD are even more susceptible to HIV, as they are approximately three times more likely to contract the virus by engaging in unprotected intercourse with an HIV-positive partner (6). Some STDs can cause mucosal or skin irritation or sores through which the virus can enter the body more easily (6). However, even STDs that do not cause skin irritation can lead to an immune response that creates more target cells for HIV to attack; thus, increasing susceptibility to the virus (6). It is important to consider sexually transmitted diseases as possible confounders when conducting HIV studies as it can affect the risk of infection.

The Gut Microbiome:

The human gut microbiota is composed of an extensive range of microbes including bacteria, fungi, and viruses that have an essential responsibility of regulating many aspects of human physiology (10). The microbiome has been shown to have a symbiotic relationship with mammalian host's immune system (10, 11). The human immune system has both innate and highly adaptive mechanisms that allow it to maintain a diverse population of microbiota, and in turn, the microbiota supports and regulates these components of the immune system (10, 12). Under ideal conditions, the immune system communicates with the gut microbiota through cytokines, pattern recognition receptor (PRR) ligands, and antimicrobial peptides. The gut microbiota and mucosal immune system function cooperatively to eliminate pathogenic microorganisms and maintain harmless or commensal microbes (10, 11). Anomalies in these interactions

between the immune system and the microbiota can feasibly contribute to the onset of a multitude of complex diseases (11, 13, 14).

Given that the gastrointestinal tract is a major hub of CD4+ T cells, the gut microbiome has become a focus of modern HIV to gain a deeper understanding of HIV transmission and pathogenesis. The virus is capable of altering the gut microbiota and the permeability of mucosal membranes, which leads to the translocation of gut microbial products into the blood stream (15). Although these microbes are native to the gastrointestinal tract, they can have pathogenic effects when they are present in the systemic circulation (15). Microbial translocation can induce chronic inflammation and immune activation, which exacerbates the progression of HIV (15).

The majority of current data investigating the link between HIV and the gut microbiome focuses on HIV serostatus and the effects of ART (9, 16-20). Previous studies analyzing the microbiome of HIV-positive individuals compared to HIV-negative individuals have noted a significant decrease in diversity and species richness among HIV-positive individuals (16-20). However, these results have been inconsistent, as some studies reported no significant difference or even an increase in diversity or richness among seropositive subjects (21-23).

The microbiota of HIV-positive subjects was demonstrated to be significantly enriched with bacteria of the genus *Prevotella* compared to HIV-negative subjects, who harbored microbiota enriched with bacteria of the genus *Bacteroides* (21, 22, 24, 25). A strong inverse relationship between these two genera was shown in numerous microbiome studies comparing seropositive and seronegative individuals by sequencing bacterial products from stool samples or rectal biopsies (16, 21, 24, 25). The relative

abundance of *Prevotella* has been found to be correlated with an increase of activated T cells and microbial translocation among HIV-positive subjects; however, this association was not seen in HIV-negative individuals (21, 26). While an increase in the relative abundance of *Prevotella* cannot be confirmed to be a product or facilitator of immune activation, studies have observed this genus to be potentially pathogenic under certain conditions (21). An increase in the relative abundance of *Prevotella* has also been associated with a number of inflammatory diseases such as rheumatoid arthritis, active ulcerative colitis, and periodontal disease (21, 27).

In animal models, a species belonging to the *Bacteroides* genus, *B. unifromis*, was shown to restore T cell proliferation deficits in "Western diet"-fed mice as well as induce higher levels of an anti-inflammatory cytokines in germ-free mice (28, 29). Other species of *Bacteroides* were observed to provide protection from several inflammatory diseases in animal models (30, 31). For these reasons, a decrease in the relative abundance of the *Bacteroides* genus may be expected among HIV-positive people who have chronic immune activation (18).

A *Prevotella* enriched microbiota is common among non-Western humans who have a predominantly plant-based diet, while a *Bacteroides* enriched microbiota is more prevalent in Westerners with diets high in fat and protein (32). Although diet plays an pivotal role in the composition of the gut microbiome, previous studies have proposed that the associations between *Prevotella* and certain immune markers indicate that HIV infection alone has the ability to alter the microbiome to some degree regardless of diet (22).

Although it is feasible that alterations in the gut microbiome are dependent on HIV serostatus, sexual behavior is often overlooked as a possible confounder. Remarkably, one study found that MSM were enriched with *Prevotella*, while non-MSM were enriched with *Bacteroides* regardless of their HIV status (16). The analysis also accounted for several variables that potentially alter the fecal microbiota including HIV risk group, HIV serostatus, ethnicity, residency, gender, and fecal consistency. Nevertheless, it was determined that HIV risk group continued to be the only statistically significant variable associated with the enrichment of *Prevotella* in a multivariate ADONIS analysis (R^2: 0.373, $p < 0.001$) (16). Only two investigations have examined the microbial differences between individuals engaging in different sexual behaviors; however, these studies have presented compelling results. In an HIV-negative cohort of men that serves as the data source for this thesis, it was concluded that the rectal microbiota of MSM engaging in condomless receptive anal intercourse (CRAI) was distinct and enriched with *Prevotella* compared to men who do not engage in anal intercourse which was enriched with *Bacteroides* (33). These findings further support the notion that sexual behavior must also be accounted for when exploring the relationship between the gut microbiome and HIV transmission and pathogenesis.

A more recent study published in 2017 showed that HIV-negative MSM and treated HIV-positive MSM significantly differed in rectal microbiota composition; however, the composition of the microbiota of HIV-negative MSM and untreated HIV-positive MSM was essentially similar (23). Since all participants in the study belong to the same HIV transmission group, it is possible that anti-HIV therapeutic agents also disrupt the composition of the rectal mucosa. Given the wide range of current knowledge

on this topic, shifts in the gut microbiome are not easily explained by a singular cause. There are many potential characteristics and risk factors that contribute to the microbial alterations seen in prior studies including HIV status, ART, and receptive anal intercourse (RAI). However, further investigation is needed to identify additional risk factors and their mechanisms of action in hopes of gaining a better understanding of the microbiota and its function in HIV pathology.

CHAPTER II: MANUSCRIPT

Introduction

Men who have sex with men (MSM) continue to be disproportionally at risk for acquiring human immunodeficiency virus (HIV) compared to any other risk group in the United States. According to the Centers for Disease Control and Prevention (CDC), 55% of people living with HIV in 2013 were gay or bisexual men (34). Receptive and insertive anal sex is the riskiest mode of sexual transmission for both acquiring and transmitting the virus; therefore, MSM who engage in anal sex are biologically at increased risk for HIV acquisition (34). Despite extensive research over a number of decades, there is no cure for HIV. However, antiretroviral therapy (ART) is available for HIV-positive patients to hinder disease progression and prevent opportunistic diseases. There are several HIV prevention methods that are widely used such as condom use, reducing the number of sexual partners, abstinence, and pre-exposure prophylaxis (PrEP). Nevertheless, multiple targeted strategies are presently needed to effectively reduce infection rates.

In recent years, some investigations have sought to better describe and understand the role of the gut microbiome on HIV infection and disease progression (9, 15-26, 33). The rectal microbiota is particularly of interest, since it is the most common site of infection among MSM, accounting for 70% HIV transmissions (35). Previous findings have established that HIV infection leads to an increase in gut mucosal permeability, which subsequently leads to microbial translocation (15). The downstream effect of microbial translocation from the gut to the systemic immune system can result in chronic immune activation and disease progression (15). Further understanding the composition of the mucosal microbiome may aid in developing new anti-HIV agents (15).

The greater part of these studies and clinical trials have been aimed at observing HIV-positive subjects and understanding how the virus alters their gut microbiota. A substantial amount of evidence has been collected showing that seropositive individuals have a significantly different microbiota relative to those without HIV. More specifically, HIV-positive subjects have been shown to have an increase in bacteria belonging to the genus *Prevotella* compared to HIV-negative subjects, who are primarily enriched with bacteria belonging to the genus *Bacteroides* (16, 21, 22, 24, 25). In particular, two species of *Prevotella* have been identified to be positively associated with HIV infection: *P. copri* and *P. stercorea*. However, conclusions about species richness and diversity comparing HIV-positive and negative individuals have been inconsistent throughout these studies. Notably, compositional changes seen in humans were not sustained in animal models who were infected with the virus (18). In simian models, no variations in the gut microbiota were found to be a direct result of simian immunodeficiency virus (36).

A limited number of studies have hypothesized that sexual behavior can meaningfully influence rectal microbial shifts in HIV-positive subjects, as opposed to the virus itself. This idea arose when Noguera-Julian et al. assessed the gut microbiota across several different HIV transmission groups including MSM, intravenous drug users, and heterosexual males and females (16). The investigators discovered that the composition of fecal microbiota clustered by HIV transmission group rather than by HIV serostatus (16). MSM were also enriched with *Prevotella*, while intravenous drug users and heterosexual subjects were enriched with *Bacteroides*, regardless of HIV status (16). This study revealed that the gut microbiota of MSM exhibited remarkably similar composition to that of HIV-positive subjects in previous analyses.

The current analysis presented here is an extension of our RM-MSM study conducted at the Hope Clinic of the Emory Vaccine Center between 2013 and 2015. The original study aimed to determine the effects of condomless receptive anal intercourse (CRAI) on various immunologic biomarkers of HIV susceptibility in the rectal mucosa among HIV-negative MSM (33). Compared to men who had never engaged in anal intercourse, the results suggested that MSM engaging in CRAI had a phenotypically distinct rectal mucosa (33). MSM in this study presented with increased levels of Th17 cells, higher CD8+ T cell proliferation and production of pro-inflammatory cytokines, increased gene expression signatures associated with mucosal injury, as well as a microbiota enriched with bacteria of the *Prevotellaceae* family (33). However, the number of shared families between MSM and controls did not differ significantly (analysis of similarity (ANOSIM) $R=0.03$, $P=0.1$) (33).

The purpose of the current analysis is to further examine the composition of the rectal mucosal microbiota of HIV-negative subjects participating in the RM-MSM study, specifically at the genus level. We hypothesize that overall differences in the microbial composition can be detected at the genus level between MSM and non-MSM as seen in prior analyses conducted at the family level. We also explore whether a single genus is driving the enrichment of *Prevotellaceae* in MSM engaging in CRAI, and examine whether the relative abundances of other genera are positively or negatively correlated with *Prevotella* and *Bacteroides* enrichment. Several measures of diversity will be calculated to evaluate the compositional similarities between study groups. The investigation will also include generalized linear models explaining the effects of CRAI on the most abundant genera found in HIV-negative MSM and non-MSM. In addition, statistical correlations between these commonly prevalent genera will be evaluated and visualized via heat maps. These findings can add to the existing body of knowledge to further comprehend the role of the microbiota in rectal HIV transmission among MSM. In future research, these data can be utilized in designing new HIV prevention methods where the composition of the gut microbiota may play a role in product efficacy (e.g. an HIV vaccine or topical microbicide).

Methods

Clinical Cohort:

The dataset used in this analysis is derived from the RM-MSM study conceived by the principal investigator, Dr. Colleen Kelley, and conducted at the Hope Clinic of the Emory Vaccine Center between 2013 and 2015. This study was approved by the Institutional Review Board at Emory University. Study subjects consisted of 62 HIV-

negative men between the ages of 18 and 45 who were recruited from the Atlanta community. The cohort consisted of two groups: MSM engaging in condomless receptive anal intercourse and men who had never engaged in receptive anal intercourse (controls). MSM subjects were in monogamous relationships of at least 45 days and engaging in CRAI with an HIV-negative partner. Inclusion criteria for the MSM group comprised of being in good health and having reported a minimum of 4 episodes of CRAI in the past month. Inclusion criteria for controls included being in good general health and no history of receptive anal intercourse regardless of sexual orientation. MSM were asked to keep an electronic or paper sex diary to report every episode of RAI throughout the study. They also recorded whether a condom, enema, or lubricant was used as well as whether ejaculation occurred inside the rectum.

The study consisted of three study visits, and participants were on study for an average of 83 days (Table 1). Subjects were screened for eligibility and tested for HIV during the first visit. Those considered by the principal investigator to be high risk of adverse effects due to study procedures were not enrolled. Participants in the MSM group were asked to abstain from engaging in CRAI for at least 72 hours prior to the second study visit. During the second visit, participants underwent peripheral blood and rectal biopsy sampling using a rigid sigmoidoscope. Mucosal samples were taken 3 to 10 cm from the anal verge. There was a washout period of 8 to 16 weeks between the second and third study visit to allow the rectal mucosa to heal from biopsy sampling. The study investigator hypothesized that the effects of CRAI on the rectal mucosa may be transitory; therefore, MSM were asked to engage in CRAI within 24 hours of study visit 3. The third study visit included repeat peripheral blood and rectal biopsy sampling.

Swabs of the rectal mucosa were obtained during the first and second biopsies (visit 2 and visit 3) for microbiota sequencing and sexually transmitted disease (STD) testing. All study visits and procedures were conducted at the Hope Clinic of the Emory Vaccine Center. Due to loss to follow-up and specimen availability, microbiota sequencing data were available from 54 participants and were included for the purposes of this analysis.

Microbiota Sequencing:

Rectal mucosal swabs were collected via rigid sigmoidoscopy during study visits 2 and 3. The swabs were stored in lysis buffer at -80˚C and subsequently used for microbiota sequencing. DNA was extracted and amplified using methods described in Halpin et al. (37). PCR product from the V1-V2 region of the 16S rRNA gene was sequenced on an Illumina MiSeq® instrument, as previously described in Kelley et al. (33). All samples were rarefied to 2,215 sequences, which encompassed full microbial diversity as defined in Kelley et al. (33). After rarefaction, 274 operational taxonomic units (OTUs) were obtained and 4 samples from the MSM group (1 sample from biopsy 1 and 3 samples from biopsy 2) were removed from the analysis due to insufficient number of reads. The Greengenes database was used to assign taxonomic levels to the genus level.

Analyses:

All downstream analyses were conducted using RStudio® (http://www.r-project.org/) (38). Cohort demographic data were measured using the tableone R package (39) and p-values were calculated using two-sample t-tests (Table 1). Alpha diversity was

measured using Chao1 and Shannon indices to estimate species richness and evenness using the phyloseq package (40). The results were displayed by boxplots generated by phyloseq and ggplot2 packages (41) (Figure 1). Wilcoxon ranked sum tests were performed to determine the change in alpha diversities between biopsies 1 and 2 within each group. Mann-Whitney-Wilcoxon tests were used to assess the differences of alpha diversities between MSM and controls during both biopsies.

Beta diversity was measured using distance based, multidimensional scaling Bray-Curtis dissimilarity analysis and visualized by a principal coordinate plot. The phyloseq package was used to create a semimetric distance matrix for principal coordinates analysis (PCoA) ordinations and to create a Bray-Curtis plot (Figure 2). Bray-Curtis dissimilarity quantifies the overall compositional variations between the two study groups (41). Differences in microbial composition between groups were statistically evaluated using the analysis of similarities (ANOSIM) test via the vegan R package (41).

Relative abundances were summed across samples to determine the most abundant OTUs in MSM, controls, and all participants. The top 10 genera were identified for the three sets; any genus that was among the top 10 in any of the three groups was included in downstream analyses (n = 15, Table 2). Additionally, *Finegoldia* (23) and *Fusobacterium* (18, 19, 23, 42) were included in statistical analyses as they have found to be increased in HIV-positive patients in prior studies.

Mean relative abundances and 95% confidence intervals for OTUs were quantified for MSM engaging in CRAI and controls at biopsy 1 and biopsy 2 using the Rmsic package (43) (Table 3). Mann-Whitney-Wilcoxon tests were used to compare the

mean relative genus abundances between MSM and controls stratified by the biopsy number. Wilcoxon ranked sum tests were performed to evaluate the difference in mean relative abundance between the two biopsies within each study group; however, these results were not included as they were not statistically significant among all genera included in Table 3.

The R package geepack was used to fit generalized linear models adjusting for repeated measurements (44). Since subjects underwent two biopsies throughout the study, the generalized estimated equation (GEE) approach was used to account for the differences between the two sample collections. Gaussian distributed GLMs with exchangeable correlation structures were used to estimate relative abundances of the most important OTUs among all subjects as well as Chao1 and Shannon indices. The models estimating Chao1 and Shannon indices included the study group variable as the main predictor, where controls represented the reference group, and they were adjusted for repeat biopsy sampling per subject. Wald tests were performed to assess the significance of study group on alpha diversity measures. The initial model estimating OTU relative abundances included study group as the main predictor (controls as the reference group), age as a confounder, and the statistical interaction between study group and age. Race was not considered as a potential confounder as it did not differ substantially between MSM and controls (P=0.172). The interaction term was not statistically significant in all models and was consequently dropped from equation. The final GLM included two variables: study group and age. P-values were calculated by Wald tests to determine the significance of the study group on the relative abundance of the top genera (Table 3).

Blue cells in Table 3 indicate that the relative abundance of the OTU was significantly lower in MSM subjects, while red cells specify that it was significantly higher.

Spearman's rank correlation coefficients were calculated using the R package Hmisc (45) to assess the relationships between the OTUs of interest among all participants, MSM engaging in CRAI, and controls. These correlations and their respective p-values, which were adjusted for multiple correlations using the Holm–Bonferroni method, were visualized using heat maps generated using the corrplot package (46) (Figures 3-5). Significant values displayed in blue and red gradients, where blue denotes a negative correlation coefficient and red denotes a positive correlation coefficient. All correlation coefficients are presented on the panels; however, non-significant Spearman correlation values (significance level = 0.05) are shown as white panels on the map.

Results

Based on previous studies mentioned (17-22, 24, 25), men who have sex with men exhibited similar microbial composition patterns to that of HIV-positive subjects, while the microbiota of men who have never engaged in CRAI structurally resembled to that of HIV-negative individuals. The top 10 OTUs present in our study groups indicate that MSM are primarily enriched with *Prevotella*, a genus within the *Prevotellaceae* family, while non-MSM are enriched with *Bacteroides* at both time points (Table 2 and 3).

Model-based estimates of relative abundances of several genera were statistically different between the two study groups. The generalized linear model determined that the relative abundance of *Prevotella* was still 11% greater among MSM compared to non-

MSM (β=0.11, P=0.003) after adjusting for the time of biopsy and age (Table 3). Additionally, MSM were enriched with an unclassified genus also belonging to the *Prevotellaceae* family compared to non-MSM subjects (β=0.02, P=0.003). The model estimated that the relative abundance of the genus *Bacteroides* was 10% lower among controls compared to MSM (β=-0.10, P=0.01). MSM had an overall decrease in *Faecalibacterium* (β=-0.03, P=0.009) and *Parabacteroides* (β=-0.01, P=0.03) relative to non-MSM (Table 3). Also noteworthy, MSM exhibited an increase in three potentially pathogenic genera, *Streptoccocus* (β=0.04, P=0.016), *Clostridium Cluster XIX* (β=0.002, P=0.013) and *Fusobacterium* (β=0.02, P=0.025). Although not statistically significant, MSM engaging in CRAI were enriched with the pathogenic genus *Escherichia/Shigella* (β=0.02, P=0.16). Notably, MSM had a marginal decrease in relative abundance compared to controls of a genus of the *Ruminococcaceae* family (β=-0.01, P=0.046), a genus of the *Lachnospiraceae* family (β=-0.04, P<0.001), a genus of the *Clostridiales* order (β=-0.01, P=0.021), and an increase in a genus of the *Prevotellaceae* family (β=0.02, P=0.003).

Changes in microbial composition between biopsy 1, where MSM were asked to abstain from CRAI for ≥72 hours, and biopsy 2, where MSM were asked to engage in CRAI 24 prior, were consistent with that of the original analysis conducted at the family level. Mean relative abundances of all genera included in this current analysis were not different between the two time points (p-values not shown).

Figures 3-5 show Spearman correlations between 15 fundamental OTUs in MSM (Figure 5), controls (Figure 4), and all subjects (Figure 3). Across all three heat maps, there was a consistent and statistically significant negative association between

Prevotella and *Bacteroides*. Among all subjects and MSM engaging in CRAI, the genus *Prevotella* was strongly negatively associated with *Bacteroides* (Spearman ρ=-0.74, P<0.001). In men who have never engaged in CRAI, the association was slightly weaker yet still noteworthy (Spearman ρ=-0.50, P=0.005). Additionally, *Prevotella* was positively correlated with another unclassified genus in the *Prevotellaceae* family among all subjects and both study groups independently. In Figure 3 including all participants, *Prevotella* was weakly negatively related to several genera containing pathogenic bacteria such as *Escherichia/Shigella* and *Finegoldia* (Spearman ρ=-0.22, P=0.041; ρ=-0.22, P=0.038). These two relationships strengthened when the data was subset into MSM subjects exclusively. *Streptococcus* and *Fusobacterium*, two genera of potentially pathogenic bacteria, were also negatively associated with *Prevotella* among the MSM group (Spearman ρ=-0.29, P=0.029; ρ=-0.28, P=0.032). Moreover, the unclassified genus under the *Prevotellaceae* family also exhibited the same negative correlations with pathogenic genera with the exception of *Fusobacterium* (Figures 3 and 5). Among controls, *Bacteroides* showed a negative relationship with *Prevotella*, an unclassified genus of the *Prevotellaceae* family, and an unclassified genus of the *Clostridiales* order (Figure 5). There were no significant correlations between the relative abundance of *Prevotella* or *Bacteroides* and age, race, frequency of enema use on study, frequency of lube use on study, and number of RAI episodes in the last 12 months.

Alpha diversity analyses presented no significant differences between study groups irrespective of the time point the sample was taken (Figure 1). There was also no change in genus diversity between biopsies 1 and 2, as Chao1 indices did not differ within MSM and controls. Correspondingly, Shannon indices were not statistically

significant across all levels of comparison. The Chao1 index is based on the amount of rare OTUs found in a sample; therefore, it will estimate higher species richness if the sample contains many rare genera. Shannon's index accounts for both species abundance and homogeneity. Model-based alpha diversity measures were not statistically different (Shannon β=-0.17, P=0.061; Chao1 β=2.86, P=0.36). Beta diversity analysis exhibited by the PCoA Bray-Curtis plot shows no clear separation between MSM and controls (Figure 2). Furthermore, ANOSIM results were also insignificant (P=0.90) (data not shown).

Discussion

In this present analysis, we examined the effects of CRAI on the microbial composition of the rectal mucosa of HIV-negative MSM. Our analyses were restricted to the 13 most abundant OTUs from our sample in addition to 2 other genera of interest. A clear difference was exhibited in the microbiota between the two study groups at the genus level. Although MSM engaged in CRAI 24 hours prior to biopsy 2, the relative abundances of the most common genera stayed constant between the two biopsies. This suggests that effects of CRAI on the rectal mucosal microbiota may be sustained; however, the ≥72-hour period of abstinence before the first biopsy may not have been long enough to observe an alteration between the two time points. Alternatively, CRAI may lead to chronic changes in the rectal microbiota.

Overall, the most abundant genus in MSM engaging in CRAI was *Prevotella*, while the most abundant genus in men who have never engaged in CRAI was *Bacteroides*. These data are consistent with the results produced by Noguera-Julian et al. (16). This exact pattern is also seen in previous data comparing HIV-positive subjects to HIV-negative subjects. Other taxa that have been found to be positively associated with

HIV infection as well as with MSM engaging in CRAI in this present study are *Clostridium* clusters and *Streptococcus* (18, 22, 42). Relative abundances of *Faecalibacterium* and *Lachnospiraceae* have been shown to be decreased in HIV-positive subjects compared to healthy controls in previous studies, yet they are also lower among the MSM group relative to the non-MSM group in this present analysis (17, 20, 21). Given that the patterns seen between HIV-positive and negative subjects are mirrored by MSM engaging in CRAI and non-MSM respectively, these data further ascertain that sexual behavior may be a stronger driving force in the rectal microbiota than HIV status.

The strong inverse relationship between *Bacteroides* and *Prevotella* found in preceding HIV studies (16, 21, 22, 24, 25) is also shown among MSM in this present analysis (Spearman ρ=-0.74). Interestingly, this correlation was only moderate among controls (Spearman ρ=-0.50), proposing that this counteraction is bolstered by CRAI. *Prevotella* was also positively correlated with an unclassified genus under the same family, *Prevotellaceae*. The unidentified genus also negatively corresponded with *Bacteroides* and was significantly increased among MSM relative to controls. Of note, two specific species of *Prevotella*, *P. copri* and *P. stercorea*, have been identified to be highly increased with HIV infection (22, 26). However, the increased prevalence of two genera under the *Prevotellaceae* family among MSM suggests more than one species, which diverge at the genus level, are enriched in MSM engaging CRAI

An inverse correlation was manifested between *Prevotella* and a number of pathogenic bacteria such as those pertaining to the *Finegoldia*, *Fusobacterium*, *Streptococcus*, and *Escherichia/Shigella* genera among MSM subjects in particular.

Markedly, the non-MSM study group did not exhibit these interactions. These associations are further reinforced by a previous study that observed a decrease in *Prevotella* among treated HIV-positive MSM along with an increase in pathogenic bacteria including *Finegoldia* (23). In summary, both *Prevotella* and pathogenic bacteria are more prevalent in MSM, yet they exhibit an inverse relationship simultaneously. We hypothesize that mucosal injury associated with CRAI allows pathogens to flourish, which consequently provokes an increase in *Prevotella* to counteract these bacteria. Alternatively, *Prevotella* may simply outcompete pathogens for resources and physical space. It cannot be confirmed whether *Prevotella* has a protective effect against certain pathogens, but these patterns warrant for further investigation on the function of this genus.

All alpha and beta diversity measures between the two study groups as well as the two study time points were not statistically different. Chao1 and Shannon indices indicate that MSM engaging in CRAI and controls have similar genus richness and evenness, meaning that the abundance of OTUs and the homogeneity of the OTU abundances between the study groups are alike. The Bray-Curtis plot shows that the overall composition of the microbiota of MSM subjects and non-MSM subjects are reasonably comparable. Although CRAI may influence important shifts in the microbiota composition, these data suggest that the microbiota of the rectal mucosa generally remains constant at the genus level even after recently engaging in CRAI.

It has been highly suspected that HIV leads to drastic changes in the gut microbiome; however, it is unclear if this due to the virus itself or because men who engage in anal intercourse have the largest burden of HIV. These data contest the former

hypothesis and support the latter. There is considerable evidence suggesting that CRAI, is largely responsible for microbial changes in the rectal mucosa; however, it would be premature to assume CRAI is the sole operator in the causal pathway. In future directions concerning HIV studies, it will be necessary to control for sexual behavior or other factors that may lead to mucosal trauma.

Given that the sample size in this analysis is relatively modest, these results should be interpreted with caution. It is plausible that the decreased strength of Spearman correlations seen in controls is due to chance. Diet was also not recorded or controlled for; however, previous findings have shown that diet has little to no effect on the relative abundance of *Prevotella* and microbiota clustering (16). The studies that have found an increase in *Prevotella* among MSM were conducted in three different geographic areas: Spain, Sweden, and the United States (16, 33). Although confounding is possible, the significant enrichment of *Prevotella* among MSM compared to non-MSM is not likely to be driven by dietary factors. It is also not certain whether the compositional changes in the rectal mucosa are a direct result of exposure to semen, since ejaculation inside the rectum occurred in the vast majority of CRAI episodes and could not be assessed as a confounder, or from mucosal microtrauma that may occur during intercourse. Additionally, the self-reported record of CRAI episodes is subject to recall and response bias. These data are mostly phenotypic and do not provide enough information to establish the implications on HIV transmission. More long-term cohort studies are needed to determine the extent to which RAI influences alterations in the rectal mucosa as well as controlling for extraneous factors such as exposure to semen and condom use. *In vivo*

and *in vitro* experiments also are necessary to observe the function of *Prevotella* in the rectal mucosa, particularly in the presence of enteric pathogens.

In conclusion, the current analysis further supported past literature observing an enrichment of the microbiota for *Prevotella* in the rectal mucosa of MSM engaging in CRAI, as opposed to *Bacteroides* in non-MSM. However, these data suggest that *Prevotella* is not the sole genus responsible for this microbial shift, as we identified two different genera under the *Prevotellaceae* family to behave in coalition. Moreover, the inverse relationships found between *Prevotella* and several potential pathogens propose a protective or interfering effect. Mucosal injury associated with CRAI may have long-lasting consequences on the rectal microbiota; however, its impact on HIV transmission is not yet clear. These findings will be beneficial in the proposal of prospective HIV transmission studies and prevention methods.

TABLES AND FIGURES

Table 1. Clinical Cohort Demographics

	Overall	Control	MSM	P-value
Number of Participants	54	20	34	
Age at Screen (mean (SD))	28.93 (5.89)	26.56 (4.48)	30.33 (6.22)	0.021*
Race (%)				0.172
White	42 (77.8)	14 (70.0)	28 (82.4)	
Black	7 (13.0)	2 (10.0)	5 (14.7)	
Latino	3 (5.6)	2 (10.0)	1 (2.9)	
Asian	2 (3.7)	2 (10.0)	0 (0.0)	
Days on Study (Mean (SD))	82.76 (26.95)	73.15 (29.95)	88.41 (23.70)	0.043*
RAI in 12 months (Mean (SD))	NA	NA	66.35 (42.93)	
Without a Condom (Mean (SD))	NA	NA	61.76 (43.00)	
With a Condom (Mean (SD))	NA	NA	4.47 (11.24)	
Sex Acts on Study (Mean (SD))	NA	NA	13.35 (10.21)	
Lube Acts on Study (Mean (SD))	NA	NA	12.06 (10.27)	
Enema Acts on Study (Mean (SD))	NA	NA	3.26 (5.53)	
*Indicates significant p-values (significance value = 0.05).				

Table 2. Top 10 genera identified in all subjects, MSM engaging in CRAI, and controls

Top 10 Genera in Rectal Mucosal Swabs

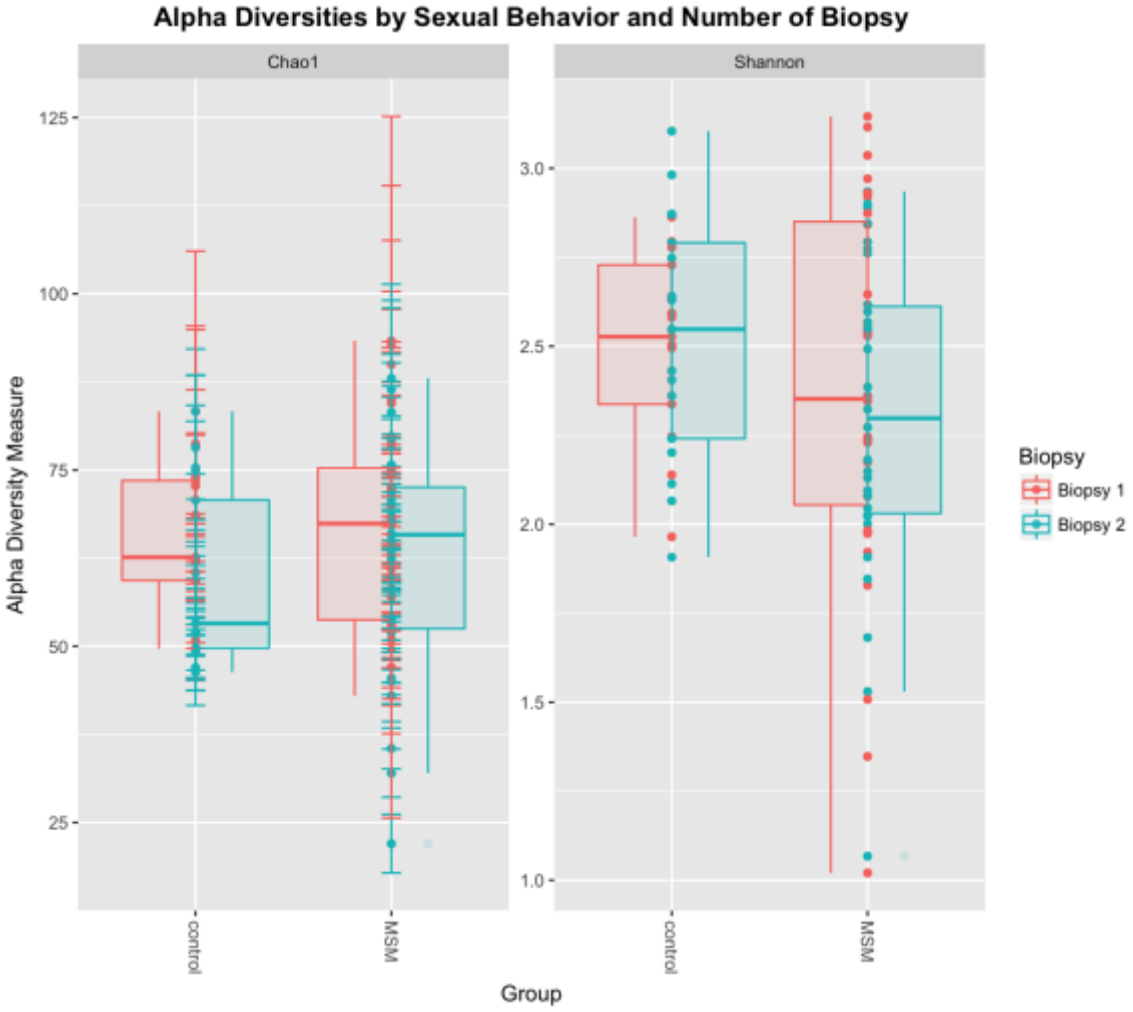
All Subjects	Controls	MSM
Bacteroides	*Bacteroides*	*Prevotella*
Prevotella	*Prevotella*	*Bacteroides*
Faecalibacterium	*Unclassified – Lachnospiraceae Family*	*Streptococcus*
Unclassified – Lachnospiraceae Family	*Faecalibacterium*	*Faecalibacterium*
Unclassified – Ruminococcaceae Family	*Unclassified – Ruminococcaceae Family*	*Unclassified – Lachnospiraceae Family*
Streptococcus	*Parabacteroides*	*Escherichia/Shigella*
Escherichia/Shigella	*Unclassified - Clostridiales Order*	*Unclassified – Prevotellaceae Family*
Unclassified – Prevotellaceae Family	*Sutterella*	*Unclassified – Ruminococcaceae Family*
Parabacteroides	*Unclassified - Enterobacteriaceae Family*	*Clostridium XIX*
Sutterella	*Escherichia/Shigella*	*Parabacteroides*

Table 3. Relative abundances of most abundant OTUs in MSM engaging in CRAI and controls and modeling results

Genus	Biopsy Number	Control Mean (95% CI)	MSM Mean (95% CI)	P-value	Overall Group P-value
Number of Participants	Biopsy 1	13	29	-	-
	Biopsy 2	17	33		
Unclassified – Lachnospiraceae Family	Biopsy 1	0.079 (0.050, 0.11)	0.040 (0.025, 0.054)	0.006*	0.0001*
	Biopsy 2	0.066 (0.046, 0.085)	0.039 (0.027, 0.050)	0.02*	
Prevotella	Biopsy 1	0.088 (0.008, 0.17)	0.20 (0.13, 0.27)	0.01*	0.003*
	Biopsy 2	0.079 (0.015, 0.14)	0.21 (0.13, 0.28)	0.02*	
Unclassified - Prevotellaceae Family	Biopsy 1	0.015 (-0.0026, 0.033)	0.023 (0.011, 0.034)	0.1	0.003*
	Biopsy 2	0.011 (0.0026, 0.019)	0.042 (0.025, 0.060)	0.04*	
Faecalibacterium	Biopsy 1	0.080 (0.044, 0.12)	0.035 (0.022, 0.049)	0.01*	0.009*
	Biopsy 2	0.063 (0.042, 0.084)	0.048 (0.032, 0.065)	0.2	
Bacteroides	Biopsy 1	0.30 (0.21, 0.38)	0.21 (0.14, 0.27)	0.08	0.01*
	Biopsy 2	0.30 (0.22, 0.38)	0.19 (0.11, 0.26)	0.02*	
Clostridium XIX	Biopsy 1	0 (0, 0)	0.022 (-0.0027, 0.046)	0.08	0.013*
	Biopsy 2	0 (0, 0)	0.017 (-0.0057, 0.040)	0.06	
Streptococcus	Biopsy 1	0.0068 (-0.0018, 0.016)	0.040 (-0.012, 0.093)	0.6	0.016*
	Biopsy 2	0.0034 (0.00066, 0.0062)	0.054 (0.010, 0.098)	0.2	
Unclassified – Clostridiales Order	Biopsy 1	0.033 (0.0033, 0.063)	0.013 (0.0071, 0.019)	0.06	0.021*
	Biopsy 2	0.024 (0.011, 0.038)	0.0075 (0.0050, 0.010)	0.005*	
Parabacteroides	Biopsy 1	0.021 (0.013, 0.030)	0.016 (0.0089, 0.022)	0.09	0.03*
	Biopsy 2	0.038 (0.022, 0.053)	0.019 (0.0096, 0.028)	0.005*	
Fusobacterium	Biopsy 1	0.00042 (-0.00041, 0.0012)	0.0077 (0.00077, 0.015)	0.04*	0.025*
	Biopsy 2	0.00024 (-0.000069, 0.00055)	0.016 (-0.0034, 0.036)	0.1	

Sutterella	Biopsy 1	0.034 (0.013, 0.055)	0.014 (0.0074, 0.021)	0.1	0.06
	Biopsy 2	0.020 (0.0058, 0.034)	0.017 (0.0064, 0.028)	0.6	
Unclassified – Ruminococcaceae Family	Biopsy 1	0.037 (0.024, 0.050)	0.032 (0.021, 0.043)	0.5	0.07
	Biopsy 2	0.044 (0.029, 0.059)	0.025 (0.018, 0.033)	0.03*	
Escherichia/Shigella	Biopsy 1	0.019 (-0.0003, 0.040)	0.045 (0.016, 0.073)	0.6	0.16
	Biopsy 2	0.018 (-0.011, 0.046)	0.027 (0.006, 0.047)	0.3	
Unclassified – Enterobacteriaceae Family	Biopsy 1	0.017 (-0.010, 0.044)	0.013 (0.0012, 0.025)	1	0.34
	Biopsy 2	0.026 (-0.0079, 0.059)	0.011 (0.0024, 0.019)	0.9	
Finegoldia	Biopsy 1	0.0092 (0.0025, 0.076)	0.016 (0.0025, 0.030)	0.2	0.68
	Biopsy 2	0.012 (0.0013, 0.022)	0.013 (0.0040, 0.022)	0.3	
* Indicates significant p-values (significance level = 0.05).					

Figure 1. Alpha diversity boxplots by study Group and biopsy number and GLM based parameter estimates



α Diversity Measure	Variable	β Estimate	Standard Error	P-value
Chao1 Index	Intercept	61.8	2.18	<2e-16*
	Study Group	2.86	3.12	0.36
Shannon Index	Intercept	2.51	0.061	<2e-16*
	Study Group	-0.17	0.091	0.061

Figure 2. Multidimensional scaling ordination plots of Bray–Curtis distances

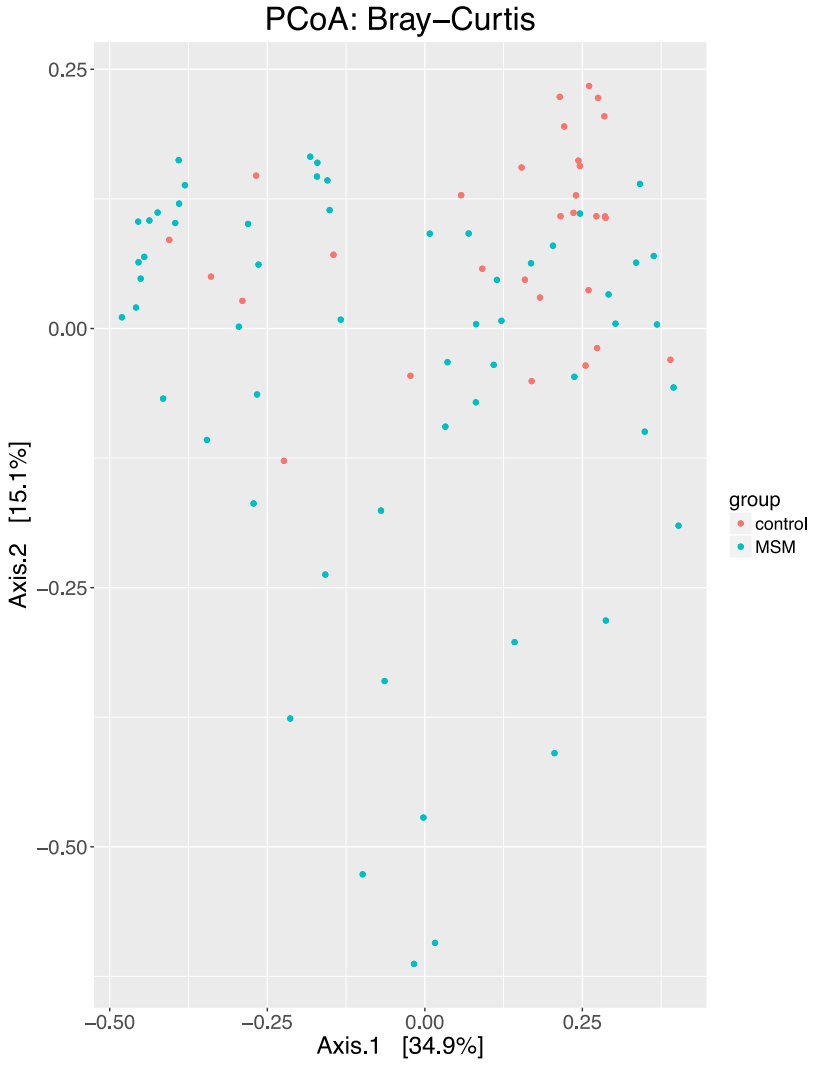


Figure 3. Spearman correlation heat map with correlation coefficients of top 20 OTUs including all subjects

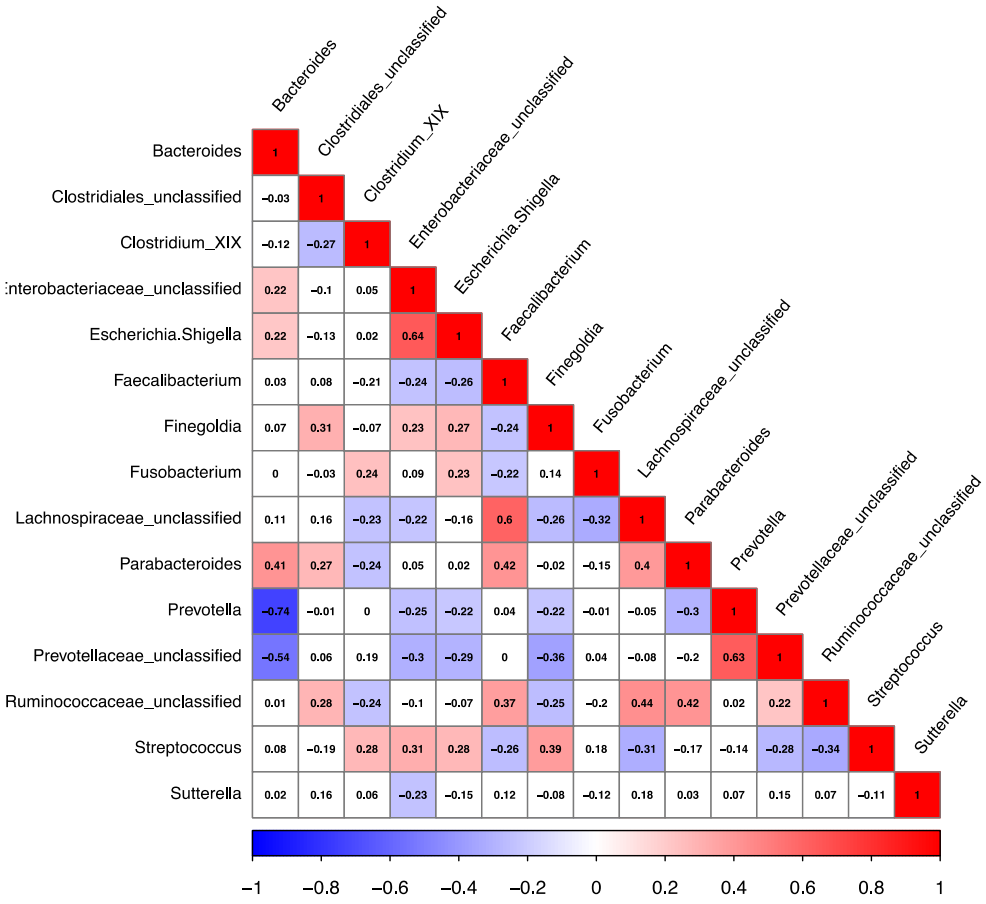


Figure 4. Spearman correlation heat map with correlation coefficients of top 20 OTUs including men who have never engaged in CRAI

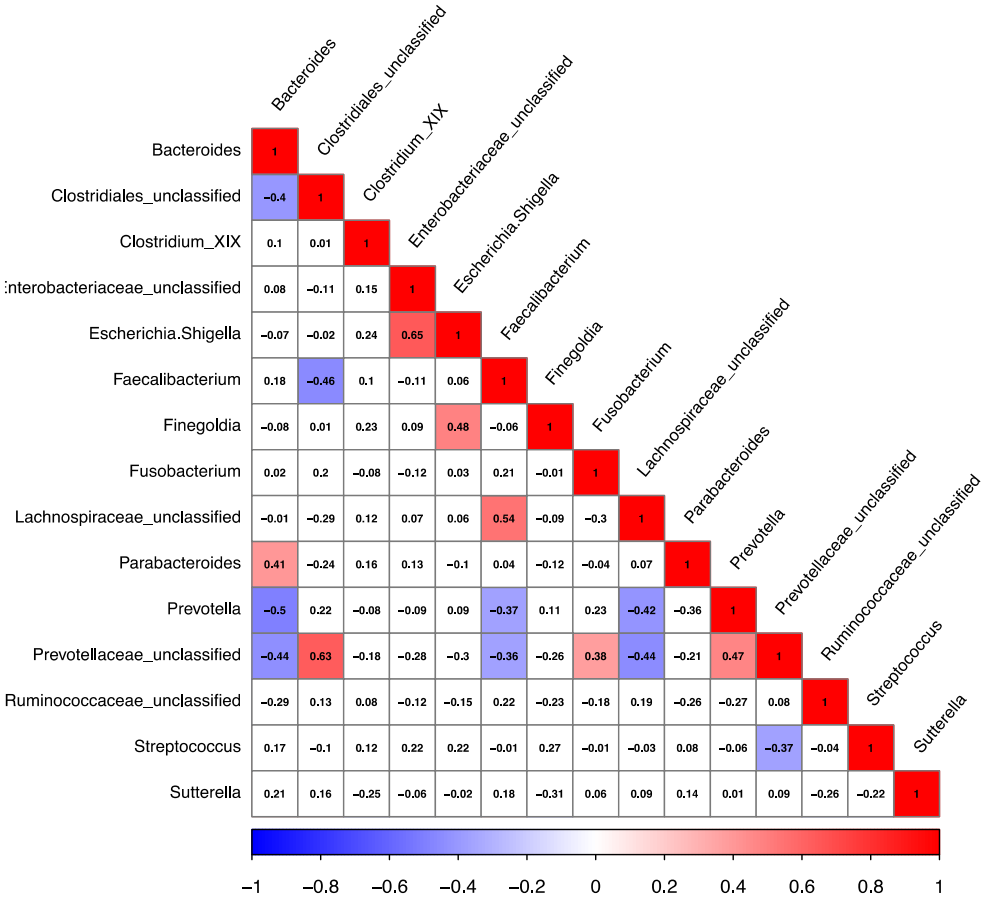
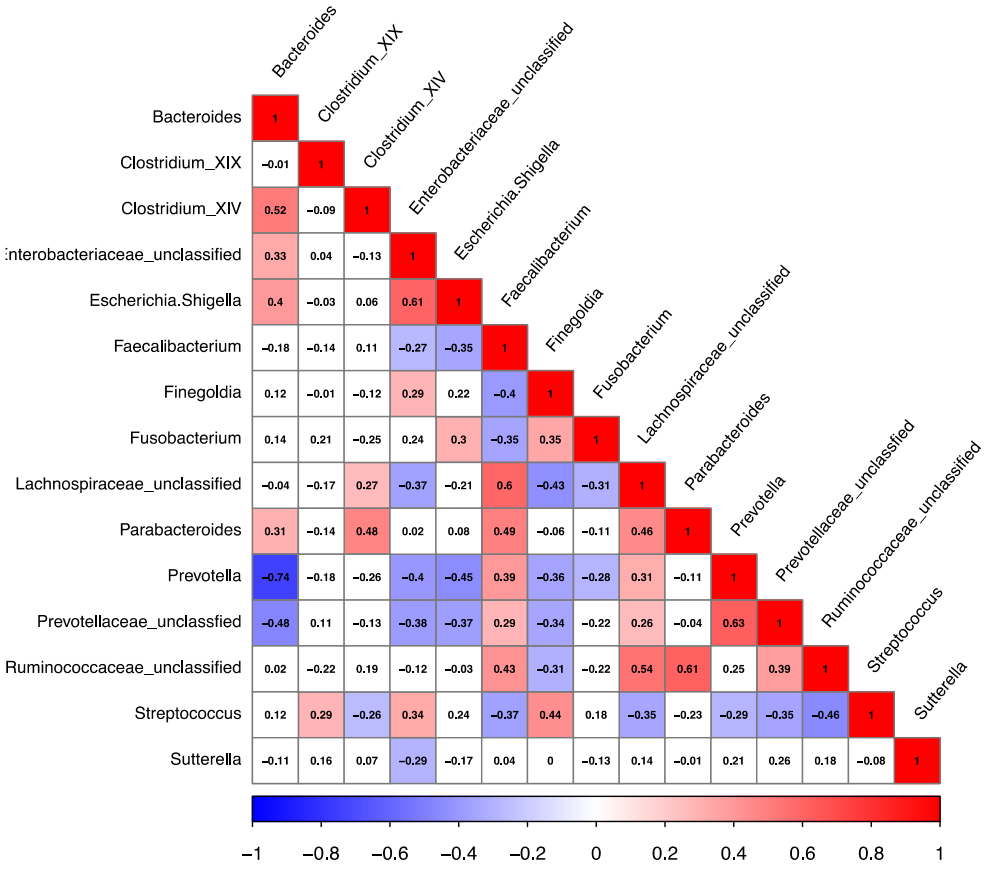


Figure 5. Spearman correlation heat map with correlation coefficients of top 20 OTUs including MSM who are engaging in CRAI



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Appendix

EMORY UNIVERSITY | Institutional Review Board

March 16, 2017

RE: Determination: No IRB Review Required
Title: ***Characteristic Shifts of the Rectal Mucosal Microbiota due to Condomless Receptive Anal Intercourse among HIV-Negative MSM***
PI: Nicole Pescatore

Dear Ms. Pescatore,
Thank you for requesting a determination from our office about the above-referenced project. Based on our review of the materials you provided, we have determined that it does not require IRB review because it does not meet the definition of research with "human subjects" as set forth in Emory policies and procedures and federal rules, if applicable. Specifically, in this project, you will using a de-identified dataset from a previous study and run several types of statistical analyses to determine the difference in Microbiota between MSM and non-MSM.

Please note that this determination does not mean that you cannot publish the results. This determination could be affected by substantive changes in the study design, subject populations, or identifiability of data. If the project changes in any substantive way, please contact our office for clarification.

Thank you for consulting the IRB.

Sincerely,

Steven J. Anzalone, M.S.
Research Protocol Analyst