Effect of Risky Sexual Behavior and Associated Gut Microbiome Changes on Susceptibility to HIV in MSM

by

Meaghan S. Price

B.S. in Microbiology, Clemson University Honors College, 2020

Submitted to the Graduate Faculty of the

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2022

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Meaghan S. Price

It was defended on

April 19, 2022

and approved by

Charles Rinaldo, PhD – Professor, University of Pittsburgh, Department of Medicine

Jeremy Martinson, PhD, Assistant Professor, University of Pittsburgh, Department of Infectious Disease and Microbiology

Huang Lin, PhD – Postdoctoral visiting fellow, National Institutes of Health, BBB

Shyamal Peddada, PhD – Senior investigator and Chief, National Institutes of Health, BBB

Thesis Advisor/Dissertation Director: Yue Chen, PhD – Associate Professor, University of Pittsburgh, Department of Infectious Diseases and Microbiology

Copyright © by Meaghan S. Price

2022

Effect of Risky Sexual Behavior and Associated Gut Microbiome Changes on Susceptibility to HIV in MSM

Meaghan S. Price, MPH

University of Pittsburgh, 2021

Background: Men who have sex with men (MSM) have been disproportionately impacted by the HIV/AIDS epidemic. Receptive anal intercourse is a prominent risk factor for HIV-1 seroconversion among MSM, and its impact on the MSM-associated gut microbiome and HIV susceptibility is of clinical and public health interest. This study aims to evaluate the association between receptive anal intercourse and gut-microbiome alterations by analyzing data from the 1984/1985 phase of the Multicenter AIDS Cohort Study (MACS).

Methods: Sexual behavior data were used to cluster 241 MACS participants into five groups based on the number of partners for receptive anal intercourse (G1: 0, G2: 1, G3: 2-3, G4: 4-8, G5: 9+). Fecal samples from study participants were collected before HIV infection and sequenced for gut microbiome analysis. Microbial alpha diversity and beta diversity were analyzed using QIIME. Microbial differential abundance was analyzed using ANCOM-BC. Subsequent HIV seroconversion rates among groups were analyzed by Chi-square.

Results: For gut microbiome beta diversity, G3 was statistically different compared to G1 at the family and genus levels (p-value < 0.05). At the microbial genus level, G5 showed significantly higher levels of *Succinivibrio, Prevotella, Desulfovibrio, Cantenibacterium*, and *Mogibacterium*, and significantly lower levels of *Alistipes* and *Akkermansia* (adjusted p-value < 0.05) compared to G1. At the species level, G5 showed significantly higher levels of *P. stercorrea*, and significantly lower levels of *A. putredinis, C. spiroforme, R. torques*, and *A. muciphila*

(adjusted p-value < 0.05) compared to G1. Subsequent HIV seroconversion rates were significantly different among sexual behaviors groups (G1: 5%, G2: 30%, G3: 52%, G4: 50%, and G5: 74%, (p-value < 0.01)).

Conclusion: Risky sexual behaviors were associated with increased HIV seroconversion rates and gut-microbiome changes, with changes in abundance of gut bacteria with pro- and anti-inflammatory properties. This suggests that receptive anal intercourse leads to pathogenic changes in the microbiome, increasing susceptibility to HIV infection in MSM.

Public health significance: Receptive anal intercourse may increase the risk of HIV-1 infection among MSM due to pathogenic impacts on the gut microbiome. The identification of such risk will allow for accurate public health guidance and policy for MSM.

Table of Contents

List of Tables

List of Figures

Preface

I wish to express my great appreciation to Dr. Yue Chen, my thesis advisor and committee chair, for her guidance, knowledge, and time spent throughout the thesis writing process. Her advice was critical in helping me develop the study design and thesis content, and her previous work was an incredible basis and resource for this paper.

I would like to thank Dr. Peddada and Dr. Lin of the NIH for their expertise and time, especially in regards to statistical analyses used in the study. They took the time to meet and work with me from the beginning of the thesis process, and their help has been invaluable.

I would also like to thank Dr. Rinaldo for his expertise and guidance throughout the thesis process. Additionally, I would like to thank him for a meeting he took with me when I was an undergraduate student visiting the University of Pittsburgh, which led me to apply to and attend the School of Public Health.

Lastly, I would like to thank my academic advisor Dr. Jeremy Martinson, who taught me as a professor throughout my time as a student and connected me to this project. I am also particularly grateful to him for being a committee member when I needed one, even with late notice.

List of Abbreviations

AIDS Acquired immunodeficiency syndrome

ANCOM-BC

ART Antiretroviral therapy

CDI *Clostridium difficile* infection

DA Differential abundance

GRID Gay-related immune deficiency

HIV Human immunodeficiency virus

KS Kaposi's sarcoma

MACS Multi-center AIDS Cohort Study

MSM Men who have sex with men

MSW Men who have sex with women

PCP Pneumocystis carinii pneumonia

PrEP Pre-exposure prophylaxis

RAI Receptive anal intercourse

SCFA Short-chain fatty acid

1.0 Introduction

1.1 HIV/AIDS Epidemic

In June of 1981, five cases of a rare lung infection known as *Pneumocystis carinii* pneumonia (PCP), were reported among previously-healthy men who have sex with men (MSM) in the United States (CDC, 1981a). Shortly following the emergence of PCP cases, a rare form of cancer known as Kaposi's sarcoma (KS) and other life-threatening opportunistic infections were reported in 26 individuals, once again among otherwise-healthy MSM (CDC, 1981b). Cases of PCP and KS are typically only seen among immunosuppressed individuals, pointing towards the possibility of an immunosuppressive disease as the common source of causation. Transmission via sexual contact was suspected as the route of transmission, as by the end of the year (1981), 270 cases and 121 deaths were seen among sexually active MSM. In early years, the disease was referred to as Gay-Related Immunodeficiency (GRID), but by September of 1982, GRID was abandoned and thereafter referred to as Acquired Immune Deficiency Syndrome (AIDS). The change in terminology was spurred by an increase in cases among the heterosexual population, particularly among intravenous drug users. This signified that the infection was not limited to the gay population nor transmission due to sexual contact alone. The emergence of PCP and KS cases among MSM would later be known as the start of the HIV/AIDS epidemic in the U.S. (Jones & Salazar, 2016).

Throughout the HIV epidemic, infected persons, especially gay men, experienced prejudice, exclusion, and negative stigma due to the complex social factors surrounding HIV

infection. As a result of pre-existing discriminatory attitudes, some felt the disease was brought upon the gay community by themselves, and was pejoratively referred to in the media as the "gay plague" (Jones & Salazar, 2016). Negative stigmas around an HIV diagnosis can lead to a lower likelihood of utilizing testing, treatment, and prevention services due to fear of discrimination (UNAIDS, 2017). Prejudice towards the gay community and HIV infected individuals shaped the course of the HIV/AIDS epidemic, and likely stymied clinical and public health advancements.

1.1.1 Multicenter AIDS Cohort Study (MACS)

The Multicenter AIDS Cohort Study (MACS) was one of the earliest studies aimed towards understanding HIV and AIDS among gay and bisexual men in the United States. Established in 1984, the MACS studied HIV infection among MSM at four sites (Baltimore, Maryland/Washington DC; Chicago, Illinois; Los Angeles, California; Pittsburgh, Pennsylvania) through participant interviews, physical examinations, and specimen collection. Resulting data were used to study the natural history of disease and elucidate risk factors associated with HIV infection and AIDS progression **(**MWCCS, n.d.**)**. Importantly, early cohorts were studied prior to the advent of antiretroviral therapy (ART), providing a unique sample population that is likely unobtainable in current times.

1.1.2 HIV Prevalence Among MSM

In the U.S., HIV continues to disproportionately impact gay and bisexual men. The most recent estimates using 2020 survey data indicate although MSM make up approximately 3% - 5% of the population (Gallup, 2021), 69% of new HIV diagnoses were among MSM in 2018 (CDC,

2020). Following male-to-male sexual contact, transmission routes of new HIV infections were attributable to heterosexual contact (23%), injection drug use (7%) and combination male-to-male sexual contact and injection drug use (4%). Among the US population by the end of 2019, an estimated 1,198,700 people (diagnosed and suspected cases) were living with HIV infection, with 13% of infections believed to be undiagnosed (CDC, 2021).

1.2 Influential Factors in HIV-1 Infection

HIV transmission occurs via the exchange of certain bodily fluids from an HIV infected individual, including blood, breast milk, semen, and vaginal secretions (WHO, 2021). Transmission routes include sexual contact across mucosal surfaces, maternal-infant exposure, and percutaneous inoculation (NIH, 2012). Factors influencing transmission among MSM include the type and frequency of sexual activities, number and HIV status of partners, and the presence or absence of pre-exposure prophylaxis (PrEP) (Hess et al., 2017). One type of sexual behavior in particular, receptive anal intercourse, contributes most to HIV transmission risk among MSM. The estimated per-act HIV transmission risk (per 10,000 exposures) is 138 for unprotected receptive anal intercourse, compared to 11 for unprotected insertive anal intercourse and 8 for unprotected receptive penile-vaginal intercourse (Patel, et al., 2014). Receptive anal intercourse (RAI) has consistently and historically been found to be a prominent risk factor. Results from the MACS showed the risk for HIV seroconversion increased in proportion to the number of RAI partners, and among MACS participants, HIV infection risk lowered upon both reduction and cessation of RAI (Kingsley et al., 1987).

1.2.1 HIV-1 and Inflammation

Physiologically, one of the reasons for receptive anal intercourse as a risk factor for HIV infection is the nature of the rectal lining and surrounding tissues. Rectal mucosa, composed of single layer columnar epithelium, is thinner than the epithelium lining of the penis or vagina and is prone to tearing during receptive anal intercourse (mechanical micro trauma). Such tearing induces inflammation of the rectal mucosa, including an inflammatory cytokine release, resulting in increased epithelial permeability and recruiting of immune cells (Kelley, 2017). Upon injury to the epithelial layer, immune cells recruited to protect against pathogen invasion include CD4+ T cells, the main target cell of the HIV virus. HIV selectively infects CD4+ T cells, particularly those expressing the HIV co-receptor CCR5 (Kelley, 2021). HIV-1 infected CD4+ T cells further spread HIV infection, and upon cell death (due to HIV-1 infection) result in the lowered CD4+ T cell counts associated with HIV immunosuppression (Vijayan et al., 2017).

1.3 Gut Microbial Dysbiosis and Susceptibility to HIV Infection

Located in the gastrointestinal tract, the gut microbiome of humans is a rich microbial environment that aids in maintaining health, and through competitive exclusion, protects against pathogenic invasion. Microbial composition varies among individuals, and can be influenced by factors such as diet, gender, host genetics, immunodeficiency, and antibiotic use. Microbial taxa in the gut microbiome are typically anaerobic, and tend to be dominated by *Firmicutes* and *Bacteroidetes* phyla members (Kriss et al., 2018). The gut microbiome is able to assist in the digestion of dietary substances unable to be digested by humans, produces factors such as short

chain fatty acids (SCFA), synthesizes vitamins, modulates xenobiotic toxicity, and interacts with host immune cells. Dysbiosis, a disturbance or shift in gut microbiome composition compared to healthy controls, is associated with certain health disorders, including inflammatory bowel disease, metabolic disorders, asthma, and neurodegenerative disease (Nair, 2019). Gut microbiota dysbiosis has also been associated with decreased levels of alpha and beta diversity, although less so for HIV infection compared to disease such as *Clostridium difficile* infection (CDI) (Vujkovic-Cvijin & Somsouk, 2019). Previously, studies have shown that HIV seroconversion among MSM is associated with distinct changes in the gut microbiome when compared to those who did not seroconvert. Additionally, the gut microbiome of MSM is distinct from men who have sex with women (MSW) regardless of HIV seroconversion (Chen et al., 2021), suggesting the microbiome of MSM could play an important role in HIV infection, and that differences in sexual behavior may influence microbial composition.

1.3.1 Short Chain Fatty Acids

Short chain fatty acids (SCFAs) are gut microbiota metabolites resulting from the fermentation of complex carbohydrates. Gut microbiota production of SCFAs include acetate, propionate, butyrate, and valerate. Typically, acetate is proportionately the most abundant SCFA product. SCFAs, in particular butyrate, are utilized as energy sources in colonic epithelial cells and can regulate epithelial cell function, differentiation, and gene expression. This includes promoting the production of molecules needed for gut barrier function, such as mucin (Martin Gallausiaux et al., 2021). SCFAs also play a role in modulating gut inflammation, as they can regulate cytokine

production in lymphocytes and can induce the differentiation of T cells. Again, butyrate in particular has been shown to have strong anti-inflammatory properties, and has been shown to promote Th17 cell differentiation (Kim et al., 2014). Largely, SCFAs promote epithelial barrier and immune cell function and provide anti-inflammatory properties in the gut, however there are results indicative of a complex relationship with inflammation. Depending on disease context and pathology, SCFAs can result in either protective or causative effects due to their cytokine and immune cell regulatory functions. For example, although the production of inflammatory cytokines aid in fighting microbial invasion, chronic mucosal inflammation can also lead to increased susceptibility to infections, including HIV (Mirmonsef et al., 2012).

1.4 Research Question

The MACS's longitudinal specimen repository provides a unique opportunity for analysis of the influential factors surrounding HIV infection among MSM. Kingsley et al. (1987) found among MACS participants, "receptive anal intercourse was the only significant risk factor for seroconversion to HIV" and the risk ratio for seroconversion was 3-fold for one partner and 18-fold for five or more partners. Chen et al. (2021) found that prior to HIV infection, there were pathogenic changes in the gut microbiome of MSM several months before seroconversion to the retrovirus. Additionally, these changes were associated with increased blood inflammatory markers and an increased risk for AIDS development.

In this study using MACS data and specimens, we have explored the relationship between sexual behavior and gut microbiome changes prior to HIV infection among MSM. The objective of this thesis is to address the following hypothesis:

> Among MSM, the number of receptive anal intercourse partners is associated with gut microbiome profiles, microbial diversity, and fecal SCFA levels in the context of subsequent HIV infection.

2.0 Methods

2.1 Participants and MACS Data Collection

Sexual behavior data and specimen samples were obtained from records and specimen cryorepositories of the MACS prospective 1984-1985 cohort study. Baseline examinations (visit 1) of participants began April 1, 1984, and follow-up examinations (visit 2) were conducted at six-month intervals, completed by November, 1985. Examinations followed a standardized protocol that included an interviewer-administered questionnaire, physical examination, laboratory tests, and specimen collection (Kingsley, 1987). For the current study, 241 MACS participants were included for analysis. All participants were seronegative at visit 1, and by visit 2, 87 participants were seropositive for HIV-1 infection, as determined by enzyme-linked immunosorbent assay (ELISA) and further confirmed by quantitative polymerase chain reaction (qPCR) assay. Only data from visit 1 were used for sexual behavior and gut microbial analysis, as this allowed specimen and data collection prior to HIV-1 seroconversion.

At each visit, participants were interviewed on sexual behaviors and practices, including the number of partners for sexual activity and intercourse, condom usage, and type of sexual behavior. For analysis in the current study, participants were grouped by sexual behavior based on the number of reported receptive anal intercourse partners in the six months prior to visit 1. Counts for RAI partners included intercourse with and without condom usage.

At each clinical research visit, MACS participants were instructed to provide stool, urine, semen, and oral wash samples, which have been preserved at -80°C without additives or

preservatives. Stool samples were obtained at home in 20 ml screw-capped glass vials and were delivered to the clinic within one day by participants (Kaslow, 1987).

2.2 Specimen Preparation

Fecal DNA was extracted using the PowerSoil DNA Extraction Kit, and the V4 variable region of the 16S rRNA gene was PCR-amplified with universal primers. Amplicons were cleaned, pooled, and sequenced on an Illumina MiSeq platform according to the manufacturer's specifications to generate paired-end reads. Following sequencing, the 16S rRNA gene data were processed using QIIME2. Raw sequences were first demultiplexed, denoised to remove noisy reads, dereplicated to reduce repetition, and clustered into amplicon sequence variants (ASVs) using the DADA2 algorithm. No ASVs were removed based on observed abundance. The taxonomic composition of bacterial communities was investigated by classifying sequences to the latest reference database using a Naive Bayes classifier (Chen et al., 2021).

To determine SCFA concentration, 50-100mg of fecal matter from each stool sample was sent to the University of Pittsburgh Health Science Metabolomics and Lipidomics Core in the Department of Pharmacology & Chemical Biology. Fecal acetate, butyrate, propionate, and valerate levels were measured using stable isotope dilution liquid chromatography mass spectrometry (Chen et al., 2021).

2.3 Statistical Analysis

241 MACS participants were divided into five groups for comparative analysis based on the reported number of RAI partners: group one (G1) contained participants with zero partners; group two (G2) contained participants with one partner; group three (G3) contained participants with two to three partners; group four (G4) contained participants with four to eight partners; and group five (G5) contained participants with nine or more partners. Sexual behavior groupings were delineated based upon the significant impact of the risky behaviors and number of participants in each group.

Gut microbial alpha diversity (within-sample) and beta diversity (between-sample) were computed using the R microbiome package. Calculations of alpha and beta diversities were based on rarefied data (subsample taxa without replacement based on the 90% of minimum library size) since the differential library sizes can have a significant impact on alpha and beta diversities (Chen et al., 2021). Two analytical methods were used to investigate alpha diversity, number of observed species (species richness) and Shannon diversity index (richness and evenness). P-values were obtained through linear regression, adjusting for age. Beta diversity was measured using Bray-Curtis dissimilarity, projected onto principal coordinates analysis (PCoA) plots. P-values were obtained using PERMANOVA. Alpha and beta diversity analysis was done at the family, genus, and species level.

Differential abundance (DA) analysis was performed using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC). A challenge of DA analysis is the compositional nature of microbiome data, as the number of reads assigned to an ASV should be interpreted relative to the total number of reads for the sample. To account for the compositional nature of microbiome data, ANCOM-BC suitably estimates and eliminates the bias introduced by

differences in sampling fractions in the observed counts. The methodology uses relative abundances to infer absolute abundances while controlling false discovery rate (Lin & Peddada, 2020).

A global test was used to compare microbial abundance among the five sexual behavior groups, based on results obtained from ANCOM-BC. The global test selects taxa significantly different in abundance among at least one of the five groups. Global test results significant at adjusted and un-adjusted p-values were visualized with a heat map of differentially abundant taxa. Bias-corrected abundances (natural log) were overlaid on each heat map in order to determine the log-fold change in between groups. ANCOM-BC pairwise comparisons (T-test) were used to further clarify differences in taxonomic abundance between groups. As opposed to the global test, which returns significance when any one of the five groups are different, the pairwise tests allow for taxa-specific comparisons between two groups. Lastly, differential abundance was compared between G1 (zero partners) and all other groups using results obtained from ANCOM-BC. Results were visualized with waterfall plots of log-fold change in bias-corrected abundance. Each analysis was performed at the family, genus, and species level. Results include both those significant at the adjusted and unadjusted p-value.

3.0 Results

3.1 Groupings and Participant Summary Characteristics

Table 1 summarizes participant characteristics among groups, including age, seroconversion status, and time to develop AIDS. No significant difference in age was seen among groups. The rate of HIV-1 seroconversion differed significantly among groups (p-value < 0.01, Chi-squared test), and tended to increase among groups with higher-number RAI partners (G1 - 5%, G2 - 30%, G3 - 52%, G4 - 50%, G5 -74%). Similarly, the time to develop AIDS among seropositive individuals differed significantly among groups (p-value < 0.01, Chi-Squared), and tended to be shorter among groups with higher-number RAI partner groups. Table 2 provides the number of receptive anal intercourse partners and the number participants in each group.

Table 1. Participant summary characteristics by group

	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Age Mean (Range)	39.84 (22-79)	40.37 (21-74)	40.86 (21-80)	41.33 (22-82)	41.87 (19-73)	0.99 (Kruskal-Wallis Rank Sum test)
Number of Receptive Anal Intercourse Partners Mean (Range)	0	1	2.46 (2 - 3)	5.12 (4-9)	28.70 (10-200)	
Conversion Status N (%)						
NC	60 (95%)	40 (70%)	27 (48%)	21 (50%)	6 (26%)	< 0.01 (Chi-square)
SC	3 (5%)	17 (30%)	29 (52%)	21 (50%)	17 (74%)	
Time to Develop AIDS N (%)						
< 5 Years	1 (2%)	5 (9%)	6 (11%)	5 (12%)	9 (39%)	< 0.01 (Chi-square)
5 - 10 Years	0 (0%)	5 (9%)	10 (18%)	6 (14%)	3 (13%)	
> 10 Years	2 (3%)	7 (12%)	13 (23%)	10 (24%)	5 (22%)	
Non-progressor	60 (95%)	40 (70%)	27 (48%)	21 (50%)	6 (26%)	

Table 2. Group classifications based on partner number for receptive anal intercourse

Group	Number of Receptive Anal Intercourse Partners	N	Mean (Range)
G1	0	63	—
G2	1	57	—
G3	2-3	56	2.46 (2-3)
G4	4-9	42	5.12 (4-9)
G5	10+	23	28.70 (10-200)

3.2 Microbial Diversity

3.2.1 Alpha Diversity

Alpha diversity measures the microbial diversity present within a sample. For this study, two diversity indices were used to compare groups at the family, genus, and species level - observed species and Shannon index. There were no significant differences in observed species or Shannon index diversity between G1 and any other group (G2- G5) at the family, genus, or species level (Figure 1).

3.2.2 Beta Diversity

Beta diversity represents between-sample differences in diversity. For this study, beta diversity at the family, genus, and species level was measured using Bray-Curtis dissimilarity and visualized with principal coordinates analysis (PCoA) plots (Figure 2). There were no significant differences between G1 and any other group (G2 - G5) in beta diversity at the species level. At the family and genus level, a statistically significant difference in beta diversity was seen between G1 and G3 (p-value = 0.03 for both family and genus beta diversity).

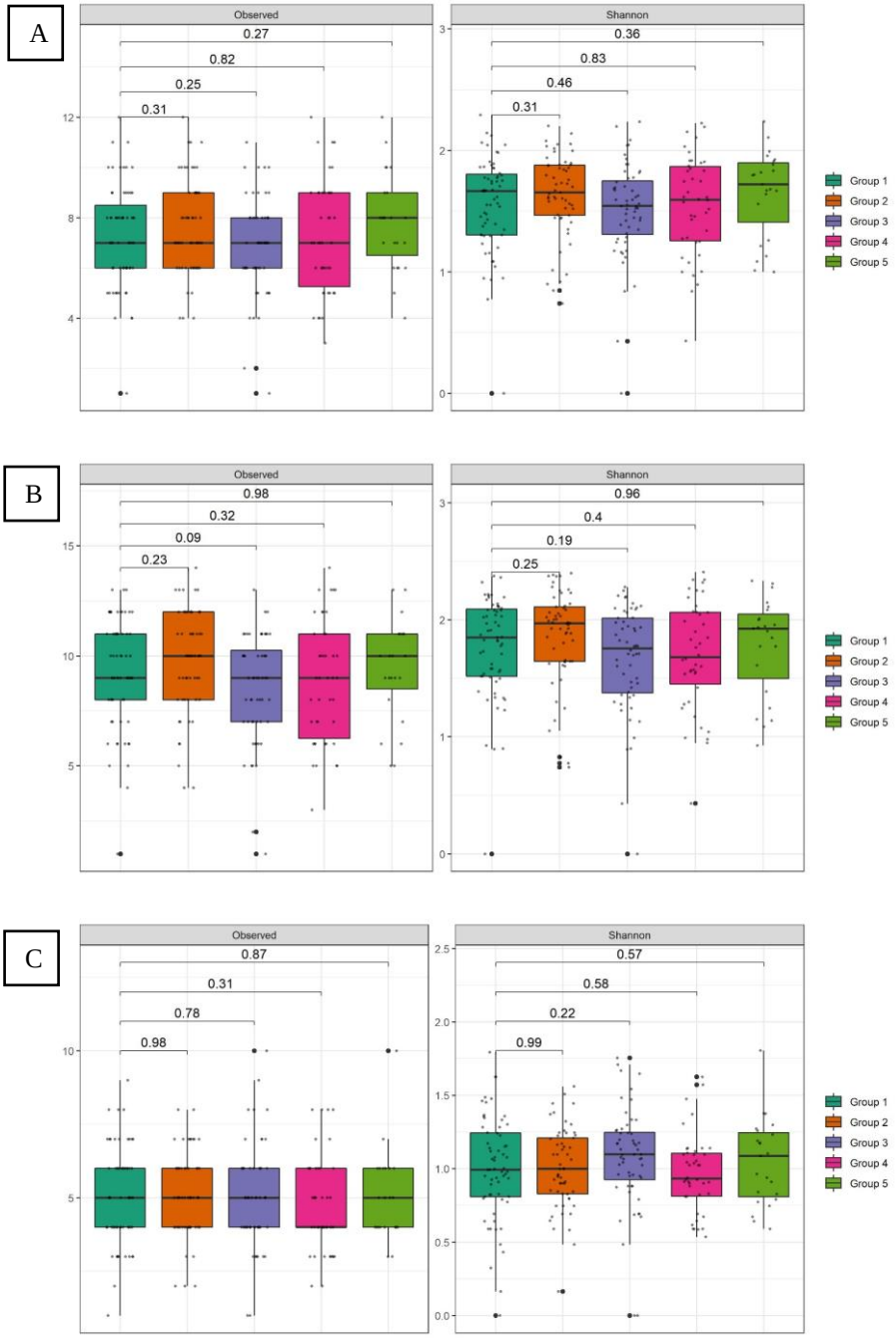
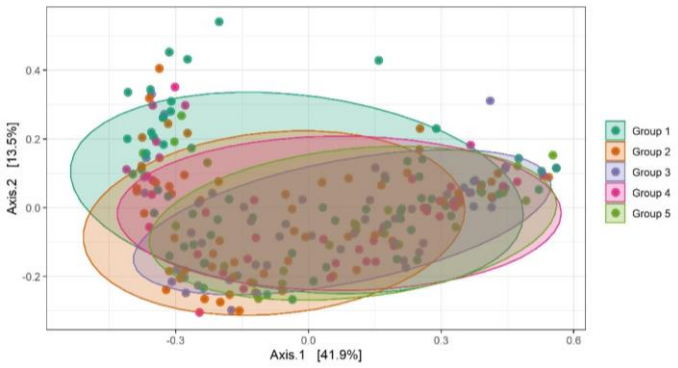


Figure 1. Alpha diversity by sexual behavior group

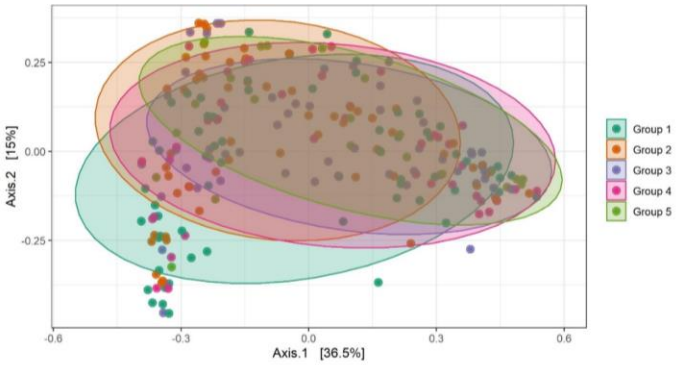
(a) Family level alpha diversity (b) Genus level alpha diversity (c) species level alpha diversity. Alpha diversity measured by observed species and Shannon diversity index. Groups represent the number of receptive anal intercourse partners as shown in Table 2. P-value obtained by linear regression adjusting for age.

A



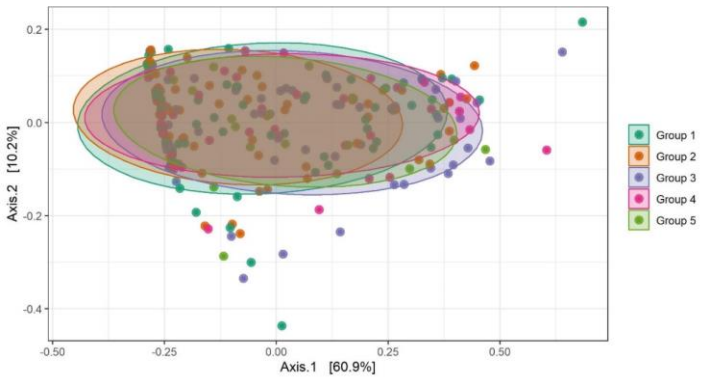
X	Y	p	q
Group 1	Group 2	0.08	0.14
Group 1	Group 3	0.03	0.12
Group 1	Group 4	0.27	0.27
Group 1	Group 5	0.10	0.14

B



X	Y	p	q
Group 1	Group 2	0.06	0.09
Group 1	Group 3	0.03	0.09
Group 1	Group 4	0.18	0.18
Group 1	Group 5	0.06	0.09

C



X	Y	p	q
Group 1	Group 2	0.42	0.56
Group 1	Group 3	0.11	0.45
Group 1	Group 4	0.65	0.65
Group 1	Group 5	0.42	0.56

Figure 2. Beta diversity by sexual behavior group

(a) Family level beta diversity (b) Genus level beta diversity (c) species level beta diversity. Beta diversity measured using Bray-Curtis dissimilarity projected onto PCoA plots. Groups represent the number of receptive anal intercourse partners as shown in Table 2. P-values obtained by PERMANOVA.

3.3 Differential Abundance

Differential abundance analysis was used to determine significant differences in relative taxa abundance between sexual behavior groups. In all heat map figures, taxa included are significant at adjusted or un-adjusted p-values based on global test results (Figure 3).

At the microbial family level, eight taxa were found to be statistically different for at least one group (Figure 4a). Family level taxa with adjusted p-value significance include *Rikenellaceae, Succinivibrionaceae, S24-7, Verrumicrobiaceae,* and *Desulfovibrionaceae.* Family level taxa with un-adjusted p-value significance include *Bacteroidaceae, Veillonellaceae,* and *Erysipelotrichaceae*. At the microbial genus level, 13 taxa were found to be statistically different for at least one group (Figure 3b). Genus level taxa with adjusted p-value significance include *Succinivibrio, Mogibacterium, Desulfovibrio, Alistipes, Akkermansia,* and *[Prevotella]*. Genus level taxa with p-value significance include *Peptococcus, Paraprevotella, Oribacterium, Dialister, Bacteroides,* and *Anaerostipes*. At the microbial species level, nine taxa were found to be significantly different for at least one group (Figure 3c). Species level taxa with significant adjusted p-values include: *Prevotella stercorea, Akkermansia muciniphila, Ruminococcus torques, Clostridium spiroforme,* and *Alistipes putredinis*. Species level taxa with significant p-values include: *Alistipes onderdonkii, Bacteroides ovatus, Bacteroides caccae,* and *Roseburia faecis*.

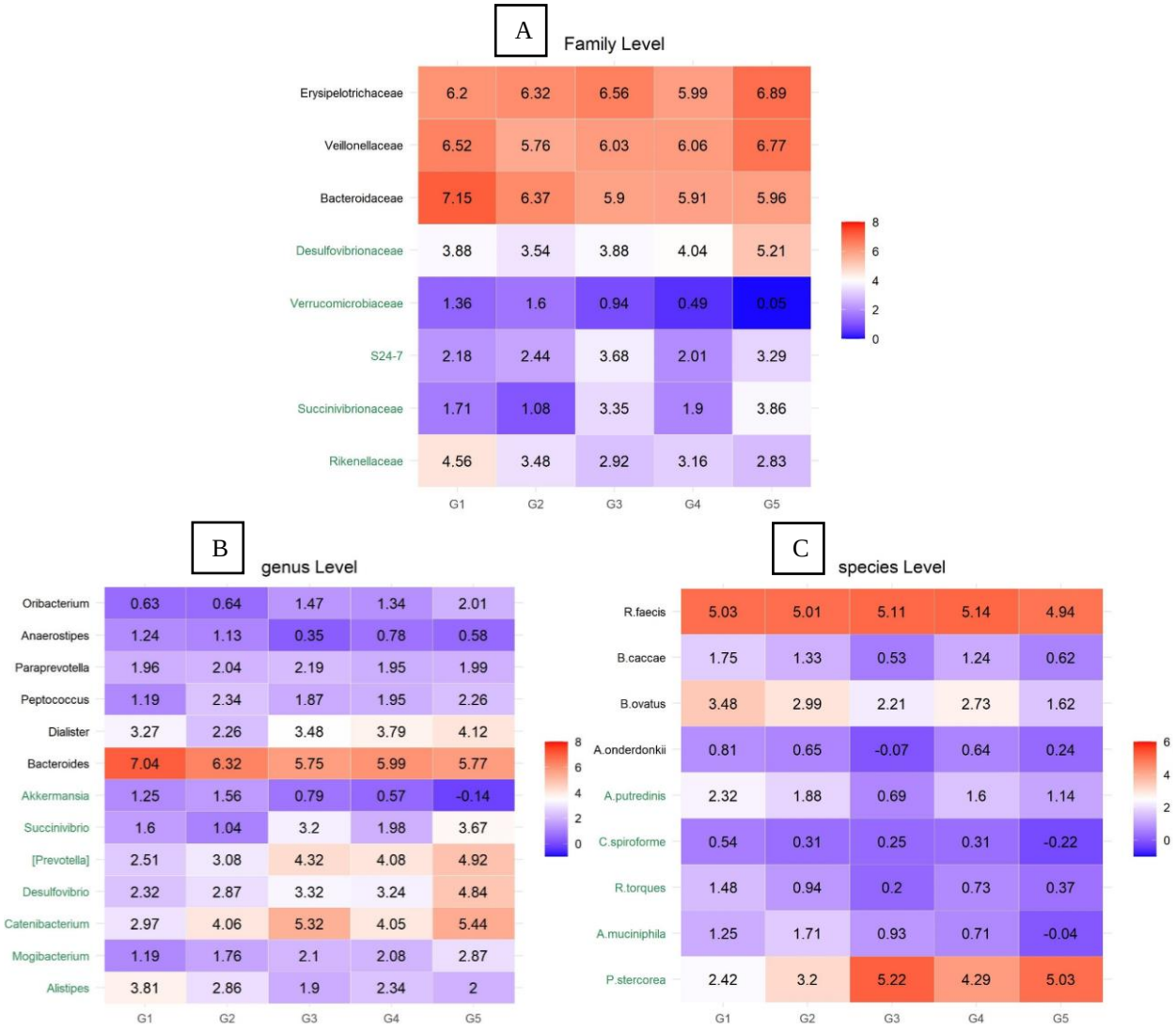


Figure 3. Heat map of differentially abundant taxa among sexual behavior groups.

(a) Family level differential abundance (b) Genus level differential abundance (c) species level differential abundance. Groups represent the number of receptive anal intercourse partners as shown in Table 2. P-value obtained by global test, adjusted p-values in green and un-adjusted p-values in black. Taxa are overlaid with bias corrected abundance, with higher relative abundance in red values and lower relative abundance in blue values.

Pairwise comparisons (T-test) between each group were made based on microbial abundance to determine significant differences in taxa abundance for any two sexual behavior groups. For clarity, results of pairwise comparisons between G1 and G5 are included below, and additional pairwise comparisons are available in supplementary Table 1. At the microbial genus

level, compared to G1, G5 showed significantly higher levels of taxa abundance for: *[Prevotella], Desulfovibrio, Catenibacterium, Mogibacterium* (adjusted p-value <0.05), *Peptococcus and Succinovibrio* (p-value <0.05). Compared to G1, G5 showed significantly lower levels of taxa abundance for: *Akkermansia, Alistipes*, and *Bacteroides* (adjusted p-value < 0.05). At the microbial species level, compared to G1, G5 showed significantly higher levels of taxon abundance for *P. stercorrea* (adjusted p-value < 0.05). Compared to G1, G5 showed significantly lower levels of abundance for: *C. spiroforme, R. torques, A. muciniphila, B. ovatus* (adjusted p-value < 0.05), and *B. Caccae* (p-value < 0.05).

Waterfall plots of log-fold (natural log) change in absolute abundance were made to visualize compositional differences between G1 and all other groups (Figure 4). Largely for family, genus, and species level analysis, taxonomic differences were most numerous between G1 and G5 (0 partners vs. 9+ partners), followed by G1 and G3 (0 partners vs. 9+ partners) and G1 vs G4 (0 partners vs. 4 – 8 partners). G2 (1 partner) was taxonomically most similar to G1 (0 partners). Taxa with adjusted p-value significance are written in blue and taxa with p-value significance are written in black.

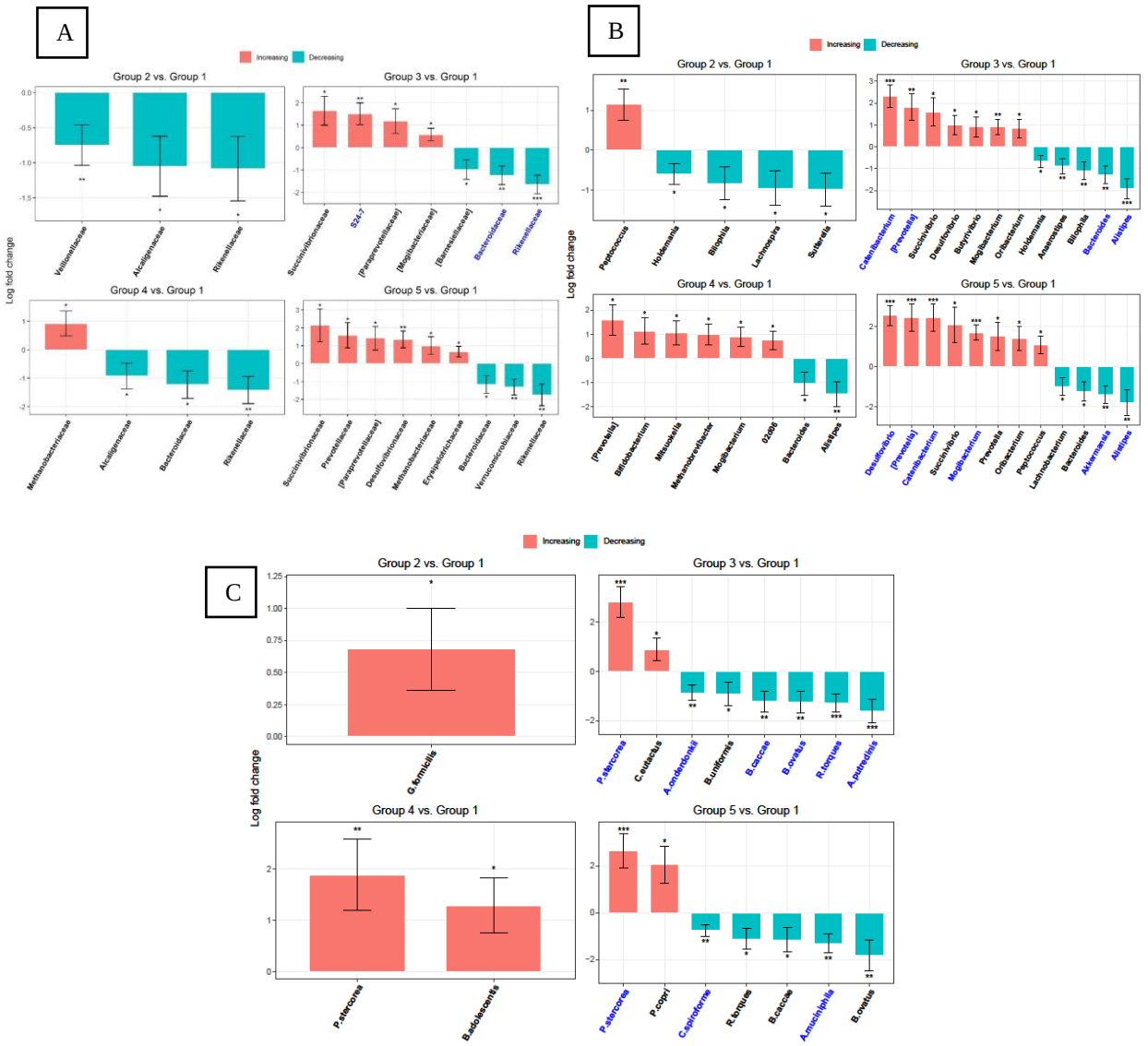


Figure 4. Waterfall plot of differentially abundant taxa

(a) Family level differential abundance of all groups compared to group 1 (b) Genus level differential abundance of all groups compared to group 1 (c) species level differential abundance of all groups compared to group 1. Bars represent log fold change (natural log) of absolute abundances for comparison groups. Groups represent the number of receptive anal intercourse partners as shown in Table 2. Data are represented by log fold changes +/- SE (shown as error bars) obtained from the ANCOM-BC model. Adjusted p-levels for corresponding taxa written in blue. *** indicates taxa significant at p-value < 0.001, ** indicates taxa significant at p-value < 0.01, * indicates taxa significant at p-value < 0.05.

3.4 Short Chain Fatty Acids

In order to evaluate differences in SCFA concentration among groups, proportional SCFA levels were plotted on a bar graph by group and seroconversion status (Figure 5). As not all participants included in microbial analysis had corresponding SCFA data, the number of participants in each group differs from Table 2 (222 versus 241 total participants). The number of participants in G1 was 58, in G2 was 52, in G3 was 50, in G4 was 40, and in G5 was 22. Analysis of seroconversion rates for MACS participants with available SCFA results (Table 3) revealed a significant different among groups (p-value < 0.001, chi-squared).

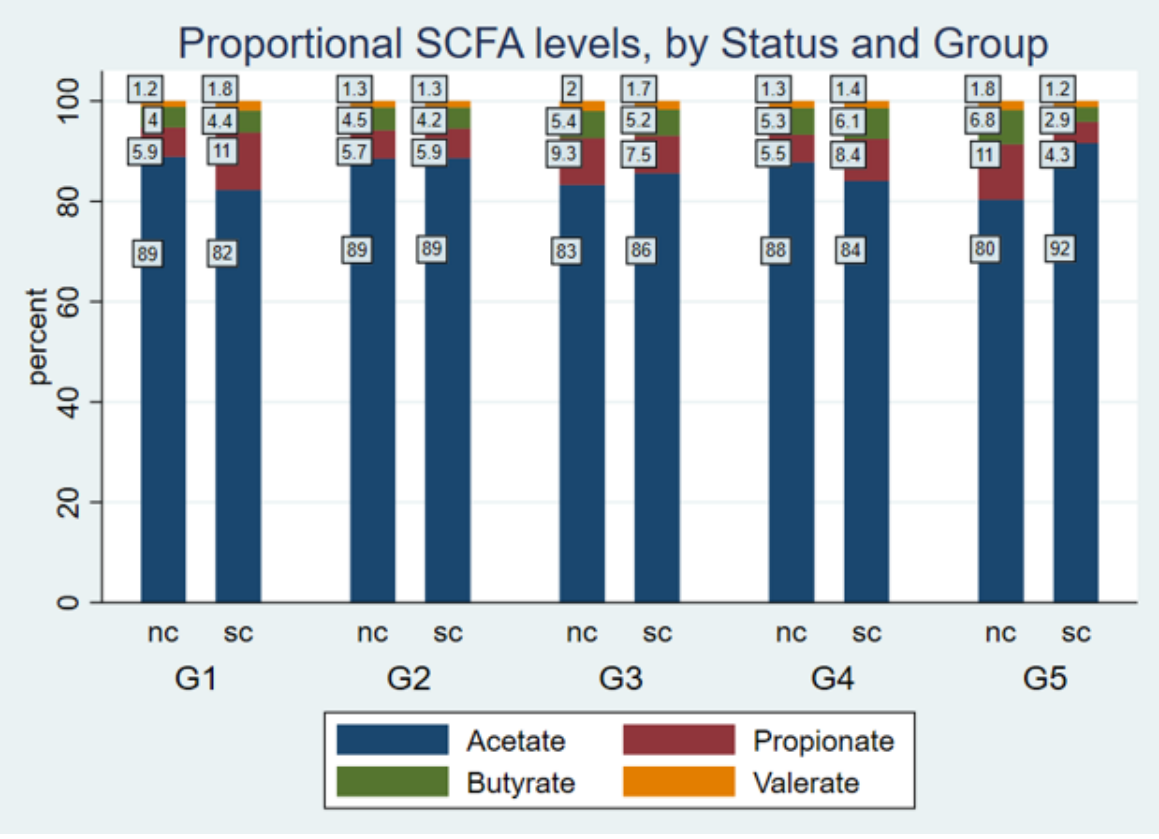


Figure 5. Proportional SCFAs by group and seroconversion status

Proportional acetate, propionate, butyrate, and valerate levels by group and seroconversion status. Groups represent the number of receptive anal intercourse partners as delineated in Table 2.

Table 3. Seroconversion status by group for participants with available SCFA samples

Seroconversion Status	**G1** (0 partners)	**G2** (1 partner)	**G3** (2-3 partners)	**G4** (4-9 partners)	**G5** (10+ partners)	**p-value**
NC n (% total NC)	55 (38.73%)	37 (26.06%)	25 (17.61%)	20 (14.08%)	5 (3.52%)	< 0.001 (Chi-squared)
SC n (% total SC)	3 (3.75%)	15 (18.75%)	25 (31.25%)	20 (25%)	17 (21.25%)	

3.5 HIV seroconversion

To analyze HIV seroconversion rate by sexual behavior group, a pie chart (Figure 6) was created using HIV status data from visit 2. Largely, seroconversion rates increase in accordance with the number of partners for RAI, with a rate of 4.8% for G1 and a rate of 74% for G5. The rate

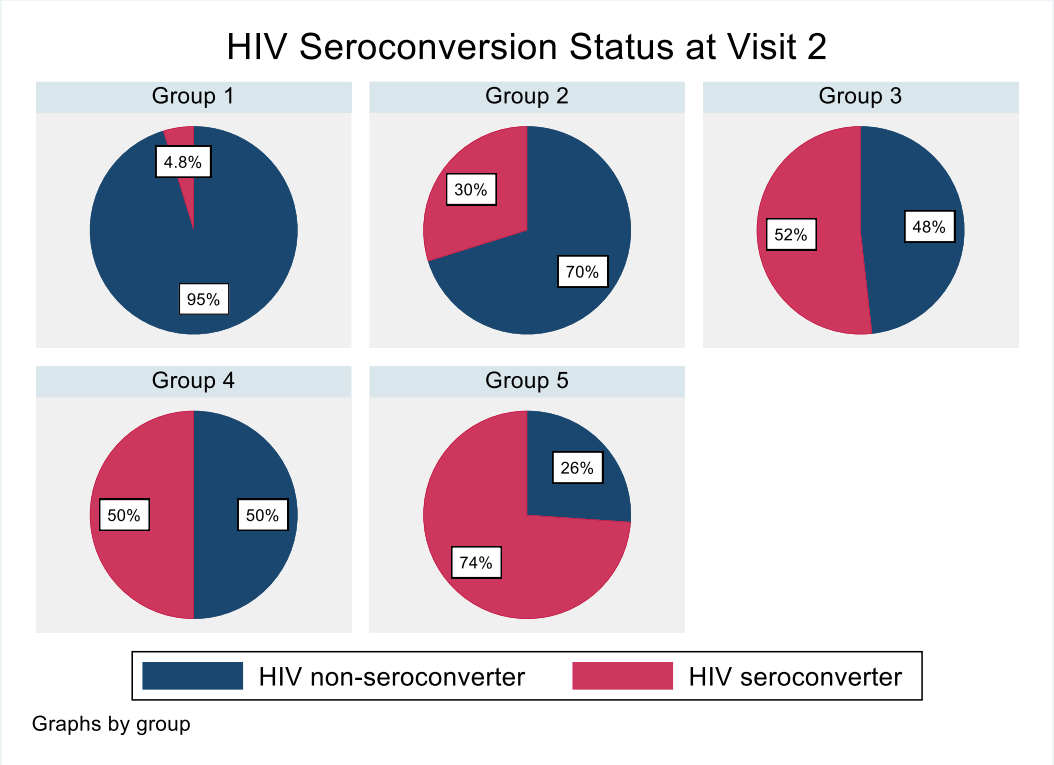


Figure 6. HIV Seroconversion rate at vist 2 by sexual behavior group.

HIV seroconversion shown in red; HIV non-seroconversion shown in blue. Groups represent the number of receptive anal intercourse partners as shown in Table 2.

4.0 Discussion

The main goal of this study was to identify associations between the number of receptive anal intercourse partners (risky sexual behavior) and the gut microbiome changes of MSM by analyzing behavior data and fecal samples from 1984-1985 MACS participants in the context of subsequent HIV seroconversion. Previous studies using MACS participants found RAI as the highest risk factor in HIV seroconversion (Kingsley et al., 1987), and found pathogenic changes in the gut microbiome of MSM were present several months prior to HIV seroconversion (Chen et al., 2021). Studies of more contemporaneous MSM sample populations find gut microbiome differences between HIV seropositive and HIV seronegative individuals (Guoqin, 2014). Rather than identifying gut microbiome changes post-HIV infection, the present study focused on changes prior to HIV infection in seronegative MSM, prior to the advent of ART or PReP.

4.1 Microbial Diversity

Findings from current literature suggest a complicated relationship between alpha diversity, MSM, disease, and HIV infection. Generally, higher alpha diversity is associated with a healthier gut microbiome, and lowered alpha diversity has been associated with disease states such as inflammatory bowel disease, *Clostridium difficile* infection, liver disease, and some types of cancer (Kriss et al., 2018). If RAI does promote gut microbiome dysbiosis and disease susceptibility, these findings suggest we should have observed the lowered alpha diversity seen among other disease states. However, compared to women, and men who have sex with women,

MSM tend to have higher alpha diversity levels (Armstrong et al., 2018). Moreover, Vujkovic-Cvijic (2020) found that this increase was linked to receptive anal intercourse in both MSM and women. These findings suggest that we should have observed increased alpha diversity among higher-partner RAI groups. However, our results show no significant difference in alpha diversity between groups. It is possible our alpha diversity results reflect the conflicting impacts of gut microbiome dysbiosis and of RAI. However, it is also likely there was no specific underlying cause, and that our sample population simply did not reflect any significant difference in alpha diversity between groups.

Beta diversity reflects the difference in taxonomic abundance between groups, and can be indicative of overall shift in community structure. Beta diversity shifts in the same direction for both men and women who engage in RAI, and for MSM who engage in RAI with and without a condom (Vujkovic-Cvijin et al., 2020). However, Armstrong et al. (2018) found that among MSM, beta diversity did not differ among those who engaged in RAI. Our results reveal a significant difference in beta diversity between G1 and G3 at the family and genus level, though there was no difference between the highest-partner RAI group (G5) and G1. While not statistically significant, beta diversity among G1 tended to differ in comparison to all other groups, which were more similar to each other. This suggests a shift in beta diversity is the result of engaging versus not engaging in RAI rather than a "dose dependent" relationship. Although not of relevance to the study sample population, current literature suggests decreased alpha diversity and shifts to beta diversity among HIV seropositive MSM compared to their HIV seronegative counterparts (Vujkovic-Cvijin et al., 2020).

4.2 Differential Abundance and Gut Microbiome Dysbiosis

Previous studies found that MSM have a gut microbiome distinct from MSW (Noguera-Julian et al., 2016), and among HIV seronegative MSM, condomless RAI is associated with changes to gut microbiome composition and systemic immune system activation (Kelley, 2017). Coleman et al. (2019) conducted *in vitro* studies comparing the impact of fecal microbiota obtained from MSM to fecal microbiota obtained from MSW in gnotobiotic mice, which are mice raised in completely sterile environments (germ-free mice) or environments in which every microorganism present is known (Dube, 2017). Results of the study (Coleman et al., 2019) showed that in mice, fecal microbiota from MSM elevated immune activation and enhanced HIV infection over fecal microbiota from MSW. Such findings indicate that the gut microbiome of MSM is impacted by sexual behavior and that these impacts result in increased HIV susceptibility. This establishes a basis for the sexual behavior focused gut microbiome analysis of MSM used in our study.

We found differential abundance among higher-partner RAI groups to be consistent with some taxa found differentially abundant in seroconverters vs. non seroconverters prior to HIV infection (Chen et al., 2021) The study also used data from 1984 -1985 MACS participants, but grouped them by seroconversion status rather than sexual behavior. Among higher-partner RAI groups, we found *Catenibacterium, Mogibacterium, Prevotella, Succinovibrio, Peptococcus, Desulfovibrio,* and *Oribacterium* to be higher in abundance, genera which were also found to be higher in abundance among HIV seroconverters prior to HIV infection. Additionally, among higher-partner RAI groups, we found *Akkermansia, Bacteroides*, and *Alistipes* to be significantly lower in abundance, genera which were also lower in abundance among HIV seroconverters prior to HIV infection. At the species level, *P. stercorea* was higher in abundance among higher partner RAI groups, and *A. onderdonkii, R. torques, B. caccae, A. putredinis, A. muciniphila,* and *B. ovatus*

to be lower in abundance among higher-partner RAI groups, again consistent with HIV seroconverters prior to HIV infection. Overall, there were shared trends in gut microbiome composition among MSM seroconverters and higher-partner RAI groups, suggesting that RAI shifts gut microbiome composition towards one susceptible to HIV infection.

Previous studies indicate that gut microbiomes of MSM are rich in *Prevotella* (Noguera-Julian et al., 2016) and higher levels of *Prevotella* are associated with RAI (Vujkovic-Cvijin et al., 2020). We found that *Prevotella* abundance increased among groups with higher numbers of RAI partners, including species *P. stercorea*. High levels of *P. stercorea* have been found in HIV seropositive MSM (Armstrong et al., 2018) and in MSM prior to HIV seroconversion (Chen et al., 2021), and has been associated with activated colonic myeloid dendritic cells among HIV-infected individuals (Dillon et al., 2016). In addition to an increase in *Prevotella* abundance, previous studies indicate a decrease in *Bacteroides* abundance among MSM (Vujkovic-Cvijin), HIV infected MSM (Armstrong et al., 2018) and MSM prior to HIV seroconversion (Chen et al., 2021). We also found the genus *Bacteroides* to be lower in abundance among higher partner RAI groups, along with species *B. caccae* and *B. ovatus*.

Certain taxa found to be differentially abundant were of interest due to their implication in inflammation and immune system activation. Family *desulfovibrionaceae* and genus *desulfovibrio* were higher in abundance in G5 compared to all lower-partner RAI groups, and differences in log fold abundance were among the highest of all significant taxa (log-fold change for G5 compared to G1 was 1.67 for *desulfovibrionaceae* and 2.52 for *desulfovibrio*). *Desulfovibrionaceae* are known to have pro-inflammatory properties, including a link to levels of innate immune system activation markers among people with HIV (Vujkovic-Cvijin et al., 2020) and peripheral T cell activation (Vujkovic-Cvijin & Somsouk, 2019). *Desulfovibrio* are producers of hydrogen sulfide,

which is believed to be toxic to epithelial cells and may contribute to gut epithelial damage and reduced mucus integrity in inflammatory bowel disease (Vujkovic-Cvijin & Somsouk, 2019). *A. Muciniphila* was found in lower abundance for G5 compared to G1 and G2 (log-fold change of -1.29 for G5 compared to G1). *A. muciniphila* resides preferentially in the mucus layer of the large intestine, uses mucus as a growth and adhesion substrate, and can promote intestinal mucosal homeostasis (Vujkovic-Cvijin et al., 2020). The combination of decreased *A. muciniphila* abundance and increased *desulfovibrio* abundance in G5 compared to lower-partner RAI groups may be indicative of a shift towards reduced gut epithelial barrier function and integrity and increased mucosal dysfunction. This has implications on HIV susceptibility, as inflammation and mucosal barrier damage can impact the number of available target cells (CD4+CCR5+ T cells) for HIV infection (Burgener et al., 2015) as CD4+ T cells are recruited to sites of epithelial inflammation. Our findings of increased pro-inflammatory and decreased anti-inflammatory bacteria among higher-partner RAI groups coordinate with findings from other studies on the impact of RAI. Kelly et al. (2017) found condomless RAI was associated with higher levels of Th17 cells, greater CD8+ T cell proliferation, production of pro-inflammatory cytokines, and molecular indicators of mucosal injury compared to MSM who had not engaged in anal intercourse.

4.3 Short-Chain Fatty Acids

Microbial communities present in the gut produce SCFAs via the fermentation of complex carbohydrates, and resultant SCFAs can influence immune function, gut homeostasis and inflammation. SCFAs are a main energy source for gut epithelial cells, which can promote gut

barrier integrity and reduce inflammation, and can promote the differentiation of both effector and regulatory T cells. Current literature reveals a complex relationship between SCFAs and inflammation, and whether increased levels have a beneficial or harmful impact on inflammation may alter based on pathological conditions (Kim et al., 2014). Previous studies have revealed that SCFAs could influence HIV susceptibility (Mirmonsef et al., 2012; Ouyang et al., 2020) due to their influence on inflammation and immune system regulation. We found differences among groups in the proportional levels of SCFAs between HIV seroconverters and non seroconverters, which may be indicative of a shift in gut microbiome functionality and subsequent susceptibility to HIV infection. In G1, proportional acetate levels were lower and proportional propionate levels were higher in seroconverters compared to non seroconverters, while in G5 the same trend was found among non seroconverters rather than seroconverters. This suggests that SCFA levels do not consistently play the same role in susceptibility to HIV infection, and rather have a complex relationship with susceptibility dependent on context.

4.4 Limitations

Limitations of this study include uneven group sizes, unavailable SCFA results for some samples, and bias introduced by participant selection and reporting. The partner delineations used in creating sexual behavior groups resulted in a group size of 23 individuals in G5, which is notably smaller than other group sizes. Not all of the participants used for diversity and microbial analysis had available results for SCFA levels, and reduced the sample population used in SCFA analysis to 222 (19 fewer than the total sample population). MACS participants freely chose to participate in the study, introducing possible self-selection bias to the sample population. Lastly, participants

self-reported sexual behavior, introducing possible self-reporting bias, and reported sexual behavior activities for a six-month period prior to visit one, introducing possible recall bias.

4.5 Future Directions

This study found distinctions in the microbial communities of sexual behavior groups, but did not consider other influential factors in gut microbiome composition. Factors such as antibiotic use, drug use, and diet would be of interest in any future studies due to possible interactions with the gut microbiome. The combined effects of such factors and sexual behavior may further modulate gut microbiome composition and resultant HIV infection susceptibility, and future studies including these factors could further clarify the relationship between sexual behavior and the gut microbiome of MSM.

Although microbial composition can be indicative of potential influences on the host by the gut microbiome, it does not provide information on the functional microbial output. Future studies would benefit from an analysis of microbial gene and protein production, although the age of MACS samples may make such analysis impossible. This would allow for a perspective on not only what microbial communities are present, but what functionalities those communities provide.

Additionally, analysis of inflammatory markers would allow for additional information on the gut microbiome's impact on HIV susceptibility among MSM. Previous studies using MACS data (Chen et al., 2021) included such analysis, but grouped participants based on seroconversion status rather than sexual behavior. Future studies could apply this approach to the groupings used in this study, clarifying the impact sexual behavior and the gut microbiome has on inflammation among MSM.

Lastly, further analysis on the relationship between SCFAs and susceptibility to HIV infection among our study population will be required to fully explore their influence. Compositional statistical analysis would provide a clearer look into that relationship, as our current results only reveal observational trends. Although not included in the current study, subsequent analysis of SCFA levels among groups was done using centered and adjacent log ratios followed by two-way ANOVA and graphical representation. These subsequent results showed acetate levels to be significant in the context of sexual behavior group and HIV seroconversion status. Additionally, G4 and G5 (4-9 and 10+ RAI partners) were combined into one group for this analysis, as this increased sample size and was still reflective of the highest risk individuals in terms of sexual behavior. Similar to the sexual behavior groups used in this study, seroconversion status was significantly different among the newly-defined groups. Future analysis should examine microbial diversity and composition in the context of these groups, as it may reveal meaningful results in the context of future studies.

5.0 Conclusions

The results of this study reveal that the number of receptive anal intercourse partners among MSM influenced gut microbiome composition, and that higher numbers of receptive anal intercourse partners (9+) were associated with changes in the abundance of pro- and anti-inflammatory taxa. These changes in gut microbiome composition may increase HIV susceptibility among MSM as increased inflammation is associated with a higher risk of HIV infection, which was evidenced by higher HIV seroconversion rates in the group with higher RAI partners. Receptive anal intercourse is known to be a critical risk factor for HIV infection, and its relation to gut microbiome alterations and subsequent HIV risk is of clinical and public health importance.

Appendix A.1 Additional Statistical Results

.

Appendix Table 1 Pairwaise comaprisons of differentially abundant taxa

Taxon	Comparison Group 1	Comparison Group 2	N1	N2	Test statistic	p-value	Adjusted p-value
Family							
Bacteroidaceae	G1	G3	62	54	3.39	0	0.01
	G1	G4	62	41	2.82	0.01	0.03
	G1	G5	62	23	2.67	0.01	0.03
	G1	G2	62	57	2.17	0.03	0.08
Desulfovibrionaceae	G2	G5	57	23	-3.73	0	0
	G1	G5	62	23	-3.06	0	0.01
	G3	G5	54	23	-3.09	0	0.01
	G4	G5	41	23	-2.67	0.01	0.03
Erysipelotrichaceae	G4	G5	41	23	-3.28	0	0.02
	G1	G5	62	23	-2.79	0.01	0.04
	G2	G5	57	23	-2.23	0.03	0.1
	G3	G4	54	41	1.96	0.05	0.13
Rikenellaceae	G1	G3	62	54	4.38	0	0
	G1	G4	62	41	3.25	0	0.01
	G1	G5	62	23	3.05	0	0.01
	G1	G2	62	57	2.61	0.01	0.03
S24-7	G1	G3	62	54	-3.35	0	0.01
	G3	G4	54	41	3.31	0	0.01
	G2	G3	57	54	-2.57	0.01	0.04
Succinivibrionaceae	G2	G3	57	54	-3.91	0	0
	G2	G5	57	23	-3.21	0	0.01
	G1	G3	62	54	-2.73	0.01	0.03
	G1	G5	62	23	-2.44	0.02	0.05
	G3	G4	54	41	2.22	0.03	0.06
	G4	G5	41	23	-2.14	0.04	0.06
Veillonellaceae	G1	G2	62	57	3.04	0	0.03
	G2	G5	57	23	-2.78	0.01	0.04
Verrucomicrobiaceae	G1	G5	62	23	3.37	0	0.01

	G2	G5	57	23	3.31	0	0.01
	G2	G4	57	41	2.16	0.03	0.11
	G3	G5	54	23	2.07	0.04	0.11
Genus							
[Prevotella]	G1	G5	62	23	-3.76	0	0
	G1	G3	62	54	-3.12	0	0.01
	G2	G5	57	23	-2.89	0.01	0.02
	G1	G4	62	41	-2.53	0.01	0.03
	G2	G3	57	54	-2.15	0.03	0.07
Akkermansia	G1	G5	62	23	3.5	0	0
	G2	G5	57	23	3.53	0	0
	G3	G5	54	23	2.15	0.04	0.12
Alistipes	G1	G3	62	54	4.63	0	0
	G1	G4	62	41	2.99	0	0.01
	G1	G5	62	23	3.03	0	0.01
	G1	G2	62	57	2.1	0.04	0.08
	G2	G3	57	54	2.12	0.04	0.08
Anaerostipes	G1	G3	62	54	3.04	0	0.03
	G2	G3	57	54	2.44	0.02	0.08
Bacteroides	G1	G3	62	54	3.44	0	0.01
	G1	G5	62	23	2.76	0.01	0.04
	G1	G4	62	41	2.34	0.02	0.07
Catenibacterium	G1	G3	62	54	-4.81	0	0
	G1	G5	62	23	-3.85	0	0
	G2	G3	57	54	-2.46	0.02	0.05
	G3	G4	54	41	2.41	0.02	0.05
	G2	G5	57	23	-2.1	0.04	0.07
	G4	G5	41	23	-2.07	0.04	0.07
Desulfovibrio	G1	G5	62	23	-5.28	0	0
	G2	G5	57	23	-4.01	0	0
	G3	G5	54	23	-3.32	0	0.01
	G4	G5	41	23	-3.16	0	0.01
	G1	G3	62	54	-2.42	0.02	0.03
Dialister	G2	G5	57	23	-3.05	0	0.03
	G2	G3	57	54	-2.62	0.01	0.03
	G2	G4	57	41	-2.71	0.01	0.03
	G1	G2	62	57	2.03	0.04	0.11

Mogibacterium	G1	G5	62	23	-4.76	0	0
	G1	G3	62	54	-2.88	0	0.02
	G2	G5	57	23	-2.86	0.01	0.02
	G1	G4	62	41	-2.47	0.02	0.04
	G3	G5	54	23	-2.07	0.04	0.09
Oribacterium	G1	G5	62	23	-2.34	0.03	0.11
	G2	G5	57	23	-2.29	0.03	0.11
	G1	G3	62	54	-2.14	0.04	0.11
	G2	G3	57	54	-2.05	0.04	0.11
Peptococcus	G1	G2	62	57	-3.23	0	0.02
	G1	G5	62	23	-2.69	0.01	0.05
Succinivibrio	G2	G3	57	54	-3.78	0	0
	G2	G5	57	23	-3.1	0	0.02
	G1	G3	62	54	-2.66	0.01	0.03
	G1	G5	62	23	-2.38	0.02	0.06
Species							
A.muciniphila	G1	G5	62	23	3.56	0	0
	G2	G5	57	23	3.99	0	0
	G3	G5	54	23	2.46	0.02	0.05
	G4	G5	41	23	2.05	0.04	0.1
A.onderdonkii	G1	G3	62	54	3.2	0	0.02
	G2	G3	57	54	2.58	0.01	0.06
	G3	G4	54	41	-2.21	0.03	0.1
A.putredinis	G1	G3	62	54	3.77	0	0
	G2	G3	57	54	2.69	0.01	0.04
	G3	G4	54	41	-2.04	0.04	0.15
B.caccae	G1	G3	62	54	3.08	0	0.03
	G1	G5	62	23	2.38	0.02	0.1
B.ovatus	G1	G3	62	54	3.03	0	0.03
	G1	G5	62	23	2.98	0	0.03
	G2	G5	57	23	2.16	0.04	0.12
C.spiroforme	G1	G5	62	23	3.69	0	0
	G2	G5	57	23	2.38	0.02	0.07
	G4	G5	41	23	2.4	0.02	0.07
	G3	G5	54	23	2.14	0.04	0.09
P.stercorea	G1	G3	62	54	-4.83	0	0
	G1	G5	62	23	-3.76	0	0

	G2	G3	57	54	-3.32	0	0
	G1	G4	62	41	-2.81	0.01	0.01
	G2	G5	57	23	-2.55	0.01	0.03
R.torques	G1	G3	62	54	4.12	0	0
	G1	G5	62	23	2.82	0.01	0.03
	G2	G3	57	54	2.39	0.02	0.06
	G1	G4	62	41	2.07	0.04	0.1

Bibliography

Armstrong, A., Shaffer, M., Nusbacher, N. M., Griesmer, C., Fiorillo, S., Schneider, J. M., Preston Neff, C., Li, S. X., Fontenot, A. P., Campbell, T., Palmer, B. E., & Lozupone, C. A. (2018). An exploration of Prevotella-rich microbiomes in HIV and men who have sex with men. *Microbiome, 6*(1), 198. https://doi.org/10.1186/s40168-018-0580-7

Bolduc, J., Hany, L., Barat, C., Ouellet, M. & Tremblay, M. (2017). Epigenetic metabolite acetate inhibits class I/II histone deacetylases, promotes histone acetylation, and increases HIV-1 integration in CD4+ T cells. *Journal of Virology, 91*(16). https://doi.org/10.1128/JVI.01943-16

Centers for Disease Control and Prevention. (1981a, June 5). Morbidity and mortality weekly report, Vol. 30, no. 21, June 5, 1981. CDC Stacks Public Health publications. https://stacks.cdc.gov/view/cdc/1261

Centers for Disease Control and Prevention. (1981b, July 3). Morbidity and mortality weekly report, Vol. 30, no. 25, July 3, 1981. CDC Stacks Public Health publications. https://stacks.cdc.gov/view/cdc/1265

Centers for Disease Control and Prevention. (2021, August 21) HIV Statistics Center. Statistics Overview. https://www.cdc.gov/hiv/statistics/index.html

Centers for Disease Control and Prevention. (2020, May). HIV surveillance report, 2018 (Updated). https://www.cdc.gov/hiv/library/reports/hiv-surveillance.html

Chen, Y., Lin, H., Cole, M., Morris, A., Martinson, J., Mckay, H., Mimiaga, M., Margolick, J., Fitch, A., Methe, B., Srinivas, V., Peddada, S. & Rinaldo, C. (2021) Signature changes in gut microbiome are associated with increased susceptibility to HIV-1 infection in MSM. *Microbiome 9***(**237). https://doi.org/10.1186/s40168-021-01168-w

Das, B. & Nair, G.B. (2019) Homeostasis and dysbiosis of the gut microbiome in health and disease. *J Biosci 44*(117). https://doi.org/10.1007/s12038-019-9926-y

Dube, P. (2017). What are germ-free mice and how are they sourced?. Taconic. Retrieved from https://www.taconic.com/taconic-insights/microbiome-and-germ-free/what-are-germ-free-mice.html

Dillon, S., Lee, E., Kotter, C. V., Austin, G. L., Gianella, S., Siewe, S., Smith, D. M., Landay, A. L., McManus, M. C., Roberston, C. E., Frank, D. N., McCarter, M. D. & Wilson, C. (2016) Gut dendritic cell activation links an altered colonic microbiome to mucosal and systemic T-cell activation in untreated HIV-1 infection. *Mucosal Immunol, 9*, 24–37 https://doi.org/10.1038/mi.2015.33

Gallup. (2021, February 24). LGBT Identification Rises to 5.6% in Latest U.S. Estimate. Gallup Politics.https://news.gallup.com/poll/329708/lgbt-identification-rises-latest-estimate.aspx

Hess, K.L., Crepaz, N., Rose, C., Purcell, D. & Paz-Bailey, G. (2017, May 29). Trends in Sexual Behavior Among Men Who have Sex with Men (MSM) in High-Income Countries, 1990–2013: A Systematic Review. *AIDS Behav, 21*, 2811–2834. https://doi.org/10.1007/s10461-017-1799-1

Jones, J. & Salazar, L. (2016). Understanding the HIV/AIDS Epidemic in the United States. Springer, Cham. Retrieved from https://doi.org/10.1007/978-3-319-34004-3_2

Kelley, C., Kraft, C., de Man, T., Duphare, C., Lee, H., Yang, J., Easley, K., Tharp, G., Mulligan, M., Sullivan, P., Bosinger, S. & Amara, R. (2017) The rectal mucosa and condomless receptive anal intercourse in HIV-negative MSM: implications for HIV transmission and prevention. *Mucosal Immunol, 10,* 996–1007. https://doi.org/10.1038/mi.2016.97

Kelley, C.F., Pollack, I., Yacoub, R., Zhu, Z., Van Doren, V.E., Gumber, S., Amara, R.R., Fedirko, V., Kraft, C.S., de Man, T.J.B., Hu, Y.-J., Grimsley Ackerley, C., Sullivan, P.S. & Bostick, R.M. (2021), Condomless receptive anal intercourse is associated with markers of mucosal inflammation in a cohort of men who have sex with men in Atlanta, Georgia. *J Int AIDS Soc., 24*: e25859. https://doi.org/10.1002/jia2.25859

Kim, C. H., Park, J., & Kim, M. (2014). Gut microbiota-derived short-chain Fatty acids, T cells, and inflammation. *Immune network, 14*(6), 277–288. https://doi.org/10.4110/in.2014.14.6.277

Kriss, M., Hazleton, K. Z, Nusbacher, N. M., Martin, C. G. & Lozupone, C. A. (2018). Low diversity gut microbiota dysbiosis: Drivers, functional implications and recovery.Current *Opinion in Microbiology, 44*, 34-40. https://doi.org/10.1016/j.mib.2018.07.003

Lin, H., & Peddada, S. D. (2020). Analysis of compositions of microbiomes with bias correction. *Nature communications*, *11*(1), 1-11. https://doi.org/10.1038/s41467-020-17041-7

Martin-Gallausiaux, C., Marinelli, L., Blottière, H., Larraufie, P., & Lapaque, N. (2021). SCFA: Mechanisms and functional importance in the gut. *Proceedings of the Nutrition Society, 80*(1), 37-49. doi:10.1017/S0029665120006916

Mirmonsef, P., Zariffard, M. R., Gilbert, D., Makinde, H., Landay, A. L., & Spear, G. T. (2012). Short-chain fatty acids induce pro-inflammatory cytokine production alone and in combination with toll-like receptor ligands. *American journal of reproductive immunology 67*(5), 391–400. https://doi.org/10.1111/j.1600-0897.2011.01089.x

MWCCS. (n.d). Our Study. Our History. Retrieved from https://statepi.jhsph.edu/mwccs/our-history/

Noguera-Julian, M., Rocafort, M., Guillén, Y., Rivera, J., Casadellà, M., Nowak, P., Hildebrand, F., Zeller, G., Parera, M., Bellido, R., Rodríguez, C., Carrillo, J., Mothe, B., Coll, J., Bravo, I., Estany, C., Herrero, C., Saz, J., Sirera, G. & Torrela, A. et al. (2016). Gut microbiota linked to sexual preference and HIV infection. *EBioMedicine, 5,* 135-146. doi: 10.1016/j.ebiom.2016.01.032

Ouyang, J., Isnard, S., Lin, J. Fombuena, B., Marette, A., Routy, B., Chen, Y. & Routy, J. (2020) Metformin effect on gut microbiota: insights for HIV-related inflammation. *AIDS Res Ther 17(*10). https://doi.org/10.1186/s12981-020-00267-2

Patel, P., Borkowf, C. Brooks, J., Lasry, A., Lansky, A. & Mermin, J. (2014). Estimating per-act HIV transmission risk, *AIDS, 28*(10), 1509-1519 doi: 10.1097/QAD.0000000000000298

Sara L. Coleman, C. Preston Neff, Sam X. Li, Abigail J.S. Armstrong, Jennifer M. Schneider, Sharon Sen, Blair Fennimore, Thomas B. Campbell, Catherine A. Lozupone & Brent E. Palmer (2020) Can gut microbiota of men who have sex with men influence HIV transmission?, *Gut Microbes, 11*(3), 610-619, DOI: 10.1080/19490976.2019.1700756

Shaw, G. M., & Hunter, E. (2012). HIV transmission. *Cold Spring Harbor perspectives in medicine, 2*(11), a006965. https://doi.org/10.1101/cshperspect.a006965

UNAIDS. (2017, October 3). UNAIDS warns that HIV-related stigma and discrimination is preventing people from accessing HIV services. UNAIDS Press Release. Retrieved from https://www.unaids.org/en/resources/presscentre/pressreleaseandstatementarchive/2017/october/20171002_confronting-discrimination

Vidya Vijayan, K. K., Karthigeyan, K. P., Tripathi, S. P., & Hanna, L. E. (2017). Pathophysiology of CD4+ T-Cell Depletion in HIV-1 and HIV-2 Infections. *Frontiers in Immunology, 8,* 580. https://doi.org/10.3389/fimmu.2017.00580

Vujkovic-Cvijin, I. & Somsouk, M. (2019). HIV and the Gut Microbiota: Composition, Consequences, and Avenues for Amelioration. *Curr HIV/AIDS Rep 16,* 204–213. https://doi.org/10.1007/s11904-019-00441-w

Vujkovic-Cvijin, I., Sortino, O., Verheij, E. Sklar, J., Wit, F. W., Koostra, N. A., Sellers, B., Brenchley, J. M., Ananworanich, J., Schim van der Loeff, M. & Belkaid, Y., Reiss, P. & Sereti, I. (2020). HIV-associated gut dysbiosis is independent of sexual practice and correlates with noncommunicable diseases. *Nat Commun 11*(2448). https://doi.org/10.1038/s41467-020-16222-8

World Health Organization. (2021, November 30). HIV/AIDS. Key Facts. Retrieved from https://www.who.int/news-room/fact-sheets/detail/hiv-aids