UCLA
UCLA Electronic Theses and Dissertations

Title
The Effects of HIV, Obesity, and Methamphetamine Use on the Gastrointestinal Microbiome of Young Men who have Sex with Men

Permalink
https://escholarship.org/uc/item/84k7h90b

Author
Cook, Ryan Robert

Publication Date
2019

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA

Los Angeles

The Effects of HIV, Obesity, and Methamphetamine Use on the Gastrointestinal Microbiome of Young Men who have Sex with Men

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Epidemiology

by

Ryan Robert Cook

2019

© Copyright by

Ryan Robert Cook

2019

ABSTRACT OF THE DISSERTATION

The Effects of HIV, Obesity, and Methamphetamine Use on the Gastrointestinal Microbiome of Young Men who have Sex with Men

by

Ryan Robert Cook
Doctor of Philosophy in Epidemiology
University of California, Los Angeles, 2019
Professor Pamina M. Gorbach, Chair

Introduction: Chronic inflammation is a major contributor to increased morbidity and mortality among people living with HIV. Research suggests that the composition of the gastrointestinal microbiome may be altered by HIV, resulting in a state of “dysbiosis” that exacerbates and perpetuates immune dysfunction. However, studies of HIV and the microbiome have thus far been limited by small sample sizes and poor control for confounding factors and have not considered potentially synergistic effects of comorbidities such as obesity and methamphetamine (MA) use. Therefore, the effects of HIV on the microbiome remain unclear. Methods: This dissertation includes biomarker, behavioral, and clinical data from 381 diverse young men who have sex with men. Microbiome composition was assessed by targeted sequencing of the V4 region of the 16S rRNA gene using rectal swab samples. In Chapter 2, we examined differences in microbiome composition between men with increasing levels of plasma HIV RNA. In Chapter 3, we split the sample into groups based on HIV and obesity and compared men who were HIV+ and obese to those with only one or neither conditions. In

Chapter 4, we explored the effects of MA use on the microbiome while testing for potential interactions between MA use and HIV status. All comparisons utilized inverse probability of treatment weighting to control for confounding by numerous behavioral and clinical factors.
Results: HIV, obesity, and MA use were all associated with shifts in microbial composition consistent with a pro-inflammatory environment. There was a dose-dependent relationship between HIV RNA level and severity of dysbiosis. Men who were HIV+ and obese had more severe dysbiosis than those with only one or neither conditions. Regardless of HIV status, MA users had higher relative abundance of many pro-inflammatory bacterial genera, with frequent users having the highest amounts.
Conclusions: As multiple comorbid conditions can negatively impact the microbiome, interventions to address dysbiosis and reduce its inflammatory consequences should consider interactions between these conditions. Future studies should utilize analytic approaches such as those employed in this dissertation in order to limit the effects of confounding and improve comparability.

The dissertation of Ryan Robert Cook is approved.

Marjan Javanbakht

Ronald S. Brookmeyer

Grace M. Aldrovandi

Pamina M. Gorbach, Committee Chair

University of California, Los Angeles

2019

Table of Contents

List of Tables vii

List of Figures viii

Acknowledgements x

Vita xiii

Chapter 1. Introduction 1
- HIV-associated chronic inflammation and comorbidities 1
- Interactions between the microbiome and the immune system 1
- Effects of HIV on the microbiome 2
- Effects of obesity and methamphetamine use on the microbiome 3

Chapter 2. Effects of HIV Viremia on the Gastrointestinal Microbiome of Young Men who have Sex with Men 5
- 2.1 Abstract 5
- 2.2 Introduction 6
- 2.3 Methods 8
- 2.4 Results 11
- 2.5 Discussion 14

Chapter 3. Combined Effects of HIV and Obesity on the Gastrointestinal Microbiome of Young Men who have Sex with Men 25
- 3.1 Abstract 25
- 3.2 Introduction 26
- 3.3 Methods 28
- 3.4 Results 31
- 3.5 Discussion 34

Chapter 4. Alterations to the Gastrointestinal Microbiome Associated with Methamphetamine Use 44
- 4.1 Abstract 44
- 4.2 Introduction 44
- 4.3 Methods 46
- 4.4 Results 50
- 4.5 Discussion 52

Chapter 5. Concluding Remarks 64

Appendix 1. Supplemental content for Chapter 2, "Effects of HIV Viremia on the Gastrointestinal Microbiome of Young Men who have Sex with Men" 67
Sample selection....67
HIV serostatus and RNA quantification. 67
Behavioral and clinical measures....67
Contaminant sequence removal procedure....68
Inverse probability of treatment weighting model selection....68
Differential abundance testing....70
ZINB model selection and analysis 70
LASSO model selection and analysis....71

Appendix 2. Supplemental content for Chapter 3, "Combined Effects of HIV and Obesity on the Gastrointestinal Microbiome of Young Men who have Sex with Men" 79
Inverse probability of treatment weight (IPTW) calculation 79
Testing for differential abundance of bacterial genera....80

Appendix 3. Supplemental content for Chapter 4, "Alterations to the Gastrointestinal Microbiome Associated with Methamphetamine Use" 91
Inverse probability of treatment weight (IPTW) calculation 91

References 99

List of Tables

Table 2.1. Participant characteristics, split by HIV viremia group, N = 383 men who have sex with men in Los Angeles, CA 19

Table 3.1. Participant characteristics, split by HIV and obesity, N = 381 men who have sex with men in Los Angeles, CA........ 38

Table 4.1. Participant characteristics, split by MA use, N = 381 men who have sex with men in Los Angeles, CA 58

Table A1.1. Covariates included in the inverse probability of treatment weight models. 73

Table A1.2. Permutational Multivariate ANOVA (PERMANOVA) of HIV category (HIV-, HIV+ undetectable, HIV+ suppressed, HIV+ viremic) on Bray-Curtis, Jaccard, and Jensen-Shannon distance matrices. 74

Table A1.3. Comparisons in alpha diversity using inverse probability of treatment weighted quantile regression (median). 75

Table A2.1. Distribution of participants characteristics included in inverse probability of treatment weight models, N = 381 men who have sex with men in Los Angeles, CA 83

Table A2.2. Permutational Multivariate ANOVA (PERMANOVA) of the combined effect of HIV and obesity on Bray-Curtis, Jaccard, and Jensen-Shannon dissimilarity statistics. 85

Table A2.3. Mean differences in alpha diversity statistics between HIV+/Obese (H+O+) and HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), HIV+/Non-obese (H+O-) participants. 86

Table A3.1. *p* and *q* values for interaction between HIV and MA use 92

List of Figures

Figure 2.1. Rectal microbial composition of study participants, N = 383. 21

Figure 2.2. Associations between HIV viremia and overall microbial composition. 22

Figure 2.3. Comparisons of individual bacterial genera between HIV viremia categories. 23

Figure 2.4. Summary of zero-inflated negative binomial (ZINB) and least absolute shrinkage and selection operator (LASSO) model results. 24

Figure 3.1. Rectal microbial composition, ordination of Bray-Curtis distances, and Chao1 diversity of study participants, N = 381. 40

Figure 3.2. Ratios of Firmicutes to Bacteroidetes and Prevotella to Bacteroides. 41

Figure 3.3. Effects of obesity on individual bacterial genera, stratified by HIV status. 42

Figure 3.4. Combined effects of HIV and obesity on individual bacterial genera. 43

Figure 4.1. Rectal microbial composition of study participants, N = 381, stratified by MA use. 60

Figure 4.2. Associations between methamphetamine (MA) use and overall microbial composition and diversity. 61

Figure 4.3. Comparisons of individual genera between methamphetamine (MA) users and non-users. 62

Figure 4.4. Dose-response analyses of bacterial counts on increasing frequency of methamphetamine (MA) use. 63

Figure A1.1. Rectal microbial composition of study participants, split by HIV viremia group. 76

Figure A1.2. Zero-inflated negative binomial effect sizes comparing HIV-infected undetectable (RNA <20 copies/mL), suppressed (RNA ≥20-200 copies/mL), and viremic (RNA > 200 copies/mL) to HIV-uninfected participants. 77

Figure A1.3. Forest plot of results of zero-inflated negative binomial models comparing genus-level bacterial counts between HIV+ suppressed (RNA ≥20-200 copies/mL) and HIV+ undetectable (RNA < 20 copies/mL, lower limit of detection) participants. 78

Figure A2.1. Rectal microbial composition of study participants, N = 383. 87

Figure A2.2. Rectal microbial composition of study participants, split by HIV and obesity status. 88

Figure A2.3. Ordination of Bray-Curtis, Jaccard, and Jensen-Shannon distances using principal coordinates analysis (PCoA). 89

Figure A2.4. Boxplots of alpha diversity statistics. 90

Figure A3.1. Rectal microbial composition of study participants, stratified by methamphetamine (MA) use and HIV status. 94

Figure A3.2. Associations between methamphetamine (MA) use and overall microbial composition and diversity, stratified by HIV status. 95

Figure A3.3. Replication of Figure 4.1, with participants' MA status determined by urine drug screen. 96

Figure A3.4. Replication of Figure 4.2, with participants' MA status determined by urine drug screen. 97

Figure A3.5. Replication of Figure 4.3, with participants' MA status determined by urine drug screen. 98

Acknowledgements

I would like to thank my advisor, Dr. Pamina Gorbach, who has been tremendously supportive during my doctoral studies and was instrumental in helping me complete this work. Dr. Gorbach truly has a gift for putting together the right people and opportunities to generate innovative, high quality research, and I sincerely thank her for her commitment to advancing my scientific career and scholarly growth. I also wish to thank Drs. Grace Aldrovandi, Jen Fulcher, Nicole Tobin, Fan Li, and the other members of Dr. Aldrovandi's lab. This dissertation would not have been possible without their scientific support and technical expertise. Working with this team has been an enlightening, enjoyable, and rewarding experience, and a model for the type of 'team science' I want to carry out in my career. I also wish to thank the other members of my committee, Drs. Marjan Javanbakht and Ron Brookmeyer, as well as Dr. Steve Shoptaw for their invaluable insights and contributions to this dissertation. I appreciate their feedback and guidance on this research and my scientific development in general. Finally, I sincerely thank all of the mSTUDY participants and staff, without whom this study would not be possible.

I also wish to thank some family and friends who have been instrumental to my education and progress as a scientist. To Drs. Deborah Jones and Steve Weiss, who introduced me to the world of research, thank you for taking a chance on the guy at the front desk of the gym. Thank you to Grandpa Joe for his persistent emphasis on the importance of education and financial support. Also, to my Mom and Dad for teaching me the value of hard work and giving me the time and space to discover where to apply it. To my wife Kiri for motivating, supporting, encouraging, and inspiring me to be the best that I can be. I honestly have no idea what I would be doing if we weren't in this journey together, but I can confidently say there wouldn't be a dissertation involved. And finally, to my daughter Mary for being the most important reason to complete this achievement, and her Nana Lili for making it possible.

Chapter two is the final, accepted version of the published manuscript: Cook RR, Fulcher JA, Tobin NH, Li F, Lee D, Javanbakht M, Brookmeyer R, Shoptaw S, Bolan R, Aldrovandi GM, Gorbach PM. Effects of HIV viremia on the gastrointestinal microbiome of young men who have sex with men. AIDS (London, England). 2019; Publish Ahead of Print. doi: 10.1097/qad.0000000000002132. RC, JF, NHT, FL, MJ, R. Brookmeyer, GMA, and PMG conceptualized and designed the study. SS, R. Bolan, PMG, and GMA were instrumental in data acquisition. Statistical and bioinformatic analyses were completed or supervised by RC, FL, DL, and R. Brookmeyer. RC drafted the manuscript, and all authors were involved in interpretation of the results and critical revision of the paper.

"Combined Effects of HIV and Obesity on the Gastrointestinal Microbiome of Young Men who have Sex with Men" (Chapter three) was submitted for publication. Co-authors on this manuscript include Jennifer Fulcher, Nicole Tobin, Fan Li, David Lee, Cora Woodward, Marjan Javanbakht, Ron Brookmeyer, Steve Shoptaw, Robert Bolan, Grace Aldrovandi, and Pamina Gorbach. RC, JF, NHT, FL, MJ, R. Brookmeyer, GMA, and PMG conceptualized and designed the study. CW, SS, R. Bolan, PMG, and GMA were instrumental in data acquisition. Statistical and bioinformatic analyses were completed or supervised by RC, FL, DL, and R. Brookmeyer. RC drafted the manuscript, and all authors were involved in interpretation of the results and critical revision of the paper.

"Alterations to the Gastrointestinal Microbiome Associated with Methamphetamine Use" (Chapter four) was submitted for publication. Co-authors on this manuscript include Jennifer Fulcher, Nicole Tobin, Fan Li, David Lee, Cora Woodward, Marjan Javanbakht, Ron Brookmeyer, Steve Shoptaw, Robert Bolan, Grace Aldrovandi, and Pamina Gorbach. RC, JF, NHT, FL, MJ, R. Brookmeyer, GMA, and PMG conceptualized and designed the study. CW, SS, R. Bolan, PMG, and GMA were instrumental in data acquisition. Statistical and bioinformatic analyses were completed or supervised by RC, FL, DL, and R. Brookmeyer. RC drafted the

manuscript, and all authors were involved in interpretation of the results and critical revision of the paper.

This dissertation was supported by the National Institute on Drug Abuse (1R36 DA046310 and 2U01 DA036267), the UCLA Center for HIV Identification, Prevention, and Treatment Services (CHIPTS; National Institute of Mental Health P30 MH58107), and the UCLA Center for Graduate Studies. Additional support provided by the UCLA AIDS Institute and UCLA CFAR Microbiome and Mucosal Immunology Core (P30 AI028697). J.A.F. was supported in part by National Institute of Allergy and Infectious Diseases (NIAID) (K08 AI124979). Overall support for the International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT) was provided by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) under Award Numbers UM1AI068632 (IMPAACT LOC), UM1AI068616 (IMPAACT SDMC) and UM1AI106716 (IMPAACT LC), with co-funding from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) and the National Institute of Mental Health (NIMH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Vita

Education

2014	MS	Public Health	University of Miami, Coral Gables
2007	BA	History	University of Michigan, Ann Arbor

Experience

2012-2013	Research Assistant Department of Psychiatry and Behavioral Sciences University of Miami, Miami, FL
2013-2017	Research Associate Department of Psychiatry and Behavioral Sciences University of Miami, Miami, FL
2015-2019	Graduate Student Researcher Department of Epidemiology/Department of Family Medicine University of California, Los Angeles, CA

Grants and Fellowships

2016	Fellowship to attend the Network Modeling for Epidemics Workshop, University of Washington at Seattle
2016	Student Research Fellowship, UCLA AIDS Institute
2016	Graduate Research Mentorship Fellowship, UCLA Graduate School
2017	UCLA Center for HIV Identification, Prevention and Treatment Services (CHIPTS) Mentored Pilot Grant
2018	Young Investigator Fellowship to attend the 2019 Conference on Retroviruses and Opportunistic Infections (CROI)
2018	Dissertation Year Fellowship, UCLA Graduate School
2018	R36 Dissertation Award, National Institute on Drug Abuse (1R36DA046310)

Honors and Awards

2013	Inductee, Delta Omega Public Health Honor Society
2014	Award of Academic Merit, University of Miami Graduate School
2017	Celia & Joseph Blann Fellowship for Academic Excellence at the Doctoral Level, UCLA Fielding School of Public Health

Selected Publications

Jones, D. L., **R. Cook,** A. Rodriguez, and D. Waldrop-Valverde (2013). Personal HIV knowledge, appointment adherence and HIV outcomes. AIDS Behav 17(1), 242–249.

Cook, R., D. Waldrop-Valverde, A. Sharma, S. Vamos, B. Mahajan, S. M. Weiss, M. Kumar, R. Nehra, and D. L. Jones (2014). Cognitive functioning, depression, and HIV medication adherence in India: a randomized pilot trial. *Health Psychol Behav Med* **2**(1), 640–652.

Cook, R., R. Hayden, S. M. Weiss, and D. L. Jones (2014). Desire for fertility among HIV-seroconcordant and -discordant couples in Lusaka, Zambia. *Cult Health Sex* **16**(7), 741–751.

Weiss, S. M., R. Zulu, D. L. Jones, C. A. Redding, **R. Cook**, and N. Chitalu (2015). The Spear and Shield Intervention to Increase the Availability and Acceptability of Voluntary Medical Male Circumcision in Zambia: A Cluster Randomized Controlled Trial. *Lancet HIV* **2**(5), e181–e189.

Cook, R., D. L. Jones, C. A. Redding, R. Zulu, N. Chitalu, and S. M. Weiss (2016). Female Partner Acceptance as a Predictor of Men's Readiness to Undergo Voluntary Medical Male Circumcision in Zambia: The Spear and Shield Project. *AIDS Behav* **20**(11), 2503–2513.

Cook, R., D. L. Jones, R. Nehra, A. M. Kumar, S. Prabhakar, D. Waldrop-Valverde, S. Sharma, and M. Kumar (2016). HIV Clade-C Infection and Cognitive Impairment, Fatigue, Depression, and Quality of Life in Early-Stage Infection in Northern Indians. *J Int Assoc Provid AIDS Care* **15**(4), 332–317.

Sandler, K., N. Shaverdian, **R. Cook**, A. Kishan, C. King, I. Yang, M. Steinberg, and P. Lee (2017). Treatment trends for patients with brain metastases: Does practice reflect the data? *Cancer* **123** (12), 2274–2282.

Gorbach, P., **R. Cook**, B. Gratzer, T. Collins, A. Parrish, J. Moore, P. Kerndt, R. Crosby, L. Markowitz, and E. Meites (2017). Human papillomavirus vaccination among young men who have sex with men and transgender women in 2 U.S. cities, 2012-1014. *Sex Transm Dis* **44** (7), 436–441.

Cook, R., B. Quinn, K. Heinzerling, and S. Shoptaw (2017). Dropout in clinical trials of pharamacologic treatment for methamphetamine dependence: the role of initial abstinence. *Addiction* **112** (6), 1077–1085.

Alcaide, M., S. Ramlagan, V. Rodriguez, **R. Cook**, K. Peltzer, S. Weiss, S. Sifunda, and D. Jones (2017). Self-report and dry blood spot measurement of antiretroviral medications as markers of adherence in pregnant women in rural South Africa. *AIDS Behav* **21** (7), 2135–2140.

Kishan, A., **R. Cook**, J. Ciezki, et al. (2018). Radical Prostatectomy, External Beam Radiotherapy, or External Beam Radiotherapy With Brachytherapy Boost and Disease Progression and Mortality in Patients With Gleason Score 9-10 Prostate Cancer. *JAMA* **319** (9), 896–905.

Hermanstyne, K., H. Green, **R. Cook**, H. Tieu, C. Dyer, L. Wilton, C. Latkin, and S. Shoptaw (2018). Social network support and decreased risk of seroconversion in Black MSM: Results of the BROTHERS (HPTN 061) Study. *J Acquir Immune Defic Syndr* **78** (2), 163–168.

Fulcher, J., S. Hussain, **R. Cook**, F. Li, N. Tobin, A. Ragsdale, S. Shoptaw, P. Gorbach, and G. Aldrovandi (2018). Effects of substance use and sexual practices on the intestinal microbiome during HIV-1 infection. *J Infect Dis* **218** (10), 1560–1570.

Cook, R., J. Fulcher, N. Tobin, F. Li, D. Lee, M. Javanbakht, R. Brookmeyer, S. Shoptaw, R. Bolan, G. Aldrovandi, and P. Gorbach (2019). Effects of HIV Viremia on the Gastrointestinal Microbiome of Young MSM. *AIDS* **33** (5), 793-804.

Chapter 1. Introduction

HIV-associated chronic inflammation and comorbidities

Despite significant progress in the treatment of HIV with antiretroviral therapy (ART), HIV-infected individuals are expected to have a shorter lifespan than their HIV-negative peers.[1-3] This reduction in life expectancy has largely been attributed to chronic inflammation associated with HIV infection,[4-6] which places infected individuals at increased risk of cardiovascular disease, metabolic disorders, neurocognitive impairment, and cancer.[7-11] Furthermore, illicit substance use disorders are highly prevalent among HIV-infected individuals,[12] and obesity is a growing problem among people living with HIV.[13,14] Like HIV, use of illicit substances and obesity have been associated with chronic inflammation.[15-21] Studies of HIV and methamphetamine use[19] and HIV and obesity[22] have shown synergistic effects of these comorbidities and HIV on inflammation.

The gastrointestinal (GI) tract is a primary site for viral replication during acute and chronic HIV-1 infection, resulting in significant loss of CD4+ T cells. GI mucosal immune dysregulation, which does not reconstitute fully with HIV treatment, is thought to be a significant driver of the persistent systemic inflammation seen in chronic HIV infection.[23] Depletion of regulatory immune cells and disruption of inflammatory cytokine pathways weakens the GI mucosal epithelium, allowing passage of microbes and microbial products into the GI tract.[24,25] It has been hypothesized that this process of microbial translocation drives turnover of CD4+ and CD8+ T cells, prematurely aging and ultimately exhausting the immune system.[25,26] Like HIV, methamphetamine use and obesity both compromise GI mucosal immunity, resulting in microbial translocation and systemic inflammation.[16-18,27,28]

Interactions between the microbiome and the immune system

The trillions of microorganisms inhabiting the GI tract, collectively called the microbiome, play a vital role in our health and the development of disease.[29] Loss of homeostasis in the

microbiome, a state of “dysbiosis,” has been associated with a broad range of illnesses, ranging from localized GI conditions to neurocognitive disorders.[29] The microbiome plays a key role in inducing and regulating nearly all aspects the immune system.[30] Interactions with microbiota beginning at birth are thought to train our immune system to respond to outside challenges and prevent autoimmune responses,[31,32] and microbiota are critical to development of lymphoid cells and structures.[33] Microbiota can induce immune regulatory capabilities; for example, *Bacteroides fragilis* have been shown to induce IL-10 producing regulatory T (T_{reg}) cells[34] and butyrate-producers such as *Clostridia* induce colonic T_{reg} cells.[35] The microbiome also plays a role in protection against pathogens by preventing colonization, producing antimicrobial peptides that directly impact pathogen survival,[36] and promoting and calibrating the innate[37] and adaptive[38] immune systems. Although the mechanisms are not always completely understood, it is clear that the microbiome plays a critical role in both localized inflammatory responses and maintenance and regulation of the entire immune system.[30]

Effects of HIV on the microbiome

There is increasing evidence that HIV disrupts the microbiome,[39-43] which may play a critical role in HIV-associated chronic inflammation and ultimately contribute to the development of serious sequelae.[7-10,44] HIV-associated dysbiosis has been correlated with biomarkers of inflammation, microbial translocation, and immune cell activation.[39,42,45] Furthermore, increased abundance of pro-inflammatory bacteria has been correlated with lower CD4+ T cell count and increased viral load, whereas the opposite has been found for increases in bacteria thought to be beneficial.[46-48] It has been hypothesized that HIV-associated immune dysregulation and dysbiosis constitute a feedback loop where immune dysregulation induces dysbiosis, and dysbiosis drives further inflammation and dysregulation.

Generally, studies have found that microbial diversity is reduced in HIV-infected individuals as compared to HIV-negative controls.[48-50] Bacteria from the phylum Proteobacteria

appear in high relative abundance among HIV-infected individuals.[46,47] Notably, the Proteobacerial family Enterobacteriaceae are elevated in HIV-infection[46,49] and have been associated with inflammation.[51,52] High abundance of certain pro-inflammatory species in the phylum Bacteroidetes, most notably *Prevotella*, is consistently found in HIV-infection,[47,49,53] while others with anti-inflammatory properties such as *Bacteroides* are decreased.[47,53] However, the majority of studies of HIV and the microbiome to date have been conducted in small samples with incomparable study cohorts and poor control for confounding factors. Additionally, no studies have examined the dose-response relationship between levels of HIV viremia and dysbiosis. Therefore, the effects of HIV on the microbiome are not well understood, and many conflicting findings appear in the literature. Chapter 2 describes the effects of increasing levels of plasma HIV RNA on the microbiome, while taking into account a host of behavioral and clinical factors that may independently impact microbial composition.

Effects of obesity and methamphetamine use on the microbiome

Dysbiosis has also been implicated in disrupted eating behavior and food intake control, [54,55] eating disorders,[56] and development of obesity and metabolic disorders.[57,58] Studies have shown that GI bacteria enable the body to extract energy from food, increase nutrient absorption, and modify taste receptors.[56] A number of commonalities between HIV-related and obesity-related dysbiosis have been observed. Reduced overall bacterial diversity[59] and high relative abundance of bacteria in the family Erysipelotrichia has been positively associated with both HIV and obesity.[60-62] In addition, HIV has been associated with decreased abundance of the family Rikenellaceae,[63,64] which has been found to be protective against obesity.[65] Despite high rates of comorbidity with HIV and a similar negative impact on the microbiome and immune system, the contribution of obesity to HIV-associated dysbiosis has not been described. Chapter 3 investigates the combined effects of HIV and obesity on the microbiome.

Despite the fact that HIV and methamphetamine (MA) are both pro-inflammatory and people living with HIV use MA at rates far higher than the general population, no studies have described the interaction between HIV and MA on the microbiome. In fact, few studies have examined the impact of MA on the microbiome at all. A study of rats found that administration of methamphetamine causes changes in the microbiome,[66] and a small cohort of HIV-infected individuals found alterations to the microbiome associated with MA use.[67] Dysbiosis has been observed in HIV-negative individuals with substance use disorders,[68] although the study included relatively few MA-dependent subjects. The microbiome may even contribute to MA use disorders. Novel research into the "gut-brain axis" suggests that dysbiosis may play a role in addiction pathology; for example, studies of cocaine reward in mice with experimentally manipulated GI bacteria suggest that dysbiosis may alter dopaminergic reward pathways and induce addictive behavior.[69] Chapter 4 describes the effects of MA on the microbiome and potential interactions between HIV and MA.

Chapter 2. Effects of HIV Viremia on the Gastrointestinal Microbiome of Young Men who have Sex with Men

2.1 Abstract

Objective: We employed a high-dimensional covariate adjustment method in microbiome analysis to better control for behavioral and clinical confounders, and in doing so examine the effects of HIV on the rectal microbiome.

Design: Three hundred eighty-three men who have sex with men were grouped into four HIV viremia categories: HIV negative (n = 200), HIV+ undetectable (HIV RNA <20 copies/mL; n = 66), HIV+ suppressed (RNA 20-200 copies/mL; n = 72) and HIV+ viremic (RNA >200 copies/mL; n = 45).

Methods: We performed 16S rRNA gene sequencing on rectal swab samples and used inverse probability of treatment-weighted marginal structural models to examine differences in microbial composition by HIV viremia category.

Results: HIV viremia explained a significant amount of variability in microbial composition in both unadjusted and covariate-adjusted analyses (R^2 = .011, p = .02). Alterations in bacterial taxa were more apparent with increasing viremia. Relative to the HIV negative group, HIV+ undetectable participants showed depletions in *Brachyspira, Campylobacter,* and *Parasutterella* while suppressed participants demonstrated depletions in *Barnesiella*, *Brachyspira* and *Helicobacter*. The microbial signature of viremic men was most distinct, showing enrichment in inflammatory genera *Peptoniphilus*, *Porphyromonas*, and *Prevotella* and depletion of *Bacteroides*, *Brachyspira,* and *Faecalibacterium*, among others.

Conclusions: Our study shows that, after accounting for the influence of multiple confounding factors, HIV is associated with dysbiosis in the gastrointestinal microbiome in a dose-dependent

manner. This analytic approach may allow for better identification of true microbial associations by limiting the effects of confounding, and thus improve comparability across future studies.

2.2 Introduction

The trillions of bacteria, viruses, and fungi inhabiting the human gastrointestinal (GI) tract have a profound impact on our health and the development of disease. Disruption in the homeostasis of the these microbes, a state of “dysbiosis,” has been associated with a broad range of illnesses, including localized GI conditions, neurocognitive disorders, cancer, autoimmune disorders, and cardiovascular disease.[29] There is tremendous variability in the diversity and composition of the microbiome, even between healthy individuals,[70] and the effects of different exposures, behaviors, and personal characteristics on the composition and function of the microbiome are incompletely understood.

Chronic inflammation is a hallmark of HIV infection and continues despite suppressive antiretroviral therapy (ART). The GI tract is a primary site for HIV replication resulting in significant loss of CD4+ T-cells vital to a healthy mucosal immune system. Depletion of regulatory immune cells and pathways leads to decreased epithelial barrier function allowing translocation of microbes and microbial products which contributes to the chronic inflammatory response.[24,25] HIV replication may also result in a state of dysbiosis,[39,40,42] which has been correlated with increases in markers of disease progression, microbial translocation, and immune activation.[39,42,45,46] It has been hypothesized that HIV-associated immune dysfunction induces this dysbiosis, and dysbiosis causes further dysfunction,[39]. thereby driving persistent systemic inflammation in HIV-infected individuals.[71]

Many studies have found that overall microbial diversity is reduced in HIV-infected individuals,[48-50] and HIV has been associated with a shift from commensals such as *Bacteroides* to pro-inflammatory taxa such as *Prevotella.*[47,49,53] However, results have been inconsistent,

with some studies showing the opposite or no effects of HIV on microbial diversity[15,63,72] and little effect on composition.[73,74] Certainly, differences in sample collection, sequencing, post-processing, and analytic methodology may be responsible for much of the heterogeneity in results.[75] While some research has focused on the role of sampling variability and type-I error on irreproducibility of findings,[76,77] comparatively little attention has been paid to sources of systematic error such as incomparable study cohorts or confounders such as sexual behavior, substance use, diet, race/ethnicity, and age. Such confounders are highly prevalent in observational human studies of the microbiome and may have a larger effect than HIV itself.[73] Due to these limitations, the specific effects of HIV on the microbiome warrant further investigation.

Increased relative abundance of pro-inflammatory bacteria has been correlated with increased viremia, whereas the opposite has been found for potentially beneficial bacteria.[46-48] Given these findings and the effects of viral replication on mucosal immunity, it stands to reason that the level of viremia may be a significant determinant of HIV-associated dysbiosis. However, the effect of viremia has not been thoroughly explored. Numerous studies comparing cohorts of HIV-infected individuals that are either on ART or ART-naïve have shown that ART does not result in full “reconstitution” of the microbiome, even if the virus is suppressed.[48,62,78] Additional studies focused on elite controllers showed that the microbial composition among individuals with controlled viremia is more similar to HIV-uninfected than viremic individuals.[79,80] However, there are likely to be important biological differences between elite controllers and other HIV-infected individuals that may limit the generalizability of these findings. In order to accurately characterize the effects of HIV on the microbiome, a more detailed examination of the effects of viremia is needed.

To this end, we compared intestinal microbial composition between HIV-uninfected, HIV-infected with undetectable viremia (HIV RNA <20 copies/ml), HIV-infected with suppressed

viremia (HIV RNA ≥20-200 copies/ml), and HIV-infected viremic individuals (HIV RNA >200 copies/ml). We utilized data from a cohort comprised entirely of men who have sex with men (MSM) and employed inverse probability of treatment weighting (IPTW) to control for a robust set of clinical and behavioral confounders. We hypothesized that alterations to the microbiome would be present in all HIV-infected subgroups as compared to HIV-uninfected controls, and the severity of dysbiosis would increase with increasing viremia.

2.3 Methods

Study Population

Specimens for this study were obtained from an ongoing prospective cohort (The mSTUDY, NIDA U01 DA036267). The mSTUDY was approved by a UCLA Institutional Review Board (IRB) and all subjects provided written informed consent at study entry. Participants are recruited from community clinics in Los Angeles and complete biannual assessments including a comprehensive physical examination and medical history, urine toxicology panel, clinical laboratory tests including plasma HIV RNA, specimen collection, and detailed behavioral questionnaire. Data presented in this manuscript were collected from baseline study visits completed between August 2014 and July 2017. Additional details on sample selection and HIV RNA quantification are provided in Appendix 1.

Specimen collection and DNA preparation

The majority (76%) of rectal swabs (FLOQSwabs, Copan Diagnostics, Murrieta, CA) were collected via anoscopy under direct mucosal visualization and without preparatory enema at approximately 8 cm from the anal verge. Due to an mSTUDY protocol change, others (24%) were participant self-collected at approximately 4-5 cm from the anal verge. Collection method was taken into account in the analysis (see Tables 2.1 and A1.1). Swabs were immediately frozen neat at -80°C until processing in bulk. For DNA processing the samples were transferred

to Lysing Matrix E tubes (MP Biomedicals, Burlingame, CA) containing RLT lysis buffer (Qiagen, Hilden, Germany) and bead-beated on a TissueLyser (Qiagen). DNA was then extracted using the AllPrep DNA/RNA/Protein kit (Qiagen) per manufacturer's protocol.

16S rRNA gene sequencing and data processing

Microbiome profiling was performed by sequencing of the V4 region of the 16S rRNA gene as previously described.[81,82] Briefly, the V4 region was amplified in triplicate reactions using Golay-barcoded primers 515F/806R. Negative controls from the DNA extraction and PCR steps, as well as independent aliquots of a bacterial mock community were processed alongside the samples to identify contaminant sequences and ensure data reproducibility. PCR products were then pooled and sequenced on the Illumina MiSeq platform using 2x150bp v2 chemistry. The sequences were demultiplexed with Golay error correction using QIIME v1.9.1,[83] and Divisive Amplicon Denoising Algorithm (DADA2) version 1.8 was used for error correction, exact sequence inference, read merging, and chimera removal.[84] Following contaminant removal (see Appendix 1), the amplicon sequence variant (ASV) table comprised 19,955,039 total merged read pairs (mean per sample = 52,375; range 10,906 to 124,889). Taxonomic assignment was performed using RDP trainset 16.[85] Rarefaction was performed at a depth of 10,906 reads for alpha diversity analyses. For all other analyses, estimates of relative library sizes ("size factors") were obtained by calculating geometric means of pairwise read count ratios.[86]

Behavioral and clinical covariates

Demographic and behavioral covariates included in the analyses were age, race/ethnicity, employment status, country of origin, a dichotomous variable for homelessness in past month, number of receptive anal intercourse (RAI) acts in past month, a dichotomous variable for RAI within the past seven days, frequency of methamphetamine, marijuana, and cocaine use in the past 6 months, tobacco smoking, and binge drinking. All demographic and behavioral data were self-reported by participants using a computer-aided self-interview (CASI);

measures are described in Appendix 1. Dichotomous variables for obesity (defined as BMI > 30 or waist circumference > 40 inches), and antibiotic use in the past month were also included in the analyses; these data were collected by clinical staff.

Statistical analyses

The primary analyses were unadjusted and inverse probability of treatment-weighted comparisons of microbiome diversity and composition between HIV-, HIV+ undetectable, HIV+ suppressed, and HIV+ viremic participants. The R package ‘phyloseq’ was used to calculate alpha diversity statistics, distance matrices, and for ordination. Differences in alpha diversity between groups were examined with Kruskal-Wallis tests followed by comparisons of median values using quantile regression (R package ‘quantreg’). Permutational Multivariate ANOVA (PERMANOVA) was used to test for overall differences in microbial composition between HIV groups (R package ‘vegan’). Zero-inflated negative binomial (ZINB) models were fit in order to test for differential abundance in bacterial genera between groups with multinomial least absolute shrinkage and selection operator (LASSO) models employed as a confirmatory analysis (R packages ‘pscl’ and ‘glmnet’). ZINB and LASSO model selection and analytic procedures are described in Appendix 1.

IPTW[87] is a method of confounder control where the study sample is re-weighted in order to create a “pseudo-population” in which treatment/exposure, here referring to the four HIV viremia groups, is independent of confounding variables (see Appendix 1). We used IPTW to control for all variables described in the Behavioral and Clinical Covariates section (Table 2.1). Weights were estimated using generalized boosted models (R package ‘twang’), and balance between groups was assessed by computing standardized mean differences for each covariate in the weighted sample (R package ‘tableone’). Table 2.1 and Table A1.1 provide information on covariate balance before and after weighting. Robust variance estimates for inference tests in weighted ZINB analyses were obtained via the sandwich estimator (R

package 'sandwich'). Additional detail about the IPTW estimation and modeling procedures is provided in Appendix 1. Missing covariate data were imputed using the Chained Equations method[88] (R package 'mice'); the proportion of missing data for each covariate is shown in Table A1.1. In order to account for multiple testing, alpha diversity and ZINB *p* values were corrected with the Benjamini-Hochberg false discovery rate (FDR) method;[89] FDR adjusted *p* values are labelled as *q* values. We utilized a threshold of two-sided *p* or *q* < 0.1 for significance testing; accordingly, we also display false coverage rate (FCR)-adjusted 90% confidence intervals[90] where relevant. All statistical analyses were completed using R v.3.4.3

2.4 Results

Sample characteristics

N = 383 participants were included in this study; 200 were HIV-, 66 were HIV+ undetectable (HIV RNA <20 copies/ml), 72 were HIV+ suppressed (HIV RNA ≥20-200 copies/ml), and 45 were HIV+ viremic (HIV RNA >200 copies/ml). All participants were MSM with an average age 31 (standard deviation = 7). Most were Hispanic (49%) or non-Hispanic Black (39%). Table 2.1 provides further detail on participant characteristics. Generally, HIV-infected participants, especially those with higher levels of viremia, were more likely to be older, unemployed, recently homeless, and to report methamphetamine use and frequent binge drinking than their HIV-uninfected peers. Among the HIV-infected participants, the mean number of years since diagnosis was 7.5 (sd = 5.7), mean $\log_{10}$ viral load was 2.0 (sd = 1.2), and mean CD4 cell count was 625 cells/mm^3 (sd = 287). No participants were ART-naïve and ninety percent of participants reported current ART.

Effects of HIV viremia on overall microbial composition

The relative composition of each individual's microbiome is displayed in Figures 2.1A and A1.1, and average composition within each HIV viremia category in Figure 2.1B. *Prevotella*

is the most highly represented bacterial genus among most participants, with increasing relative amounts of *Bacteroides, Bifidobacteria,* and *Fusobacteria* in those towards the right side of the axis (Figure 2.1A). Higher levels of *Alloprevotella* and *Porphyromonas* are apparent in the HIV+ viremic group, and lower levels of *Bacteroides* are apparent in all HIV+ groups relative to HIV- controls (Figure 2.1B).

To quantitatively examine the influence of HIV viremia on differences in microbial composition between-subjects we used PERMANOVA with Bray-Curtis distance. HIV viremia explained a significant amount of variability in microbial composition in both unadjusted (R^2 = .014, p = .001) and covariate-adjusted analyses (R^2 = .011, p = .017) (Table A1.2). Figure 2.2A displays ordination of the samples by principal coordinates analysis (Bray-Curtis distance), where HIV- and HIV+ viremic groups are distinct while HIV+ undetectable and HIV+ suppressed are more similar.

Comparisons of alpha diversity suggest a tendency for HIV+ individuals to have higher diversity in metrics that do not account for evenness (observed count and Chao1 statistic) (Figure 2.2B). Kruskal-Wallis analyses revealed significant differences in observed and Chao1 richness by HIV group (Table A1.3). Quantile regression was further used to investigate these differences and revealed higher median observed and Chao1 values for HIV+ suppressed versus HIV- individuals (q = .022 in IPTW-adjusted analyses). No other significant differences were found in any group. Shannon and Simpson indices did not vary greatly between groups.

Differences in specific bacterial taxa associated with HIV viremia

Zero-inflated binomial (ZINB) models were utilized to identify bacterial genera that were differentially abundant among the HIV viremia groups. HIV+ undetectable showed significant enrichment in *Finegoldia* and *Streptococcus* and depletion in *Bacteroides, Brachyspira, Campylobacter, Helicobacter, Parasutterella,* and *Turicibacter* when compared to HIV- uninfected. After IPTW adjustment for behavioral and clinical confounders, depletions in

Campylobacter, Parasutterella and *Brachyspira* remained significant (Figure 2.3A). HIV+ suppressed participants (HIV RNA ≤200 copies/ml) had increased *Pseudoflavonifractor* and decreased *Bacteroides, Barnsiella, Brachyspira, Campylobacter, Escherichia/Shigella, Flaonifractor, Helicobacter, Oxalobacter, Parabacteroides, Turicibacter,* and *Victivallis* relative to HIV-negative subjects. Following IPTW adjustment, depletions in *Barnesiella, Helicobacter,* and *Brachyspira* remained significant (Figure 2.3B).

HIV+ viremic men (HIV RNA >200 copies/ml) had the most distinct microbial signature relative to HIV-negative, showing significant enrichment of *Corynebacterium, Dietzia, Finegolda, Murdochiella, Negativicoccus, Peptoniphilus, Porphyromonas,* and *Prevotella* as well as depletion of *Arcanobacterium, Brachyspira, Bacteroides, Campylobacter, Faecalibacterium, Helicobacter* and *Succinivibrio*. With IPTW adjustment, enrichment of *Murdochiella, Peptoniphilus, Porphyromonas,* and *Prevotella* and depletion of *Arcanobacterium, Bacteroides*, *Brachyspira, Faecalibacterium,* and *Helicobacter* were significant (Figure 2.3C). For some bacteria, including *Faecalibacterium, Peptoniphilus, Porphyromonas*, *Prevotella*, and *Streptococcus,* effect size (i.e., degree of enrichment or depletion) increased with increasing viremia (Figure A1.2).

An IPTW-adjusted multinomial least absolute shrinkage and selection operator (LASSO) model was also used as an additional method of feature selection to compare with ZINB findings. Among HIV+ participants with undetectable or suppressed viremia, no genera were significant in both the adjusted ZINB and LASSO models. However, among the HIV+ viremic group, differences in *Bacteroides, Peptoniphilus, Porphyromonas, and Prevotella* were consistent across analytic strategies (Figure 2.4).

It was also of interest to determine whether HIV+ participants with low levels of viremia (HIV RNA < 200 copies/mL) had distinct microbial signatures from those who were HIV+ but undetectable (HIV RNA <20 copies/mL). One genus, *Sneathea,* was significantly different

between these groups in adjusted ZINB analyses ($q < .1$). The LASSO model identified depletions in *Gemmiger* in HIV+ suppressed as compared to undetectable participants (Figure A1.3).

2.5 Discussion

In this study examining the effects of HIV on the rectal microbiome in a cohort of 383 young, mostly minority MSM, we found important differences in microbial composition between HIV-uninfected and HIV-infected men which varied depending on level of viremia. HIV viremia category accounted for about 1% of the variability in microbiome composition, an effect size that is consistent with previous studies.[73] As hypothesized, microbiome perturbations were most evident among HIV+ viremic men, and least evident in HIV+ men with undetectable viremia. Importantly, we utilized IPTW to account for multiple confounding factors in our analyses, which decreased the likelihood that the results we report are attributable to clinical or behavioral covariates affecting the microbiome such as sexual behavior, substance use, or obesity.

High diversity is generally associated with a healthy rectal microbiome,[70] and reduced richness and diversity has been reported in studies comparing HIV-infected and uninfected persons.[49,50,78,91-93] Still other studies report no differences in diversity associated with HIV-infection,[15,63] while others have suggested that differences in diversity may be related to sampling location[47] or HIV-treatment status.[62,92] We found few significant differences in diversity metrics in our study, and findings did not follow a clear dose-response pattern with level of viremia. As we were able to adjust for multiple confounders in our analyses, we can be reasonably confident that previously reported determinants of diversity such as sexual behavior[73] and substance use[68] had limited influence on our findings. Our results suggest that once these confounding factors are taken into account, bacterial diversity and richness may not be substantially impacted by HIV infection itself.

One of the more consistent findings across studies of HIV and the microbiome has been enrichment in *Prevotella* and depletion in *Bacteroides* among both untreated and treated HIV-infected individuals.[46,47,49] *Prevotella* species are considered pro-inflammatory,[64,94] while *Bacteroides* species have been shown to induce regulatory T-cell differentiation and IL-10 production.[34,95] Previous work has suggested that observed alterations to the *Prevotella/Bacteroides* ratio may have been due to sexual behavior rather than HIV;[73] however, others have shown decreased *Bacteroides* among HIV-infected MSM who were matched with MSM controls.[46] Our study examined exclusively MSM and controlled for recency and frequency of receptive anal intercourse in our analyses, therefore, our study provides additional evidence that HIV may directly alter the *Prevotella/Bacteroides* ratio independent of sexual behavior. Although we found decreased *Bacteroides* in all HIV-infected individuals, the effect was similar between undetectable and suppressed participants and only statistically significant after adjustment for confounding in the viremic group. In addition, we found increasing relative amounts of *Prevotella* with increasing levels of viremia, which were only significant in the viremic group. Our findings are consistent with previous research showing that *Prevotella* may normalize with ART[48] whereas depletions in *Bacteroides* persist even with therapy.[62]

Of the 78 genera tested, ZINB and LASSO models identified *Porphyromonas* as the genus with the largest difference between HIV+ viremic and HIV- individuals. *Porphyromonas* is a well-known modifier of inflammatory cytokines.[96] In fact, *Porphyromonas gingivalis* has been identified as a potential cause of systemic inflammation and metabolic disorders associated with periodontal disease[97] and implicated in inflammatory processes leading to the development of atherosclerosis.[98] Furthermore, administration of *P. gingivalis* to mice was shown to induce GI dysbiosis and contribute to intestinal permeability.[99] The association between increasing levels of HIV viremia and *Porphyromonas* may therefore represent an important mechanism behind HIV-associated chronic inflammation deserving of further study.

Of particular interest in our study is the examination of low level viremia individuals who are not undetectable (HIV RNA ≥20-200 copies/ml). It is notable that this group, while distinct from HIV-uninfected, was virtually superimposed with the undetectable viremia (HIV RNA <20 copies/ml) group in ordination analysis. Only a single genus, *Sneatha*, was statistically significantly different between these two groups when directly compared. Clinically, persistent low level viremia may increase risk of subsequent virologic failure,[100] but a recent large study showed no difference in progression to AIDS or incidence of non-AIDS events in persons with low level viremia compared to undetectable.[101] Our analysis suggests that microbial composition is similar between those with low level and undetectable viremia, but remains distinguishable from HIV-uninfected individuals. While those with low level viremia can still have microbial translocation and inflammatory biomarkers,[102] the overall decreased dysbiosis in low level viremia may correspond to reduced chronic inflammation which lessens clinical progression.

Our results should be interpreted with consideration of the following limitations. First, we did not have diet information on our cohort. We included race/ethnicity, country of origin, employment status, obesity, and homelessness (all of which may impact dietary intake) in our covariate adjustment set to mitigate this limitation to the best of our ability. Second, the IPTW procedure will only achieve perfect balance between exposure groups in nonparametric settings with large sample sizes relative to the number of relevant confounders, and there is potential for residual confounding even by variables we controlled for in our study. However, we note that many of the most significant confounders (e.g. age, antibiotic use, sexual behavior, alcohol use, obesity) were well-balanced after weighting. The ability of 16S sequencing to provide species-level resolution is limited, thus we conducted analyses at the genus level. We acknowledge that differentially abundant genera do not necessarily indicate differences in functionally important species. Finally, we did not have time since ART initiation for our cohort, and thus cannot

determine if participants were viremic because they recently started treatment or because treatment was failing.

Our study also has numerous strengths. Primarily, we utilized data from a large cohort of regionally, socioeconomically, and behaviorally similar individuals, increasing the internal validity of our findings. We employed a novel technique, IPTW, to incorporate a large amount of clinical and behavioral data into our analyses. With IPTW, analyses are “marginal structural models” instead of conditional on covariates, as in multiple regression. Modeling microbiome data marginally offers several advantages including the ability to control for many confounding factors without inducing overfitting bias[103] or losing efficiency due to overstratification.[104] Addressing sources of systematic error using IPTW may improve reproducibility in future studies of HIV and the microbiome, We also stratified our HIV-infected participants by level of viremia, allowing us to examine differences between HIV-uninfected and HIV+ undetectable, suppressed, and viremic individuals. This stratification leads to better understanding of the relationship between active viral replication and dysbiosis, namely, that dysbiosis increases with increasing viremia. Finally, we were able to replicate our major findings using two distinct analytic strategies. Genera identified as differentially abundant in both analyses may be more likely to be true discoveries.

This study contributes to a growing body of literature describing the effects of HIV on microbial dysbiosis. We show that, even when taking into account the influence of multiple confounding factors, HIV is associated with intestinal dysbiosis in a dose-dependent manner. Although great strides have been made in the management of chronic HIV infection, the life expectancy among HIV-infected individuals remains reduced relative to their HIV-negative peers.[105] This reduction has largely been attributed to increased rates of inflammation-related comorbidities observed among people living with HIV,[106] and the microbiome likely plays a key role in modulating interactions between HIV and the immune system. Therefore, understanding

the ways in which HIV and the microbiome interact may be a crucial step towards developing intervention strategies to reduce the burden of HIV-associated morbidity and mortality.

Table 2.1. Participant characteristics, split by HIV viremia group, N = 383 men who have sex with men in Los Angeles, CA

	HIV-Negative mean (sd)/n (%)	Undetectable (HIV RNA < 20 copies/mL)	Suppressed (HIV RNA ≥20 - 200 copies/mL)	Viremic (HIV RNA > 200 copies/mL)	SMD[a] (unweighted)	SMD (weighted)
n	200	66	72	45		
Age	28.91 (6.43)	33.41 (6.55)	34.10 (6.36)	33.18 (6.86)	.41	.17
Employment					.54	.25
Student	26 (13.0)	5 (7.6)	3 (4.2)	1 (2.2)		
Unemployed	61 (30.5)	23 (34.8)	50 (69.4)	27 (60.0)		
Full/part time	113 (56.5)	38 (57.6)	19 (26.4)	17 (37.8)		
Race/Ethnicity					.48	.37
Black Non-Hispanic	82 (41.0)	17 (25.8)	25 (34.7)	26 (57.8)		
Hispanic	98 (49.0)	37 (56.1)	35 (48.6)	19 (42.2)		
Other Non-Hispanic	20 (10.0)	12 (18.2)	12 (16.7)	0		
Country of origin					.12	.08
United States	171 (85.5)	53 (80.3)	55 (76.4)	36 (80.0)		
Other	29 (14.5)	13 (19.7)	17 (23.6)	9 (20.0)		
Homeless in past 6 months	65 (32.5)	20 (30.3)	21 (29.2)	22 (48.9)	.21	.18
RAI in past 7 days	88 (44.0)	32 (48.5)	30 (41.7)	19 (42.2)	.08	.07
Number of RAI acts in past month	2.06 (4.19)	2.48 (4.68)	2.33 (5.45)	4.42 (8.03)	.19	.07
Methamphetamine use in past 6 months					.54	.20
Daily/Weekly	21 (10.5)	14 (21.2)	18 (25.0)	21 (46.7)		
Monthly/less	32 (16.0)	21 (31.8)	19 (26.4)	9 (20.0)		
Never	147 (73.5)	31 (47.0)	35 (48.6)	15 (33.3)		
Marijuana use					.30	.14
Daily/Weekly	71 (35.5)	19 (28.8)	21 (29.2)	19 (42.2)		
Monthly/less	57 (28.5)	10 (15.2)	16 (22.2)	10 (22.2)		
Never	72 (36.0)	37 (56.1)	35 (48.6)	16 (35.6)		
Cocaine use					.16	.09
At least once	53 (26.5)	11 (16.7)	21 (29.2)	13 (28.9)		
Never	147 (73.5)	55 (83.3)	51 (70.8)	32 (71.1)		
Tobacco smoking					.26	.15
>1 pack/day	10 (5.0)	3 (4.5)	5 (6.9)	2 (4.4)		
<1 pack/day	68 (4.0)	24 (36.4)	31 (43.1)	25 (55.6)		
Nonsmoker	122 (61.0)	39 (59.1)	36 (50.0)	18 (40.0)		
Binge drinking in					.40	.15

past 6 months[b]						
Weekly	24 (12.0)	15 (22.7)	8 (11.1)	8 (17.8)		
Monthly/less	111 (55.5)	26 (39.4)	20 (27.8)	16 (35.6)		
Never	65 (32.5)	25 (37.9)	44 (61.1)	21 (44.7)		
Obese[c]	64 (32.0)	16 (24.2)	13 (18.1)	10 (22.2)	.17	.15
Antibiotic use	11 (5.5)	5 (7.6)	7 (9.7)	8 (17.8)	.21	.08
Sample collection method					.21	.08
Anoscopy	152 (77.0)	47 (71.2)	53 (73.6)	39 (86.7)		
Self-collected	46 (23.0)	19 (28.8)	19 (26.4)	6 (13.3)		
Years since HIV diagnosis [d]	N/A	7 (6)	7 (5)	8 (6)	N/A	N/A
HIV RNA copies/mL (median, IQR)	N/A	N/A	20 (30)	15,730 (48,680)	N/A	N/A
CD4 cells/mm^3	N/A	708.95 (279.6)	645.21 (262.9)	470.02 (280.1)	N/A	N/A
ART regimen					N/A	N/A
INSTI + NRTI	0	30 (45.5)	30 (41.7)	8 (17.8)		
NNRTI + NRTI	0	21 (31.8)	20 (27.8)	7 (15.6)		
NRTI + PI		10 (15.2)	11 (15.3)	9 (20)		
Other	0	5 (7.6)	8 (10.2)	4 (8.8)		
Missing/Not reported/NA	166 (83)	0	3 (4.2)	17 (37.8)		
PrEP user[e]	37 (19)	N/A	N/A	N/A	N/A	N/A

SMD = Standardized mean difference; RAI = Receptive anal intercourse; ART = Antiretroviral therapy; INSTI = Integrase strand transfer inhibitor; NRTI = Nucleoside reverse transcriptase inhibitor; NNRTI = Non-nucleoside reverse transcriptase inhibitor; PI = Protease inhibitor

[a]Binge drinking defined as 6 or more drinks on one occasion.

[b]Obese defined as BMI > 30 or BMI > 25 and waist circumference > 40 inches.

[c]HIV RNA, CD4 cell count and ART regimen were not included in the inverse probability of treatment weight model (as they are generally not relevant to HIV negative participants), all other variables in the table were included.

[d]HIV negative men taking tenofovir disoproxil fumarate/emtricitabine for pre-exposure prophylaxis (PrEP).

[e]SMD is a measure of imbalance across groups; higher SMDs indicate greater imbalance. Average SMD before weighting = .29, after weighting = .15.

Figure 2.1. Rectal microbial composition of study participants, N = 383. (A) Columns represent the relative composition of each subject's microbiome at the genus level. HIV status of the subjects is indicated by a colored line below their microbial composition. Subjects are ordered by the first principal coordinate of a Bray-Curtis pairwise distance matrix. Genera representing less than 1% of the composition on average across samples were combined into "Other." (B) Average microbial composition within each HIV viremia category. Unadjusted and inverse probability of treatment weighted compositions are shown. Bacterial genera representing less than 1% of the overall relative composition or present in less than 20% of the samples were grouped into "Other."

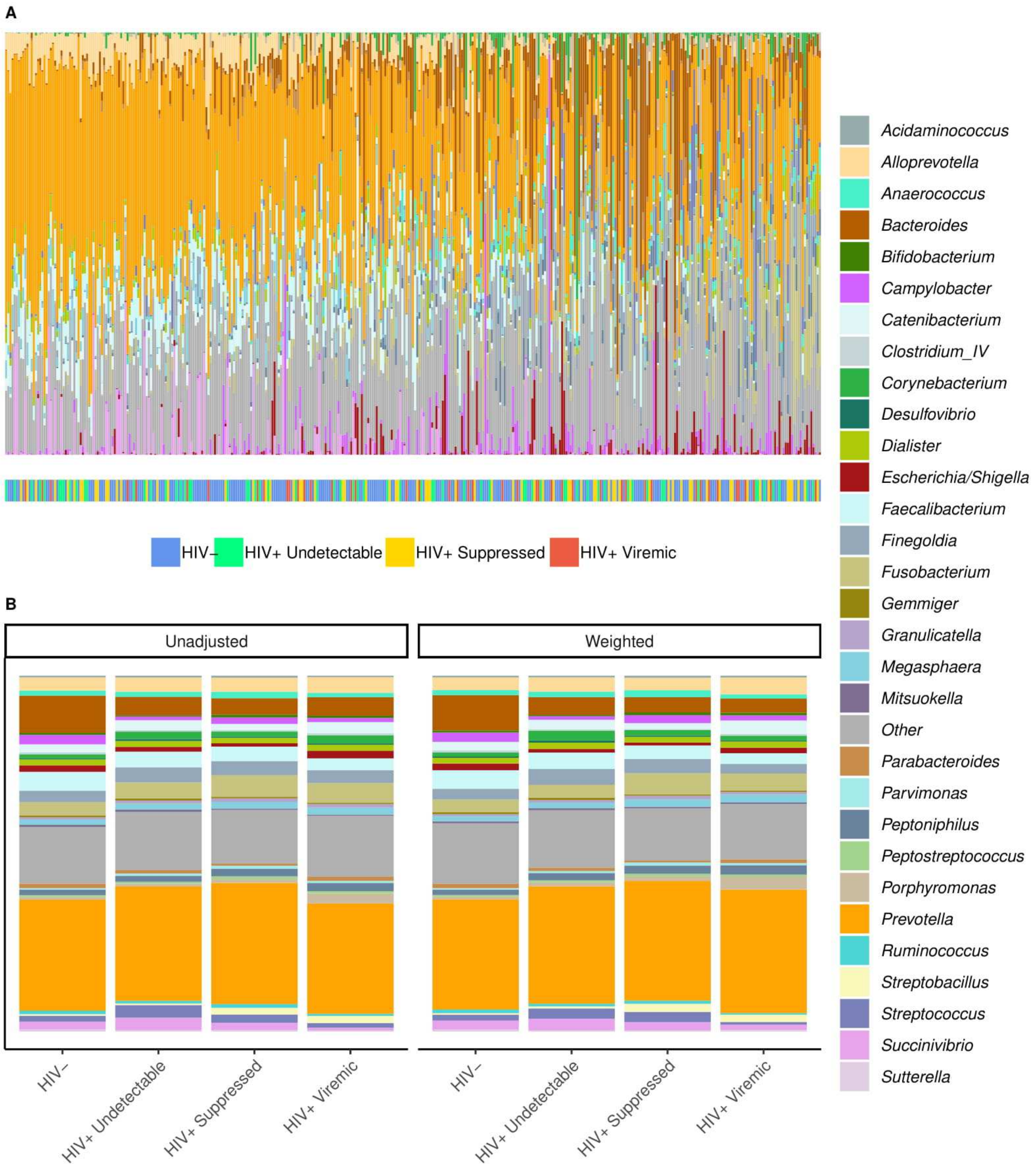


Figure 2.2. Associations between HIV viremia and overall microbial composition. (A) Ordination of the Bray-Curtis distance between samples using principal coordinates analysis. PCoA = Principal coordinate axis. Ellipses are 95% confidence regions for each group assuming points follow a multivariate *t* distribution. (B) Boxplots of richness metrics. Boxes represent the lower, median, and upper quartile of the data and whiskers are 1.5*interquartile range.

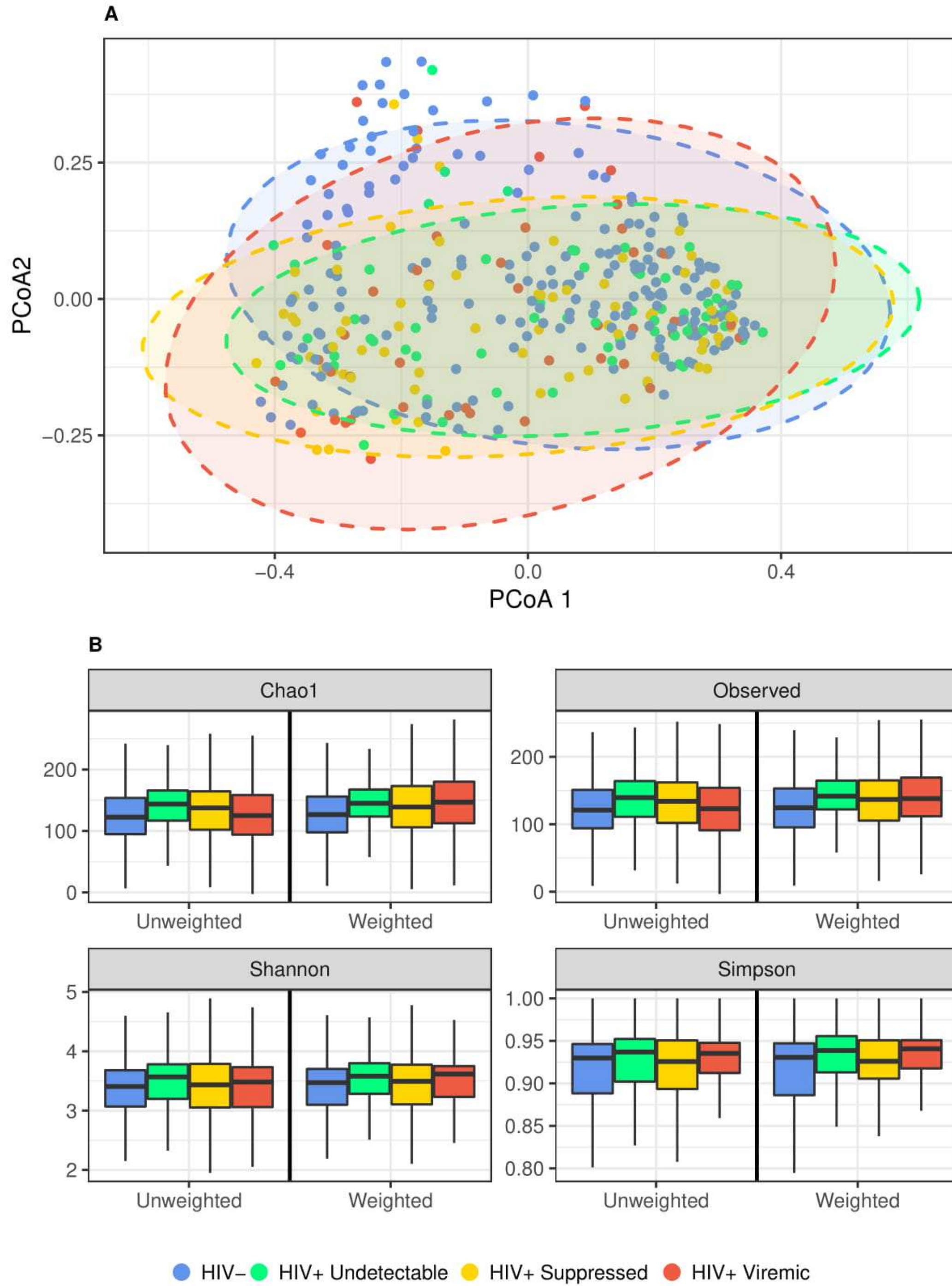


Figure 2.3. Comparisons of individual bacterial genera between HIV viremia categories. Forest plots of results of zero-inflated negative binomial models comparing genus-level bacterial counts between HIV-negative and (A) HIV+ undetectable (HIV RNA <20 copies/ml), (B) HIV+ suppressed (HIV RNA >20 and ≤200 copies/ml) and (C) HIV+ viremic (HIV RNA >200 copies/ml) participants. Inverse probability of treatment-weighted effect sizes and 90% confidence intervals (truncated at -4, 4) are plotted, with statistical significance (*q* < 0.1) indicated in color. Effect sizes are log ratios of normalized genera counts.

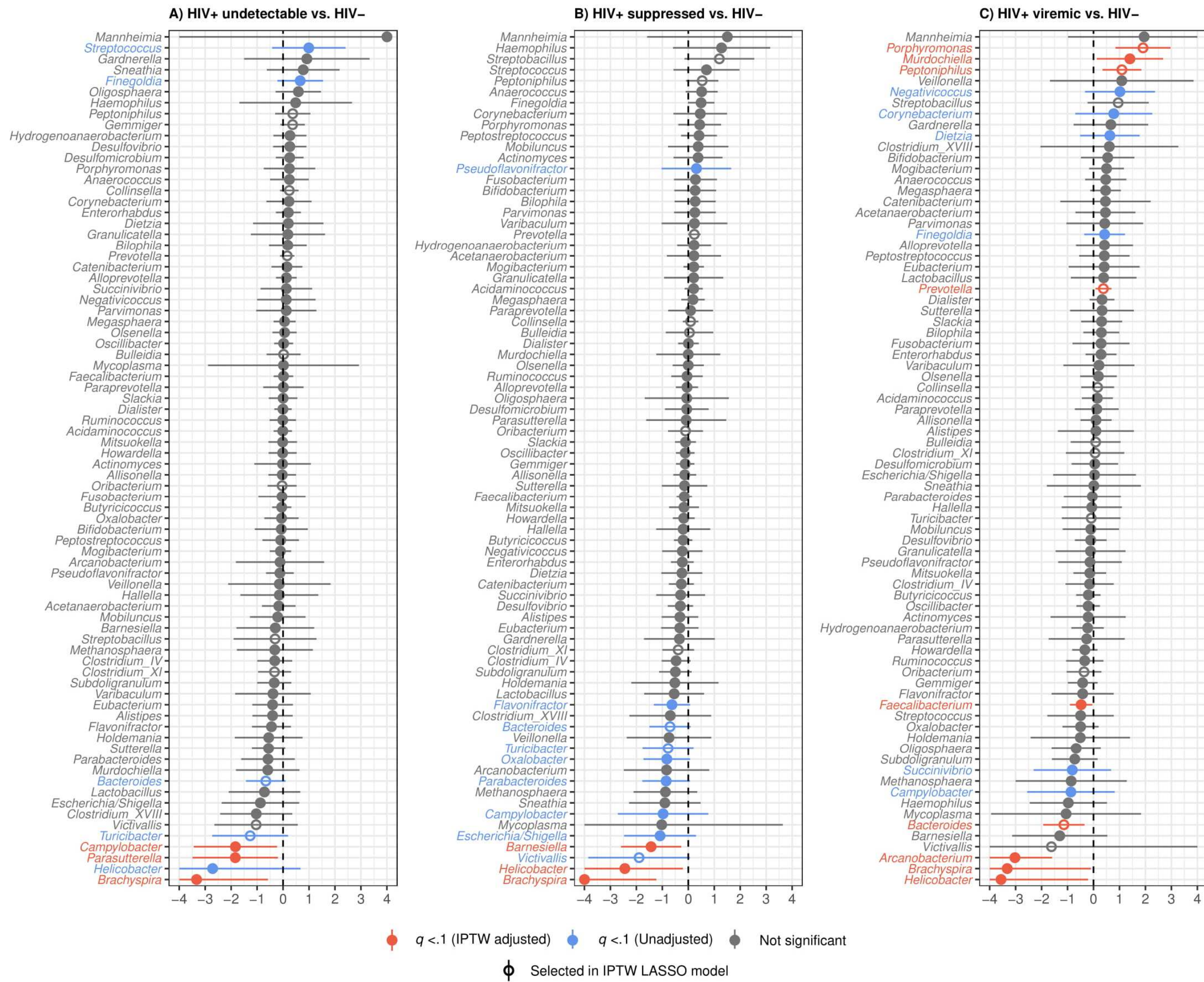
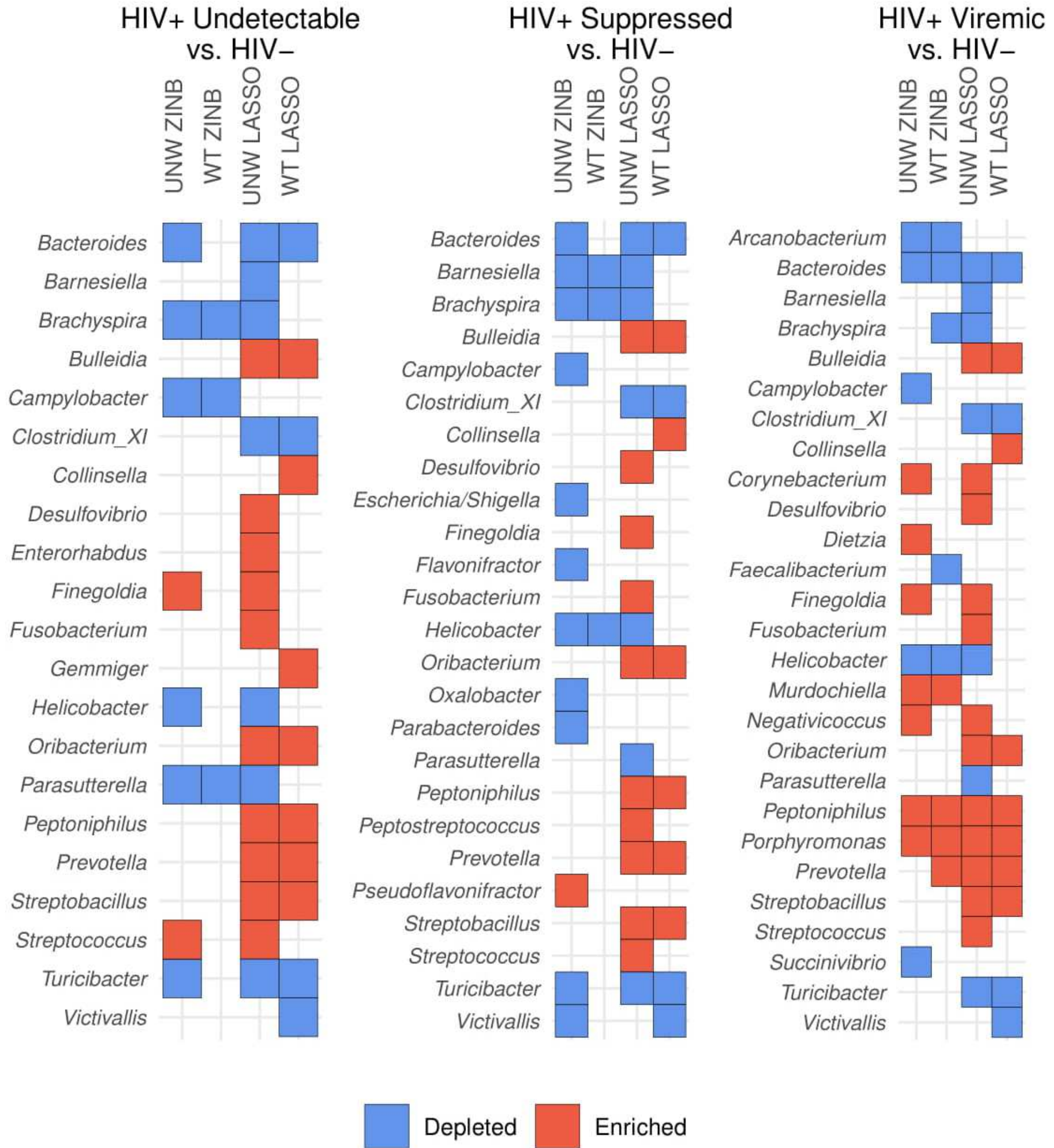


Figure 2.4. Summary of zero-inflated negative binomial (ZINB) and least absolute shrinkage and selection operator (LASSO) model results. Enriched taxa are those with positive effect sizes (relative to HIV-), depleted are those with negative effect sizes. Genera with no effect in either analysis are not shown. UNW = unadjusted, WT = IPTW adjusted.



Chapter 3. Combined Effects of HIV and Obesity on the Gastrointestinal Microbiome of Young Men who have Sex with Men

3.1 Abstract

Introduction. The prevalence of obesity is rising among people living with HIV, with racial/ethnic minority populations disproportionately affected. Both obesity and HIV infection are associated with immune dysregulation and may work synergistically to increase the risk of inflammation-associated sequelae. Disruption of gut bacterial communities may be one of the key drivers of this inflammation; however, the combined effects of HIV and obesity on the microbiome have not been explored.

Methods. Our study included rectal swab samples from 381 members of an ongoing cohort of diverse young men who have sex with men. Thirty-nine were HIV+ and obese (H+O+), 143 were HIV+ and non-obese (H+O-), 64 were HIV- and obese (H-O+), and 135 were HIV- and non-obese (H-O-). Obesity was defined as BMI > 30 or waist circumference > 40 inches. Microbiome composition was assessed by targeted sequencing of the V4 region of the 16S rRNA gene. We used inverse probability of treatment-weighted marginal structural models to investigate differences in microbial composition between HIV and obesity groups while controlling for a large number of clinical and behavioral covariates.

Results. Significant variability in microbial composition was explained by the combination of HIV and obesity (R^2 for the marginal contribution of the H+/O+ group = .008, *p* = .001). H+O+ participants had the highest ratios of *Prevotella* to *Bacteroides,* a pro-inflammatory enterotype that has been described in HIV and obesity independently. H+O+ participants had lower levels of *Bacteroides* and *Veillonella* than all other groups, suggesting a synergistic effect of HIV and obesity on these genera. Conversely, *Dietzia* and *Finegoldia* were reduced and *Faecalibacterium* was enriched in H+O+ compared to H+O- and H-O+, but not compared to H-

O- controls, suggesting that HIV and obesity may have some antagonistic effects on these genera.

Conclusions. Our findings support the hypothesis that HIV and obesity act together to disrupt gut microbial communities, which may help explain higher levels of generalized inflammation among people living with both HIV and obesity. Interactions between conditions altering the microbiome, such as HIV and obesity, should be taken into consideration when designing interventions to address dysbiosis and reduce its inflammatory consequences.

3.2 Introduction

Surveillance data from 2015-2016 suggests that 40 percent of United States adults are obese and at high risk of heart disease, stroke, diabetes, and cancer.[107]The obesity epidemic is a worsening public health crisis, with rates increasing over the last 15 years and projected to affect 50% of U.S. adults by the year 2030.[108] Obesity is of increasing concern among people living with HIV, partially because of improved life expectancy and quality of life due to antiretroviral therapy (ART) and partially as a side effect of the drugs themselves.[109] The prevalence of obesity among people living with HIV is similar to the general population and rates are rising rapidly.[13,14] HIV and obesity disproportionately affect racial and ethnic minorities in the United States; the highest age-adjusted prevalences of obesity are found among Hispanics (47.0%) and non-Hispanic blacks (46.8%),[107] and the highest rates of HIV are among non-Hispanic Blacks (43.6 per 100,000) and Hispanics (17 per 100,000).[110]

Studies suggest that obesity plays a role in HIV pathogenesis and the development of comorbid illnesses.[14] Both obesity and long-term HIV infection are associated with cytokine disruption, immune dysregulation, and chronic inflammation, and may work synergistically to increase the risk of inflammation-associated sequelae. Higher levels of generalized inflammation and monocyte activation have been observed in HIV-infected individuals who are

obese as compared to non-obese,[111] and these immune parameters have been correlated with weight gain following ART initiation.[112] The consequences of combined inflammatory effects may be severe; one study found the prevalence of multimorbidity was nearly 80% in people with HIV and obesity,[113] and inflammatory biomarkers are associated with mortality in HIV-infected individuals.[5]

Numerous studies have shown that HIV disrupts the composition and function of the gastrointestinal (GI) microbiome.[39-43] This disruption, termed “dysbiosis,” is characterized by a shift from commensals to pro-inflammatory and potentially pathogenic bacteria. Dysbiosis has also been observed in obese individuals[59,114-116] and a number of commonalities between HIV-associated and obesity-associated dysbiosis have been observed. Reduced overall bacterial diversity has been described in both HIV and obesity.[39,40,59] An elevated *Firmicutes* to *Bacteroidetes* ratio has been described in obese individuals[114,115] as well as those living with HIV.[117] Within *Bacteroidetes*, increased relative abundance of *Prevotella* and decreased *Bacteroides* is one of the most common enterotypes associated with HIV infection,[40] and similar findings have been described in obese individuals.[116] In both HIV and obesity, dysbiosis may be one of the key drivers of chronic inflammation.[16,18,39,42,45] HIV and obesity have independently been shown to disrupt barrier function of the mucosal epithelium, allowing translocation of microbes and microbial products into the GI tract.[17,25] This process is highly inflammatory and may exacerbate dysbiosis.

Despite the role of the microbiome in mediating inflammation and data showing that HIV and obesity may synergistically contribute to immune dysfunction and ultimately increase the risk of non-AIDS related morbidity and mortality,[22,111,118] the joint effect of HIV and obesity on microbial dysbiosis has not been described. Therefore, we examined the combined effects of HIV and obesity on dysbiosis in a cohort of young, mostly racial/ethnic minority men who have sex with men (MSM). We hypothesized that HIV and obesity would act synergistically to

decrease overall bacterial diversity and increase the abundance of pro-inflammatory bacterial taxa while simultaneously decreasing those with anti-inflammatory properties.

3.3 Methods

Study population. Specimens were obtained from an ongoing cohort, the Minority Men who have Sex with Men Cohort at UCLA Linking Infections Noting Effects (MASCULINE, or mSTUDY, NIDA U01 DA036267). Both the current study and the mSTUDY were approved by a UCLA Institutional Review Board and all study procedures were done in accordance with ethical standards for research involving human subjects. Sample selection procedures have been previously described (Appendix 1).[119]

Specimen collection and DNA preparation. Included specimens were rectal swabs (FLOQSwabs, Copan Diagnostics, Murrieta, CA), the majority (76%) of which were collected via anoscopy under direct mucosal visualization and without preparatory enema at approximately 8 cm from the anal verge. Due to an mSTUDY protocol change, others (24%) were participant self-collected at approximately 4-5 cm from the anal verge. Collection method was taken into account in the analysis (Tables 1 and S1). Swabs were immediately frozen neat at -80°C until processing in bulk. For DNA processing the samples were transferred to Lysing Matrix E tubes (MP Biomedicals, Burlingame, CA) containing RLT lysis buffer (Qiagen, Hilden, Germany) and bead-beated on a TissueLyser (Qiagen). DNA was then extracted using the AllPrep DNA/RNA/Protein kit (Qiagen) per manufacturer’s protocol.

16S rRNA gene sequencing and data processing. Microbiome profiling was performed by sequencing of the V4 region of the 16S rRNA gene as previously described.[81,119,120] Briefly, the V4 region was amplified in triplicate reactions using Golay-barcoded primers 515F/806R. PCR products were then pooled and sequenced on the Illumina MiSeq platform using 2x150bp v2 chemistry. The sequences were demultiplexed with Golay

error correction using QIIME v1.9.1,[83] and Divisive Amplicon Denoising Algorithm (DADA2) version 1.8 was used for error correction, exact sequence inference, read merging, and chimera removal.[84] The resultant amplicon sequence variant (ASV) table comprised 19,955,039 total merged read pairs (mean per sample = 52,375; range 10,906 to 124,889). Taxonomic assignment was performed using RDP trainset 16 (https://doi.org/10.5281/zenodo.810827). Rarefaction was performed at a depth of 10,906 reads for alpha diversity analyses. To normalize all other analyses, estimates of relative library sizes (“size factors”) were obtained by calculating geometric means of pairwise read count ratios.[86]

HIV serostatus, obesity, and covariates. HIV testing was conducted using the OraQuick Advance® HIV 1/2 (OraSure Technologies, Bethlehem, PA) and plasma HIV RNA was quantified using a standard clinical laboratory assay (Cobas® AmpliPrep/Cobas® TaqMan® HIV-1 Test, Version 2.0). Anthropometrics including height, weight, and waist circumference were gathered by trained clinical staff, and participants were classified as obese if they had BMI > 30 or waist circumference > 40 inches. Measurement of waist circumference is recommended by the National Heart, Lung, and Blood Institute as part of an obesity-related risk assessment;[121] for men, a waist circumference > 40 inches indicates high risk for the development of obesity-related health conditions.

Demographic and behavioral covariates included in the analyses were age, race/ethnicity, country of origin, a dichotomous variable for homelessness in past six months, number of receptive anal intercourse (RAI) acts in past month, number of sex partners in the past 6 months, positive PCR test for STI (rectal gonorrhea, rectal chlamydia, or syphilis), frequency of methamphetamine and marijuana use in the past 6 months, tobacco smoking, and binge drinking. All demographic and behavioral data were self-reported by participants using a computer-aided self-interview; measures have been previously described (Appendix 1).[119]

Antibiotic use in the past month as well as drugs currently used for ART were also controlled in the analyses. These data were collected by trained clinic staff.

Statistical analyses. To explore the combined effects of HIV and obesity on the microbiome, most analyses in this study compare the “index” group of HIV-infected obese (H+O+) participants with three reference groups of HIV-infected non-obese (H+O-), HIV-uninfected obese (H-O+), and HIV-uninfected non-obese (H-O-) participants. We also compare obese to non-obese participants within strata of HIV status. As we examined the effects of HIV on the microbiome in detail in a previous study,[119] we do not make this comparison here. Analyses utilize inverse probability of treatment weighting (IPTW) to control for confounding. In an IPTW analysis, the study sample is re-weighted to balance treatment/exposure groups with respect to covariates used to calculate the weights, creating a “pseudo-population” where these covariates no longer act as confounders. See Tables 1 and S1 for a list of covariates included in the IPTW models. IPTW were estimated using generalized boosted models (R package ‘twang’) and robust standard errors for IPTW-adjusted analyses were obtained using the sandwich estimator (R package ‘sandwich’). See Appendix 2 for a description of the IPTW calculation process.

Prior to analysis, differences in clinical and behavioral covariates between the four HIV and obesity groups were described using standardized mean differences and tested for significance using Chi-square, Kruskal-Wallis, or multinomial logistic regression models. The R package ‘phyloseq’ (version 1.24.2) was used to calculate alpha diversity statistics, distance matrices, and to create ordination plots. Permutational multivariate ANOVA (PERMANOVA) was used to test for overall differences in microbial composition between groups (R package ‘vegan’). IPTW-adjusted linear regression analyses were utilized to test for mean differences in alpha diversity, *Firmicutes/Bacteroidetes* and *Prevotella/Bacteroides* ratios between HIV and

obesity groups. These analyses utilized a threshold of $p < .05$ to determine statistical significance and Wald-type 95% confidence intervals are displayed where appropriate.

Zero-inflated negative binomial models (ZINB) with IPTW adjustment were used to test for differential abundance of specific genera; see Appendix 2 for an overview of the ZINB model selection and analytic procedures. A pre-filtering step excluded genera appearing in less than 10% of samples as well as those with less than 100 total reads across all samples, resulting in 78 genera included in ZINB analyses. In order to account for the large amount of tests, *p* values obtained from ZINB models were corrected using Benjamini & Hochberg's False Discovery Rate (FDR) method.[89] FDR-adjusted *p* values are labelled as *q* values, and $q < .1$ was used as a threshold to determine statistical significance. Accordingly, we display 90% false coverage rate (FCR)-adjusted confidence intervals[90] to accompany these analyses. All statistical analyses were performed using R v.3.5.1.

3.4 Results

Demographics and clinical characteristics. Three hundred eighty-one participants were included; 39 were HIV+ and obese (H+O+), 143 were HIV+ and non-obese (H+O-), 64 were HIV- and obese (H-O+), and 135 were HIV- and non-obese (H-O-). All participants were MSM, their average age was 31, and most were Hispanic (49%) or non-Hispanic Black (39%). Among the obese participants, the mean BMI was 34.8 and waist circumference was 43.7 inches. Obese participants had less frequent RAI, fewer sex partners, and were less likely to test positive for a rectal STI than their non-obese peers. Among the HIV+ participants, the mean (log 10) plasma RNA level was 2.0 and CD4 cell count was 626 cells/mm^3. As compared to HIV- participants, HIV+ men were older and more reported using methamphetamine and binge drinking in the past 6 months (Tables 3.1 and A2.1).

Effects of HIV and obesity on overall microbial composition. Figure 3.1A displays the average microbial composition within each group defined by HIV and obesity status after adjustment with IPTW. The H+O+ group shows the lowest relative amounts of *Bacteroides, Campylobacter,* and *Escherichia/Shigella* and the highest relative amounts of *Bifidobacterium* and *Prevotella*. In contrast, the H-O- group shows higher levels of *Bacteroides*, *Escherichia/Shigella,* and *Parabacteroides* and lower levels of *Succinivibrio* than all other groups. Individual-level microbial compositions are shown in Figures A2.1 and A2.2.

We calculated Bray-Curtis, Jaccard, and Jensen-Shannon dissimilarity statistics to quantitatively examine differences in overall composition between the HIV and obesity groups. Figure 3.1B displays ordination of the Bray-Curtis distance by principal coordinates analysis, which shows clustering of H+O+ subjects. PERMANOVA models suggest that HIV and obesity combined explain a significant amount of between-subject variation in the microbiome, over and above each factor alone (Using Bray-Curtis distance, R^2 for the marginal contribution of H+/O+ = .008, p = .001; additional results in table A2.2 and figure A2.3).

We also calculated and compared measures of alpha diversity between groups. Figure 3.1C displays boxplots of Chao1 index values, split by HIV and obesity status. HIV+ individuals generally showed higher diversity than HIV-, with little difference by obesity. Results of a linear regression analysis provide support for these observations. Mean Chao1 diversity among the H+O+ group was higher than the H-O+ group (mean difference = 18.5, p = .036) and the H-O- group (mean difference = 15.9, p = .069), while the difference between H+O+ and H+O- participants was minor (mean difference = 4.9, p = .6). Results were consistent for metrics that account for evenness (e.g. Shannon index); additional results are presented in table A2.3 and figure A2.4.

Differences in Firmicutes/Bacteroidetes and Prevotella/Bacteroides ratios. Figure 3.2A displays boxplots of the (natural log) *Firmicutes* to *Bacteroidetes* ratios within each HIV

and obesity group. No significant differences were seen on a regression analysis between the H+O+ group and any other group (Figure 3.2B). Boxplots of *Prevotella* to *Bacteroides* ratios are shown in Figure 3.2C, which show H+O+ participants with the highest values of this ratio. H-O+ and H+O- groups appear to have similar values, and the *Prevotella* to *Bacteroides* ratio is lowest among H-O- participants. A regression analysis confirms that the *Prevotella* to *Bacteroides* ratio is significantly higher among H+O+ participants compared to H-O- (mean difference in log ratio = 1.84, $p < .001$); however, the H+O+ group was not different than the H-O+ or H+O- groups (Figure 3.2D).

Differences in specific genera associated with obesity, stratified by HIV status. In the absence of HIV, there were few significant differences in relative abundance due to obesity (Figure 3.3). Obese participants showed enrichment in *Allisonella, Finegoldia,* and *Succinivibrio* and depletion in *Arcanobacterium* and *Mannheimia* relative to non-obese participants. However, within the HIV+ stratum, the microbial signature of obese participants was more distinct, showing enrichment in *Bifidobacterium, Butyricicoccus, Clostridium* cluster XI and *Faecalibacterium* and depletion in *Bacteroides, Escherichia/Shigella, Finegoldia* and *Gardnerella,* among others, relative to those without obesity (Figure 3.3).

Effects of HIV and obesity together on microbial abundance. Finally, we compared the abundance of each taxa between the H+O+ group and H+O-, H-O+, and H-O- groups. We first conducted a joint test of the three comparisons, which indicated that there was at least one significant difference ($q < .1$) between H+O+ and the others in 22 genera including *Bacteroides, Bifidobacterium, Brachyspira, Escherichia/Shigella, Faecalibacterium, Porphyromonas,* and *Prevotella,* among others. We then examined individual comparisons for those genera, and as can be seen in Figure 3.4, different interactions are evident. H+O+ participants had lower levels of *Bacteroides* and *Veillonella* than all other groups, suggesting a synergistic effect of HIV and obesity on these genera. *Dietzia* and *Finegoldia* were reduced and *Faecalibacterium* was

enriched in H+O+ compared to H+O- and H-O+, but not compared to H-O- controls. This suggests that HIV and obesity may have antagonistic effects on these genera, e.g., perhaps both conditions alter the relative abundance of other taxa enough to “normalize” levels of *Dietzia*, *Finegoldia* and *Faecalibacterium*. Finally, *Barnesiella, Peptoniphilus,* and *Succinivibro* were altered by obesity, but only in the HIV+ stratum, and *Porphyromonas* was altered by HIV only in the obese stratum.

3.5 Discussion

In this study, we explored the combined effects of HIV and obesity on the gastrointestinal microbiome of young, mostly racial/ethnic minority MSM. Analyses of overall microbial composition revealed significant differences between H+O+ participants and those without HIV and/or obesity; findings were supported by PERMANOVA models showing significant variability in microbial composition explained by the combination of HIV and obesity. HIV and obesity did not jointly alter the Firmicutes/Bacteroidetes ratio, but H+O+ subjects did have the highest *Prevotella/Bacteroides* ratios. We found that obesity altered the abundance of several genera only in the presence of HIV (i.e., only in the HIV+ stratum). Finally, we also noted that HIV and obesity acted synergistically to decrease *Bacteroides* and *Veillonella*. In general, these findings support the hypothesis that microbial composition is altered by the combination of HIV and obesity over and above the contributions of each condition alone.

Contrary to our hypothesis, neither HIV nor obesity was associated with reduced alpha diversity; in fact, HIV-infected participants appeared to have greater diversity than HIV-uninfected. With regards to obesity, our results are consistent with the findings of a meta-analysis by Sze and Schloss.[59] Although a significant difference was noted in only two of ten studies in the meta-analysis, Sze and Schloss showed that obesity did in fact reduce richness and evenness with small effect sizes that were unlikely to be detected in any individual cohort.

Although many studies have previously reported a decrease in richness and diversity due to HIV,[49,50,78,91-93] others have shown that ART may normalize this difference[62,92] and 90% of HIV-infected individuals in our study were taking ART and had high CD4 counts. Generally, a healthy rectal microbiome should be highly diverse,[70] and our findings may reflect a return to health following successful treatment.

We also hypothesized that HIV and obesity would increase the ratio of *Firmicutes* to *Bacteroidetes*, which was not supported by our data. Support for this hypothesis largely comes from animal models,[122] experimental studies showing that the ratio of these phyla decreased as obese individuals lost weight,[114] and observations in other HIV-infected cohorts.[117] However, several re-analyses and meta-analyses have failed to establish that this ratio is affected by obesity in humans,[59,123,124] and our findings suggest that the addition of HIV does not change these results. Furthermore, we were able to control for a number of clinical and behavioral confounders such as sexual behavior, which may have exaggerated or spuriously generated previously described differences. On the other hand, we found a significantly higher *Prevotella* to *Bacteroides* ratio among H+O+ individuals compared to H-O- controls. As *Bacteroides* species have immune-regulatory properties[95] and *Prevotella* are considered pro-inflammatory,[64,94] this finding is consistent with the theory that HIV and obesity may act synergistically to increase inflammation. Dominance of either *Prevotella* or *Bacteroides* constitutes a microbial enterotype that may be useful in predicting susceptibility to HIV infection[125] as well as response to diabetes medications[126] and weight-loss diets,[127] making this ratio highly important to individuals living with HIV and obesity.

We found that *Bifidobacterium* was increased in H+O+ individuals relative to both non-obese groups, a surprising finding, as *Bifidobacterium* is thought to be protective against obesity based on observed associations with weight loss, better glycemic control, reduced adiposity and ability to counteract leptin resistance.[128] Interestingly, there may be some interaction between

the shift from *Bacteroides* to *Prevotella* associated with HIV and obesity and the protective effects of *Bifidobacterium*: One study showed that increasing *Bifidobacterium* resulted in improved metabolic parameters in *Bacteroides* but not *Prevotella*-rich subjects[126] and another found a negative association between obesity and *Bifidobacterium,* but only among study subjects of the *Bacteroides*-dominant microbial enterotype.[129] Therefore, it is possible that the joint effects of HIV and obesity on the *Prevotella*/*Bacteroides* ratio inhibit the potential metabolic benefits of increased *Bifidobacterium*. We also found that *Faecalibacterium* was increased in H+O+ relative to H+O- and H-O+, but unchanged relative to H-O- controls, a pattern suggesting antagonism between HIV and obesity on the relative abundance of *Faecalibacterium.* A randomized study of 6 weeks of prebiotic therapy among HIV-infected individuals found a compositional shift in favor of *Faecalibacterium,* which correlated strongly with butyrate production and reduction in inflammatory biomarkers.[130] However, if HIV and obesity are truly antagonistic with respect to increasing *Faecalibacterium,* HIV-infected individuals who are also obese may fail to benefit from such therapy. Validating these suppositions would require an experimental study, but these interactions are examples of insights that may be gained by studying the simultaneous effects of multiple diseases on the microbiome.

Our study is subject to a number of limitations. Primarily, diet information was not available for this cohort. Diet has been shown to have little impact on the microbiome relative to HIV and other confounding factors (e.g. sexual behavior);[73] however, diet is undoubtedly a major determinant of obesity status. We adjusted our analyses for race/ethnicity, country of origin, and homelessness in order to mitigate this limitation; however, diet remains an important omitted confounder. Although most of our behavioral data were self-reports of sensitive topics (e.g. substance use, sexual behavior), we utilized a computer-aided self-interview to reduce social desirability bias. Additionally, IPTW adjustment does not achieve perfect covariate balance between exposure groups in most real-world research applications, and residual

confounding is possible. However, our IPTW achieved excellent balance on many of the most important covariates (e.g. antibiotic use, alcohol drinking, sexual behavior), increasing the likelihood that our findings are truly attributable to HIV and obesity. The ability to integrate a large amount of clinical and behavioral data into our analyses using IPTW is a significant strength of this study. Finally, our study was conducted exclusively in young MSM. Restricting to this group increases internal validity by preventing the influence of some confounders (e.g. gender differences, sexual preferences) but may limit the generalizability of our findings to women or other HIV risk groups. Despite this, our large cohort included adequate numbers of HIV-infected and obese participants to examine the joint effects of both exposures. Although it is widely accepted that many factors simultaneously impact microbial composition, there have been few studies examining the combined effects of multiple exposures or behaviors on the microbiome.

This study of a diverse group of young MSM identified numerous alterations to the gastrointestinal microbiome among H+O+ individuals relative to those with only one or neither conditions. We found that the alterations could be synergistic, antagonistic or have no effect; it is important to take bacteria-specific interactions into account when evaluating interventions to address dysbiosis and ameliorate its inflammatory consequences. HIV and obesity disproportionally affect racial and ethnic minorities[107,110,131] and their joint inflammatory effects may partially explain why HIV-infected minorities experience higher rates of non-AIDS related chronic diseases than Whites.[132] Therefore, interventions to reduce microbial dysbiosis in this vulnerable population could have added benefit.

Table 3.1. Participant characteristics, split by HIV and obesity, N = 381 men who have sex with men in Los Angeles, CA

	HIV-/ Non-obese mean (sd)/ n (%)	HIV-/Obese	HIV+/ Non-obese	HIV+/ Obese	*p*[¶]
n	135	64	143	39	
Age	28.1 (6.3)	30.5 (6.6)	33.1 (6.7)	35.6 (5.4)	<.001
Race/Ethnicity					.263
Black Non-Hispanic	53 (39.3)	29 (45.3)	54 (37.8)	14 (35.9)	
Hispanic	63 (46.7)	33 (51.6)	70 (49.0)	20 (51.3)	
Other Non-Hispanic	19 (14.1)	2 (3.1)	19 (13.3)	5 (12.8)	
Country of origin					.150
United States	113 (83.7)	58 (90.6)	115 (80.4)	29 (74.4)	
Other	22 (16.3)	6 (9.4)	28 (19.6)	10 (25.6)	
Homeless in past 6 months	50 (37.0)	16 (25.0)	52 (36.4)	11 (28.2)	
Number of RAI acts in past month	2.3 (4.7)	1.3 (2.6)	3.2 (6.5)	2.0 (3.1)	.175
Number of anal sex partners in past 6 months	7.3 (8.1)	6.2 (7.2)	8.0 (9.2)	5.9 (8.2)	.182
Positive for STI by PCR test [†]	18 (13.3)	3 (4.7)	23 (16.1)	3 (7.7)	.101
Methamphetamine use in past 6 months					<.001
Daily/Weekly	16 (11.9)	6 (9.4)	46 (32.2)	7 (17.9)	
Monthly/less	35 (11.1)	17 (26.6)	40 (28.0)	9 (23.1)	
Never	104 (77.0)	41 (64.1)	57 (39.9)	23 (59.0)	
Marijuana use					.289
Daily/Weekly	49 (36.3)	21 (32.8)	48 (33.6)	11 (28.2)	
Monthly/less	38 (28.1)	19 (29.7)	29 (20.3)	7 (17.9)	
Never	48 (35.6)	24 (37.5)	66 (46.2)	21 (53.8)	
Tobacco smoker	53 (39.3)	25 (39.1)	74 (51.7)	16 (41.0)	.140
Binge drinking in past 6 months [‡]					.002
Weekly	18 (13.3)	6 (9.4)	26 (18.2)	5 (12.8)	
Monthly/less	70 (51.9)	41 (64.1)	50 (35.0)	13 (33.3)	
Never	48 (34.8)	17 (26.6)	67 (46.9)	21 (53.8)	
Antibiotic use	9 (6.7)	2 (3.1)	15 (10.5)	5 (12.8)	.191
Sample collection strategy					.463
Anoscopy	107 (79.3)	46 (71.9)	111 (77.6)	27 (69.2)	
Self-collected	28 (20.7)	18 (28.1)	32 (22.4)	12 (30.8)	
HIV RNA $\log_{10}$ copies/mL (median, IQR) [§]			1.6 (1.9)	1.5 (1.5)	N/A
CD4 cells/mm^3 (median, IQR) [§]			603 (341)	632 (419)	N/A
CD4 cells/mm^3 < 200			11 (0.08)	3 (0.08)	N/A
ART regimen					N/A
NRTI + INSTI			51 (35.7)	17 (43.6)	

NRTI + NNRTI			43 (30.1)	5 (12.8)	
NRTI + PI			22 (15.4)	8 (20.5)	
Other			11 (7.7)	5 (12.8)	
Missing/Not reported/NA			16 (11.2)	4 (10.3)	
Tenofovir disoproxil fumarate /emtricitabine for pre-exposure prophylaxis (PrEP)	24 (17.8)	12 (18.8)			N/A
BMI §	24.3 (2.9)	34.8 (5.7)	24.4 (3.0)	35.0 (7.3)	N/A
Waist circumference (inches) §	33.2 (3.1)	43.6 (6.0)	34.5 (3.1)	43.8 (5.9)	N/A

RAI = Receptive anal intercourse; STI = Sexually transmitted infection; ART = Antiretroviral therapy; INSTI = Integrase strand transfer inhibitor; NRTI = Nucleoside reverse transcriptase inhibitor; NNRTI = Non-nucleoside reverse transcriptase inhibitor; PI = Protease inhibitor

†Sexually transmitted infections include rectal gonorrhea and chlamydia as well as syphilis.

‡Binge drinking defined as 6 or more drinks on one occasion.

§HIV RNA, CD4 cell count, waist circumference, and BMI were not included in the inverse probability of treatment weight model, all other variables in the table were included.

¶*p* values are from Kruskal-Wallis tests, Chi-square tests, or multinomial logistic regression models depending on variable distributions. If the latter, the p value represents a likelihood ratio test of all model coefficients vs. an intercepts-only model.

Figure 3.1. Rectal microbial composition, ordination of Bray-Curtis distances, and Chao1 diversity of study participants, N = 381. (A) Average microbial composition within each HIV and obesity category, adjusted for behavioral and clinical confounders using inverse probability of treatment weighting. Groups are HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), HIV+/Non-obese (H+O-), and HIV+/Obese (H+O+). Bacterial genera representing less than 1% of the overall relative composition or present in less than 20% of the samples were grouped into "Other." (B) Ordination of Bray-Curtis distances between samples using principal coordinates analysis. PCoA = Principal coordinate axis. Ellipses are 95% confidence regions for each group assuming points follow a multivariate *t* distribution. (C) Boxplots of Chao1 index vales. Boxes represent the lower, median, and upper quartile of the data and whiskers are 1.5*interquartile range.

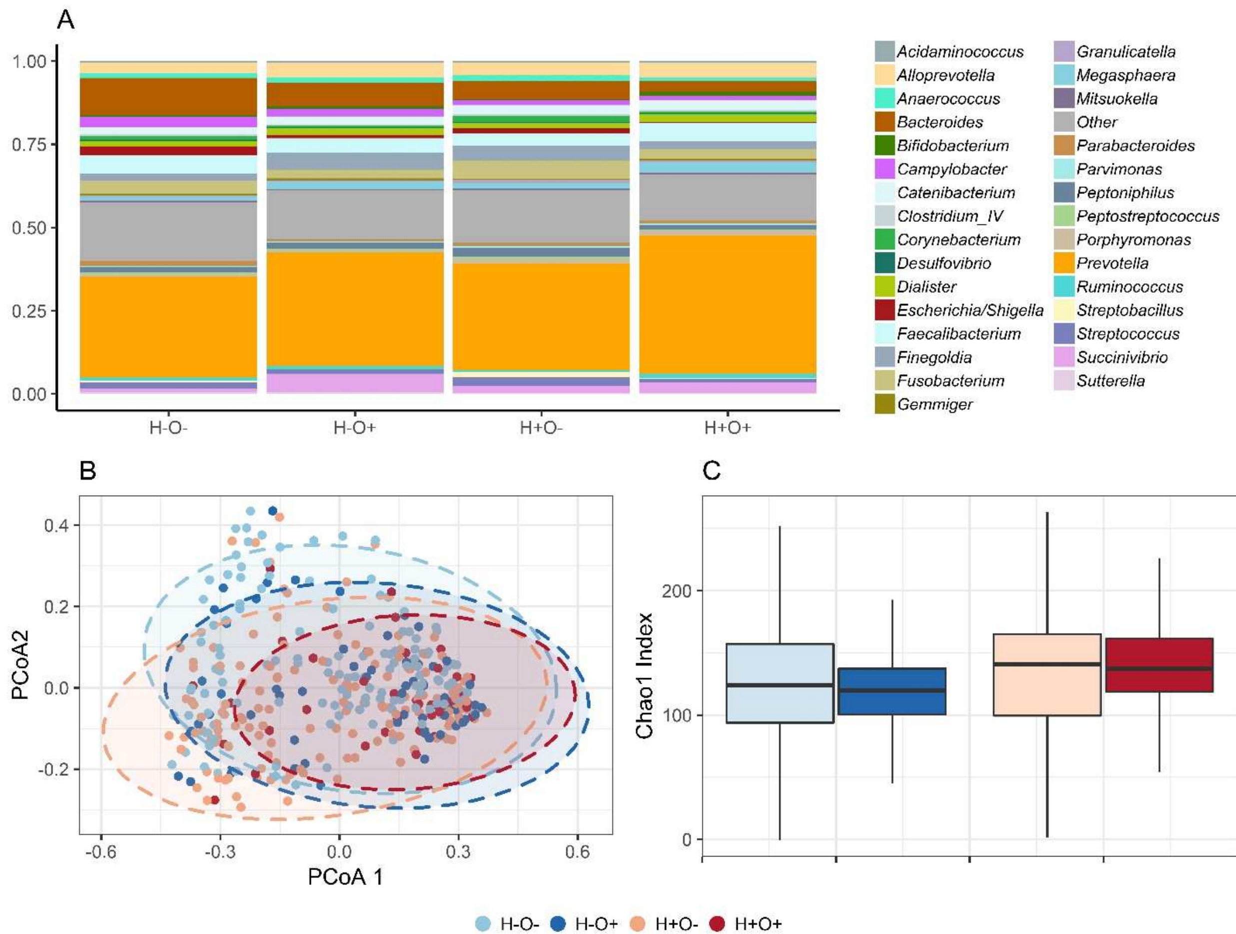


Figure 3.2. Ratios of Firmicutes to Bacteroidetes and Prevotella to Bacteroides. (A) Boxplots of log *Firmicutes* to *Bacteroidetes* ratios. Boxes represent the lower, median, and upper quartile of the data and whiskers are 1.5*interquartile range. (B) Mean differences in ratios and Wald 95% confidence intervals, adjusted for behavioral and clinical confounders using inverse probability of treatment weighting (IPTW). The HIV+/Obese (H+O+) group is compared to the HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), and HIV+/Non-obese groups (H+O-). (C) Boxplots of log *Prevotella* to *Bacteroides* ratios. (D) IPTW-adjusted mean differences in ratios and 95% confidence intervals.

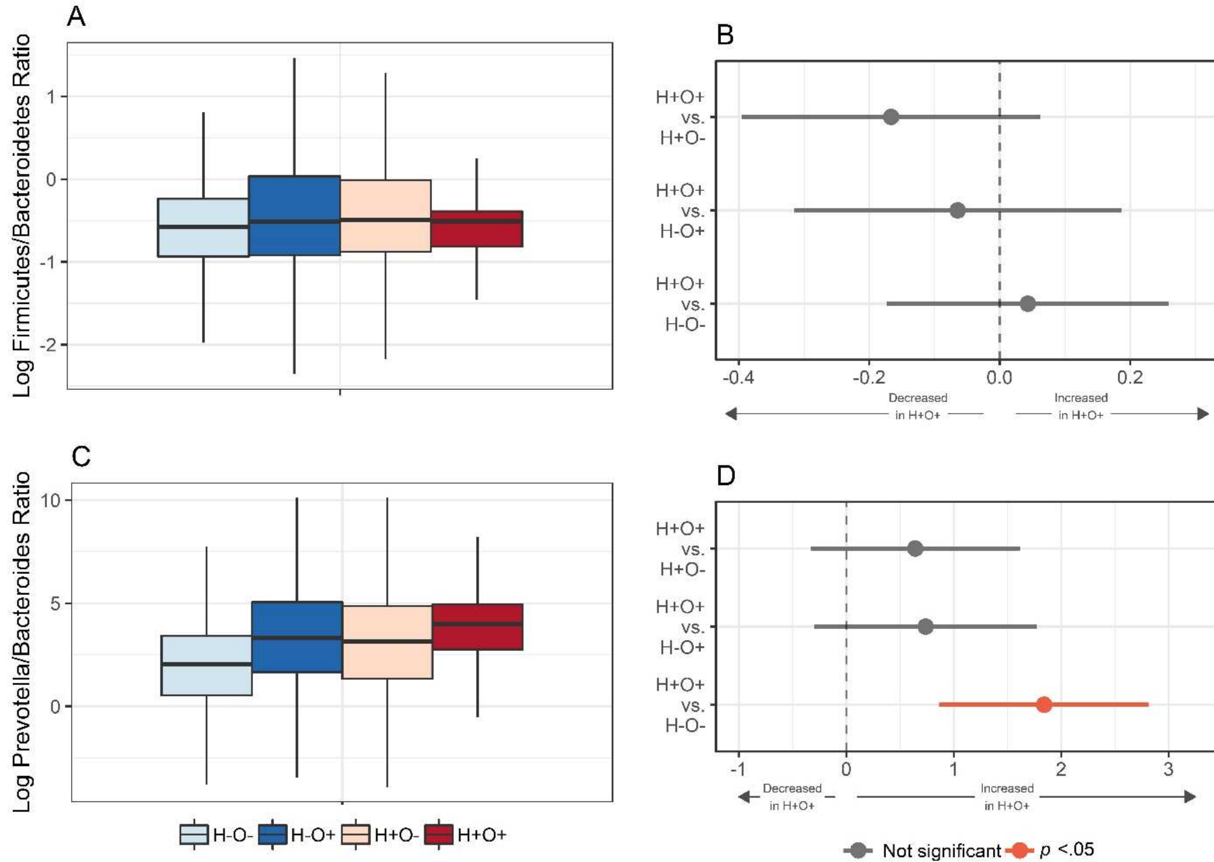


Figure 3.3. Effects of obesity on individual bacterial genera, stratified by HIV status. Forest plots of results of zero-inflated negative binomial models comparing genus-level bacterial counts between obese and non-obese participants, stratified by HIV status. Inverse probability of treatment-weighted effect sizes and false coverage rate (FCR)-adjusted 90% confidence intervals (truncated at -6, 6) are plotted, with statistical significance ($q < 0.1$) indicated in red. Effect sizes are log ratios of normalized genera counts.

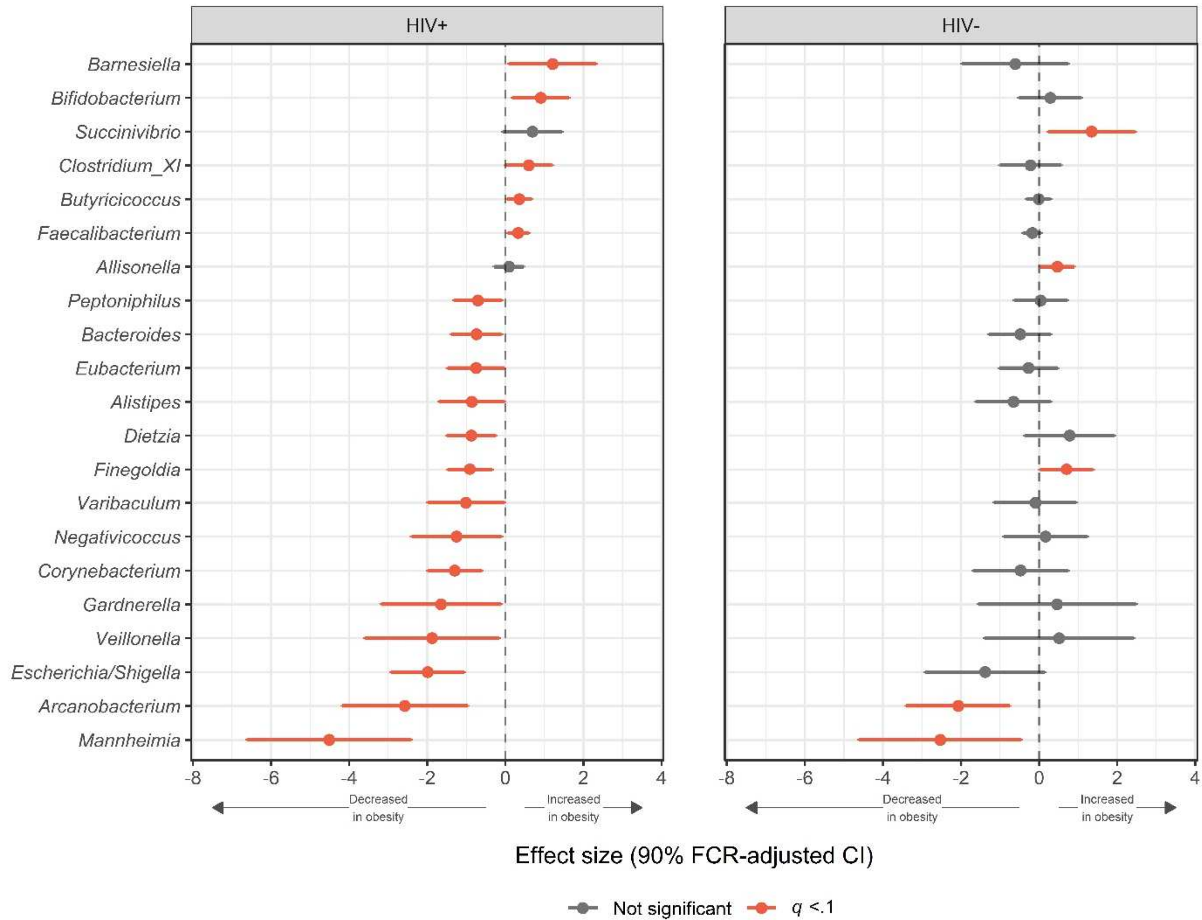
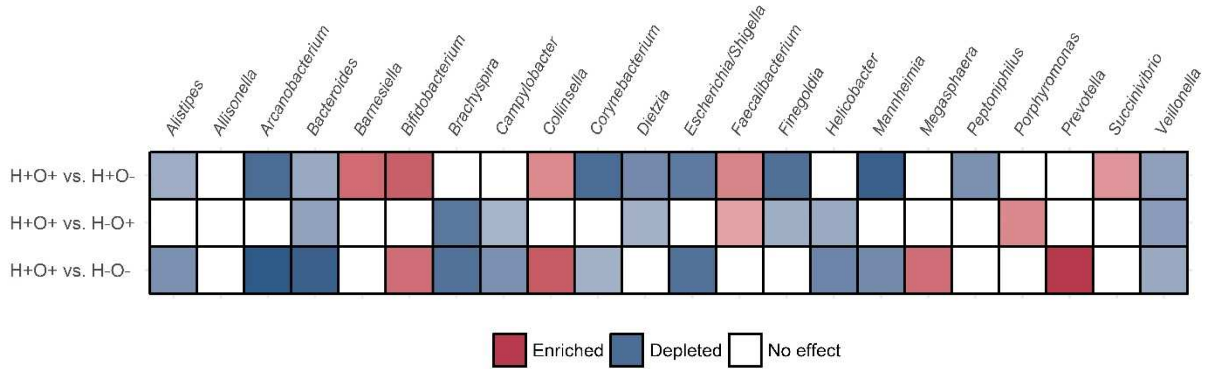


Figure 3.4. Combined effects of HIV and obesity on individual bacterial genera. Heatmap of results of zero-inflated negative binomial models comparing genus-level bacterial counts between HIV+/Obese (H+O+) and HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), and HIV+/Non-obese (H+O-) participants. Statistically significant results ($q < .1$) are colored with intensity proportional to effect size; "no effect" indicates $q > .1$.



Chapter 4. Alterations to the Gastrointestinal Microbiome Associated with Methamphetamine Use

4.1 Abstract

Methamphetamine (MA) use is a major public health problem in the United States, especially among people living with HIV. Many MA-induced neurotoxic effects are mediated by inflammation, and gut microbiota may play a role in this process. However, few studies have examined the effects of MA on the microbiome, and none have considered potential interactions with HIV. We performed 16S gene sequencing on rectal swab samples from 381 men who have sex with men. We assessed MA use with the NIDA-modified ASSIST and compared microbial diversity and composition between MA users and non-users while testing for interactions with HIV. All analyses utilized inverse probability of treatment weighting to control for numerous behavioral and clinical confounders, including demographics, sexual behavior and use of other drugs. Forty-one percent of individuals in our study used MA and 48% were HIV-infected. MA use explained significant variation in overall microbial composition (PERMANOVA, Bray-Curtis distance, R^2=.005, p=.008). Regardless of HIV status, MA users had higher levels of *Finegoldia, Parvimonas, Peptoniphilus,* and *Porphyromonas,* and lower levels of *Butyricicoccus* and *Faecalibacterium,* among others. Other genera, including *Actinomyces* and *Streptobacillus,* were increased in HIV+ MA users compared to HIV+ non-users. Relative abundance of *Finegoldia* and *Peptoniphilus* increased with increasing frequency of MA use, while *Butyricicoccus* decreased. In summary, MA use was associated with an imbalance in gut microbial composition favoring pro-inflammatory, potentially pathogenic bacteria, including some with neuroactive potential. Additional investigations into mechanisms linking dysbiosis with MA toxicity and poor HIV outcomes among people who use MA are warranted.

4.2 Introduction

The 2017 National Survey on Drug Use and Health estimated that nearly one million people in the United States were current users of methamphetamine (MA) or had a MA use disorder in the past year.[133] MA use is a major public health concern with myriad negative health consequences ranging from anxiety and confusion to psychosis and violent behavior; chronic abuse may even result in severe and lasting structural changes in the brain affecting emotional regulation and cognition.[134] MA use is much more prevalent among people living with HIV, with rates of recent use 30 times higher and rates of dependence 33 times higher than the general population (0.3% vs. 9% recent use and 0.4% vs. 13% dependence[12,133,135]). MA increases susceptibility to HIV infection by altering immune activity,[136,137] inhibiting neurocognitive processes involved in judgement and decision-making,[138] and increasing the frequency of risky sex acts.[12,139] In two large cohort studies of MSM, the attributable fraction for HIV incidence due to MA use was 0.16[140] and 0.32,[141] both of which signal the possibility for biological processes that might explain this heightened incidence. MA use among people living with HIV is associated with reduced likelihood of achieving viral suppression,[142] faster disease progression,[143-145] and increased risk of transmission to others.[146]

Many MA-induced neurotoxic effects are mediated by inflammation,[136,147] and the microbiome, which is involved in inducing and regulating the immune system,[30] may play a role in this process. Exposure to MA impacts both innate and adaptive immunity, increasing production of inflammatory cytokines, inhibiting T-cell proliferation, altering gene expression of immune cells, modifying cytokine signaling pathways, and increasing blood-brain barrier permeability.[136,148,149] MA damages gut wall integrity and increases intestinal permeability,[149] leading to the translocation of microbiota into the body. This process disrupts symbiotic interactions between the host immune system and microbiota, inducing an immune response that may cyclically exacerbate intestinal permeability and further inflammation[24]. Microbial translocation has been cited as one of the key drivers of chronic inflammation described in

many other diseases, including HIV, and may play a similar role in MA-induced inflammation and toxicity. Furthermore, mediated by inflammation, disruption of gut bacterial communities (termed “dysbiosis”) may be a mechanistic link between methamphetamine use and HIV transmission and disease progression.

Dysbiosis has been described in individuals with substance use disorders,[68] chronic prescription opioid[150] and cocaine users,[15] as well as people living with HIV[39,40] and those practicing receptive anal intercourse (RAI).[73] We recently showed that MA use was associated with microbiome changes in a small sample of HIV-infected MSM.[67] However, no large studies into the effects of MA on the microbiome has been completed, and no studies have examined the potential role of HIV in MA-induced dysbiosis. In order to address this gap, we studied the effects of MA on the gastrointestinal microbiome in a cohort of young men who have sex with men (MSM), approximately half of whom were HIV-infected, and all of whom were engaging in anal intercourse. We hypothesized that MA use would be associated with increased relative abundance of pro-inflammatory and pathogenic bacterial taxa as well as alterations to taxa with neurologic effects. We also hypothesized that MA and HIV would interact, such that higher levels of dysbosis would be observed among HIV+ MA users.

4.3 Methods

Study population. Specimens and data for this study were drawn from a larger cohort, the NIDA-funded Minority Men who have Sex with Men Cohort at UCLA Linking Infections Noting Effects (MASCULINE, or mSTUDY). Subject selection procedures have been previously described.[119] Briefly, participants were all men born males, aged 18-45, with one-half of the sample purposefully included due to current substance use (the other half non-substance users) and one-half of the sample purposefully included due to HIV-infection (the other half being HIV-negative). Both the current study and the mSTUDY were approved by a UCLA Institutional

Review Board. All participants provided written informed consent prior to participation and all study procedures were done in accordance with ethical principles for human subjects research.

Specimen collection and DNA preparation. As previously described,[119] samples included in this study were rectal swabs (FLOQSwabs, Copan Diagnostics, Murrieta, CA). The majority (76%) were collected via anoscopy under direct mucosal visualization and without preparatory enema at approximately 8 cm from the anal verge. Due to an mSTUDY protocol change, others (24%) were participant self-collected at approximately 4-5 cm from the anal verge. Collection method was taken into account in the analysis (see Table 4.1). Swabs were immediately frozen neat at -80°C until processing in bulk. For DNA processing the samples were transferred to Lysing Matrix E tubes (MP Biomedicals, Burlingame, CA) containing RLT lysis buffer (Qiagen, Hilden, Germany) and bead-beated on a TissueLyser (Qiagen). DNA was then extracted using the AllPrep DNA/RNA/Protein kit (Qiagen) per manufacturer's protocol.

16S rRNA gene sequencing and data processing. Microbiome profiling was performed by sequencing of the V4 region of the 16S rRNA gene as previously described.[81,119,120] Briefly, the V4 region was amplified in triplicate reactions using Golay-barcoded primers 515F/806R. PCR products were then pooled and sequenced on the Illumina MiSeq platform using 2x150bp v2 chemistry. The sequences were demultiplexed with Golay error correction using QIIME v1.9.1,[83] and Divisive Amplicon Denoising Algorithm (DADA2) version 1.8 was used for error correction, exact sequence inference, read merging, and chimera removal.[84] The resultant amplicon sequence variant (ASV) table comprised 19,955,039 total merged read pairs (mean per sample = 52,375; range 10,906 to 124,889). Taxonomic assignment was performed using RDP trainset 16 (https://doi.org/10.5281/zenodo.810827). Rarefaction was performed at a depth of 10,906 reads for alpha diversity analyses. To normalize all other analyses, estimates of relative library sizes ("size factors") were obtained by calculating geometric means of pairwise read count ratios.[86]

Measurement of MA use. MA use was measured using an adapted version of the NIDA-modified ASSIST.[151] Participants were asked how often they used MA in the previous six months; response choices were “Daily”, “Weekly”, “Monthly”, “Less often”, “Once”, and “Never.” For most analyses we categorized participants as MA users if they indicated any use in the past six months, and non-users if they responded “Never.” For the dose-response analysis, we combined “Monthly”, “Less often”, and “Once” into “Monthly or less often,” given that infrequent exposures are likely to have similar effects on the microbiome. In addition, participants were screened for MA use via urinalysis [Fastect® II (Branan Medical Corporation); iScreen® Dip Card (Alere)]. We did not use the urinalysis results as our primary exposure variable because the detection window for MA is 48-72 hours and no exposure quantification (and thus dose-response analysis) could be done. We instead compared the self-report and urinalysis results as a sensitivity analysis (Figures A3.3, A3.4, and A3.5).

Behavioral and clinical covariates. Analyses controlled for a large set of behavioral and clinical covariates including age, race/ethnicity, homelessness in past six months, number of receptive anal intercourse (RAI) acts in past month, number of sex partners in past six months, an indicator for RAI in the past seven days, an indicator for a positive STI test (including PCR tests for rectal gonorrhea and chlamydia and serology for primary or secondary syphilis), self-reported use of marijuana and cocaine, tobacco smoking, and binge drinking (defined as 6+ drinks on more than one occasion) in the past six months, and use of antibiotics in the past month. We also controlled for type of antiretroviral therapy (including use of pre-exposure prophylaxis if HIV-) and an indicator for CD4 cell count <200. Measures and assays have been previously described.[119]

Statistical analyses. Prior to completing the primary analyses for this study, we compared clinical and behavioral characteristics between MA users and non-users using descriptive statistics, Wilcoxon or Chi-square tests, and standardized mean differences (see

Appendix 1). All analyses of microbiome outcomes were adjusted for clinical and behavioral confounders using inverse probability of treatment weighting (IPTW). IPTW is a technique in which the study sample is re-weighted to achieve balance between exposure groups (here, MA users vs. non-users) on important covariates so that they no longer act as confounders.[87] Covariates included in the IPTW model are listed in Table 4.1, and further information about the IPTW calculation and adjustment process is available in Appendices 1 and 3. IPTW were estimated using generalized boosted models (R package 'twang') and robust standard errors for IPTW-adjusted analyses were obtained using the sandwich estimator (R package 'sandwich'). All analyses in this study proceeded by first testing for interactions between MA use and HIV status using multiplicative interaction terms. A threshold of $p < .1$ was used as a cutoff for significance of interaction tests; if significant, comparisons of MA users vs. non-users are presented stratified by HIV status (retaining HIV status and the interaction term in the model). If no significant interaction was detected, comparisons of MA users vs. non-users were completed controlling for HIV status (retaining HIV status as a covariate but dropping the interaction term).

The R package 'phyloseq' was used to calculate distance matrices, alpha diversity metrics, and for ordination. Permutational multivariate ANOVA (PERMANOVA; R package 'vegan') was used to test for differences in overall microbial composition and linear regression was used to test for differences in diversity between MA users and non-users. Zero-inflated negative binomial (ZINB) models were used to test for differences in individual bacterial genera between groups. We employed a previously described model selection strategy[119] to choose the optimal ZINB model for each genus. A pre-filtering step excluded genera appearing in less than 10% of samples as well as those with less than 100 total reads across all samples, resulting in 78 genera included in ZINB analyses. Dose-response analysis was completed by regressing bacterial counts on frequency of MA use using orthogonal polynomial coding of linear and quadratic curves. As sensitivity analyses, all analyses were repeated redefining MA use

according to urine toxicology results (except for dose-response, owing to the qualitative nature of urine toxcology).

PERMANOVA, alpha diversity, and dose-response analyses utilized a threshold of $p < .05$ to determine statistical significance. In order to account for the large amount of genera tested, p values obtained from ZINB models were corrected using Benjamini & Hochberg's False Discovery Rate (FDR) method.[89] FDR-adjusted p values are labelled as q values, and $q < .1$ was used as a threshold to determine statistical significance. Accordingly, we display 90% false coverage rate (FCR)-adjusted confidence intervals[90] to accompany these analyses. All statistical analyses were performed using R v.3.5.1.

4.4 Results

Participant characteristics. This study included 381 participants, 156 MA users (41%) and 225 non-users (59%). All participants were MSM, the mean age was 31, and most were Hispanic (49%) or non-Hispanic Black (39%). Sixty-five percent of MA users were HIV+ as compared to 36% of non-users. MA users were also older than non-users, were more likely to have experienced homelessness, had RAI more frequently, had more anal sex partners, were more likely to have recently used cocaine, and were more likely to be tobacco smokers. See Table 4.1 for additional details.

Effects of MA use on overall microbial composition and diversity. PERMANOVA analyses with Bray-Curtis, Jaccard, and Jensen-Shannon distances did not reveal significant evidence supporting an interaction between MA and HIV on overall microbial composition (all $p > .1$; Table A3.1). Therefore, we described and compared overall composition between MA users and non-users while controlling for HIV status. Descriptive barplots suggested increased *Finegoldia, Fusobacterium, Peptoniphilus, Porphyromonas, Streptobacillus,* and *Streptococcus* and decreased *Bacteroides, Faecalibacterium,* and *Succinivibrio* in MA users compared to non-

users (Figures 4.1A and 4.1B). Ordination of Bray-Curtis, Jaccard, and Jensen-Shannon distances by principal coordinates analyses revealed differences in overall composition by MA status (Figure 4.2A), which were supported by PERMANOVA analyses showing that MA explained significant variation in overall microbial composition (Bray-Curtis $R^2 = .005$, $p = .008$; additional results in Figure 4.2A). No significant interactions between MA and HIV were detected in observed diversity or Chao1, Shannon, or Simpson indices (all $p > .1$; Table A3.1), and no differences in diversity were detected between MA users and non-users in any metric (Figure 4.2B). Despite lack of evidence for an interaction between HIV and MA use, we display descriptive, ordination and alpha diversity plots stratified by HIV status in figures A3.1 and A3.2.

Effects of MA use on specific genera. Using ZINB models with IPTW adjustment, we found differences between MA users and non-users in multiple genera. For some, there was no evidence for an interaction between MA and HIV: Regardless of HIV status, MA users had higher levels of *Finegoldia, Fusobacterium, Parvimonas, Peptoniphilus, Peptostreptococcus,* and *Porphyromonas,* and lower levels of *Butyricicoccus* and *Faecalibacterium,* among others (Figure 4.3). For four genera, a significant ($q < .1$; Table A3.1) interaction between HIV and MA was detected. *Actinomyces, Mannheimia, Negativicoccus,* and *Streptobacillus* were increased in HIV+ MA users compared to HIV+ non-users, but no difference was found in the absence of HIV. No genera were significant only in the HIV- stratum.

Dose-response analysis of bacterial counts on increasing frequency of MA use. From all genera showing significant differences between MA users and non-users, we chose a subset of nine for IPTW-adjusted dose-response analysis based on previously published associations with MA use, HIV, inflammation, or relevance to MA toxicity. Of the 156 MA users in the study, 40 were daily users, 35 used weekly, and 81 used monthly or less often. Counts of *Finegoldia* and *Peptoniphilus* increased linearly with increasing frequency of MA use, while counts of *Butyricicoccus* decreased. *Porphyromonas* also appeared to increase and

Faecalibacteirum to decrease, but these trends were not statistically significant. Clear dose-response trends in *Actinomyces, Fusobacterium, Parvimonas,* and *Peptostreptococcus* were not apparent, and no significant quadratic dose-response curves were noted in any genera (Figure 4.4).

Sensitivity analysis using urine toxicology screening to define MA use. Our findings, which were based on participant self-report of MA use, were consistent when we re-defined MA use using urine drug screening results. Fourteen percent of study participants (n = 52) tested positive for MA including 3 individuals who self-reported no MA use (49/52 who tested positive also self-reported using MA). One hundred seven self-reported using MA, but tested negative, likely because their last use was outside the drug screen window of detection.

In biomarker analyses, MA use was still a significant driver of variation in overall microbial composition (Bray-Curtis R^2 = .008, p = .008), and no differences in alpha diversity were noted between MA users and non-users. Many of the same genera were elevated in MA users, e.g., *Finegoldia, Fusobacterium, Peptoniphilus, Peptostreptococcus,* and *Porphyromonas*, and elevations in *Streptobacillus* in the HIV+ stratum were noted in both analyses. Depletion in *Faecalibacterium* was consistent across analyses; the biomarker analysis also identified depletions in *Clostridium* cluster XI and *Lactobacillus* in MA users. Results from this sensitivity analysis are presented in figures A3.3, A3.4, and A3.5.

4.5 Discussion

This study of 381 young, diverse MSM who were either HIV-infected or at high risk for HIV acquisition found that MA use significantly impacted gut microbial composition after controlling for multiple clinical and behavioral confounders. Measures of overall composition were altered by MA use, but measures of diversity were not, and the associations between MA and overall composition and diversity did not depend on participants' HIV status. Several genera

were increased in MA users regardless of HIV status, many of them considered pro-inflammatory and pathogenic, while others were increased among HIV+ participants only. We found dose-response relationships between the abundance of several bacterial taxa and frequency of MA use, such that the abundance of pro-inflammatory taxa increased and commensals decreased with increasing frequency of MA use. Finally, we were able to replicate our findings using a biomarker confirming recent MA use (urine drug screen). MA effect sizes were slightly larger in the biomarker analysis, likely because the window of detection for MA is short, making frequent users more likely to test positive. Our analyses utilized a novel method of confounder control, IPTW, to account for several factors that have previously been associated with dysbiosis (e.g. RAI,[73] cocaine use,[15] and alcohol use[152]), making our findings more likely to be truly attributable to MA use.

Although little research has been done on the effects of MA on the microbiome, our results are mostly consistent with previously published literature. A study of 37 HIV+ individuals from the same cohort as the current data[67] (none of the individuals in the current sample were included in this previous study) reported a MA effect size (PERMANOVA R^2) of .1, larger than the effect we found. As in our study, there were no significant differences in diversity between MA users and non-users. Enrichment in *Porphyromonas* in MA users was consistent across studies. As a well-known modifier of inflammatory cytokines[96] and a potential cause of intestinal permeability[99] and systemic inflammation associated with periodontal and cardiovascular diseases,[97] *Porphyromonas* may play a role in MA-associated inflammation and deserves further investigation. Another study in which MA was administered to rats reported an overall effect of MA that is consistent with our findings (R^2 of .008).[66] This study also reported higher alpha diversity in the MA-conditioned group, which was not replicated in our study, and taxonomic differences that do not overlap with our findings, likely because of differences between the mouse model and a human cohort. Finally, a study comparing individuals with

substance use disorders (SUDs) to healthy controls found a large effect of SUD (R^2 of .067), higher observed diversity among individuals with SUDs, and differences in specific genera that do not match our findings.[68] However, participants with MA use disorder only accounted for 30% of the SUD group, and the study did not control for large differences in lifestyle and clinical confounders between individuals with SUDs and healthy controls.

MA use is associated with increases in production and alterations in gene expression of many pro-inflammatory cytokines,[137,153] which may contribute to neurological deficits, anxiety, and impaired memory.[154] Many of the bacterial genera that were elevated in MA users, such as *Porphyromonas,*[96] *Veillonella,*[155] and *Fusobacterium*[156] have been correlated with increases in pro-inflammatory cytokines. MA also exacerbates systemic inflammation by damaging gastrointestinal barrier integrity and inducing permeability, allowing the translocation of microbes and microbial products into the body. Our study identified depletions in the butyrate-producing genera *Faecalibacterium* and *Butyricicoccus* in MA users, which have been inversely correlated with biomarkers of microbial translocation.[49,157] A study of patients with alcohol use disorder showed that those with higher levels of gut permeability had lower levels of *Bifidobacterium* and *Faecalibacterium* species and exhibited more symptoms of alcohol dependence and cravings.[158]

Emerging preclinical research has demonstrated a complex interplay between the microbiome and the central nervous system,[159,160] leading to inquiries about the role of dysbiosis in addiction pathology. Gut bacteria produce neuroactive substances, including serotonin, epinephrine and dopamine,[161] which may access the brain's reward centers via gut-innervating vagal neurons.[162] *Streptococcus,* which was elevated in MA users in our study, can produce serotonin,[161] and *Lactobacillus*, which was depleted in MA users (in the biomarker analysis), can produce gamma-aminobutyric acid (GABA).[161] The connection between dysbiosis and addiction pathology has been demonstrated in laboratory experiments of other drugs of abuse. For example, increased sensitivity to cocaine reward through alterations in dopaminergic pathways

has been observed in mice with experimentally disrupted microbiome.[69] Another study showed that manipulation of the microbiome resulted in several characteristics of opioid dependence in mice, such as reduced opioid analgesic potency and impaired reward behavior.[163] In addition, there is some preclinical evidence that “repairing” the microbiome might alleviate addiction pathology, e.g., *Lactobacillus* species restored chemically-depressed dopamine levels in the prefrontal cortex when administered to rats as a probiotic.[164]

Potential mechanisms linking dysbiosis to the pathology of MA dependence remain largely unexplored, especially in humans. There is limited clinical evidence linking dysbiosis with symptoms associated with MA dependence, such altered stress response and increased depression. Butyrate-producing *Faecalibacterium*, which was decreased in MA users in our study, has been associated with reduced depression and higher quality of life.[165] Common symptoms of MA withdrawal, including depression, anxiety, and fatigue, have been correlated with imbalances in gut microbiota[166] and probiotics have been used to successfully reduce anxiety and depression in clinical trials.[167] It is plausible that targeting dysbiosis may ease these symptoms among individuals undergoing treatment for MA use disorders.

We also found a number of genera that were impacted by MA which have previously been shown to play a role in HIV acquisition and pathogenesis. Increased abundances of *Finegoldia* and *Peptoniphilus* in the penile microbiome have been associated with elevated risk for HIV seroconversion in men,[168] and *Parvimonas* has been shown to increase genital tract inflammation[169] and the risk of HIV acquisition[170] in women. The implications of enrichment of these bacteria in the rectal microbiome have not been explored; however, it is likely that inflammation exacerbated by dysbiosis underlies the increase in seroconversion risk, which would be highly relevant to at-risk MSM. We also found that MA use increased *Fusobacterium*, which has been correlated with decreased CD4+ T-cell count and increased T-cell activation in HIV+ individuals as well as reduced T-cell recovery following ART initiation.[171] HIV and MA

interacted to multiplicatively increase *Actinomyces*, which may play a role in reactivating HIV in latently infected cells.[172] *Parvimonas* and *Peptostreptococcus* are oral pathogens that have been implicated in periodontal infections among HIV+ individuals,[173,174] and their damaging effects may be heightened in MA users due to MA's proclivity to reduce saliva production.[175] Finally, increased abundance of *Veillonella* has been linked with HIV-associated pulmonary diseases.[176] Our study, showing that MA impacted the relative abundance of each of these genera, may highlight mechanisms underlying the relationship between MA use, HIV acquisition and transmission risk, and HIV disease progression which warrant further investigation.

Our results should be interpreted considering the following limitations. Primarily, no diet data is collected for this cohort. We controlled for race/ethnicity and homelessness, which may impact diet and thus mitigate the effects of this limitation; however, we were unable to fully account for the effects of diet in our analyses. Using IPTW, our study accounts for a plethora of other clinical and behavioral confounders which may have masked true findings or generated spurious associations in previous studies. However, IPTW cannot achieve perfect balance between exposure groups in real-world research applications, and thus we cannot rule out residual confounding even by variables included in our analyses. Our study was also conducted in a cohort comprised entirely of MSM, all of whom were practicing anal intercourse, which increases internal validity by eliminating the effects of some important confounders (e.g. gender, sexual behavior[73]). However, this may limit the generalizability of our findings to other groups, such as women who use MA. Finally, because the ability of 16S gene sequencing to identify bacterial species is limited, we conducted our analyses at the genus level. We caution that differences in genera do not necessarily correspond to differences in functionally important species.

MA use remains a significant public health challenge, especially among people living with HIV. Our study found that MA use was associated with an imbalance in gut microbial

composition favoring pro-inflammatory, potentially pathogenic bacteria, including some with neuroactive potential. There is currently no accepted pharmaceutical treatment for MA use disorder and limited evidence for the effectiveness of cognitive-behavioral therapy; further research into changes in the microbiome associated with MA use may hold therapeutic potential for individuals with MA use disorder. Moreover, increases in multiple taxa that have been previously associated with poor HIV outcomes or HIV transmission and acquisition are particularly concerning in our study population of MSM who were either HIV-infected or at high risk for infection. Additional investigation into the mechanisms underlying these associations may improve HIV prognosis and prevent future infections among this vulnerable group.

Table 4.1. Participant characteristics, split by MA use, N = 381 men who have sex with men in Los Angeles, CA

	MA- negative n = 225 mean (sd), median n (%)	MA-positive n = 156	*P*[d]	SMD[e] (pre, post IPTW)
Age	30.17 (6.85), 29	32.58 (6.75), 33	<.001	.35, .16
HIV+	80 (35.6)	102 (65.4)	<.001	N/A
Race/ethnicity			.6	.1, .07
Black-Non Hispanic	93 (41.3)	57 (36.5)		
Hispanic	107 (47.6)	79 (50.6)		
Other-Non Hispanic	25 (11.1)	20 (12.8)		
Homeless in past 6 months	52 (23.1)	77 (49.4)	<.001	.57, .28
Had RAI in last 7 days	102 (45.3)	65 (41.7)	.5	.07, .03
Number of RAI acts in past month	2.09 (4.94), 0	2.88 (5.33), 1	<.001	.15, .03
Number of anal sex partners in past 6 months	6.17 (7.60), 3	8.79 (9.28), 5	<.001	.31, .18
Positive for STI[a]	19 (8.4)	28 (17.9)	.006	.28, .18
Marijuana use in past 6 months			.1	.23, .21
Daily/Weekly	69 (30.7)	60 (38.5)		
Monthly/less	52 (23.1)	41 (26.3)		
Never	104 (46.2)	55 (35.3)		
Cocaine use in past 6 months	40 (17.8)	60 (38.5)	<.001	.47, .24
Tobacco smoker	73 (32.4)	95 (60.9)	<.001	.6, .38
Binge drinking in past 6 months[b]	138 (61.3)	91 (58.3)	.6	.06, .04
Antibiotic use in past month	15 (6.7)	16 (10.3)	.2	.13, .07
Sample collection strategy			.5	.07, .01
Anoscopy	169 (75.1)	122 (78.2)		
Self-collected	56 (24.9)	34 (21.8)		
Type of ART			<.001	.56, .28
INSTI + NRTI	30 (13.3)	39 (25.0)		
NNRTI + NRTI	25 (11.1)	23 (14.7)		
NRTI + PI	15 (6.7)	15 (9.6)		
Other	4 (1.8)	12 (7.7)		
HIV+ and missing ART data	6 (2.7)	14 (9.0)		
HIV- pre-exposure prophylaxis (PrEP) user	30 (13.3)	7 (4.5)		
HIV-, no PrEP	115 (51.1)	47 (30.1)		
Among HIV+ participants only				
HIV RNA log_{10} copies /mL (median, IQR) c	1.03 (0.7)	1.03 (1.7)		N/A

CD4 cells/mm^3 (median, IQR) [c]	590.5 (267)	635 (424.3)		N/A
CD4 cells/mm^3 < 200	5 (2.2)	9 (5.8)		.18, .15

MA = Methamphetamine; SMD = Standardized mean difference; RAI = Receptive anal intercourse; STI = Sexually transmitted infection; ART = Antiretroviral therapy; INSTI = Integrase strand transfer inhibitor; NRTI = Nucleoside reverse transcriptase inhibitor; NNRTI = Non-nucleoside reverse transcriptase inhibitor; PI = Protease inhibitor

[a]Sexually transmitted infections include rectal gonorrhea, rectal chlamydia as well as primary/secondary syphilis.

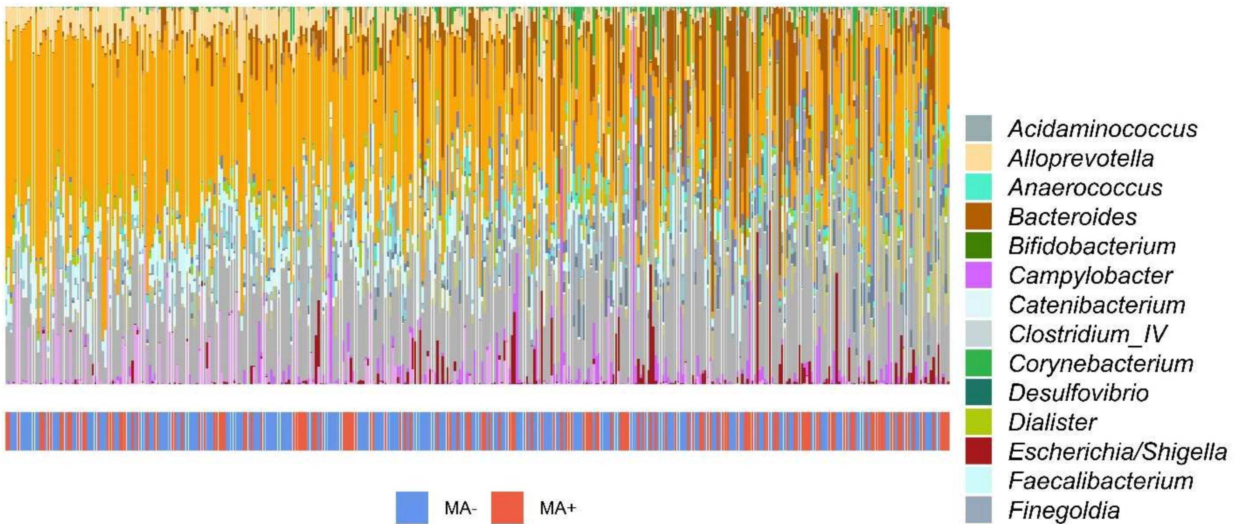
[b]Binge drinking defined as 6 or more drinks on one occasion.

[c]HIV RNA and continuous CD4 cell count were not included in the inverse probability of treatment weight model, all other variables in the table were included.

[d]*p* values are from Wilcoxon tests or Chi-square tests.

[e]SMD is a measure of imbalance across groups; higher SMDs indicate greater imbalance. Average SMD before weighting = .28, after weighting = .14.

Figure 4.1. Rectal microbial composition of study participants, N = 381, stratified by MA use. (A) Columns represent the relative composition of each subject's microbiome at the genus level. Methamphetamine (MA) use by the subjects is indicated by a colored line below their composition. Subjects are ordered by the first principal coordinate of a Bray-Curtis pairwise distance matrix. Genera representing less than 1% of the composition on average across samples were combined into "Other." (B) Average microbial composition within each MA use group. Bacterial genera representing less than 1% of the overall relative composition or present in less than 10% of the samples were grouped into "Other."



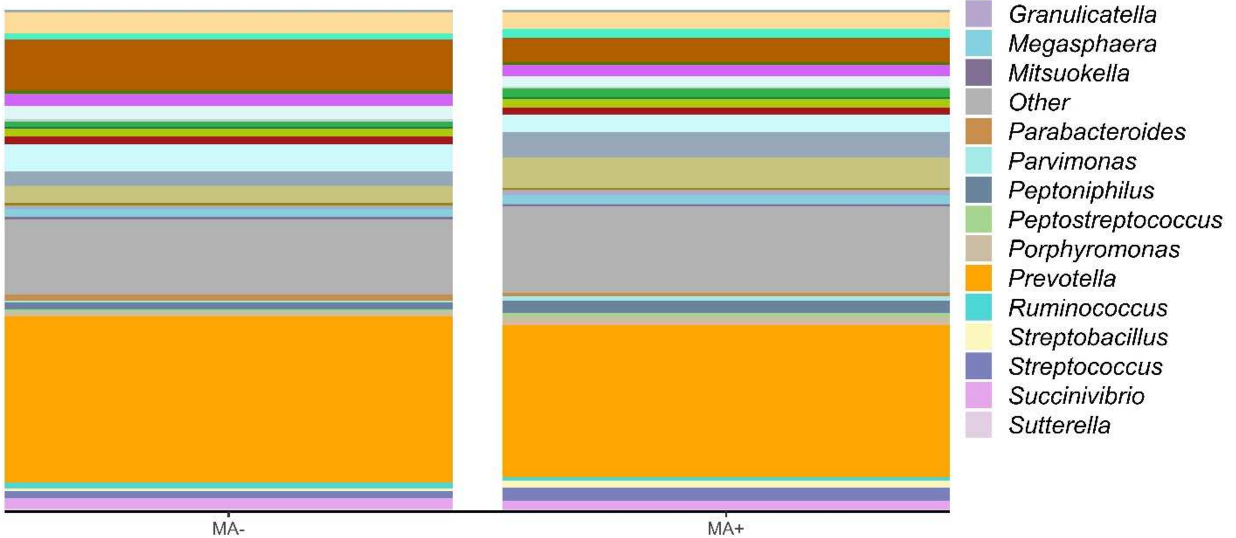


Figure 4.2. Associations between methamphetamine (MA) use and overall microbial composition and diversity. (A) Ordination of the samples using principal coordinates analysis. PCoA = Principal coordinate axis. Ellipses are 95% confidence regions for each group assuming points follow a multivariate t distribution. R^2 and p values are from PERMANOVA analyses of distance metrics. (B) Boxplots of diversity metrics. Boxes represent the inverse probability of treatment weight-adjusted lower, median, and upper quartiles of the data and whiskers are 1.5*interquartile range. p values are from IPTW-adjusted linear regression analyses comparing diversity metrics between MA users and non-users.

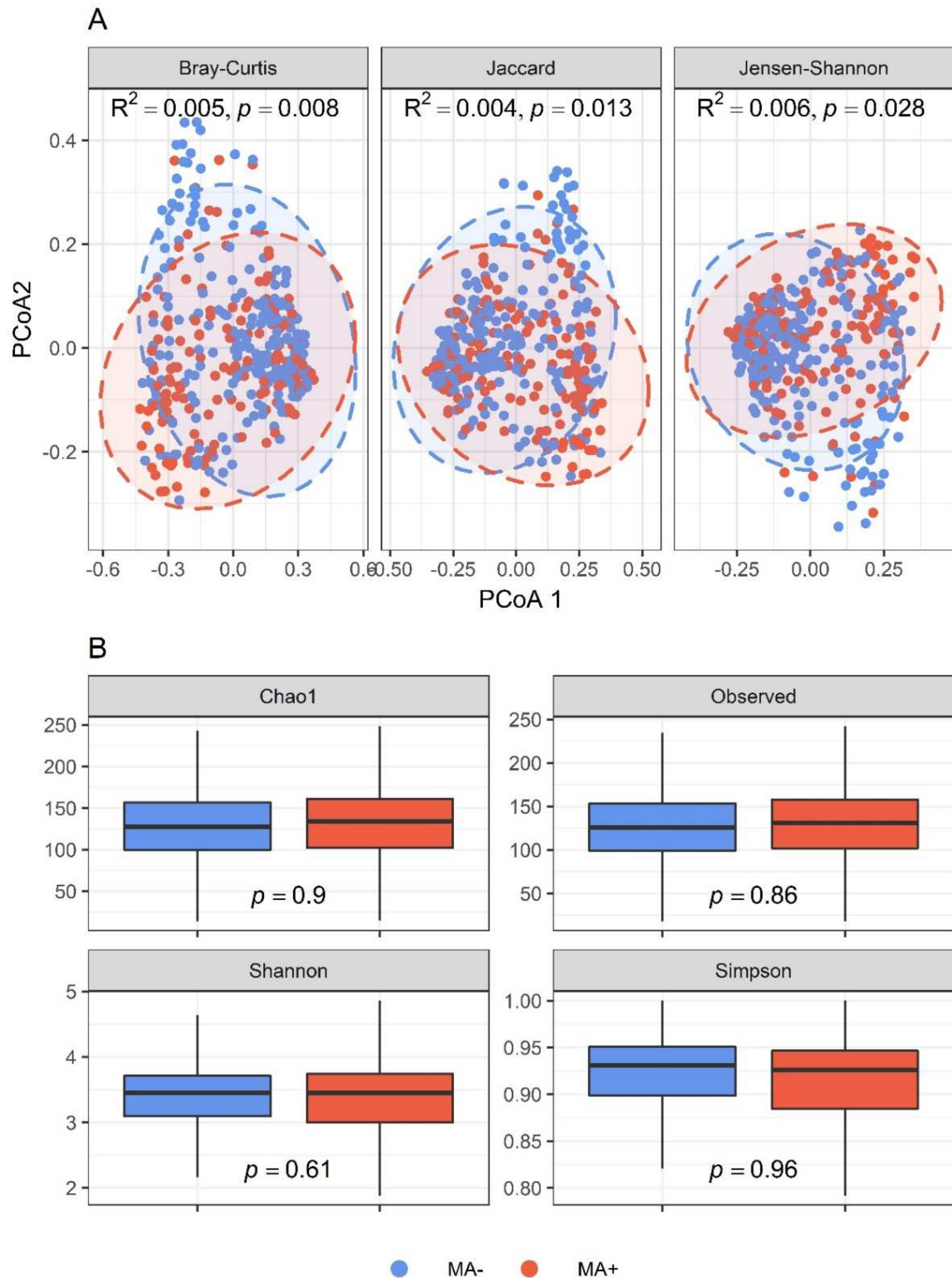


Figure 4.3. Comparisons of individual genera between methamphetamine (MA) users and non-users. Forest plots of results of zero-inflated negative binomial models comparing genus-level bacterial counts between methamphetamine (MA) users and non-users. Inverse probability of treatment-weighted effect sizes (log normalized count ratios) and false coverage rate (FCR)-adjusted 90% confidence intervals are plotted, with statistical significance ($q < 0.1$) indicated in black. Genera with no effect are not shown. Dots are sized proportionally to overall mean abundance across samples, i.e., genera with larger dots are, on average, more abundant.

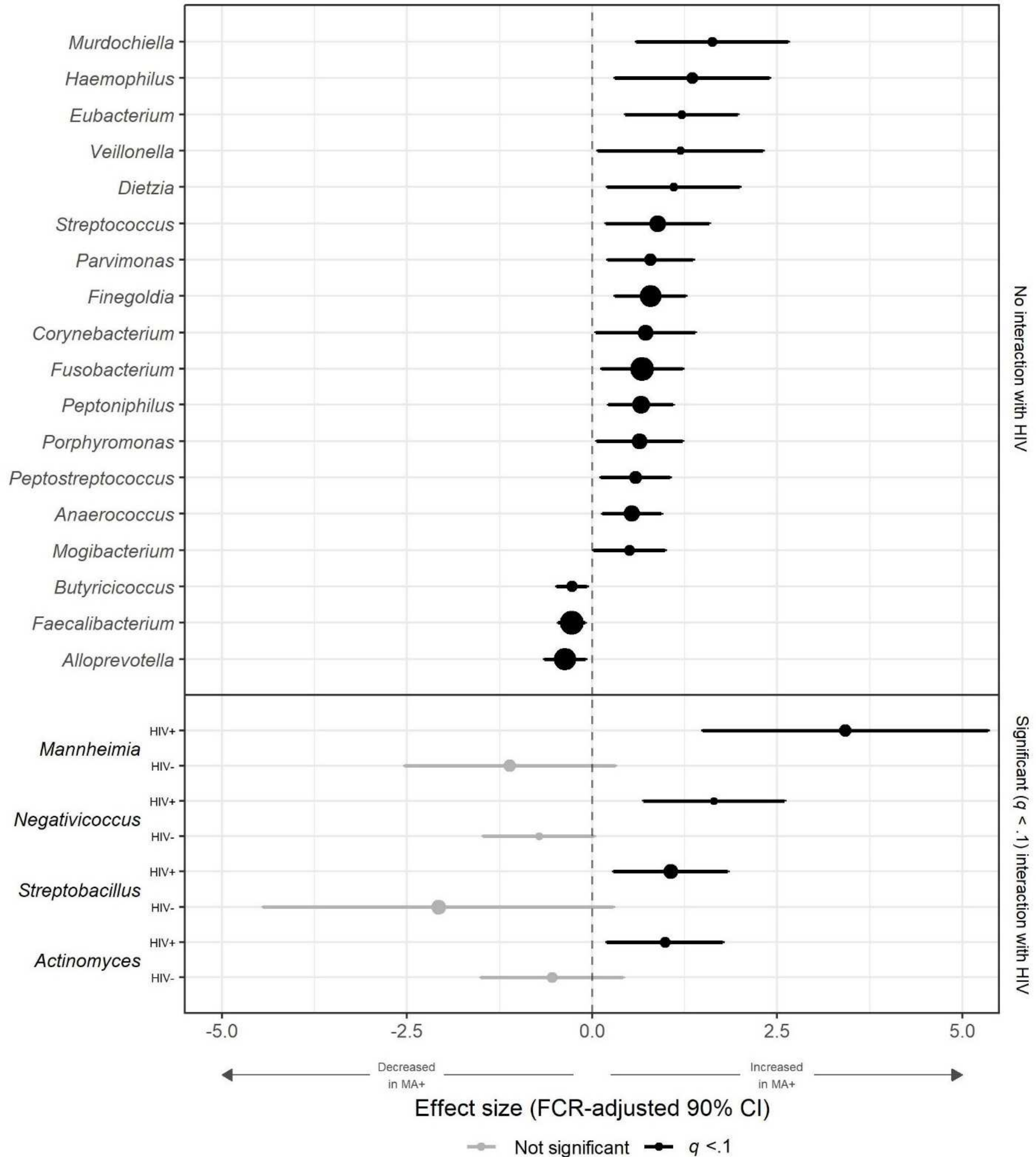
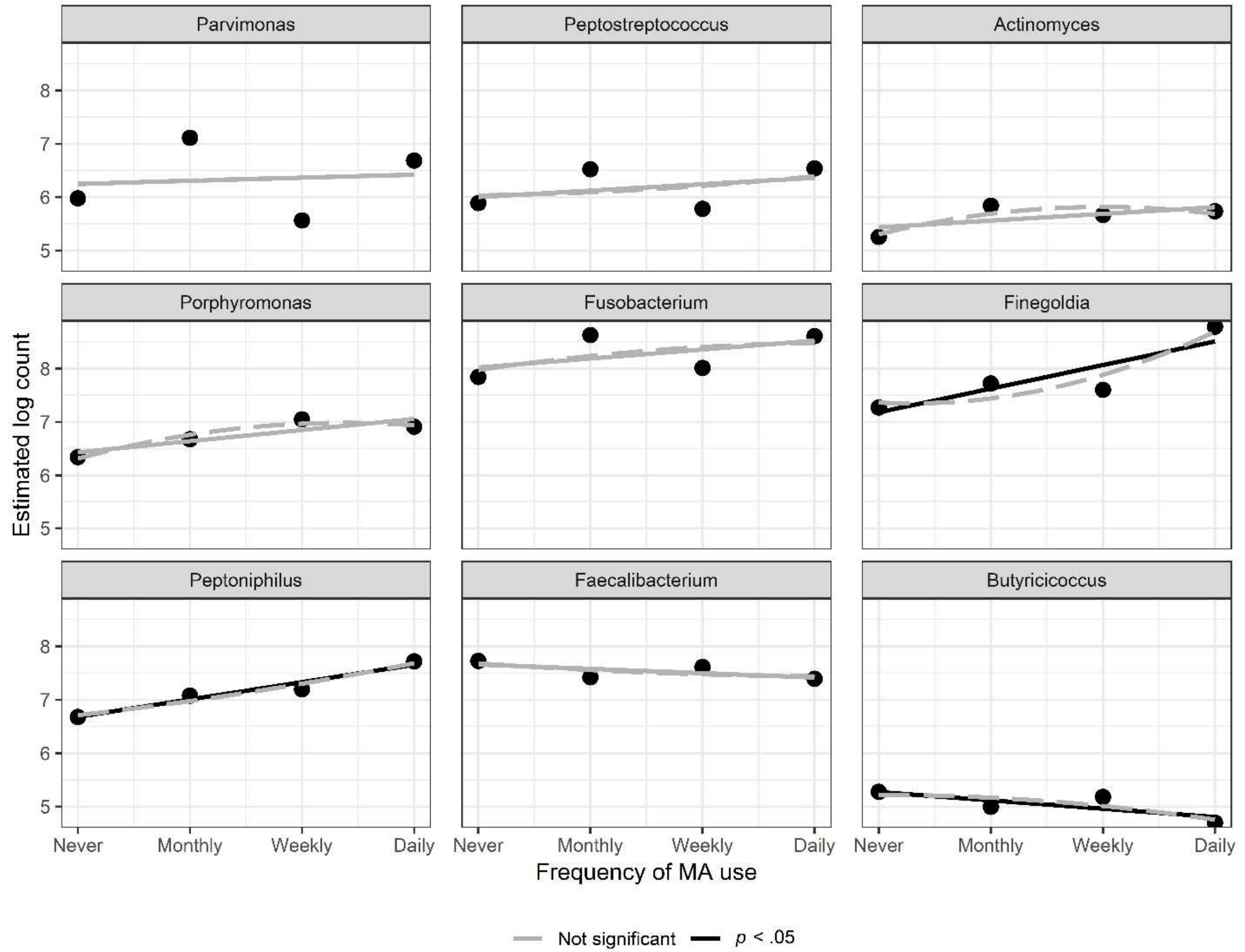


Figure 4.4. Dose-response analyses of bacterial counts on increasing frequency of methamphetamine (MA) use. Plots show expected log bacterial counts at each level of MA use with linear (solid) and quadratic (dashed) dose-response curves drawn to fit the points. Significance of the curves was assessed using zero-inflated negative binomial models with orthogonal polynomial coding of MA use.



Chapter 5. Concluding Remarks

The microbiome represents one of the most exciting, promising, and challenging frontiers in HIV research. As shown in this dissertation, HIV and its associated comorbidities have a profound negative impact on the composition of the gut microbiome. Shifts attributable to HIV, obesity, and methamphetamine (MA) use largely favored pro-inflammatory, potentially pathogenic bacterial taxa at the cost of those with beneficial, immune regulatory properties. Because of the multidimensional, symbiotic relationship between the microbiome and the immune system, this "dysbiosis" likely plays a significant role in HIV-associated chronic inflammation and the development of related diseases.

This dissertation addresses several factors which have contributed to inconsistencies in the literature on HIV and the microbiome. Most significantly, this is the first study to account for several behaviors and characteristics that are common among people living with HIV and have been independently shown to affect the microbiome. Using inverse probability of treatment weighting (IPTW) to adjust for this robust set of covariates is an innovative solution to the challenge of integrating questionnaire and clinical data into microbiome analyses. Furthermore, this is the largest study of HIV and the microbiome to date, and undoubtedly one of the few with adequate power to account for the dimensional and distributional challenges presented by microbiome data while adjusting for confounding. In addition, this dissertation includes several novel findings. Chapter two describes for the first time a dose-response relationship between levels of HIV viremia and dysbiosis. Chapter three includes an exploration of the combined effects of two exposures, HIV and obesity, on the microbiome; although it is widely accepted that many factors impact microbial composition, few studies have considered more than one at a time. Chapter four is one of only two human studies of the effects of MA on the microbiome, and the first to consider interactions between MA use and HIV. Each of these innovations relies

on a technique or principle that may be applied to future microbiome research in order to increase scientific validity and improve reproducibility.

Research into the clinical implications of dysbiosis is in its infancy; however, there is reason to believe that intervening to "repair" the microbiome may have implications for immune reconstitution and reduction in HIV pathogenesis. Studies in Nigeria,[177] Tanzania,[178,179] and Canada[180] have shown that administration of *Lactobacillus* as a probiotic increases CD4 count among people living with HIV. In one within-person study adjusted for length of time using antiretroviral therapy (ART), the magnitude of increase attributable to the probiotic was 63% of that attributable to ART (0.17 CD4+ T-cells/µL/day attributable to probiotic yogurt vs. 0.27 cells/µL/day attributable to ART).[178] These studies also report reduced gastrointestinal symptoms (e.g. diarrhea, nausea) in the probiotic groups. Another study found slower CD4+ T-cell decline in untreated HIV-infected individuals randomized to a prebiotic supplement.[181] Other research has focused on the effects of pre/probiotics on markers of inflammation and microbial translocation, such as one study which found reduced levels of soluble CD14 and activated CD4+ T-cells in ART-naïve individuals treated with a prebiotic.[182] Another study found that probiotic intervention resulted in reduced D-dimer, C-reactive protein, and interleukin-6 (IL-6) levels.[183] Finally, a randomized trial of virally suppressed HIV-infected individuals found that probiotics decreased levels of lipopolysaccharide-binding protein (LPS) and IL-6.[184,185] In each of these studies, reductions in inflammatory markers correlated with shifts in the microbiome from pro-inflammatory to commensal bacterial taxa. Aside from pre/probiotics, fecal microbiota transplant (FMT) is the other major method of intervention under study. FMT has been tested in simian immunodeficiency virus (SIV)-infected rhesus macaques; the treatment was well-tolerated and showed some immunological benefits, despite not having a large impact on microbial composition.[186] Because of the tremendous between-person variability in the gut

microbiome and its resilience to alterations, therapeutic treatments for dysbosis are challenging to evaluate.

This dissertation showed significant shifts in the composition of the microbiome associated with HIV, obesity, and MA use. However, the *function* of the microbiome, rather than the composition, may be more relevant to our health and the development of disease. The number of unique microbial genes present in the gut is over 150 times greater than the human genome,[187] and the metabolic activity of the microbiome has been estimated to be roughly comparable to the liver.[49] Sequencing the entire bacterial genome rather than targeted 16S sequencing, as well as employing additional -*omics* techniques (e.g. transcriptomics, proteomics), may reveal important differences in bacterial functional pathways driving immune dysfunction which are not reflected in compositional differences. In addition, although most research has focused on bacteria, the microbiome contains trillions of viruses, fungi, and other microorganisms that interact with the immune system and may be relevant to HIV pathogenesis.

Even in the era of ART, HIV remains a significant public health challenge. Huge racial, ethnic, and socioeconomic disparities exist across the HIV care continuum,[188] and consequently, in life expectancy among people living with HIV.[1,105] This research was conducted in a group of young, mostly low-income, minority men who have sex with men, who are representative of a high proportion of individuals affected by HIV the United States. Therefore, the findings of this dissertation have the potential to significantly improve the health of a large and particularly vulnerable population. Although microbiome research, and -*omics* research in general, is fraught with methodologic challenges, future research in this area is rife with opportunity to better understand HIV pathogenesis and ultimately improve the lives of those living with HIV.

Appendix 1. Supplemental content for Chapter 2, “Effects of HIV Viremia on the Gastrointestinal Microbiome of Young Men who have Sex with Men”

Sample selection

Of the 462 participants enrolled in the mSTUDY at the time our study was conducted, we selected a random subsample of 383 based on achieving approximately balanced numbers of HIV+ and HIV- substance users and non-users. The mSTUDY cohort was started to study the effects of non-injection drug use on HIV acquisition and treatment outcomes among Racial/Ethnic minority MSM. As such, substance users are highly represented and we wanted to balance this confounding factor between HIV+ and HIV- groups as well as possible. The total sample size was set in order to achieve 90% power to detect a PERMANOVA effect size of $\omega^2<0.005$ at $\alpha=0.1$, comparing HIV-infected and -uninfected groups (ω^2 is a bias-corrected R^2 statistic; power analysis was conducted using the R package 'micropower'[189]). Two samples had read counts less than the rarefication depth or did not meet the minimum number of nonzero pairwise read ratios, 10, for inclusion in the size factor calculation and were excluded from alpha diversity, PERMANOVA, ZINB, and LASSO analyses.

HIV serostatus and RNA quantification.

HIV negative status was confirmed with the OraQuick Advance® HIV 1/2 (OraSure Technologies, Bethlehem, PA). Among infected participants, plasma HIV RNA was quantified using a standard clinical laboratory assay (Cobas® AmpliPrep/Cobas® TaqMan® HIV-1 Test, Version 2.0, assay range of 20−10,000,000 copies/mL).

Behavioral and clinical measures

Demographic data collected included age, Race/Ethnicity, country of origin (United States vs. other countries), employment status, and housing status (homeless or stably

housed). Self-reported frequency of methamphetamine, marijuana, and cocaine use in the past six months was measured using the NIDA-modified ASSIST.[151] Due to the relatively low frequency of daily methamphetamine/marijuana use, daily users were combined with weekly users. Cocaine use (including both powder and 'crack' cocaine) was overall infrequent, and thus cocaine was treated as a dichotomous variable. Alcohol use in the past six months was measured with the NIAAA recommended alcohol questions,[190] assessing frequency and amount of alcohol "bingeing," consuming six or more drinks on one occasion. Tobacco smoking was measured with the Multicenter AIDS Cohort Study (MACS) questionnaire[191] and sexual behavior, including recency and frequency of receptive anal intercourse, was measured with the American Men's Internet Survey questionnaire.[192] Obesity was determined by clinician-gathered anthropometrics (height, weight, and waist circumference) and individuals were classified as obese if they had BMI > 30 or waist circumference > 40 inches. Antibiotics used in the past month were also assessed by trained clinical staff. Prior to analysis, we used multiple imputation with the chained equations method[88] to fill in missing covariate data. Amounts of missing data for each covariate are displayed in Table A1.1.

Contaminant sequence removal procedure

Removal of contaminant sequence variants (SVs) was performed by a calculating a 'contaminant score' $S_i = \frac{\sum_J c_{ij}}{\sum c_i}$ for sequence *i*, set of negative control samples *J*, and read count of sequence *i* in sample *j* c_{ij}. Scores range from 0 for sequences that are only observed in "true" samples to 1 for sequences that are only observed in negative controls. Intermediate values are interpreted as an estimate of the likelihood that a given sequence variant was derived from negative controls (i.e. contamination). We used a threshold of $S_i \geq 0.1$ to identify and remove contaminant SVs prior to all further analysis.

Inverse probability of treatment weighting model selection

Inverse probability of treatment weighting (IPTW) is a balancing procedure used to render the treatment/exposure under study (i.e., HIV viremia group) independent of confounding variables. In essence, weighting by the inverse probability of treatment (or in this case, exposure) transforms the study sample into a "pseudo-population" where exposure status is independent of variables used in the IPTW calculation. It has been shown that if the model used to estimate the weights includes all relevant confounders and is not misspecified, in a weighted population, the effect estimate of exposure on outcome is unbiased.[87] With IPTW, the outcome models are "marginal structural models" instead of conditional on covariates, as in multiple regression. Modeling microbiome data marginally offers several advantages including the ability to control for many confounding factors without inducing overfitting bias[103] or losing efficiency due to overstratification.[104]

Mathematically, the IPTW is defined as $\frac{P(X=x)}{P(X=x|\boldsymbol{C})}$ for exposure levels $x \in 1 \ldots n$ ($n = 4$ in this study, corresponding to the four HIV viremia categories) and set of confounder variables $\boldsymbol{C}$. If the treatment probabilities are known (as in, for example, a randomized trial), no estimation is necessary; however, in observational studies the IPTW must be estimated from data. We utilized two methods to estimate the IPTW: a generalized boosted model[193] and multinomial logistic regression. Our covariate set $\boldsymbol{C}$ included all variables described in the behavioral and clinical covariates section above (see Table 2.1 and Table A1.1 for more details).

Prior to fitting the IPTW models, we calculated the standardized mean difference (SMD) in each confounding variable across HIV viremia groups, which measures the severity of imbalance in that variable (higher SMDs indicate greater imbalance; see the R package 'tableone' vignette for calculation details: https://cran.r-project.org/web/packages/tableone/tableone.pdf). After fitting each model, we re-calculated the SMD while applying the weights. Table A1.1 presents SMDs before and after weighting for each variable in the IPTW model plus descriptive statistics of the IPTW themselves. If the IPTW can

be flawlessly estimated, all covariates included in the IPTW model will be perfectly balanced across exposure groups in a weighted sample (i.e., SMD = 0). In practice, perfect balance is only achieved in fully nonparametric settings with large amounts of data relative to the number of variables in ***C***, and the SMD measures the severity of the remaining unbalance. We selected the method that produced the lowest average SMD across all confounders, which was the generalized boosted model (Table A1.1), and used this model to estimate the IPTW.

Differential abundance testing

To test for differential abundance of bacterial taxa between HIV viremia groups [HIV-, HIV+ undetectable [HIV RNA < 20 copies/mL (lower limit of detection)], HIV+ suppressed (RNA between 20 and 200 copies/mL), and HIV+ viremic (RNA > 200 copies/mL)], we utilized zero-inflated negative binomial (ZINB) or negative binomial (NB) models with multinomial least absolute shrinkage and selection operator (LASSO) models used as a confirmatory strategy. First, a pre-filtering step excluded genera with less than 100 total reads across all samples or present in less than 10% of samples; after filtering, 78 genera were tested in ZINB and LASSO models. Estimates of relative library sizes ("size factors") were obtained by calculating geometric means of pairwise read count ratios.[86] ZINB models were normalized by including log size factors as an offset term, and LASSO models utilized a normalized ASV matrix generated by dividing read counts by size factors.

ZINB model selection and analysis

Our model selection procedure was as follows: We fit six statistical models to each of 78 bacterial Genus variables. Two were ZINB models, the first including HIV viremia category as a predictor in both the count and zero-inflation components of the model, and the second including HIV only in the count component. We also fit a NB model without a zero-inflation component. Finally, we fit Poisson versions of each of the aforementioned models. All models included HIV viremia group as a categorical predictor and log "size factor" (estimate of relative

library size) as an offset term.[86] We selected the optimal model according to minimum AIC, which has been shown to outperform other methods of model selection for microbiome data.[194] ZINB was the selected model for 75/78 taxa (96%) with NB selected to model *Bacteroides, Prevotella,* and *Streptococcus*. Positive counts of these three taxa were observed in nearly all samples (97%, 100%, and 87%, respectively). As has been previously observed,[194] the Poisson models were a poor fit for these data, and none were selected as the best model by AIC. Using the optimal statistical model for each taxa, we obtained unadjusted coefficient estimates, standard errors, and *p* values, and corrected all *p* values using FDR. If the first ZINB model was selected (using HIV group in both the count and zero-inflation model components), the coefficients we report are from the count portion of the model. Next, we utilized IPTW to obtain covariate-adjusted estimates. Model-based standard errors are incorrect in inverse probability of treatment-weighted models; therefore, we used the sandwich estimator to obtain robust standard error estimates. Finally, we calculated false coverage rate (FCR)-adjusted 90% confidence intervals[90] for each IPTW-adjusted parameter estimate. FCR adjustment preserves the connection between statistical significance and confidence interval coverage after selection using the FDR method. Adjustment ensures that genera with statistically significant *q* values will have confidence intervals excluding the null value of zero and those that are nonsignificant will include zero.

LASSO model selection and analysis

We utilized two multinomial LASSO regression models, one unadjusted and one IPTW-adjusted, as confirmatory methods of feature selection. The models included the same 78 genera from ZINB testing as predictors, and HIV viremia category was the multinomial outcome variable. Prior to fitting the models, each predictor variable (count of bacterial genus) was standardized to have a mean of zero and standard deviation of one. Microbiome data are often on very different scales: Some taxa vary comparatively little across samples, while others may

be absent in many samples, but present in huge abundance in others. Standardization was performed so that the scale of each variable did not impact the likelihood of inclusion in the LASSO model.

For each model, the optimal value of the LASSO penalty coefficient λ was chosen using 500 repetitions of 10-fold cross-validation (CV). Briefly, for each repetition i = 1 to 500, the data were split into 10 randomly selected groups, or folds. Then, for folds k = 1 to 10, the kth was held out and the other 9 were used to fit the LASSO model. The fitted model was used to predict the holdout data and the mean squared error was recorded. This process was repeated for 100 values of λ, ranging from very small (admitting all 78 genera into the model) to comparatively large (allowing no predictors except an intercept term). Results were averaged across the 10 folds, resulting in a CV error curve for repetition i. There is significant uncertainty in CV error estimates because of the random selection of folds, so the process was repeated 500 times and the curves were aggregated to obtain the mean CV error for each value of λ as well as its standard error. As is standard practice in elastic net regression,[195,196] the optimal value of λ was chosen as the minimum value (which is the best fitting model for the data) plus 1 standard error. This choice is recognized as the most parsimonious model that lies within a reasonable degree of uncertainty from the “best” model.

The chosen values of λ were then used to fit unweighted and inverse probability of treatment weighted LASSO models, and taxa with nonzero coefficients were recorded as “selected” in the models. Because of the standardization, LASSO effect sizes are not directly comparable with ZINB effect sizes; however, we display in Figure 2.4 whether each nonzero effect was negative (indicating depletion of that genus) or positive (indicating enrichment).

Table A1.1. Covariates included in the inverse probability of treatment weight models. Standardized mean differences (SMDs) are given before and after weighting. Percent missing is prior to multiple imputation. GBM = generalized boosted model, LRM = logistic regression model, IQR = interquartile range.

Variable	Percent missing	SMD (before)	SMD (GBM)	SMD (LRM)
Age	0	.405	.171	.245
Employment status	2.9	.544	.254	.336
Race/Ethnicity	2.1	.484	.372	.364
Country of origin	0.5	.118	.080	.109
Homelessness	0.3	.214	.176	.227
Recent RAI	4.7	.075	.070	.138
Frequency of RAI	8.9	.192	.066	.133
Methamphetamine use	0.5	.539	.202	.213
Marijuana use	0.5	.296	.139	.263
Cocaine use	0.5	.159	.091	.134
Tobacco smoking	2.3	.262	.150	.212
Binge drinking	0.5	.397	.145	.213
Obesity	0	.171	.150	.051
Antibiotic use	0	.209	.077	.080
Sample collection type	0	.206	.078	.162
Average SMD		**.285**	**.148**	**.192**
	Range	Median	IQR (Q3 – Q1)	
IPTW (GBM)	0.14 – 3.72	0.66	0.33 (0.55 – 0.89)	
IPTW (LRM)	0.14 – 11.74	0.71	0.49 (0.57 – 1.06)	

Table A1.2. Permutational Multivariate ANOVA (PERMANOVA) of HIV category (HIV-, HIV+ undetectable, HIV+ suppressed, HIV+ viremic) on Bray-Curtis, Jaccard, and Jensen-Shannon distance matrices. Unadjusted and covariate adjusted results are shown.

Distance metric	Type	F	R^2	*p*
Bray-Curtis	Unadjusted	1.75	.014	.001
	Adjusted	1.39	.011	.017
Jaccard	Unadjusted	1.44	.011	.005
	Adjusted	1.24	.010	.030
Jensen-Shannon	Unadjusted	2.28	.018	.001
	Adjusted	1.61	.013	.019

Table A1.3. Comparisons in alpha diversity using inverse probability of treatment weighted quantile regression (median). Values are expected median differences between the group in the 'Coefficient' column and the HIV negative reference group.

Statistic	Coefficient	Value	SE	t	*p*	*q*
Observed[a]	Undetectable	11.00	9.43	1.16	0.245	0.374
Observed	Suppressed	18.00	5.93	3.03	0.003	0.022
Observed	Viremic	15.00	11.41	1.31	0.190	0.374
Chao1[b]	Undetectable	10.30	10.38	1.09	0.277	0.374
Chao1	Suppressed	18.80	6.44	2.92	0.004	0.022
Chao1	Viremic	20.80	12.68	1.64	0.102	0.305
Shannon[c]	Undetectable	0.016	0.099	0.16	0.873	0.873
Shannon	Suppressed	0.118	0.070	1.68	0.094	0.305
Shannon	Viremic	0.102	0.119	0.86	0.392	0.471
Simpson[d]	Undetectable	-0.005	0.008	-0.60	0.552	0.602
Simpson	Suppressed	0.007	0.007	1.08	0.280	0.374
Simpson	Viremic	0.008	0.007	1.14	0.255	0.374

[a]Kruskal-Wallis test for difference between groups $p = .025$. Kruskal-Wallis tests are not adjusted for confounding.
[b]Kruskal-Wallis $p = .025$
[c]Kruskal-Wallis $p = .332$
[d]Kruskal-Wallis $p = .325$

Figure A1.1. Rectal microbial composition of study participants, split by HIV viremia group. Columns represent the relative composition of each subject's microbiome at the genus level. Within HIV viremia groups, subjects are ordered by the first principal coordinate of a Bray-Curtis pairwise distance matrix. Genera representing less than 1% of the composition on average across samples were combined into "Other."

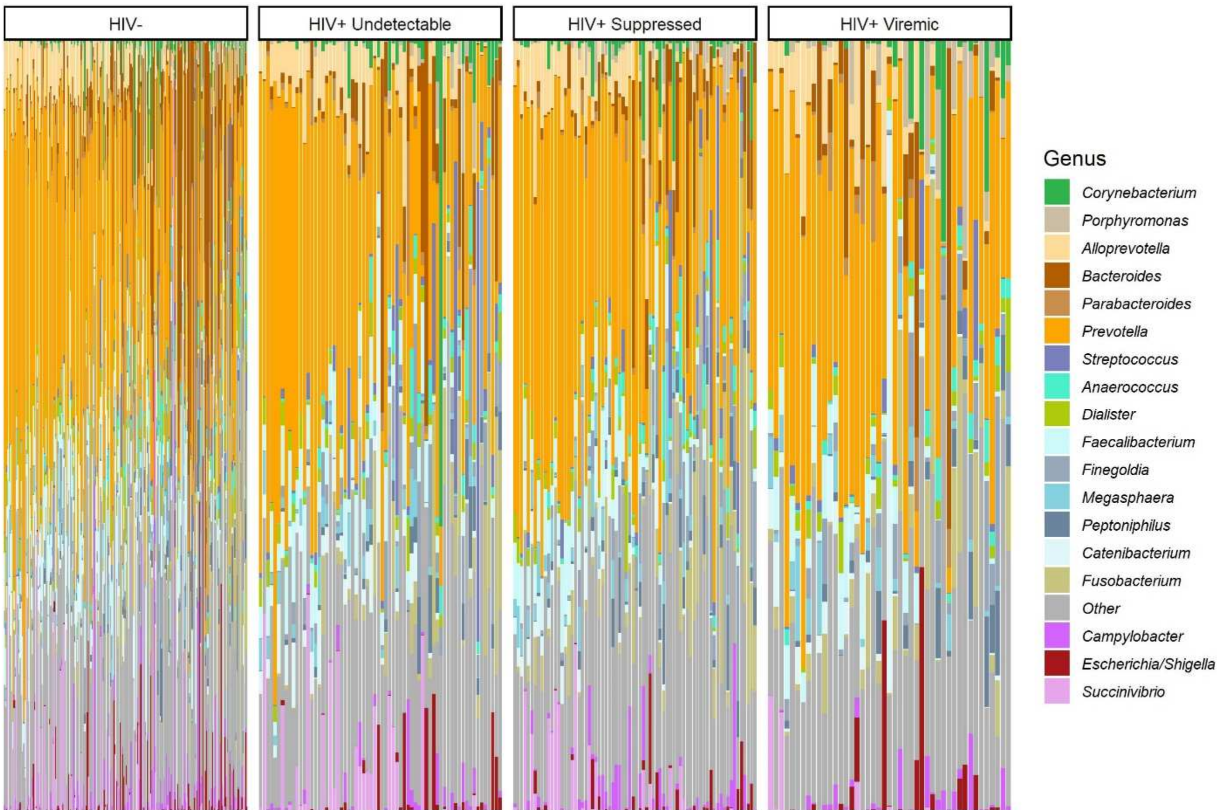


Figure A1.2. Zero-inflated negative binomial effect sizes comparing HIV-infected undetectable (RNA <20 copies/mL), suppressed (RNA ≥20-200 copies/mL), and viremic (RNA > 200 copies/mL) to HIV-uninfected participants. All taxa with at least one significant difference between groups are shown. Inverse probability of treatment-weighted effect sizes and 90% false coverage rate-adjusted confidence intervals (truncated at -4, 4) are plotted, with statistical significance ($q < 0.1$) indicated in color. Effect sizes are log ratios of normalized genera counts.

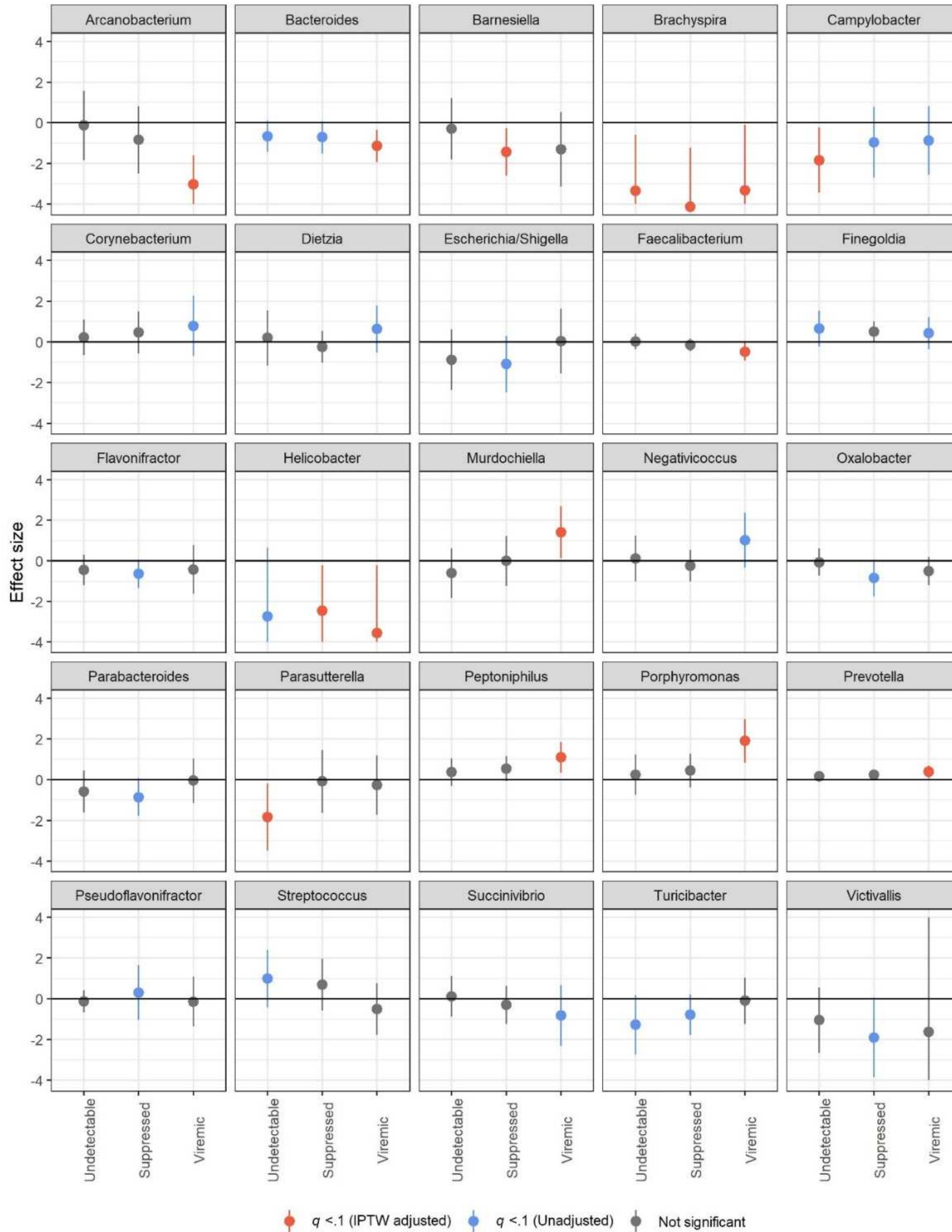
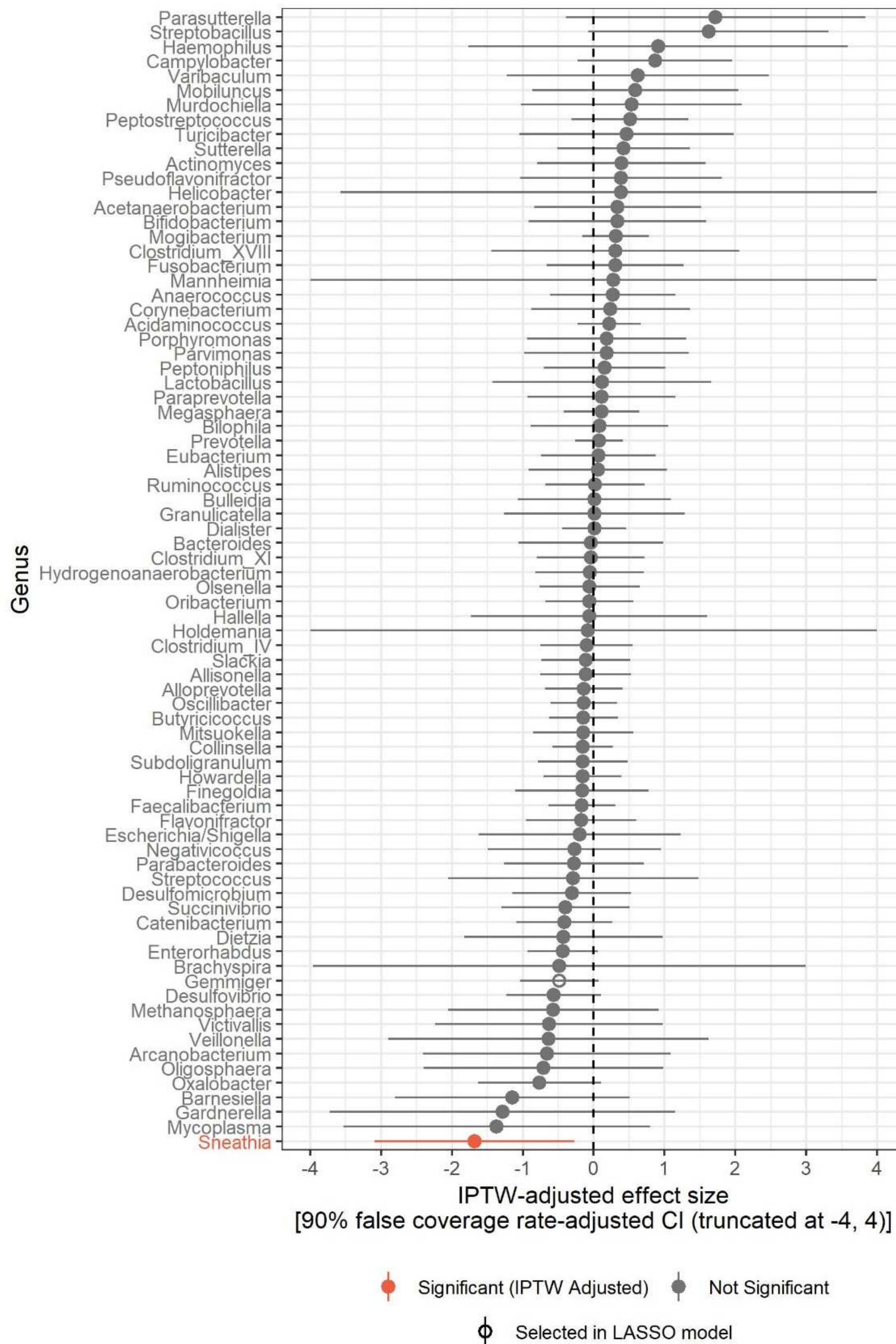


Figure A1.3. Forest plot of results of zero-inflated negative binomial models comparing genus-level bacterial counts between HIV+ suppressed (RNA ≥20-200 copies/mL) and HIV+ undetectable (RNA < 20 copies/mL, lower limit of detection) participants. Inverse probability of treatment-weighted effect sizes and 90% false coverage rate-adjusted confidence intervals (truncated at -4, 4) are plotted, with statistical significance ($q < 0.1$) indicated in color. Effect sizes are log ratios of normalized genera counts.



Appendix 2. Supplemental content for Chapter 3, "Combined Effects of HIV and Obesity on the Gastrointestinal Microbiome of Young Men who have Sex with Men"

Inverse probability of treatment weight (IPTW) calculation

Inverse probability of treatment weighting (IPTW) is a balancing procedure used to render the treatment or exposure under study independent of confounding variables. In essence, weighting by the inverse probability of treatment/exposure transforms the study sample into a "pseudo-population" where exposure status is independent of variables used in the IPTW calculation. It has been shown that if the model used to estimate the weights includes all relevant confounders and is not misspecified, in a weighted population, the effect estimate of exposure on outcome is unbiased.[87] With IPTW, the outcome models are "marginal structural models" instead of conditional on covariates, as in multiple regression. Modeling microbiome data marginally offers several advantages including the ability to control for many confounding factors without inducing overfitting bias[103] or losing efficiency due to overstratification.[104]

For a two-level exposure variable *X*, the IPTW is defined as $\frac{P(X=x)}{P(X=x|\boldsymbol{C})}$ where *x* equals 0 or 1, unexposed or exposed, and ***C*** is a (possibly high-dimensional) set of confounding variables. We have two such exposures of interest in this study: HIV and obesity, both of which are treated as dichotomous variables. As outlined by VanderWeele (2009),[197] the IPTW needed for estimation of the effects of two exposures depends on whether one wishes to study effect modification (the effect of one exposure within strata of another) or interaction (the joint, simultaneous, or combined effects of both exposures). In this study we examine both the effects of obesity on the microbiome within strata of HIV status (effect modification) and the combined effects of HIV and obesity on the microbiome (interaction); accordingly, two sets of weights were

generated. IPTW for effect modification were defined as $\frac{P(O=o|H=h)}{P(O=o|H=h,\boldsymbol{C_1})}$ for obesity status *O,* HIV status *H,* and set of covariates $\boldsymbol{C_1}$. For a list of covariates included in $\boldsymbol{C_1}$, see Tables 1 and S1. IPTW for interaction were defined as $\frac{P(O=o)P(H=h)}{P(O=o|\boldsymbol{C_1})P(H=h|\boldsymbol{C_2})}$, which is the product of the two exposure-specific weights $\frac{P(O=o)}{P(O=o|\,\boldsymbol{C_1})}$ and $\frac{P(H=h)}{P(H=h|\,\boldsymbol{C_2})}$. Covariate sets $\boldsymbol{C_1}$ and $\boldsymbol{C_2}$ were the same with the exception of two variables, ART type and an indicator for CD4 cell count < 200, which were included in the set for obesity (i.e. included in $\boldsymbol{C_1}$) but not for HIV, given that these variables are generally not applicable to HIV- participants.

In theory, weighting by the IPTW will perfectly balance all covariates used to create the weights across exposure groups, thereby eliminating the possibility that these covariates can act as confounders. Because of finite sample sizes and the need for parametric models, perfect balance is never achieved in practice, except in the most trivial cases. The absolute value of the standardized mean difference (SMD), which is the mean difference in a covariate between groups in units of its standard deviation, is a metric commonly used to measure the performance of IPTW estimates. SMDs for each covariate in the IPTW model are calculated before and after weighting, and reductions in covariate-specific SMDs represent reductions in confounding by that covariate. A substantial reduction in the average SMD across all covariates indicates the IPTW, when applied during the exposure-outcome analysis, will significantly reduce confounding. There is no agreed-upon “cut-off” SMD value indicating adequate confounding control; some have suggested 0.1,[198] but others have indicated that is probably unnecessarily conservative.[199] Table A2.1 shows the SMD for each covariate included in the IPTW models for HIV and obesity, before and after weighting, as well as the average SMD before and after applying each IPTW. We achieve an average SMD of .09 for balance across HIV status, and .12 for balance across obesity.

Testing for differential abundance of bacterial genera

We utilized zero-inflated negative binomial (ZINB) or negative binomial (NB) models to test for differential abundance of bacterial genera by HIV and obesity status.[194] Prior to analysis, we conducted a pre-filtering step to exclude genera present in less than 10% of samples and those comprised of less than 100 total reads across all samples. After this pre-filtering step, 78 genera were included in differential abundance analyses. A model selection procedure that has been previously described (Appendix 1)[119] was used to determine the optimal statistical model for each genus. Briefly, we fit Poisson and negative binomial models with IPTW adjustment, plus or minus zero-inflation components, to each genus and selected the best fitting model by AIC. HIV status, obesity, and their interaction were included as predictors in the count portion of every model. As part of the model selection process, we also considered their inclusion in the zero-inflation component; however, the coefficients we report come from the count portion of the model even if the optimal model also included HIV and obesity in the zero-inflation component. All ZINB models included (log) estimates of relative library sizes ("size factors", calculated using the geometric mean of pairwise read ratios method[86]) as offset terms. All analyses adjusted for confounding using IPTW, and standard errors for comparisons of interest were obtained using the sandwich estimator.

Differences in specific genera associated with obesity, stratified by HIV status. Using the optimal statistical model, we estimated the effect of obesity on each of 78 bacterial genera within strata of HIV status: HIV+, Obese vs. Non-obese and HIV-, Obese vs. Non-obese. We applied a false discovery rate (FDR)[89] correction to the resulting vector of 156 p values (two comparisons for each genus) and considered effects with $q < 0.1$ (q being the FDR-corrected p value) as statistically significant. In accordance with the FDR correction, we also calculated 90% false coverage rate (FCR)-adjusted confidence intervals[90]. Results are shown graphically in Figure 3.3.

Effects of HIV and obesity together on microbial abundance. Using the optimal statistical model for each genus, we first conducted a joint test of the three hypotheses of interest: HIV+/Obese (H+O+) vs. HIV+/Non-obese (H+O-), H+O+ vs. HIV-/Obese (H-O+), and H+O+ vs. HIV-/Non-obese (H-O-). We applied an FDR correction to the vector of 78 *p* values resulting from these joint tests and retained genera with $q < 0.1$, indicating that there was a significant difference between the H+O+ group and at least one other group, for further investigation. We then performed each individual comparison of interest (i.e., H+O+ vs. H+O-, H+O+ vs. H-O+, and H+O+ vs. H-O-) for the twenty-one genera that were retained and applied another FDR correction to the 63 resulting *p* values. Results of these comparisons are graphically displayed in Figure 3.4.

Table A2.1. Distribution of participants characteristics included in inverse probability of treatment weight models, N = 381 men who have sex with men in Los Angeles, CA

	Obese Mean (sd)/n (%)	Non-Obese	SMD (Pre, Post)	HIV+	HIV-	SMD (Pre, Post)
n	103	278		182	199	
Age	32.46 (6.6)	30.68 (7.0)	.26, .14	33.63 (6.5)	28.90 (6.5)	.73, .19
Race/ Ethnicity			.23, .13			.10, .05
Black-Non Hispanic	43 (41.7)	107 (38.5)		68 (37.4)	82 (41.2)	
Hispanic	53 (51.5)	134 (48.2)		90 (49.5)	97 (48.7)	
Other-Non Hispanic	7 (6.8)	37 (13.3)		24 (13.2)	20 (10.1)	
Country of origin			.07, .06			.18, .16
US	87 (84.5)	227 (81.7)		144 (79.1)	170 (85.4)	
Other						
Homeless in past 6 months	27 (26.2)	101 (36.3)	.23, .09	63 (34.6)	65 (32.7)	.03, .07
Number of R AI acts in pas t month	1.60 (2.89)	2.79 (5.73)	.27, .15	2.92 (5.99)	2.07 (4.19)	.19, .04
Number of sex partners i n past 6 months	6.08 (7.6)	7.68 (8.7)	.20, .06	7.55 (9.0)	6.97 (7.8)	.07, .02
Positive for rectal STI	6 (5.8)	41 (14.7)	.30, .24			.11, .07
Methamph-et amine use in past 6 months			.26, .16			.63, .14
Daily/ Weekly	13 (12.6)	61 (21.9)		53 (29.1)	21 (10.6)	
Monthly/ less	26 (25.2)	55 (19.8)		49 (26.9)	32 (16.1)	
Never	64 (62.1)	162 (58.3)		80 (44.0)	146 (73.4)	
Marijuana use			.08, .10			.26, .10
Daily/ Weekly	32 (31.1)	97 (34.9)		59 (32.4)	70 (35.2)	
Monthly/ less	26 (25.2)	67 (24.1)		36 (19.8)	57 (28.6)	
Never	45 (43.7)	114 (41.0)		87 (47.8)	72 (36.2)	
Tobacco smoker	41 (39.8)	126 (45.3)	.12, .07	90 (49.5)	77 (38.7)	.21, .03
Binge drinking in past 6 months[b]			.21, .11			.44, .12
Weekly	11 (10.7)	43 (15.5)		31 (17.0)	23 (11.6)	

Monthly/ less	54 (52.4)	119 (42.8)		62 (34.1)	111 (55.8)	
Never	38 (36.9)	116 (41.7)		89 (48.9)	65 (32.7)	
Antibiotic use	7 (6.8)	24 (8.6)	.07, .05	20 (11.0)	11 (5.5)	.20, .13
Sample collection strategy			.17, .10			.03, .04
Anoscopy	73 (70.9)	218 (78.4)		138 (75.8)	153 (76.9)	
Self-collected	30 (29.1)	60 (21.6)		44 (24.2)	46 (23.1)	
CD4 count < 200	3 (2.9)	11 (4)	.06, .07			N/A
ART regimen			.38, .24			N/A
INSTI + NRTI	17 (16.5)	52 (18.7)				
NNRTI + NRTI	5 (4.9)	43 (15.5)				
NRTI + PI	8 (7.8)	22 (7.9)				
NRTI[c]	12 (11.7)	26 (9.4)				
Other	5 (4.9)	11 (4.0)				
Missing/ Not reported/None	56 (56.4)	124 (44.6)				
Average SMD			.19, .12			.24, .09

SMD = Standardized mean difference; RAI = Receptive anal intercourse; STI = Sexually transmitted infection; ART = Antiretroviral therapy; INSTI = Integrase strand transfer inhibitor; NRTI = Nucleoside reverse transcriptase inhibitor; NNRTI = Non-nucleoside reverse transcriptase inhibitor; PI = Protease inhibitor

[a]Sexually transmitted infections include gonorrhea, chlamydia, and syphilis.

[b]Binge drinking defined as 6 or more drinks on one occasion.

[c]HIV negative men taking tenofovir disoproxil fumarate/emtricitabine for pre-exposure prophylaxis (PrEP).

Table A2.2. Permutational Multivariate ANOVA (PERMANOVA) of the combined effect of HIV and obesity on Bray-Curtis, Jaccard, and Jensen-Shannon dissimilarity statistics. Values represent the marginal effect of HIV plus obesity after accounting for the variation explained by each factor separately. Unadjusted and covariate adjusted results are shown.

Distance metric	Type	F	R2	p
Bray-Curtis	Unadjusted	2.80	.007	.002
	Adjusted	3.06	.008	.001
Jaccard	Unadjusted	2.20	.006	.001
	Adjusted	2.39	.006	.001
Jensen-Shannon	Unadjusted	3.60	.009	.002
	Adjusted	3.82	.01	.002

Table A2.3. Mean differences in alpha diversity statistics between HIV+/Obese (H+O+) and HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), HIV+/Non-obese (H+O-) participants. Analyses were adjusted for confounding using inverse probability of treatment weighting.

Contrast	Statistic	Mean difference	Standard error	*t*	*p*
H+O+ vs. H-O-	Observed	15.4	8.3	1.85	0.066
	Chao1	15.9	8.7	1.82	0.069
	Shannon	0.2	0.1	1.82	0.069
	Simpson	0.03	0.02	2.18	0.030
H+O+ vs. H-O+	Observed	16.9	8.4	2.00	0.046
	Chao1	18.5	8.8	2.10	0.036
	Shannon	0.18	0.1	1.81	0.071
	Simpson	0.019	0.011	1.68	0.094
H+O+ vs. H+O-	Observed	4.7	8.2	0.58	0.6
	Chao1	4.9	8.7	0.57	0.6
	Shannon	0.06	0.09	0.72	0.5
	Simpson	0.01	0.01	1.03	0.3

Figure A2.1. Rectal microbial composition of study participants, N = 383. Columns represent the relative composition of each subject's microbiome at the genus level. HIV and obesity status of the subjects is indicated by a colored line below their microbial composition [HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), HIV+/Non-obese (H+O-), and HIV+/Obese (H+O+)]. Subjects are ordered by the first principal coordinate of a Bray-Curtis pairwise distance matrix. Genera representing less than 1% of the composition on average across samples were combined into "Other."

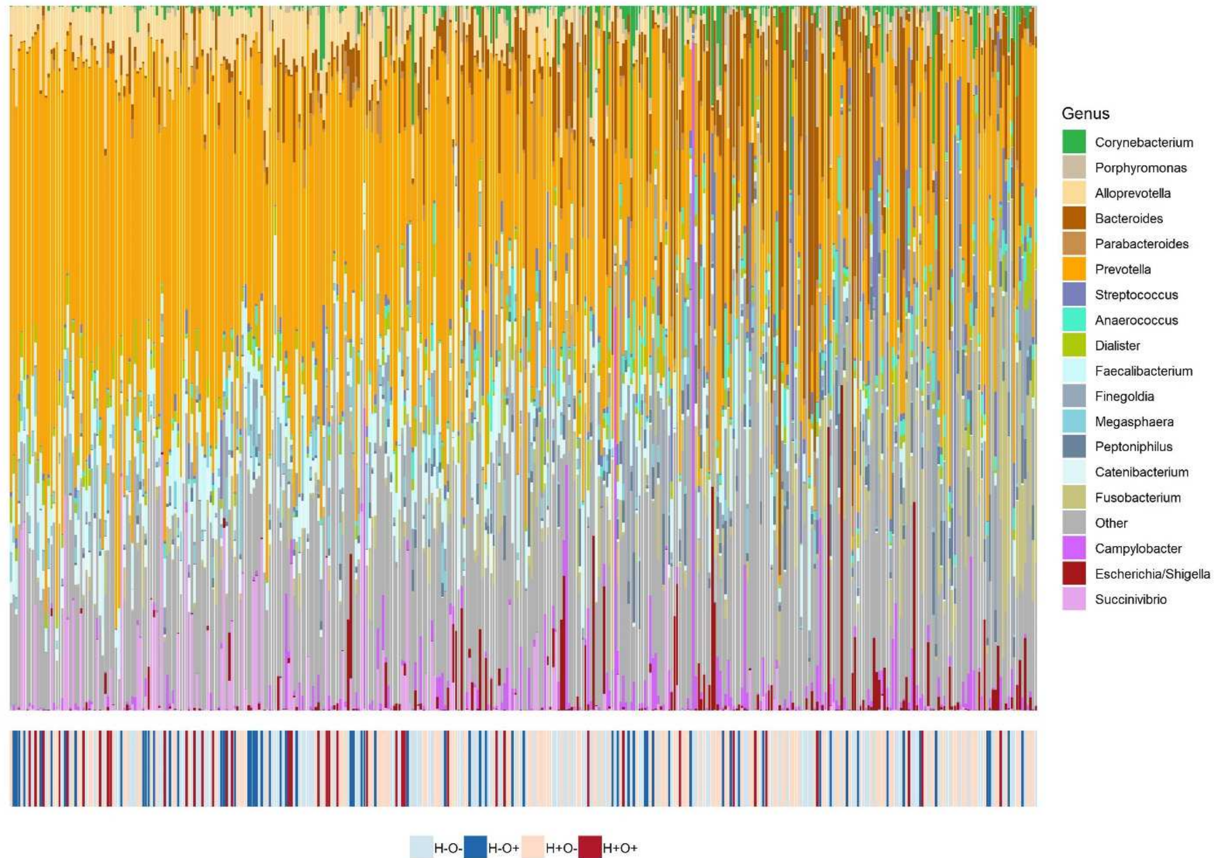


Figure A2.2. Rectal microbial composition of study participants, split by HIV and obesity status. Columns represent the relative composition of each subject's microbiome at the genus level. Within HIV and obesity groups, subjects are ordered by the first principal coordinate of a Bray-Curtis pairwise distance matrix. Genera representing less than 1% of the composition on average across samples were combined into "Other."

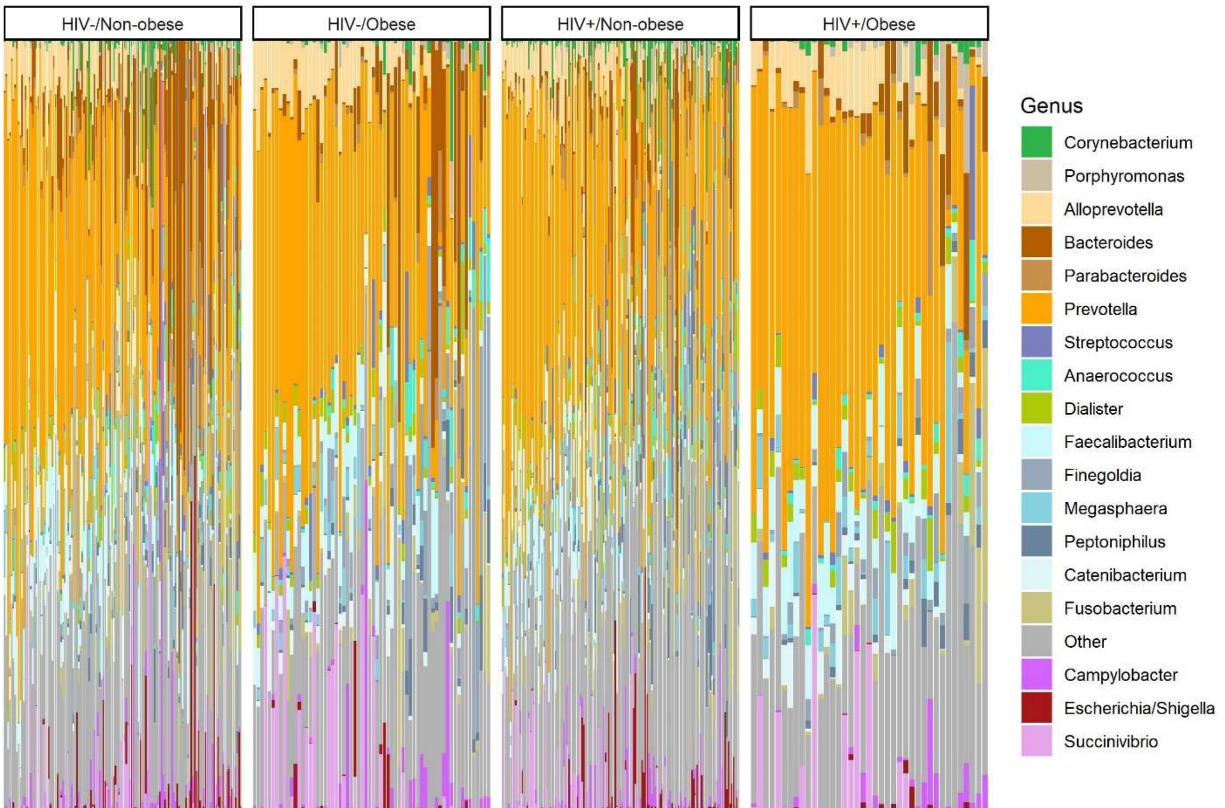


Figure A2.3. Ordination of Bray-Curtis, Jaccard, and Jensen-Shannon distances using principal coordinates analysis (PCoA). Groups are HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), HIV+/Non-obese (H+O-), and HIV+/Obese (H+O+). Ellipses are 95% confidence regions for each group assuming points follow a multivariate *t* distribution.

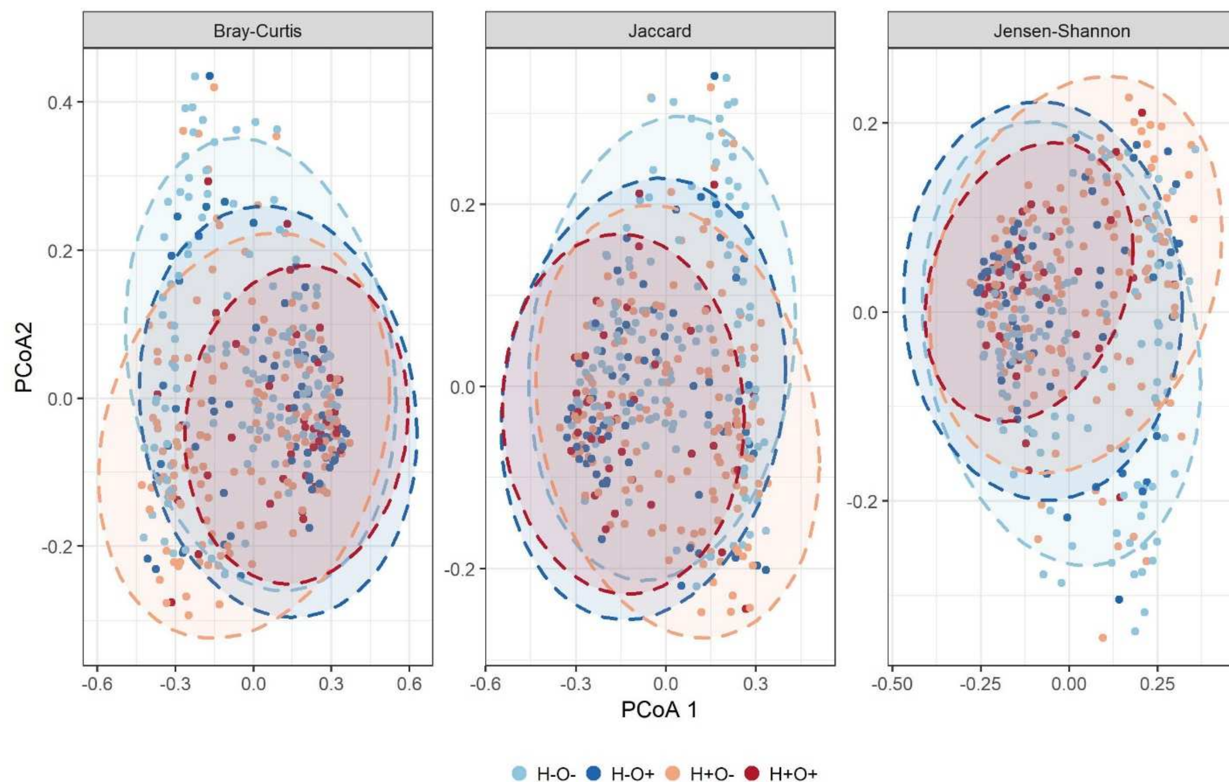
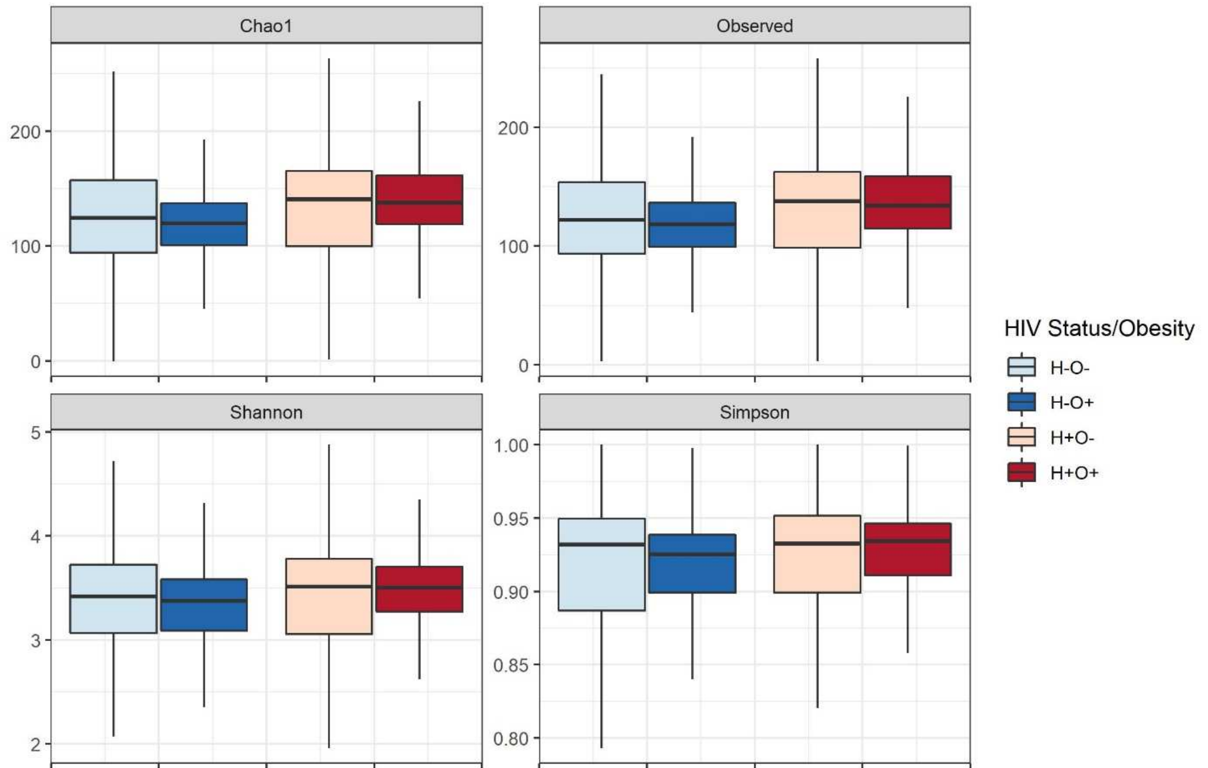


Figure A2.4. Boxplots of alpha diversity statistics. Groups are HIV-/Non-obese (H-O-), HIV-/Obese (H-O+), HIV+/Non-obese (H+O-), and HIV+/Obese (H+O+). Boxes represent the lower, median, and upper quartile of the data and whiskers are 1.5*interquartile range.



Appendix 3. Supplemental content for Chapter 4, "Alterations to the Gastrointestinal Microbiome Associated with Methamphetamine Use"

Inverse probability of treatment weight (IPTW) calculation

For a two-level exposure variable *X*, the IPTW is defined as $\frac{P(X=x)}{P(X=x|C)}$ where *x* equals 0 or 1, unexposed or exposed, and *C* is a (possibly high-dimensional) set of confounding variables. In this paper, we consider potential interactions between two such variables: HIV status and MA use. As outlined by VanderWeele (2009)[197], the IPTW needed for estimation of the effects of two exposures depends on whether one wishes to study effect modification (the effect of one exposure within strata of another) or interaction (the joint, simultaneous, or combined effects of both exposures). In this study we examine the effects of MA use on the microbiome within strata of HIV status (effect modification); accordingly, IPTW were defined as $\frac{P(M=m|H=h)}{P(M=m|H=h,C)}$ for MA use status *M* (user, non-user), HIV status *H* (HIV+, HIV-), and set of covariates *C*. For a list of covariates included in *C*, see Table 4.1. For the dose-response analyses, where MA use was treated as a four-category variable, IPTW were defined as $\frac{P(M=m)}{P(M=m|C)}$ for MA use frequency $M \in$ {Never, Monthly or less, Weekly, Daily. HIV status was included as an additional covariate in dose-response ZINB models. IPTW were estimated using generalized boosted models[193].

Table 4.1 shows the SMD for each covariate included in the IPTW model, before and after weighting, as well as the average SMD before and after applying the weights. Prior to weighting, the average SMD for balance across MA use was 0.28; after applying IPTW, it was 0.14.

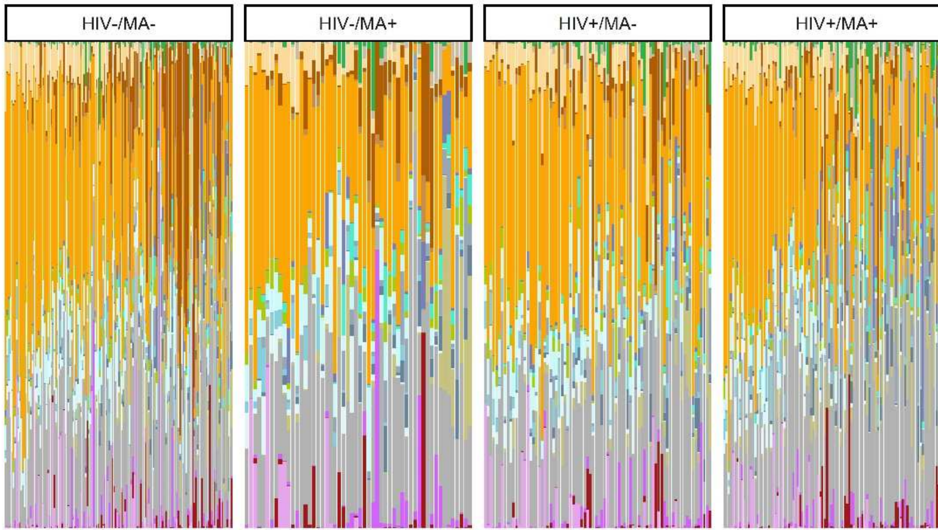
Table A3.1. *p* and *q* values for interaction between HIV and MA use

Overall composition (PERMANOVA)	
Statistic	Interaction *p* value
Bray-Curtis	.193
Jaccard	.252
Jensen-Shannon	.115
Alpha diversity	
Statistic	Interaction *p* value
Observed	.236
Chao1	.285
Shannon	.465
Simpson	.909
Individual genera	
Genus	Interaction *q* value
Acetanaerobacterium	0.732
Acidaminococcus	0.873
Actinomyces	0.095
Alistipes	0.801
Allisonella	0.924
Alloprevotella	0.791
Anaerococcus	0.873
Arcanobacterium	0.924
Bacteroides	0.786
Barnesiella	0.791
Bifidobacterium	0.904
Bilophila	0.791
Brachyspira	0.924
Bulleidia	0.095
Butyricicoccus	0.904
Campylobacter	0.857
Catenibacterium	0.505
Clostridium_IV	0.904
Clostridium_XI	0.34
Clostridium_XVIII	0.783
Collinsella	0.267
Corynebacterium	0.924
Desulfomicrobium	0.924
Desulfovibrio	0.924
Dialister	0.445
Dietzia	0.924
Enterorhabdus	0.791
Escherichia/Shigella	0.924
Eubacterium	0.791
Faecalibacterium	0.857
Finegoldia	0.791
Flavonifractor	0.791
Fusobacterium	0.873
Gardnerella	0.873
Gemmiger	0.791
Granulicatella	0.924
Haemophilus	0.873
Hallella	0.857
Helicobacter	0.527

Holdemania	0.267
Howardella	0.305
Hydrogenoanaerobacterium	0.791
Lactobacillus	0.791
Mannheimia	0.001
Megasphaera	0.873
Methanosphaera	0.402
Mitsuokella	0.857
Mobiluncus	0.924
Mogibacterium	0.873
Murdochiella	0.904
Mycoplasma	0.924
Negativicoccus	0.001
Oligosphaera	0.106
Olsenella	0.549
Oribacterium	0.924
Oscillibacter	0.857
Oxalobacter	0.873
Parabacteroides	0.783
Paraprevotella	0.267
Parasutterella	0.924
Parvimonas	0.904
Peptoniphilus	0.913
Peptostreptococcus	0.924
Porphyromonas	0.791
Prevotella	0.857
Pseudoflavonifractor	0.791
Ruminococcus	0.857
Slackia	0.267
Sneathia	0.791
Streptobacillus	0.095
Streptococcus	0.873
Subdoligranulum	0.924
Succinivibrio	0.627
Sutterella	0.791
Turicibacter	0.873
Varibaculum	0.857
Veillonella	0.791
Victivallis	0.873

Figure A3.1. Rectal microbial composition of study participants, stratified by methamphetamine (MA) use and HIV status.

A



B

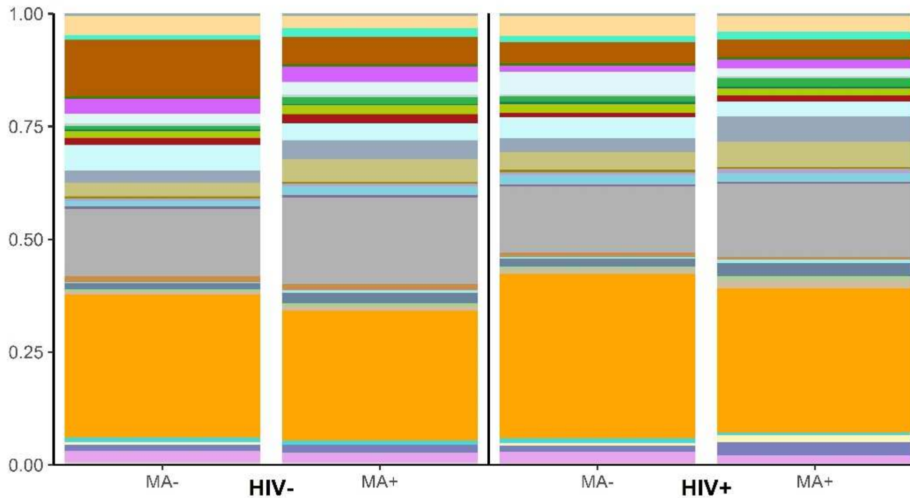


Figure A3.2. Associations between methamphetamine (MA) use and overall microbial composition and diversity, stratified by HIV status.

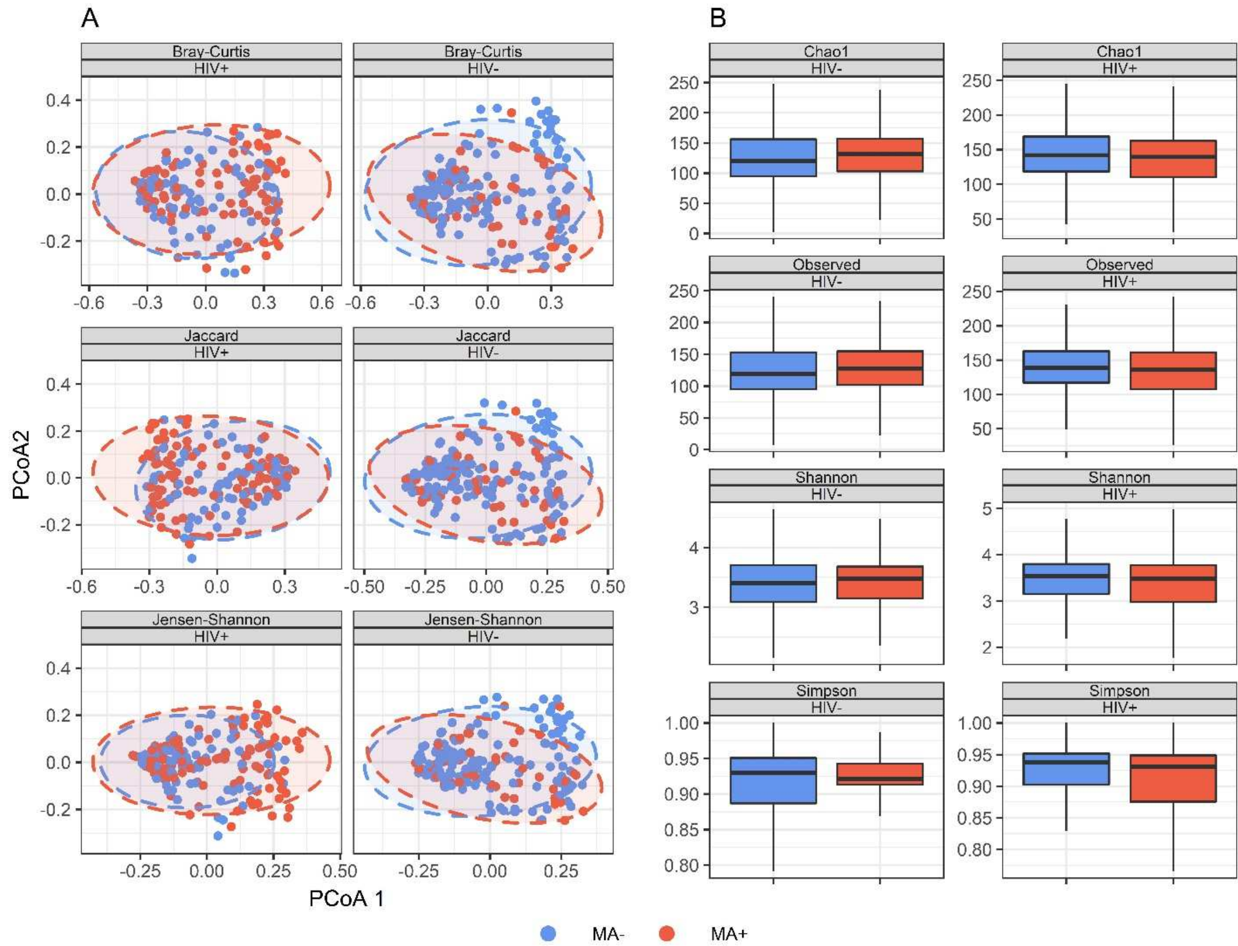
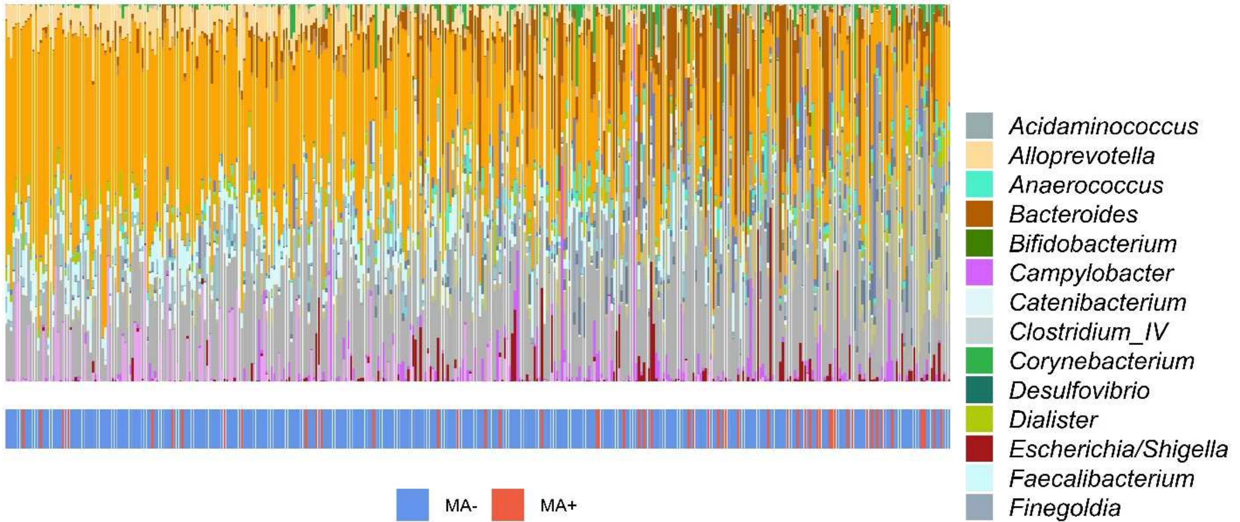


Figure A3.3. Replication of Figure 4.1, with participants' MA status determined by urine drug screen.

A



B

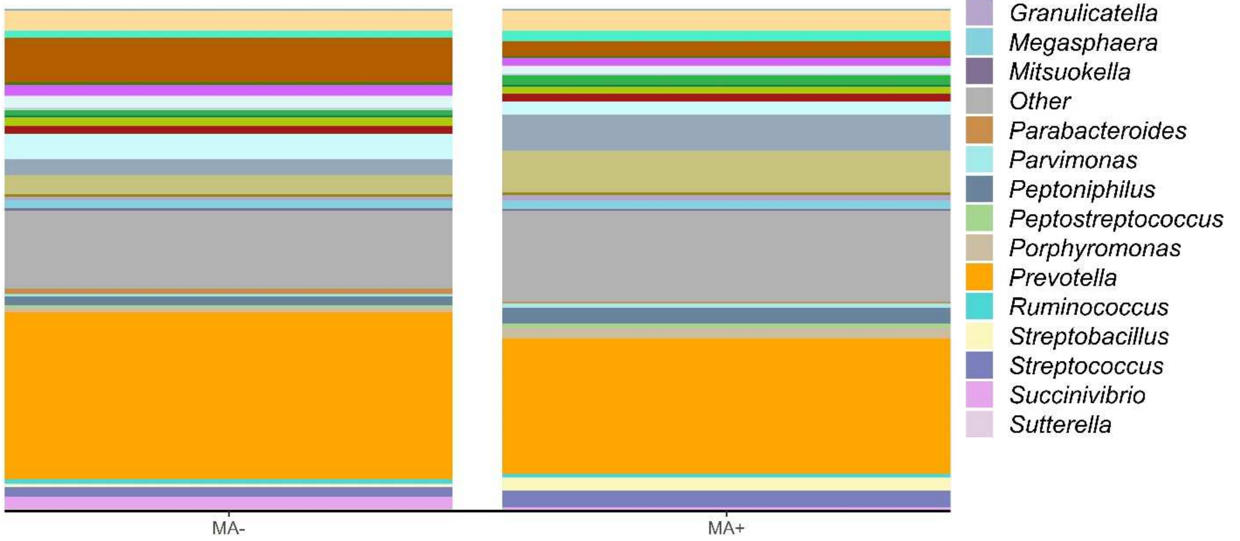


Figure A3.4. Replication of Figure 4.2, with participants' MA status determined by urine drug screen.

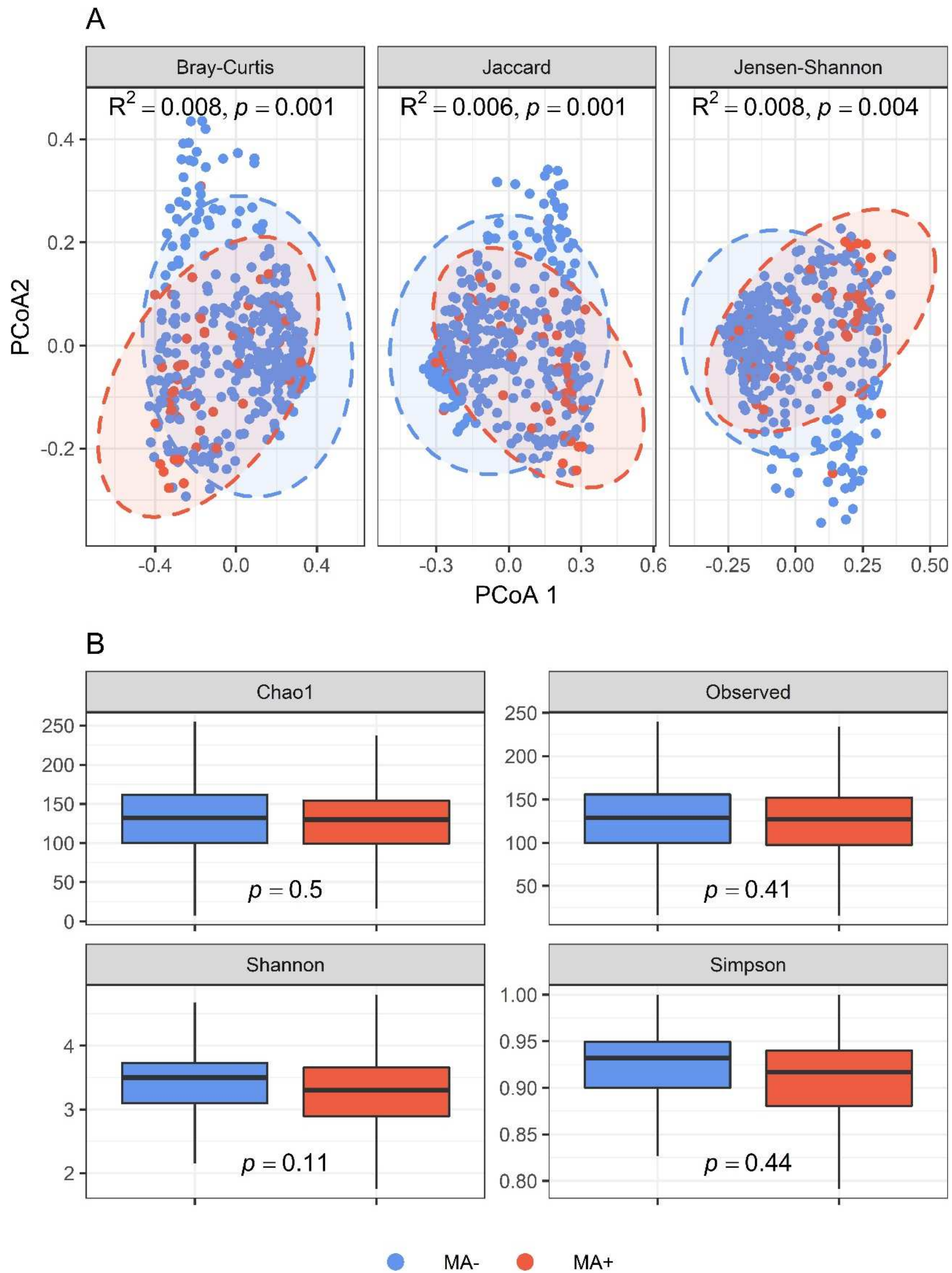
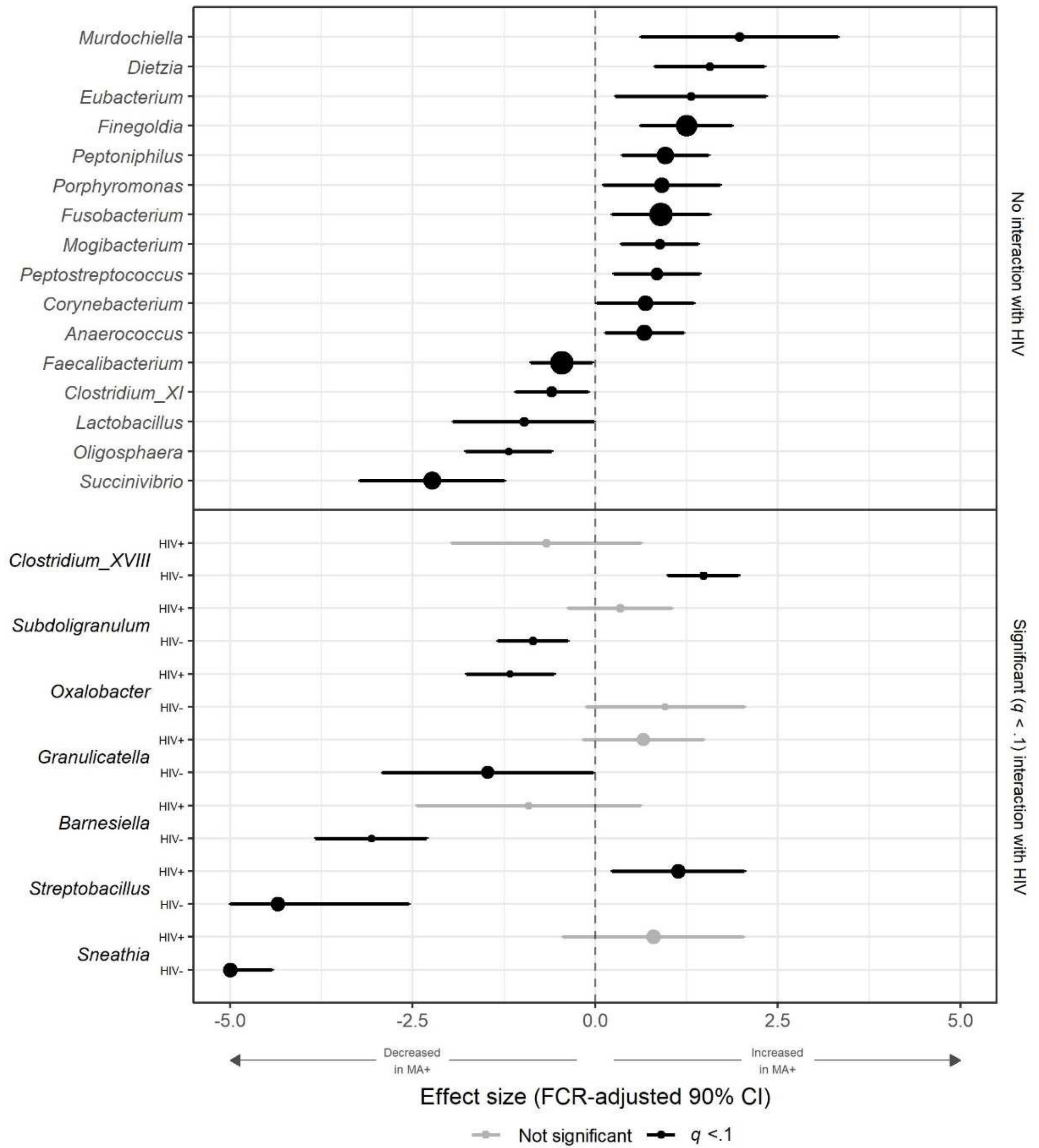


Figure A3.5. Replication of Figure 4.3, with participants' MA status determined by urine drug screen.



References

1. Siddiqi AE, Hall HI, Hu X, Song R. Population-Based Estimates of Life Expectancy After HIV Diagnosis: United States 2008-2011. *Journal of acquired immune deficiency syndromes (1999).* 2016;72(2):230-236.

2. Siddiqi AE, Hu X, Hall HI. Mortality among blacks or African Americans with HIV infection--United States, 2008-2012. *MMWR Morbidity and mortality weekly report.* 2015;64(4):81-86.

3. Wandeler G, Johnson LF, Egger M. Trends in life expectancy of HIV-positive adults on antiretroviral therapy across the globe: comparisons with general population. *Current opinion in HIV and AIDS.* 2016;11(5):492-500.

4. So-Armah KA, Tate JP, Chang CH, et al. Do Biomarkers of Inflammation, Monocyte Activation, and Altered Coagulation Explain Excess Mortality Between HIV Infected and Uninfected People? *Journal of acquired immune deficiency syndromes (1999).* 2016;72(2):206-213.

5. Kuller LH, Tracy R, Belloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS medicine.* 2008;5(10):e203.

6. Sandler NG, Wand H, Roque A, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *The Journal of infectious diseases.* 2011;203(6):780-790.

7. Clifford DB. HIV-associated neurocognitive disorder. *Current opinion in infectious diseases.* 2017;30(1):117-122.

8. Goncalves PH, Montezuma-Rusca JM, Yarchoan R, Uldrick TS. Cancer prevention in HIV-infected populations. *Seminars in oncology.* 2016;43(1):173-188.

9. Gutierrez J, Albuquerque ALA, Falzon L. HIV infection as vascular risk: A systematic review of the literature and meta-analysis. *PloS one.* 2017;12(5):e0176686.

10. Willig AL, Overton ET. Metabolic Complications and Glucose Metabolism in HIV Infection: A Review of the Evidence. *Current HIV/AIDS reports.* 2016;13(5):289-296.

11. Losina E, Hyle EP, Borre ED, et al. Projecting 10-yr, 20-yr and Lifetime Risks of Cardiovascular Disease in Persons Living with HIV in the US. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2017.

12. Mimiaga MJ, Reisner SL, Grasso C, et al. Substance use among HIV-infected patients engaged in primary care in the United States: findings from the Centers for AIDS Research Network of Integrated Clinical Systems cohort. *American journal of public health.* 2013;103(8):1457-1467.

13. Crum-Cianflone N, Roediger MP, Eberly L, et al. Increasing rates of obesity among HIV-infected persons during the HIV epidemic. *PloS one.* 2010;5(4):e10106.

14. Erlandson KM, Lake JE. Fat Matters: Understanding the Role of Adipose Tissue in Health in HIV Infection. *Current HIV/AIDS reports.* 2016;13(1):20-30.

15. Volpe GE, Ward H, Mwamburi M, et al. Associations of cocaine use and HIV infection with the intestinal microbiota, microbial translocation, and inflammation. *Journal of studies on alcohol and drugs.* 2014;75(2):347-357.

16. Cavalcante-Silva LH, Galvao JG, da Silva JS, de Sales-Neto JM, Rodrigues-Mascarenhas S. Obesity-Driven Gut Microbiota Inflammatory Pathways to Metabolic Syndrome. *Frontiers in physiology.* 2015;6:341.

17. Cox AJ, West NP, Cripps AW. Obesity, inflammation, and the gut microbiota. *The lancet Diabetes & endocrinology.* 2015;3(3):207-215.

18. Sanz Y, Moya-Perez A. Microbiota, inflammation and obesity. *Advances in experimental medicine and biology.* 2014;817:291-317.

19. Mata MM, Napier TC, Graves SM, Mahmood F, Raeisi S, Baum LL. Methamphetamine decreases CD4 T cell frequency and alters pro-inflammatory cytokine production in a model of drug abuse. *European journal of pharmacology.* 2015;752:26-33.

20. Gao B, Li L, Zhu P, et al. Chronic administration of methamphetamine promotes atherosclerosis formation in ApoE-/- knockout mice fed normal diet. *Atherosclerosis.* 2015;243(1):268-277.

21. Costello EJ, Copeland WE, Shanahan L, Worthman CM, Angold A. C-reactive protein and substance use disorders in adolescence and early adulthood: a prospective analysis. *Drug and alcohol dependence.* 2013;133(2):712-717.

22. Koethe JR, Heimburger DC, PrayGod G, Filteau S. From Wasting to Obesity: The Contribution of Nutritional Status to Immune Activation in HIV Infection. *The Journal of infectious diseases.* 2016;214 Suppl 2:S75-82.

23. Mudd JC, Brenchley JM. Gut Mucosal Barrier Dysfunction, Microbial Dysbiosis, and Their Role in HIV-1 Disease Progression. *The Journal of infectious diseases.* 2016;214 Suppl 2:S58-66.

24. Brenchley JM, Douek DC. Microbial translocation across the GI tract. *Annual review of immunology.* 2012;30:149-173.

25. Marchetti G, Tincati C, Silvestri G. Microbial translocation in the pathogenesis of HIV infection and AIDS. *Clinical microbiology reviews.* 2013;26(1):2-18.

26. Torres B, Guardo AC, Leal L, et al. Protease inhibitor monotherapy is associated with a higher level of monocyte activation, bacterial translocation and inflammation. *Journal of the International AIDS Society.* 2014;17:19246.

27. Leclercq S, Cani PD, Neyrinck AM, et al. Role of intestinal permeability and inflammation in the biological and behavioral control of alcohol-dependent subjects. *Brain, behavior, and immunity.* 2012;26(6):911-918.

28. Monnig MA. Immune activation and neuroinflammation in alcohol use and HIV infection: evidence for shared mechanisms. *The American journal of drug and alcohol abuse.* 2017;43(1):7-23.

29. Lynch SV, Pedersen O. The Human Intestinal Microbiome in Health and Disease. *The New England journal of medicine.* 2016;375(24):2369-2379.

30. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell.* 2014;157(1):121-141.

31. Olszak T, An D, Zeissig S, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science (New York, NY).* 2012;336(6080):489-493.

32. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in immunology.* 2007;19(2):59-69.

33. Bauer H, Horowitz RE, Levenson SM, Popper H. The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. *The American journal of pathology.* 1963;42:471-483.

34. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature.* 2008;453(7195):620-625.

35. Furusawa Y, Obata Y, Fukuda S, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature.* 2013;504(7480):446-450.

36. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. *Nature immunology.* 2013;14(7):685-690.

37. Franchi L, Kamada N, Nakamura Y, et al. NLRC4-driven production of IL-1beta discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nature immunology.* 2012;13(5):449-456.

38. Ivanov, II, Frutos Rde L, Manel N, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell host & microbe.* 2008;4(4):337-349.

39. Dillon SM, Frank DN, Wilson CC. The gut microbiome and HIV-1 pathogenesis: a two-way street. *AIDS (London, England).* 2016;30(18):2737-2751.

40. Gootenberg DB, Paer JM, Luevano JM, Kwon DS. HIV-associated changes in the enteric microbial community: potential role in loss of homeostasis and development of systemic inflammation. *Current opinion in infectious diseases.* 2017;30(1):31-43.

41. Zilberman-Schapira G, Zmora N, Itav S, Bashiardes S, Elinav H, Elinav E. The gut microbiome in human immunodeficiency virus infection. *BMC medicine.* 2016;14(1):83.

42. Zevin AS, McKinnon L, Burgener A, Klatt NR. Microbial translocation and microbiome dysbiosis in HIV-associated immune activation. *Current opinion in HIV and AIDS.* 2016;11(2):182-190.

43. Liu J, Williams B, Frank D, Dillon SM, Wilson CC, Landay AL. Inside Out: HIV, the Gut Microbiome, and the Mucosal Immune System. *Journal of immunology (Baltimore, Md : 1950).* 2017;198(2):605-614.

44. Hileman CO, Funderburg NT. Inflammation, Immune Activation, and Antiretroviral Therapy in HIV. *Current HIV/AIDS reports.* 2017;14(3):93-100.

45. d'Ettorre G, Ceccarelli G, Giustini N, et al. Probiotics Reduce Inflammation in Antiretroviral Treated, HIV-Infected Individuals: Results of the "Probio-HIV" Clinical Trial. *PloS one.* 2015;10(9):e0137200.

46. Vujkovic-Cvijin I, Dunham RM, Iwai S, et al. Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Science translational medicine.* 2013;5(193):193ra191.

47. Dillon SM, Lee EJ, Kotter CV, et al. An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal immunology.* 2014;7(4):983-994.

48. Nowak P, Troseid M, Avershina E, et al. Gut microbiota diversity predicts immune status in HIV-1 infection. *AIDS (London, England).* 2015;29(18):2409-2418.

49. Mutlu EA, Keshavarzian A, Losurdo J, et al. A compositional look at the human gastrointestinal microbiome and immune activation parameters in HIV infected subjects. *PLoS pathogens.* 2014;10(2):e1003829.

50. Yu G, Fadrosh D, Ma B, Ravel J, Goedert JJ. Anal microbiota profiles in HIV-positive and HIV-negative MSM. *AIDS (London, England).* 2014;28(5):753-760.

51. Guentzel MN. Escherichia, klebsiella, enterobacter, serratia, citrobacter, and proteus. 1996.

52. Lupp C, Robertson ML, Wickham ME, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell host & microbe.* 2007;2(2):119-129.

53. Vazquez-Castellanos JF, Serrano-Villar S, Latorre A, et al. Altered metabolism of gut microbiota contributes to chronic immune activation in HIV-infected individuals. *Mucosal immunology.* 2015;8(4):760-772.

54. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. *Nature reviews Gastroenterology & hepatology.* 2012;9(10):577-589.

55. Alcock J, Maley CC, Aktipis CA. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. *BioEssays : news and reviews in molecular, cellular and developmental biology.* 2014;36(10):940-949.

56. Temko JE, Bouhlal S, Farokhnia M, Lee MR, Cryan JF, Leggio L. The Microbiota, the Gut and the Brain in Eating and Alcohol Use Disorders: A 'Menage a Trois'? *Alcohol and alcoholism (Oxford, Oxfordshire).* 2017;52(4):403-413.

57. Anhe FF, Varin TV, Le Barz M, et al. Gut Microbiota Dysbiosis in Obesity-Linked Metabolic Diseases and Prebiotic Potential of Polyphenol-Rich Extracts. *Current obesity reports.* 2015;4(4):389-400.

58. Duranti S, Ferrario C, van Sinderen D, Ventura M, Turroni F. Obesity and microbiota: an example of an intricate relationship. *Genes & nutrition.* 2017;12:18.

59. Sze MA, Schloss PD. Looking for a Signal in the Noise: Revisiting Obesity and the Microbiome. *mBio.* 2016;7(4).

60. Greiner T, Backhed F. Effects of the gut microbiota on obesity and glucose homeostasis. *Trends in endocrinology and metabolism: TEM.* 2011;22(4):117-123.

61. Zietak M, Kovatcheva-Datchary P, Markiewicz LH, Stahlman M, Kozak LP, Backhed F. Altered Microbiota Contributes to Reduced Diet-Induced Obesity upon Cold Exposure. *Cell metabolism.* 2016;23(6):1216-1223.

62. Lozupone CA, Rhodes ME, Neff CP, Fontenot AP, Campbell TB, Palmer BE. HIV-induced alteration in gut microbiota: driving factors, consequences, and effects of antiretroviral therapy. *Gut microbes.* 2014;5(4):562-570.

63. Dinh DM, Volpe GE, Duffalo C, et al. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. *The Journal of infectious diseases.* 2015;211(1):19-27.

64. Dillon SM, Lee EJ, Kotter CV, et al. Gut dendritic cell activation links an altered colonic microbiome to mucosal and systemic T-cell activation in untreated HIV-1 infection. *Mucosal immunology.* 2016;9(1):24-37.

65. Harris K, Kassis A, Major G, Chou CJ. Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? *Journal of obesity.* 2012;2012:879151.

66. Ning T, Gong X, Xie L, Ma B. Gut Microbiota Analysis in Rats with Methamphetamine-Induced Conditioned Place Preference. *Frontiers in microbiology.* 2017;8:1620.

67. Fulcher JA, Hussain SK, Cook R, et al. Effects of Substance Use and Sex Practices on the Intestinal Microbiome During HIV-1 Infection. *The Journal of infectious diseases.* 2018;218(10):1560-1570.

68. Xu Y, Xie Z, Wang H, et al. Bacterial Diversity of Intestinal Microbiota in Patients with Substance Use Disorders Revealed by 16S rRNA Gene Deep Sequencing. *Scientific reports.* 2017;7(1):3628.

69. Kiraly DD, Walker DM, Calipari ES, et al. Alterations of the Host Microbiome Affect Behavioral Responses to Cocaine. *Scientific reports.* 2016;6:35455.

70. Human Microbiome Project Consortum. Structure, function and diversity of the healthy human microbiome. *Nature.* 2012;486(7402):207-214.

71. Deeks SG. HIV infection, inflammation, immunosenescence, and aging. *Annual review of medicine.* 2011;62:141-155.

72. Lozupone CA, Li M, Campbell TB, et al. Alterations in the gut microbiota associated with HIV-1 infection. *Cell host & microbe.* 2013;14(3):329-339.

73. Noguera-Julian M, Rocafort M, Guillen Y, et al. Gut Microbiota Linked to Sexual Preference and HIV Infection. *EBioMedicine.* 2016;5:135-146.

74. Hoel H, Hove-Skovsgaard M, Hov JR, et al. Impact of HIV and Type 2 diabetes on Gut Microbiota Diversity, Tryptophan Catabolism and Endothelial Dysfunction. *Scientific reports.* 2018;8(1):6725.

75. Pollock J, Glendinning L, Wisedchanwet T, Watson M. The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. *Applied and environmental microbiology.* 2018;84(7).

76. Thorsen J, Brejnrod A, Mortensen M, et al. Large-scale benchmarking reveals false discoveries and count transformation sensitivity in 16S rRNA gene amplicon data analysis methods used in microbiome studies. *Microbiome.* 2016;4(1):62.

77. Hawinkel S, Mattiello F, Bijnens L, Thas O. A broken promise: microbiome differential abundance methods do not control the false discovery rate. *Briefings in bioinformatics.* 2017.

78. McHardy IH, Li X, Tong M, et al. HIV Infection is associated with compositional and functional shifts in the rectal mucosal microbiota. *Microbiome.* 2013;1(1):26.

79. Manner IW, Baekken M, Kvale D, et al. Markers of microbial translocation predict hypertension in HIV-infected individuals. *HIV medicine.* 2013;14(6):354-361.

80. Vesterbacka J, Rivera J, Noyan K, et al. Richer gut microbiota with distinct metabolic profile in HIV infected Elite Controllers. *Scientific reports.* 2017;7(1):6269.

81. Bender JM, Li F, Martelly S, et al. Maternal HIV infection influences the microbiome of HIV-uninfected infants. *Science translational medicine.* 2016;8(349):349ra100.

82. Pannaraj PS, Ly M, Cerini C, et al. Shared and Distinct Features of Human Milk and Infant Stool Viromes. *Frontiers in microbiology.* 2018;9:1162.

83. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature methods.* 2010;7(5):335-336.

84. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods.* 2016;13(7):581-583.

85. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology.* 2007;73(16):5261-5267.

86. Chen L, Reeve J, Zhang L, Huang S, Wang X, Chen J. GMPR: A robust normalization method for zero-inflated count data with application to microbiome sequencing data. *PeerJ.* 2018;6:e4600.

87. Robins JM, Hernan MA, Brumback B. Marginal structural models and causal inference in epidemiology. *Epidemiology (Cambridge, Mass).* 2000;11(5):550-560.

88. Van Buuren S, Brand JPL, Groothuis-Oudshoorn CGM, Rubin DB. Fully conditional specification in multivariate imputation. *Journal of Statistical Computation and Simulation.* 2006;76(12):1049-1064.

89. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the royal statistical society Series B (Methodological).* 1995:289-300.

90. Benjamini Y, Yekutieli D. False Discovery Rate–Adjusted Multiple Confidence Intervals for Selected Parameters. *Journal of the American Statistical Association.* 2005;100(469):71-81.

91. Sun Y, Ma Y, Lin P, et al. Fecal bacterial microbiome diversity in chronic HIV-infected patients in China. *Emerging microbes & infections.* 2016;5:e31.

92. Monaco CL, Gootenberg DB, Zhao G, et al. Altered Virome and Bacterial Microbiome in Human Immunodeficiency Virus-Associated Acquired Immunodeficiency Syndrome. *Cell host & microbe.* 2016;19(3):311-322.

93. Dubourg G, Lagier JC, Hue S, et al. Gut microbiota associated with HIV infection is significantly enriched in bacteria tolerant to oxygen. *BMJ open gastroenterology.* 2016;3(1):e000080.

94. Scher JU, Sczesnak A, Longman RS, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *eLife.* 2013;2:e01202.

95. Troy EB, Kasper DL. Beneficial effects of Bacteroides fragilis polysaccharides on the immune system. *Frontiers in bioscience (Landmark edition).* 2010;15:25-34.

96. Wang PL, Ohura K. Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists.* 2002;13(2):132-142.

97. Arimatsu K, Yamada H, Miyazawa H, et al. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Scientific reports.* 2014;4:4828.

98. Choi J, Chung SW, Kim SJ, Kim SJ. Establishment of Porphyromonas gingivalis-specific T-cell lines from atherosclerosis patients. *Oral microbiology and immunology.* 2001;16(5):316-318.

99. Nakajima M, Arimatsu K, Kato T, et al. Oral Administration of P. gingivalis Induces Dysbiosis of Gut Microbiota and Impaired Barrier Function Leading to Dissemination of Enterobacteria to the Liver. *PloS one.* 2015;10(7):e0134234.

100. Laprise C, de Pokomandy A, Baril JG, Dufresne S, Trottier H. Virologic failure following persistent low-level viremia in a cohort of HIV-positive patients: results from 12 years of observation. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2013;57(10):1489-1496.

101. Bernal E, Gomez JM, Jarrin I, et al. Low level viremia is associated with clinical progression in HIV-infected patients receiving antiretroviral treatment. *Journal of acquired immune deficiency syndromes (1999).* 2018.

102. Reus S, Portilla J, Sanchez-Paya J, et al. Low-level HIV viremia is associated with microbial translocation and inflammation. *Journal of acquired immune deficiency syndromes (1999).* 2013;62(2):129-134.

103. Babyak MA. What you see may not be what you get: a brief, nontechnical introduction to overfitting in regression-type models. *Psychosomatic medicine.* 2004;66(3):411-421.

104. De Stavola BL, Cox DR. On the consequences of overstratification. *Biometrika.* 2008;95(4):992-996.

105. Harrison KM, Song R, Zhang X. Life expectancy after HIV diagnosis based on national HIV surveillance data from 25 states, United States. *Journal of acquired immune deficiency syndromes (1999).* 2010;53(1):124-130.

106. Guaraldi G, Orlando G, Zona S, et al. Premature age-related comorbidities among HIV-infected persons compared with the general population. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2011;53(11):1120-1126.

107. Centers for Disease Control and Prevention. Overweight & Obesity. https://www.cdc.gov/obesity/data/adult.html. Accessed July 10, 2018.

108. Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet (London, England).* 2011;378(9793):815-825.

109. Taramasso L, Ricci E, Menzaghi B, et al. Weight Gain: A Possible Side Effect of All Antiretrovirals. *Open forum infectious diseases.* 2017;4(4):ofx239.

110. Centers for Disease Control and Prevention. HIV Surveillance Report. 2016; https://www.cdc.gov/hiv/pdf/library/reports/surveillance/cdc-hiv-surveillance-report-2016-vol-28.pdf. Accessed 2018, April 18.

111. Conley LJ, Bush TJ, Rupert AW, et al. Obesity is associated with greater inflammation and monocyte activation among HIV-infected adults receiving antiretroviral therapy. *AIDS (London, England).* 2015;29(16):2201-2207.

112. Laidlaw E, Gouel-Cheron A, Kuriakose S, et al. Weight Gain After ARV Initiation Correlates with Increased Inflammatory Biomarkers. Paper presented at: 22nd International AIDS Conference; July 23-27, 2018; Amsterdam, Netherlands.

113. Kim DJ, Westfall AO, Chamot E, et al. Multimorbidity patterns in HIV-infected patients: the role of obesity in chronic disease clustering. *Journal of acquired immune deficiency syndromes (1999).* 2012;61(5):600-605.

114. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. *Nature.* 2006;444:1022.

115. Armougom F, Henry M, Vialettes B, Raccah D, Raoult D. Monitoring Bacterial Community of Human Gut Microbiota Reveals an Increase in Lactobacillus in Obese Patients and Methanogens in Anorexic Patients. *PloS one.* 2009;4(9):e7125.

116. Zhang H, DiBaise JK, Zuccolo A, et al. Human gut microbiota in obesity and after gastric bypass. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(7):2365-2370.

117. Ling Z, Jin C, Xie T, Cheng Y, Li L, Wu N. Alterations in the Fecal Microbiota of Patients with HIV-1 Infection: An Observational Study in A Chinese Population. *Scientific reports.* 2016;6:30673.

118. Lake JE. The Fat of the Matter: Obesity and Visceral Adiposity in Treated HIV Infection. *Current HIV/AIDS reports.* 2017;14(6):211-219.

119. Cook RR, Fulcher JA, Tobin NH, et al. Effects of HIV viremia on the gastrointestinal microbiome of young men who have sex with men. *AIDS (London, England).* 9000;Publish Ahead of Print.

120. Bender JM, Li F, Adisetiyo H, et al. Quantification of variation and the impact of biomass in targeted 16S rRNA gene sequencing studies. *Microbiome.* 2018;6(1):155.

121. National Heart Lung and Blood Institute. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: The evidence report. 1998; https://www.nhlbi.nih.gov/files/docs/guidelines/ob_gdlns.pdf. Accessed July 13, 2017.

122. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A.* 2005;102(31):11070-11075.

123. Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS letters.* 2014;588(22):4223-4233.

124. Finucane MM, Sharpton TJ, Laurent TJ, Pollard KS. A taxonomic signature of obesity in the microbiome? Getting to the guts of the matter. *PloS one.* 2014;9(1):e84689.

125. Sui Y, Dzutsev A, Venzon D, et al. Influence of gut microbiome on mucosal immune activation and SHIV viral transmission in naive macaques. *Mucosal immunology.* 2018;11(4):1219-1229.

126. Gu Y, Wang X, Li J, et al. Analyses of gut microbiota and plasma bile acids enable stratification of patients for antidiabetic treatment. *Nature communications.* 2017;8(1):1785.

127. Hjorth MF, Roager HM, Larsen TM, et al. Pre-treatment microbial Prevotella-to-Bacteroides ratio, determines body fat loss success during a 6-month randomized controlled diet intervention. *International journal of obesity (2005).* 2018;42(2):284.

128. Mulders RJ, de Git KCG, Schele E, Dickson SL, Sanz Y, Adan RAH. Microbiota in obesity: interactions with enteroendocrine, immune and central nervous systems. *Obesity reviews : an official journal of the International Association for the Study of Obesity.* 2018;19(4):435-451.

129. Nakayama J, Watanabe K, Jiang J, et al. Diversity in gut bacterial community of school-age children in Asia. *Scientific reports.* 2015;5:8397.

130. Serrano-Villar S, Vazquez-Castellanos JF, Vallejo A, et al. The effects of prebiotics on microbial dysbiosis, butyrate production and immunity in HIV-infected subjects. *Mucosal immunology.* 2017;10(5):1279-1293.

131. Ogden CL, Lamb MM, Carroll MD, Flegal KM. Obesity and socioeconomic status in adults: United States, 2005-2008. *NCHS data brief.* 2010(50):1-8.

132. Buchacz K, Baker RK, Palella FJ, Jr., et al. Disparities in prevalence of key chronic diseases by gender and race/ethnicity among antiretroviral-treated HIV-infected adults in the US. *Antiviral therapy.* 2013;18(1):65-75.

133. Substance Abuse and Mental Health Services Administration. Key Substance Use and Mental Health Indicators in the United States: Results from the 2017 National Survey on Drug Use and Health. 2018; https://www.samhsa.gov/data/sites/default/files/cbhsq-reports/NSDUHFFR2017/NSDUHFFR2017.pdf. Accessed February 4, 2019.

134. National Institute on Drug Abuse. Research Report Series: Methamphetamine. 2013; https://d14rmgtrwzf5a.cloudfront.net/sites/default/files/methrrs.pdf. Accessed February 4, 2019.

135. Hartzler B, Dombrowski JC, Crane HM, et al. Prevalence and Predictors of Substance Use Disorders Among HIV Care Enrollees in the United States. *AIDS Behav.* 2017;21(4):1138-1148.

136. Salamanca SA, Sorrentino EE, Nosanchuk JD, Martinez LR. Impact of methamphetamine on infection and immunity. *Frontiers in neuroscience.* 2014;8:445.

137. Fulcher JA, Shoptaw S, Makgoeng SB, et al. Brief Report: Recent Methamphetamine Use Is Associated With Increased Rectal Mucosal Inflammatory Cytokines, Regardless of HIV-1 Serostatus. *Journal of acquired immune deficiency syndromes (1999).* 2018;78(1):119-123.

138. Droutman V, Xue F, Barkley-Levenson E, et al. Neurocognitive decision-making processes of casual methamphetamine users. *NeuroImage Clinical.* 2018:101643.

139. Maxwell S, Shahmanesh M, Gafos M. Chemsex behaviours among men who have sex with men: A systematic review of the literature. *The International journal on drug policy.* 2018;63:74-89.

140. Koblin BA, Husnik, M.J., Colfax, G., Huang, Y., Madison, M., Mayer, K., Barresi, P.J., Coates, T.J., Chesney, M.A., Buchbinder, S. Risk factors for HIV infection among men who have sex with men. *AIDS.* 2006;20:731-739.

141. Plankey MW, Ostrow, D.G., Stall, R., Cox, C., Li, X., Peck, J.A., Jacobson, L.P. The relationship between methamphetamine and popper use and risk of HIV seroconversion in the multicenter AIDS cohort study. *Journal of Acquired Immune Deficiency Syndrome.* 2007;45(1):85-92.

142. Hood JE, Buskin SE, Golden MR, Glick SN, Banta-Green C, Dombrowski JC. The Changing Burden of HIV Attributable to Methamphetamine Among Men Who Have Sex with Men in King County, Washington. *AIDS Patient Care STDS.* 2018;32(6):223-233.

143. Ellis RJ, Childers ME, Cherner M, Lazzaretto D, Letendre S, Grant I. Increased human immunodeficiency virus loads in active methamphetamine users are explained by reduced effectiveness of antiretroviral therapy. *The Journal of infectious diseases.* 2003;188(12):1820-1826.

144. Shoptaw S, Stall R, Bordon J, et al. Cumulative exposure to stimulants and immune function outcomes among HIV-positive and HIV-negative men in the Multicenter AIDS Cohort Study. *Int J STD AIDS.* 2012;23(8):576-580.

145. Carrico AW, Shoptaw S, Cox C, et al. Stimulant use and progression to AIDS or mortality after the initiation of highly active antiretroviral therapy. *Journal of acquired immune deficiency syndromes (1999).* 2014;67(5):508-513.

146. Scott HM, Vittinghoff E, Irvin R, et al. Age, race/ethnicity, and behavioral risk factors associated with per contact risk of HIV infection among men who have sex with men in the United States. *Journal of acquired immune deficiency syndromes (1999).* 2014;65(1):115-121.

147. Loftis JM, Janowsky A. Neuroimmune basis of methamphetamine toxicity. *International review of neurobiology.* 2014;118:165-197.

148. Potula R, Haldar B, Cenna JM, Sriram U, Fan S. Methamphetamine alters T cell cycle entry and progression: role in immune dysfunction. *Cell death discovery.* 2018;4:44.

149. Prakash MD, Tangalakis K, Antonipillai J, Stojanovska L, Nurgali K, Apostolopoulos V. Methamphetamine: Effects on the brain, gut and immune system. *Pharmacological research.* 2017;120:60-67.

150. Banerjee S, Sindberg G, Wang F, et al. Opioid-induced gut microbial disruption and bile dysregulation leads to gut barrier compromise and sustained systemic inflammation. *Mucosal immunology.* 2016;9(6):1418-1428.

151. National Institute on Drug Abuse. NIDA-Modified ASSIST V2.0. 2012; https://www.drugabuse.gov/sites/default/files/pdf/nmassist.pdf. Accessed April 12, 2018.

152. Mutlu EA, Gillevet PM, Rangwala H, et al. Colonic microbiome is altered in alcoholism. *American journal of physiology Gastrointestinal and liver physiology.* 2012;302(9):G966-978.

153. Li X, Wu F, Xue L, et al. Methamphetamine causes neurotoxicity by promoting polarization of macrophages and inflammatory response. *Human & experimental toxicology.* 2018;37(5):486-495.

154. Grigoleit JS, Kullmann JS, Wolf OT, et al. Dose-dependent effects of endotoxin on neurobehavioral functions in humans. *PloS one.* 2011;6(12):e28330.

155. Abe K, Takahashi A, Fujita M, et al. Dysbiosis of oral microbiota and its association with salivary immunological biomarkers in autoimmune liver disease. *PloS one.* 2018;13(7):e0198757.

156. Proenca MA, Biselli JM, Succi M, et al. Relationship between Fusobacterium nucleatum, inflammatory mediators and microRNAs in colorectal carcinogenesis. *World journal of gastroenterology.* 2018;24(47):5351-5365.

157. Dillon SM, Kibbie J, Lee EJ, et al. Low abundance of colonic butyrate-producing bacteria in HIV infection is associated with microbial translocation and immune activation. *AIDS (London, England).* 2017;31(4):511-521.

158. Leclercq S, Matamoros S, Cani PD, et al. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proc Natl Acad Sci U S A.* 2014;111(42):E4485-4493.

159. Burokas A, Moloney RD, Dinan TG, Cryan JF. Microbiota regulation of the Mammalian gut-brain axis. *Advances in applied microbiology.* 2015;91:1-62.

160. Reardon S. Gut-brain link grabs neuroscientists. *Nature.* 2014;515(7526):175-177.

161. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature reviews Neuroscience.* 2012;13(10):701-712.

162. Han W, Tellez LA, Perkins MH, et al. A Neural Circuit for Gut-Induced Reward. *Cell.* 2018;175(3):665-678.e623.

163. Lee K, Vuong HE, Nusbaum DJ, Hsiao EY, Evans CJ, Taylor AMW. The gut microbiota mediates reward and sensory responses associated with regimen-selective morphine

dependence. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology.* 2018;43(13):2606-2614.

164. Wei CL, Wang S, Yen JT, et al. Antidepressant-like activities of live and heat-killed Lactobacillus paracasei PS23 in chronic corticosterone-treated mice and possible mechanisms. *Brain research.* 2019.

165. Valles-Colomer M, Falony G, Darzi Y, et al. The neuroactive potential of the human gut microbiota in quality of life and depression. *Nature Microbiology.* 2019.

166. Stevens BR, Goel R, Seungbum K, et al. Increased human intestinal barrier permeability plasma biomarkers zonulin and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety or depression. *Gut.* 2018;67(8):1555-1557.

167. Pirbaglou M, Katz J, de Souza RJ, Stearns JC, Motamed M, Ritvo P. Probiotic supplementation can positively affect anxiety and depressive symptoms: a systematic review of randomized controlled trials. *Nutrition research (New York, NY).* 2016;36(9):889-898.

168. Liu CM, Prodger JL, Tobian AAR, et al. Penile Anaerobic Dysbiosis as a Risk Factor for HIV Infection. *mBio.* 2017;8(4).

169. Lennard K, Dabee S, Barnabas SL, et al. Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. *Infection and immunity.* 2018;86(1).

170. McClelland RS, Lingappa JR, Srinivasan S, et al. Evaluation of the association between the concentrations of key vaginal bacteria and the increased risk of HIV acquisition in African women from five cohorts: a nested case-control study. *The Lancet Infectious diseases.* 2018;18(5):554-564.

171. Lee SC, Chua LL, Yap SH, et al. Enrichment of gut-derived Fusobacterium is associated with suboptimal immune recovery in HIV-infected individuals. *Scientific reports.* 2018;8(1):14277.

172. Huang CB, Emerson KA, Gonzalez OA, Ebersole JL. Oral bacteria induce a differential activation of human immunodeficiency virus-1 promoter in T cells, macrophages and dendritic cells. *Oral microbiology and immunology.* 2009;24(5):401-407.

173. Brito LC, Sobrinho AP, Teles RP, et al. Microbiologic profile of endodontic infections from HIV- and HIV+ patients using multiple-displacement amplification and checkerboard DNA-DNA hybridization. *Oral diseases.* 2012;18(6):558-567.

174. Paster BJ, Russell MK, Alpagot T, et al. Bacterial diversity in necrotizing ulcerative periodontitis in HIV-positive subjects. *Annals of periodontology.* 2002;7(1):8-16.

175. Hamamoto DT, Rhodus NL. Methamphetamine abuse and dentistry. *Oral diseases.* 2009;15(1):27-37.

176. Shenoy MK, Lynch SV. Role of the lung microbiome in HIV pathogenesis. *Current opinion in HIV and AIDS.* 2018;13(1):45-52.

177. Anukam KC, Osazuwa EO, Osadolor HB, Bruce AW, Reid G. Yogurt containing probiotic Lactobacillus rhamnosus GR-1 and L. reuteri RC-14 helps resolve moderate diarrhea and increases CD4 count in HIV/AIDS patients. *Journal of clinical gastroenterology.* 2008;42(3):239-243.

178. Irvine SL, Hummelen R, Hekmat S, Looman CW, Habbema JD, Reid G. Probiotic yogurt consumption is associated with an increase of CD4 count among people living with HIV/AIDS. *Journal of clinical gastroenterology.* 2010;44(9):e201-205.

179. Hummelen R, Changalucha J, Butamanya NL, et al. Effect of 25 weeks probiotic supplementation on immune function of HIV patients. *Gut microbes.* 2011;2(2):80-85.

180. Hemsworth JC, Hekmat S, Reid G. Micronutrient supplemented probiotic yogurt for HIV-infected adults taking HAART in London, Canada. *Gut microbes.* 2012;3(5):414-419.

181. Cahn P, Ruxrungtham K, Gazzard B, et al. The immunomodulatory nutritional intervention NR100157 reduced CD4+ T-cell decline and immune activation: a 1-year multicenter randomized controlled double-blind trial in HIV-infected persons not receiving antiretroviral therapy (The BITE Study). *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2013;57(1):139-146.

182. Gori A, Rizzardini G, Van't Land B, et al. Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: results of the "COPA" pilot randomized trial. *Mucosal immunology.* 2011;4(5):554-563.

183. Stiksrud B, Nowak P, Nwosu FC, et al. Reduced Levels of D-dimer and Changes in Gut Microbiota Composition After Probiotic Intervention in HIV-Infected Individuals on Stable ART. *Journal of acquired immune deficiency syndromes (1999).* 2015;70(4):329-337.

184. Villar-Garcia J, Guerri-Fernandez R, Moya A, et al. Impact of probiotic Saccharomyces boulardii on the gut microbiome composition in HIV-treated patients: A double-blind, randomised, placebo-controlled trial. *PloS one.* 2017;12(4):e0173802.

185. Villar-Garcia J, Hernandez JJ, Guerri-Fernandez R, et al. Effect of probiotics (Saccharomyces boulardii) on microbial translocation and inflammation in HIV-treated patients: a double-blind, randomized, placebo-controlled trial. *Journal of acquired immune deficiency syndromes (1999).* 2015;68(3):256-263.

186. Hensley-McBain T, Zevin AS, Manuzak J, et al. Effects of Fecal Microbial Transplantation on Microbiome and Immunity in Simian Immunodeficiency Virus-Infected Macaques. *Journal of virology.* 2016;90(10):4981-4989.

187. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature.* 2010;464(7285):59-65.

188. Dailey AF, Johnson AS, Wu B. HIV Care Outcomes Among Blacks with Diagnosed HIV - United States, 2014. *MMWR Morbidity and mortality weekly report.* 2017;66(4):97-103.

189. Kelly BJ, Gross R, Bittinger K, et al. Power and sample-size estimation for microbiome studies using pairwise distances and PERMANOVA. *Bioinformatics (Oxford, England).* 2015;31(15):2461-2468.

190. National Institute on Alcohol Abuse and Alcoholism. NIAAA Recommended Alcohol Questions. 2003; https://www.niaaa.nih.gov/research/guidelines-and-resources/recommended-alcohol-questions. Accessed April 12, 2018.

191. Multicenter AIDS Cohort Study. MACS Section 4 Questionnaire - Visit 15. 1991; http://www.statepi.jhsph.edu/macs/Questionnaires/S4forms/v15_s4.pdf. Accessed April 12, 2018.

192. Sanchez T, Zlotorzynska M, Sineath C, Kahle E, Sullivan P. The Annual American Men's Internet Survey of Behaviors of Men Who have Sex with Men in the United States: 2014 Key Indicators Report. *JMIR public health and surveillance.* 2016;2(1):e23.

193. McCaffrey DF, Griffin BA, Almirall D, Slaughter ME, Ramchand R, Burgette LF. A tutorial on propensity score estimation for multiple treatments using generalized boosted models. *Statistics in medicine.* 2013;32(19):3388-3414.

194. Xu L, Paterson AD, Turpin W, Xu W. Assessment and Selection of Competing Models for Zero-Inflated Microbiome Data. *PloS one.* 2015;10(7):e0129606.

195. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of statistical software.* 2010;33(1):1-22.

196. Krstajic D, Buturovic LJ, Leahy DE, Thomas S. Cross-validation pitfalls when selecting and assessing regression and classification models. *Journal of Cheminformatics.* 2014;6(1):10.

197. VanderWeele TJ. On the distinction between interaction and effect modification. *Epidemiology (Cambridge, Mass).* 2009;20(6):863-871.

198. Normand ST, Landrum MB, Guadagnoli E, et al. Validating recommendations for coronary angiography following acute myocardial infarction in the elderly: a matched analysis using propensity scores. *J Clin Epidemiol.* 2001;54(4):387-398.

199. Austin PC. Balance diagnostics for comparing the distribution of baseline covariates between treatment groups in propensity-score matched samples. *Statistics in medicine.* 2009;28(25):3083-3107.