

**PRE AND POST HIV SEROCONVERSION GUT MICROBIOME IN HIV-INFECTED  
INDIVIDUALS**

by

**Mariah Lynn Cole**

BS Biology, University of Pittsburgh, 2015

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the Department of Infectious Diseases and Microbiology  
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This thesis was presented

by

Mariah L. Cole

It was defended on

August 7, 2017

and approved by

Alison Morris, MD, MS  
Professor, Director, Vice Chair and Chair  
Pulmonary, Allergy, and Critical Care  
Center for Medicine & Microbiome  
Department of Medicine  
University of Pittsburgh

Jeremy J. Martinson, DPhil  
Assistant Professor  
Infectious Diseases and Microbiology  
Graduate School of Public Health  
University of Pittsburgh

**Thesis Advisor:** Charles R. Rinaldo, PhD  
Professor and Chair  
Infectious Diseases and Microbiology  
Graduate School of Public Health  
University of Pittsburgh

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**ABSTRACT**

Intensive research on human immunodeficiency virus (HIV) is being conducted in efforts to understand HIV pathogenesis. In the past decade, the development of innovative bioinformatics technology has focused research on the human gut microbiome and its potential role in the pathogenesis of HIV. Recent research has shown that gut microbial imbalance, or dysbiosis, may lead to microbial translocation and chronic inflammation in HIV-infected individuals, further enhancing HIV progression, potentially towards the development of AIDS. Gut microbiota in untreated men who have sex with men (MSM) with HIV can have an over-representation of pro-inflammatory Proteobacteria, associated with mucosal and systemic immune activation. My research aims to investigate the gut microbiome of 16 untreated HIV-infected men who have sex with men (MSM) at time-points that are ~6 months pre-seroconversion and ~6 months post-seroconversion to assess bacterial changes that may make individuals more susceptible to the development of AIDS, using Multicenter AIDS Cohort Study (MACS) fecal samples from 1984-1985. Using high throughput sequencing technology, bacterial 16s rRNA genes were amplified, sequenced, and then clustered into operational taxonomic units using QIIME software. Results showed that fecal samples from both non-HIV infected controls and HIV-infected MSM in 1984-85 had dominant taxa from the phyla Firmicutes, Bacteroidetes and Proteobacteria. Both visits non-infected controls showed a relative abundance of Firmicutes (35.3%), Bacteroidetes (56%)

and Proteobacteria (5.53%); similarly, both visits seroconverters showed a relative abundance of Firmicutes (39.93%) Bacteroidetes (50.58%), and Proteobacteria (5.41%). Genera level abundance of seroconverters (SC) both visits vs non-HIV infected controls both visits showed an increase in *Prevotella* (51.2% SC; 38% controls) and a decrease in *Bacteroides* (14.1% SC; 27.11% controls). These results suggest that an increase in *Prevotella* within the six month post-seroconversion to HIV, with microbial translocation of *Prevotella* or its metabolites, could be a factor in subsequent development of AIDS. Alpha diversity and beta diversity are currently being analyzed to provide the statistical significance of these findings. This pilot study sets a strong foundation for building further research in the MACS assessing the effects of the microbiome in HIV infection. This study is important for public health because it will help further develop an understanding of and how microbial composition and microbial products influence the pathogenesis of progressive HIV infection; potentially formulating improved ancillary treatments to improve the long-term health of HIV infected persons on ART.

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## **PREFACE**

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## **1.0 INTRODUCTION**

### **1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV)**

Human immunodeficiency virus (HIV) is a serious public health issue and since its discovery, has been a great focus of academic, biotechnological, and clinical research. Despite over 30 years of research, there is still little known about the pathogenesis of HIV.

Approximately 36.7 million people worldwide, with 850,000 to 950,000 individuals living in the United States, have been diagnosed with human immunodeficiency virus (HIV); which can eventually cause acquired immunodeficiency syndrome (AIDS) (1). 1.1 million individuals worldwide died of AIDS-related illnesses in 2015 (1). In parts of Africa, there is a 4.4% HIV prevalence rate, which is dramatically higher than any other country (Americas have a 0.5% prevalence) (1). Outside of Africa, populations at highest risk include those with the highest amount of risky behavior such as intravenous drug users, prisoners, sex workers and men who have sex with men (MSM) (2). In 2014, MSM made up more than half of the new HIV diagnoses in the United States (3). However, any individual participating in any risky behavior (i.e. unprotected sex) can acquire HIV and be unaware until tested, providing the opportunity to spread to other individuals (4). Once infected with HIV, combination anti-retroviral therapy (cART) or anti-retroviral therapy (ART) can be used to suppress viral replication, and reduce acquired immunodeficiency syndrome (AIDS)-related mortality and morbidity, but ART

treatment is a life-long treatment with damaging side effects and is overall undesirable for an individual. When ART is stopped, replication of the virus begins again within weeks. Current HIV/AIDS research focuses on searching for innovative therapies and preventative measures to provide better treatment and care to HIV-infected individuals. HIV disease progression depends on a variety of known and unknown factors.

## 1.2 HIV VIROLOGY

### 1.2.1 Classification

HIV is from the family *Retroviridae*, genus *Lentivirus*, and can lead to the development of AIDS; a progressive immune system failure in which an individual becomes susceptible to opportunistic infections and cancers (5). HIV is a single stranded, positive sense, enveloped RNA virus(6). HIV's DNA is stored within a capsid and a lipid envelope. The virus is spherical with a diameter of ~100-120 nm. The viral genome consists of two copies of RNA and code for nine genes: Gag, Pol, Env (encode for structural proteins), Tat and Rev (encode for regulatory proteins), and Vpu, Vpr, Vif, and Nef (encode for accessory proteins)(6). HIV has the ability to integrate its own DNA into the human host's genome, and for this reason, it is very difficult to eliminate.

### **1.2.2 Entry and Replication**

Virus acquisition occurs through contact and exposure of infected bodily fluids and blood between individuals. Initially, precursor glycoprotein (gp) 160 is cleaved by the cellular protease furin into two subunits, resulting in a surface subunit glycoprotein 120 (gp120) and a transmembrane subunit gp41. To enter a cell, docking must occur (7). Docking occurs when gp120 interacts with the CD4 receptor and a coreceptor (CCR5 and/or CXCR4) primarily on T lymphocytes (T-helper cells), macrophages (CCR5), and microglial cells (8). After interaction occurs, the viral envelope fuses with the host cell membrane through an unknown mechanism and releases viral contents into the host cell's cytosol (8). Viral reverse transcriptase (RT) generates a double stranded (ds) complementary DNA (cDNA) and imports this DNA into the cell nucleus. After undergoing modifications, HIV transcripts are released and translated into the cytoplasm, where they are prepared for virion assembly in lipid rafts located on cellular membranes (8). Assembly occurs at different areas on different cell types; T-lymphocyte virion assembly occurs at the cell surface; whereas, in macrophages and dendritic cells, assembly occurs on endosomal membranes and buds off from the membrane (8). After budding, virions search for new target cells to infect and the cycle is repeated.

### **1.2.3 Pathogenesis**

HIV infection has three distinct stages: acute infection, clinical latency (chronic infection), and AIDS (symptomatic stage) [(5), (9)]. Acute infection occurs between 2 to 4 weeks after HIV infection, some flu-like symptoms may be presented, but most infected individuals do not show symptoms during this stage. Exponential amounts of virus are produced during this

time, destroying CD4<sup>+</sup> T cells in the process. Viral stability occurs within 3 months and is termed viral set point; when the host's immune system develops antibodies for the virus. This antibody development is identified as seroconversion. During the clinical latency, or chronic HIV infection, virus continues to replicate in lymph nodes and blood, slowly damaging the infected host's immune system (5). This period can last for several years before progressing to AIDS. Progression to AIDS is quicker in individuals who are not regularly taking ART; however, some individuals may never progress to AIDS. AIDS is defined by increased invasion of opportunistic infections (i.e., *Toxoplasmosis*, *Salmonella septicemia* and *Candidiasis*) and cancers (i.e., Kaposi's sarcoma and lymphomas) that arise and thrive due to an overwhelmed immune system(5). During this stage, and infected individual's CD 4 count drops below 200 cells per cubic millimeter (cells/mm<sup>3</sup>) of blood. Without antiretroviral therapy (ART), AIDS mortality is on average 3 years (5). Progression through each stage is dependent upon a variety of factors, age, HIV subtype, co-infections, nutrition status, stress level, genetic background, and ART (5).

Pathogenesis of HIV infection and AIDS development is still being studied. It is believed that several types of viral and host factors (receptors/coreceptors) are involved in HIV disease progression (9). A 32-nt deletion in CCR5 commonly presented in Caucasian populations, protects individuals from being infected with HIV (homozygous CCR5 delta32/delta32), or slow the rate of development towards AIDS (heterozygous genotype) (10). Macrophages (MP) play a major role in immune regulation, and have been implicated in functioning in dissemination of HIV to various tissues in the body (11). MP's primary function is to uptake free or opsonized virus (phagocytize) and destroy the virus through lysosome fusion/intracellular mechanisms (11) MP's also have other functions during viral immunity: MP's help facilitate antigen presentation through expression of high levels of major histocompatibility antigens (MHC) and T-lymphocyte

costimulatory molecules, MP's secrete bioactive molecules which affect immunoregulatory functions via proinflammatory cytokines, as well as, secrete chemokines and other factors involved in viral infection and replication repression (11). Based on MP's functions, it has been implicated as a potential factor in the pathogenesis of HIV towards AIDs (11). Programmed death-1 (PD-1), a negative regulator of T cell function expressed on cytotoxic T lymphocytes (CTL), has been implicated in being responsible for CTL functional defects (12, 13). This suggests PD-1 as a potential therapeutic target in reversing T-cell dysfunctions during HIV infection.

#### **1.2.4 Tropism**

HIV tropism is dependent on viral protein interactions with cell surface receptors; CD4 receptor and a seven transmembrane coreceptor CCR5 or CXCR4. CD4 is expressed on T helper (Th) cells, regulatory T-cells, monocytes, macrophages and dendritic cells (DC) (14). Memory T cells, activated CD4<sup>+</sup> lymphocytes, gut associated lymphoid tissues (GALT), macrophages, DC and microglia express the CCR5 co receptor; whereas, naïve, resting CD4<sup>+</sup> lymphocytes, CD8 cells, B-cells, neutrophils and eosinophils express the CXCR4 coreceptor (14). The majority of newly transmitted HIV infections use the CCR5 coreceptor. HIV strains can be divided into three main trophic groups: macrophage-tropic (M-tropic), T-cell line tropic (T-tropic) or dual-tropic (9). M-tropic strains, also known as R5 (CCR5 binding) virus, infect peripheral blood mononuclear cells (PBMC), monocytes, macrophages and T-lymphocytes; Mucosal epithelial cells express the coreceptor CCR5 allowing for R5 viruses to infect sexually; mainly through the rectal route into the intestinal tract. (15). T-tropic viruses (X4 virus), target CXCR4 coreceptor cells, preferentially CD4<sup>+</sup> T cells, and only infect low levels in the GALT [(9), (16)].

### 1.3 GUT ASSOCIATED LYMPHOID TISSUES (GALT) AND HIV

The gut associated lymphoid tissues (GALT), harbor more than 50% of the human's total T lymphocytes; these lymphocytes are mostly activated in effort to fight pathogens (16). CD4<sup>+</sup> T cells function in immune protection by communicating with B cells to produce antibodies, enhancing macrophage lineage cell production, and recruiting other immune cells such as neutrophils, eosinophils, and basophils to the site of infection (17). Th17 cells play a role in bacterial and fungal defense at mucosal surfaces and helps maintain enterocyte homeostasis (18). High-level HIV viral replication occurs in the gut mucosal tissues. CD4<sup>+</sup> T cells (TH17) are depleted early on in infection, mostly through apoptosis, but also through another programmed cell death process known as pyroptosis (19); decreasing immune efficiency and leading to the inability to control HIV infection. Furthermore, research conducted by Sankaran *et al* 2005, showed a downregulation of mucosal genes related to digestion and lipid metabolism, cell cycle, and cell growth regulation in HIV-infected individuals (20).

Extreme depletion of CD4<sup>+</sup> T cells leads to increased intestinal wall permeability and systemic translocation of microbes and microbial products; subsequently leading to an increased, constant immune activation. HIV can deplete CD4<sup>+</sup> T cells through direct (*cytopathogenic*), indirect (*generation of incomplete reverse transcripts, leading to an extreme inflammatory response*) or negative (*reduces the immune system's ability to regenerate new CD4<sup>+</sup> T cells*) mechanisms (2)

Anti-retroviral therapy (ART) has drastically improved HIV patient's lives, but is not a cure. In high HIV prevalent areas, such as Sub-Saharan Africa, ART is incomplete or unobtainable; other therapeutics and treatments need to be developed and implemented. A promising focus for a co-therapeutic is the gut microbiome. Without ART, HIV/AIDS patients are more susceptible



to microbial/viral infections (21, 22). It has been shown that even when HIV-infected individuals are receiving ART, their gut microbiota does not reconstitute fully (23-25).

#### **1.4 THE GUT MICROBIOME**

The human microbiome is defined as all the metagenome of bacteria, viruses, fungi and archaea living on or in the human body. The human body is composed mostly of microbial cells, with microbial cells consisting of approximately 90% of the cells (26). Gut homeostasis is maintained greatly by gut microbiota. Gut bacteria process nutrients and provide metabolites important for nutrition, as well as, a variety of small molecules such as short chain fatty acids (SCFAs) that provide energy for enterocytes and have immunomodulatory effects (27). Microorganisms have been shown to play an important role in modulating the human host immune system and metabolism (28). The gut microbiome protects the host's intestines from invasion or colonization of exogenous pathogens through what is known as colonization resistance (29). These commensal microorganisms can protect through the production of antimicrobial peptides and IgA, or by enhancing the host's innate/adaptive immune responses (26). The microbiota plays an important role in the human immune system, with potential involvement with diseases and their pathogenesis. In recent years, the microbiota has been shown to be correlated with metabolic diseases such as obesity, diabetes, and luminal diseases such as irritable bowel diseases (IBD); Crohn's disease, celiac disease, and irritable bowel Syndrome (IBS), as well as HIV/AIDS (21, 26, 30-40).

In the recent years with the development of new sequencing techniques and promising results, there has been a major focus on the microbiome and its relationship to disease pathogenesis. A pubmed search of “microbiome” shows **34,311** articles have been published since 1971, and a pubmed search of “HIV and the microbiome” shows **412** articles published since 1991; However, there are no studies to date that focuses on the pre- and post-seroconversion microbiota in HIV infected individuals. This is due to the difficulty in delineating such HIV seroconversions in this era of relatively low numbers of primary infections in developed countries.

## **1.5 MICROBIAL COMPOSITION INSIDE THE HUMAN GUT**

The healthy gut microbiota consists of primarily the Phyla Firmicutes and Bacteroidetes. Phyla Actinobacteria and Verrucomicrobia are also abundant. The level of diversity, distribution and number of bacteria varies greatly throughout the human gut. *Streptococcus* is the dominant genus in the distal esophagus, duodenum and jejunum; whereas, *Helicobacter* dominates the stomach [(41, 42)] *Helicobacter* also determines the diversity of the gastric flora; if *Helicobacter* inhabits as a commensal, the dominant genera of the gastric flora include *Streptococcus*, *Prevotella*, *Veillonella* and *Rothia*; however, if *Helicobacter* inhabits as a pathogenic phenotype, the richness of the gastric genera decreases drastically. Over 70% of all the microbes found in the human body are constituted in the large intestine. This is the primary focus for research on disease states and pathogenesis, (i.e., HIV), when using fecal data. Firmicutes and Bacteroidetes are the primary phyla of the large intestine. In a healthy gut microbiome, there are constant stable levels of pathogenic microorganisms ( $\leq 0.01\%$  abundance), including *Salmonella enterica*,

*Campylobacter jejuni*, *Vibrio cholera*, *Escherichia coli* (E. coli) and *Bacteroides fragilis*.

Largely, low abundance of phyla Proteobacteria, with high abundance of *Bacteroides*, *Prevotella*, and *Ruminococcus* are characteristics of a healthy gut microbiome (43). Microbes that are characteristically identified in stool and can be used for luminal microbiota analysis include *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterobacteriaceae*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* (44).

Colonic organisms function in nutrient metabolism; for example, *Bacteroides*, *Roseburia*, *Bifidobacterium*, *Faecalibacterium*, and *Enterobacteria* produce short chain fatty acids (SCFA) such as butyrate, propionate, and acetate that provide energy to the host (45). Lipid metabolism is another function of some colonic organisms (i.e *Bacteroides thetaiotaomicron*), as well as, lipid hydrolysis (46). Gut microbial proteinases and peptidases work together with human proteinases for efficient protein metabolizing; the L-histidine to histamine conversion is an example of this cooperative function (47). Some *Bacteroides* members have been shown to synthesize conjugated linoleic acid (CLA) and have immunomodulatory properties; *Bacteroides intestinalis* can also deconjugate primary bile acids (48-51). In the large intestine, a two-tiered mucus layer prevents luminal microbes from epithelial contact and therefore antimicrobial proteins are limitedly produced here (52). Antimicrobial proteins (i.e., cathelicidins, C-type lectins and [pro] defensins) function mainly in the small intestine and are produced by Paneth cells through induction via certain gut microbiota (e.g., *Bacteroides thetaiotaomicrobe* and *Lactobacillus* sp.) (53, 54).

Gut microbiota have been shown to manipulate the innate and adaptive immune systems in mammals: *Bacillus fragilis* produces a symbiosis factor, polysaccharide A, which induces T-regulatory cells through TLR2 signaling, further enhancing immunologic tolerance (55). MyD88

(an adaptor molecule important for toll like receptor signaling) dependent mechanisms, important for maturation of IL1 $\beta$  in response to pathogenic organisms, are induced by commensal organisms (55). A study in murine intestines showed short filamentous bacteria inducing CD4<sup>+</sup> T helper cells, which in return produce interleukin (IL)-17 and IL-22 in the lamina propria; resulting in an increased expression of inflammatory genes and antimicrobial defenses, further protecting against intestinal pathogens (56, 57). Macrophages recently were shown (in mice) to be dependent on cross talking with microbes in the intestinal tract to produce IL-1 $\beta$  and promote intestinal immune homeostasis (58).

## **1.6 THE GUT MICROBIOME AND HIV**

Recent discovery of the “leaky gut theory,” has been implicated in HIV progression towards AIDS. It is known that HIV over activates the immune system, but the mechanism is still unclear. The leaky gut theory may be associated with this immune over activation. This theory implies that bacteria/bacterial products such as lipopolysaccharides (LPS), translocate out of the gastrointestinal tract (GIT), due to an increased permeability of the GIT and overall decreased mucosal barrier integrity (e.g., tight junctions decline), and into the blood, causing a systemic chronic immune activation (59, 60). Chronic immune activation is detrimental to individuals infected with HIV. Increased T cell turnover creates an imbalance in the immune homeostasis and results in T-cell half-life decrease, T cell clonal exhaustion, and possibly depletion of memory T cell pools; additionally, chronic immune activation leads to constant T cell generation, and subsequently driving viral replication (18)

An assortment of research has shown drastic differences in the enteric microbiome composition of HIV-infected and non-HIV infected individuals (24, 33). Among these studies, untreated HIV-infected individual stools were analyzed resulting in a major increase in the bacterial genus *Prevotella* and a significant decrease in *Bacteroides* (24, 30, 33, 34). A study conducted in China evaluated uninfected and chronically HIV-infected human stool samples for alpha (*diversity within samples*) and beta diversity (*diversity between samples*) and discovered an increase in the phyla *Firmicutes* and *Proteobacteria* in chronic HIV infected patients, in comparison to non-HIV infected controls (61). In the same study, an increase of *Bacteroides* and *Arabacteroides* were also observed in chronically infected patients. There have been conflicting results on the changes of the microbiome regarding HIV infection. Several studies, (23, 30, 33, 34, 62) showed an increase of *Prevotella* and a loss of *Bacteroides* in HIV infected individuals; whereas, other studies have shown the opposite effects (63) or no difference in these two genera (31, 63, 64).

HIV disrupts the overall immune system by destroying CD4<sup>+</sup> T cells and allowing for opportunistic infections to occur, eventually leading to the development of AIDS. However, it is not fully understood what makes an individual susceptible to developing AIDS or the exact sequence of pathogenesis from HIV towards the development of AIDs. This research study therefore focuses on pre-and post-seroconversion fecal sample analysis from the Multicenter AIDS Cohort Study (MACS). This longitudinal cohort study of the natural history of HIV infection in men who have sex with men (MSM) has collected pre and post seroconversion fecal, serum, and plasma samples since 1984. My study uses 1984-1985 fecal specimens. Here I address the hypothesis that the *pre-seroconversion* microbiome is compromised in some MSM, making them more vulnerable to developing AIDS *after* HIV infection, OR a subset of MSM

develop such a microbiomic risk factor for AIDS *after* (post-) HIV seroconversion. Thus, in either case the microbiome enhances susceptibility of an individual to progress towards AIDS. I propose that this is mediated by massive immune activation caused by microbial product translocation from the gut to the blood.

## 1.7 ANALYZING THE MICROBIOME

There are two main techniques used for analyzing the microbiome: metagenomics and 16S rRNA gene. In my study, 16S rRNA approach was used. 16S ribosomal RNA (rRNA) sequencing is common in the microbiome field. The 16S rRNA is a conserved gene found only in bacteria and archaea. Amplifying the 16S rRNA helps to identify and compare bacteria that are present within a sample. The V4 region of the 16S rRNA gene is a variable region that is targeted in microbiome studies because it has the lowest geodesic distance (*location on the 16S rRNA gene*) and is part of the major function of the 16S rRNA gene than other variable regions; it is therefore more sensitive and reliable as a marker for bacterial and phylogenetic analysis (65). Data resulting from sequencing the 16S rRNA can be analyzed using bioinformatic approaches. One popular bioinformatic approach is the QIIME pipeline (Quantitative Insight Into Microbial Ecology).

Since microbiome research generates large datasets, bioinformatic pipelines, such as QIIME and MOTHUR are essential in analyzing large microbiome datasets. QIIME is an open-source bioinformatics pipeline used to analyze large, raw microbiome data. Once sample 16S rRNAs are amplified and sequenced, raw bacterial sequences are subjected to quality control (i.e. clean up (*demultiplexed* and *read quality*), read count restrictions (*<1000 read counts samples*

*are rejected from the study*), OTU table generation, taxonomy assignment (i.e *greengenes*, *RDP*), phylogenetic analysis, alpha and beta diversity analyses and many more microbiome analyses.

Alpha and beta diversity measure the amount of diversity within and between samples. Alpha diversity measures the richness and evenness of a sample. The richness of a sample is the number of species that are genetically related to each other, measured using operational taxonomic units (OTUs); whereas, the evenness is the relative abundance of the species richness in the sample. Beta diversity is the comparison of diversity of samples to each other, by measuring distance or dissimilarity between each sample pair (phylogenetic measurement). Beta diversity addresses the question of which sample, A, B or C are more similar in composition to one another.

## **2.0 PUBLIC HEALTH IMPORTANCE**

Understanding what drives systemic immune activation is critical for understanding HIV pathogenesis and subsequent AIDS development. This study will help further develop an understanding if and how microbial composition and microbial products influence the pathogenesis of progressive HIV infection. They could help formulate improved ancillary treatments to improve the long-term health of HIV infected persons on ART.

### **2.1 STATEMENT OF PROJECT AND HYPOTHESIS**

My project focuses on analyzing the pre-seroconversion and post-seroconversion gut microbiota using fecal samples from MACS individuals from 1984 to 1985, to address the hypothesis that bacterial changes from pre-to post seroconversion may make individuals susceptible to developing AIDS. There are three timepoints we will be analyzing in regard to AIDS development: development of AIDS within 2-3 years after seroconversion (SC), development within 5-7 years of SC and development of AIDS 10 years after SC. DNA extraction, amplification of the bacterial 16s rRNA genes through PCR, high-throughput sequencing and bioinformatic analysis will be used to assess this hypothesis. QIIME software will be used to analyze the raw sequence data and PRISM will be used to analyze data provided from QIIME. Sequences will be clustered into operational taxonomic units using QIIME, and alpha and beta analyses will be compared between individuals who developed AIDS versus those who did not. Bar charts will be used to represent data visually. Using these techniques, *we hypothesize that microbial composition*



*will show decreased microbiome diversity after HIV seroconversion, with more diversity decrease in those who developed AIDS, indicating a possible role in the susceptibility to the development of AIDS.* Results from this thesis project will be used for future analysis of the gut microbiome among these MACS individuals to evaluate biomarkers, as well as, potential metabolites that may play a role in HIV pathogenesis towards AIDS

## **3.0 METHODS**

### **3.1 SAMPLING AND SAMPLE COLLECTION**

MACs fecal samples (n=55) were chosen from the repository of stored samples in 1984 and 1985. Fecal samples were stored at -80°C. Fecal samples were chosen at two timepoints for the same individual: Visit 1 (*0 months*): Non-HIV infected controls and pre-seroconverters, and visit 2 (*~6 months*): Non-HIV infected controls and ~6 month post-seroconversion. Samples were chosen from categories: seronegative (SN) and seroconverters (SC). Seroconverters who developed AIDS were chosen based on three time points: development of AIDS within 2-3 years after seroconversion (SC), development within 5-7 years of SC and development of AIDS 10 years after SC.

### **3.2 DNA EXTRACTION**

DNA was isolated using the MO Bio PowerSoil® DNA Isolation Kit (Qiagen, United States) was performed in accordance to MO Bio Incorporated's instructions, (PowerSoil® DNA Isolation Kit 2016, United States) with an additional step of heating tubes for 10 minutes. A negative control was run for each DNA extraction set, totaling to six negative DNA extraction controls.

### 3.3 PCR AND SEQUENCING

From extracted DNA, PCR amplification of the bacterial 16S rRNA gene V3-V4 hypervariable region was performed using Caporaso *et al* primers and the Q5 HS-High-Fidelity polymerase (NEB). Thermocycler conditions were 98C for 30s, (98C for 10s, 57C for 30s, 72C for 30s) repeated for 30 cycles, 72C for 2 min and hold at 4 degrees. One negative PCR control was run and one positive control consisting of 8 different known bacterial DNA.

For amplified DNA confirmation, agarose gels were run at 250V for 10-15 minutes, using 5.0ul of amplified DNA and 5.0 of 2X marker (*totaling 10ul in each well*). Unconfirmed samples were re-amplified using 0.5ul extracted DNA to prevent PCR crossover and run again on an agarose gel. Repeats which failed two times were re-extracted from the stool sample.

The V3-V4 regions were pooled and mixed in equal concentrations and then sequenced using the Illumina MiSeq (San Diego, California).

### 3.4 BIOINFORMATICS ANALYSIS / QIIME

Reads were demultiplexed into their corresponding samples based on the assigned barcodes (*short sequences attached to the sample for sample identification*) using QIIME pipeline. All the primers and barcoded sequences were removed. Biom tables were constructed in QIIME using a closed reference approach and quality score of 25. Low output samples (*with less than 1000 reads*) were removed: 9 samples in my study were removed.

Operational taxonomic units (OTU) were chosen based on a 97% similarity using greengenes reference database. Reads that did not cluster were removed from analysis. Relative abundances of taxa at the phylum and genus level were estimated.

## 4.0 RESULTS

### 4.1 STATISTICAL SUMMARY OF DATA

Forty-one individual samples were categorized into 2 groups; Non-HIV controls (25 samples) and Seroconverter AIDS (16 samples) (SC). Samples were then categorized into 4 subgroups within the SC AIDS group, based on their time of AIDS development post seroconversion (**Table 1**): AIDS development within 2 to 5 years post seroconversion, AIDS development within 5 to 7 years post seroconversion, AIDS development within 7 to 10 years post seroconversion and AIDS development after 10 years post seroconversion. 2 of the 16 SC AIDS samples have not developed AIDS. None of the participants were on anti-retroviral therapy at the time of sample collection. All participants were white, except 1 in the subgroup >10 years who was black (**Table 1**). Ages ranged from 21-39 years old (**Table 1**).

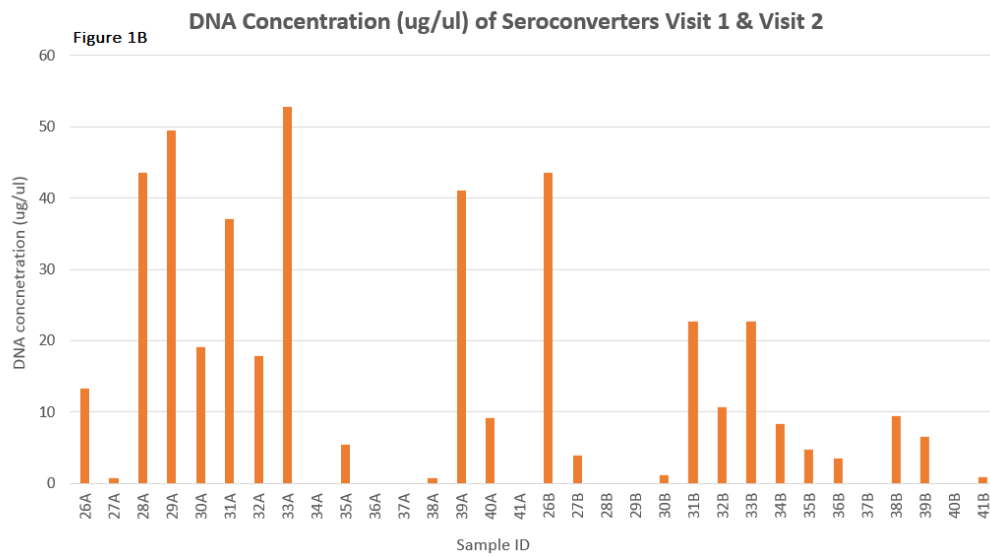
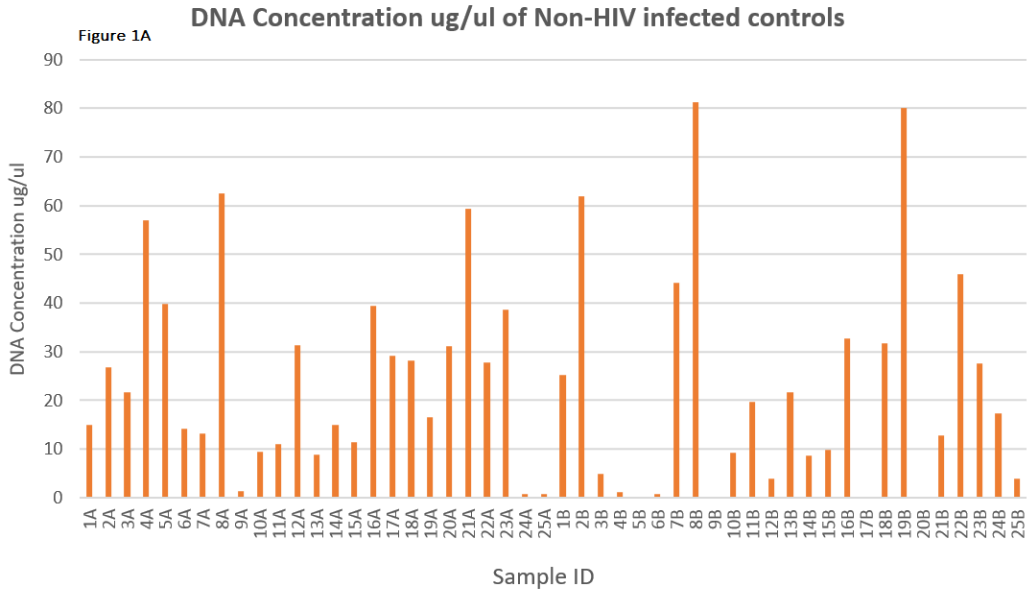
**Table 1 Summarized Demographics of Samples**

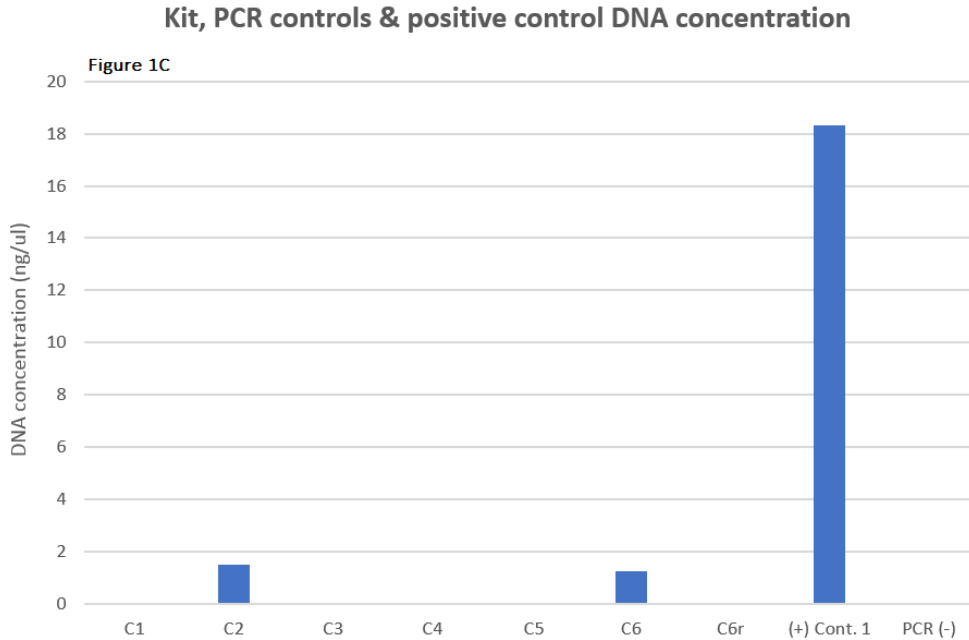
<b>Table 1: Demographics</b>	<b>Controls</b>	<b>SC AIDS</b>	<b>2-5 years</b>	<b>5-7 years</b>	<b>7-10 years</b>	<b>&gt; 10 years</b>
Number of Subjects	26	16	3	2	4	7
Gender	All Male	All Male	All Male	All Male	All Male	All Male
Age (Mean $\pm$ SD)	33.33 $\pm$ 5.76	30.44 $\pm$ 4.66	34.33 $\pm$ 2.89	34 $\pm$ 1.41	28.25 $\pm$ 2.75	29 $\pm$ 5.48
Ethnicity (white)	26	15	3	2	4	6
Time diagnosed HIV since VI (years)	0	0.5	0.5	0.5	0.5	0.5
Years lived with AIDS	0	8.56 $\pm$ 9.93	1.67 $\pm$ 2.08	1 $\pm$ 1.41	6.75 $\pm$ 12.2	14.7 $\pm$ 9.03
Has smoked/Currently Cig Smoker	19	12	2	2	4	4
Used Pot Before Visit 1	13	15	3	2	4	6
Used Pot Since Visit 1 at Visit 2	18	13	3	2	3	5
# of Sexual partners (Mean $\pm$ SD)	28.6 $\pm$ 32	67.4 $\pm$ 65.2	81.7 $\pm$ 36.2	115 $\pm$ 120.2	66.3 $\pm$ 58	48.3 $\pm$ 69.3

**Table 1. Summarized demographics of non-HIV controls and seroconverter samples.** W: White, B: Black, Y: Yes, N: No. Time to develop AIDS post-seroconversion (2-5, 5-7, 7-10, >10) *Note: 2 individuals have not developed AIDS.*

## 4.2 DNA QUANTITATION

We quantitated 3.0ul of purified amplified DNA, from each sample and each control, using the Qubit Fluorimeter. DNA concentration was measured in ug/ul and concentration values ranged from 0.733 ug/ul to 81.3 ug/ul (Table 2 & Figure 1A-1C).





**Figure 1 Qubit DNA Quantitation Data**

**Figure 1: Qubit DNA quantitation of samples and controls.**

**A)** HIV-Uninfected Controls DNA concentration for visit 1 (1A-25A) and visit 2 (1B-25B) . **B)** Seroconverter DNA concentration for visit 1 (26A-41A) and visit 2 (26A-41A) **C)** Kit Controls. C1-C6: MoBio Power fecal negative controls, C6r: Repeated, (+) Control: 8 known bacterial DNA- positive control; PCR (-): distilled water. DNA concentration is measured in ng/ul.

**Table 2 DNA Concentration Summary Table**

Table 2. DNA Concentration summary table for all samples									
Sample ID	DNA Conc. (ug/ul)	Sample ID	DNA Conc. (ug/ul)	Sample ID	DNA Conc. (ug/ul)	Sample ID	DNA Conc. (ug/ul)	Sample ID	DNA Conc. (ug/ul)
1A	15	21A	59.3	16B	32.7	36A	0	40B	0
2A	26.7	22A	27.7	17B	0	37A	0	41B	0.84
3A	21.6	23A	38.7	18B	31.7	38A	0.74	C1	0
4A	57	24A	0.707	19B	80	39A	41	C2	1.5
5A	39.9	25A	0.807	20B	0	40A	9.13	C3	0
6A	14.1	1B	25.2	21B	12.8	41A	0	C4	0
7A	13.1	2B	61.9	22B	45.9	26B	43.5	C5	0
8A	62.4	3B	4.97	23B	27.6	27B	3.92	C6	1.22
9A	1.33	4B	1.15	24B	17.3	28B	0	C6r	0
10A	9.4	5B	0	25B	3.92	29B	0	(+) Cont. 1	18.3
11A	11	6B	0.733	26A	13.3	30B	1.11	PCR (-)	0
12A	31.4	7B	44.1	27A	0.807	31B	22.7		
13A	8.73	8B	81.3	28A	43.5	32B	10.7		
14A	15	9B	0	29A	49.5	33B	22.7		
15A	11.3	10B	9.27	30A	19.1	34B	8.27		
16A	39.4	11B	19.6	31A	37	35B	4.76		
17A	29.1	12B	3.93	32A	17.8	36B	3.44		
18A	28.2	13B	21.6	33A	52.8	37B	0		
19A	16.5	14B	8.53	34A	0	38B	9.4		
20A	31.1	15B	9.8	35A	5.46	39B	6.6		

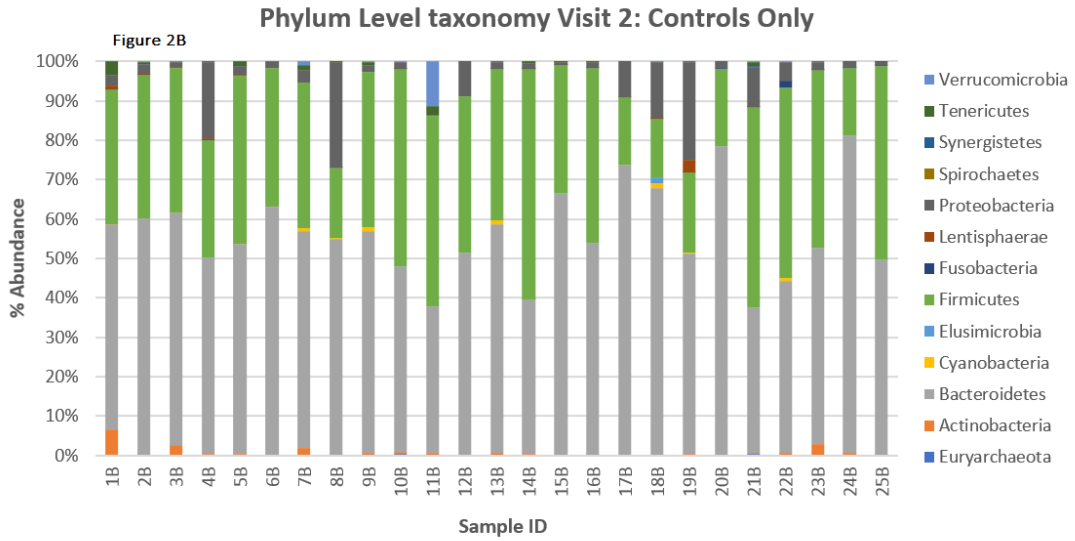
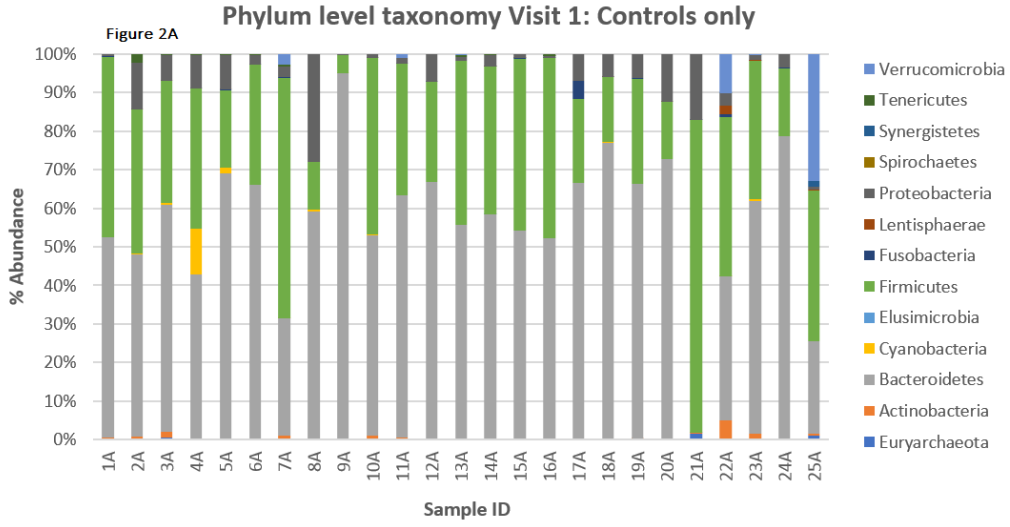
**Table 2. Summarized Qubit Quantitation of DNA concentration in samples and controls.**

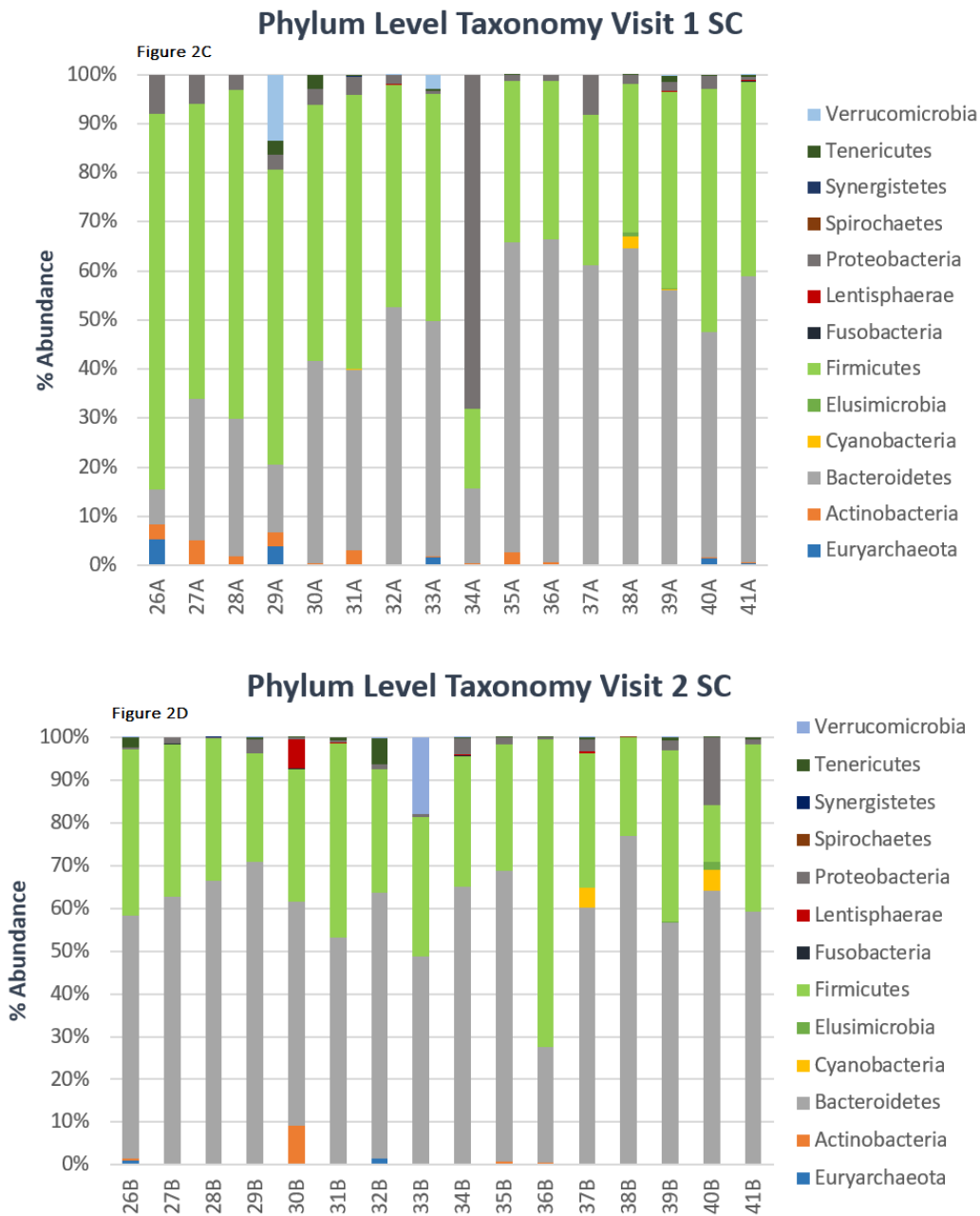
C1-C6: MoBio Power fecal negative controls, C6r: Repeated, (+) Control: 8 known bacterial DNA- positive control; PCR (-): distilled water. DNA concentration is measured in ng/ul. Out of range: below the level of detection (>0.50ug/ul).

### 4.3 PHYLUM LEVEL TAXONOMY ANALYSIS

To examine differences in bacterial composition in samples, 100% stacked bar charts were created at the phylum level. The three top dominant phyla in both visits for non-HIV infected samples were Bacteroides (56%), Firmicutes (35.3%), and Proteobacteria (5.53%). For controls Visit 1 samples Bacteroides composition constituted of (56.19%) of total bacteria, Firmicutes constituted (34.30%) and Proteobacteria constituted (5.71%) of total bacteria. Controls visit 2 samples consisted of (53.4%), (34.8%), (5.77%) respectively (**Figure 2A-2B**). The three top dominant phyla in both visits for seroconverter AIDS were Bacteroides (50.58%), Firmicutes (39.93)%, and Proteobacteria (5.41%). Visit one SC AIDS constituted of Bacteroides (44%), Firmicutes 37.10%), and Proteobacteria (7.81%); whereas Visit 2 SC AIDS constituted of Bacteroides (59.25%), Firmicutes (34.34%) and Proteobacteria (2.25%) (**Figures 2C-2D**). Bacteroides, Firmicutes and Proteobacteria are summarized in **Figures 3A-5B**, comparing controls and seroconverters AIDS groups.



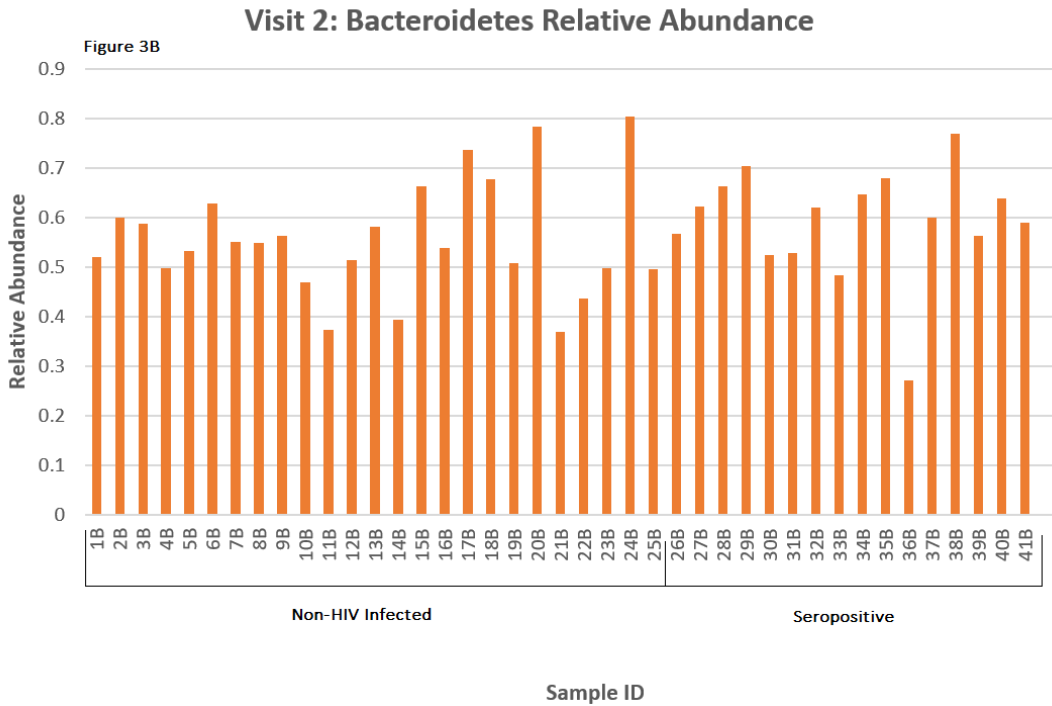
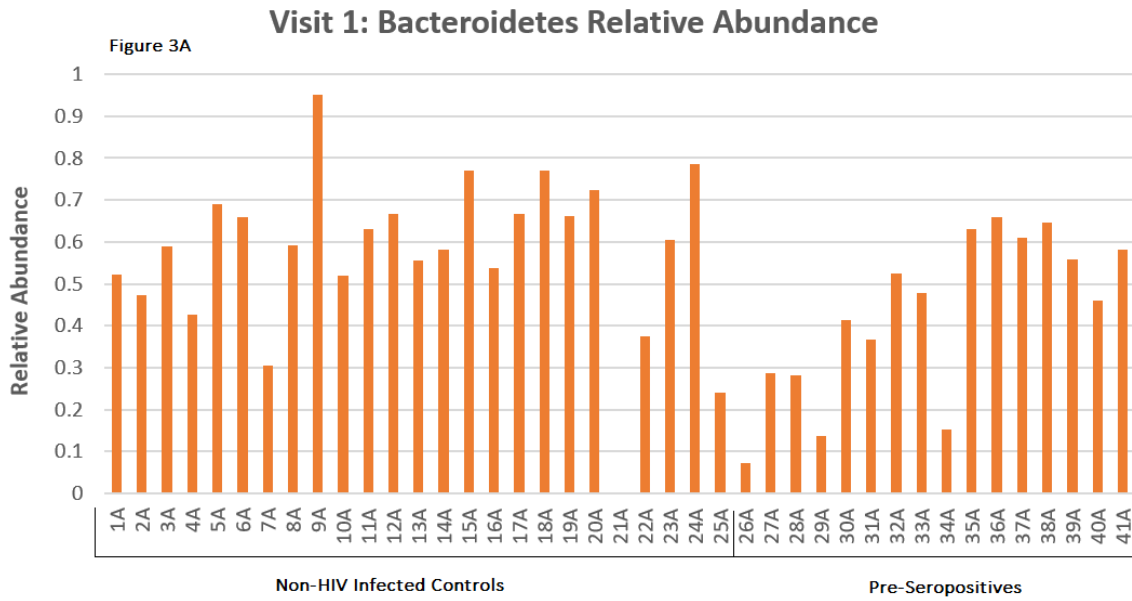




**Figure 2 Phylum Level Taxonomy Bar Charts**

**Figure 2: Phylum-level taxon distribution in non-HIV infected controls.**

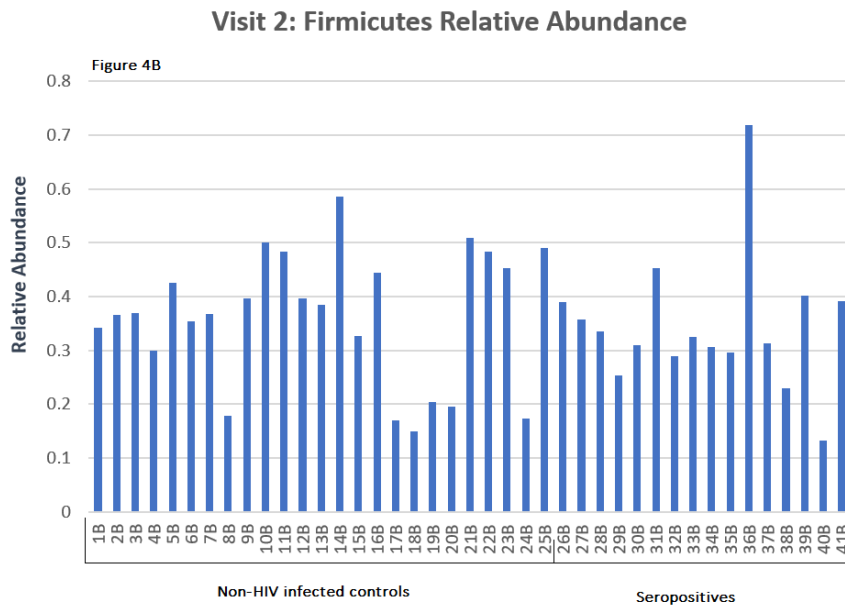
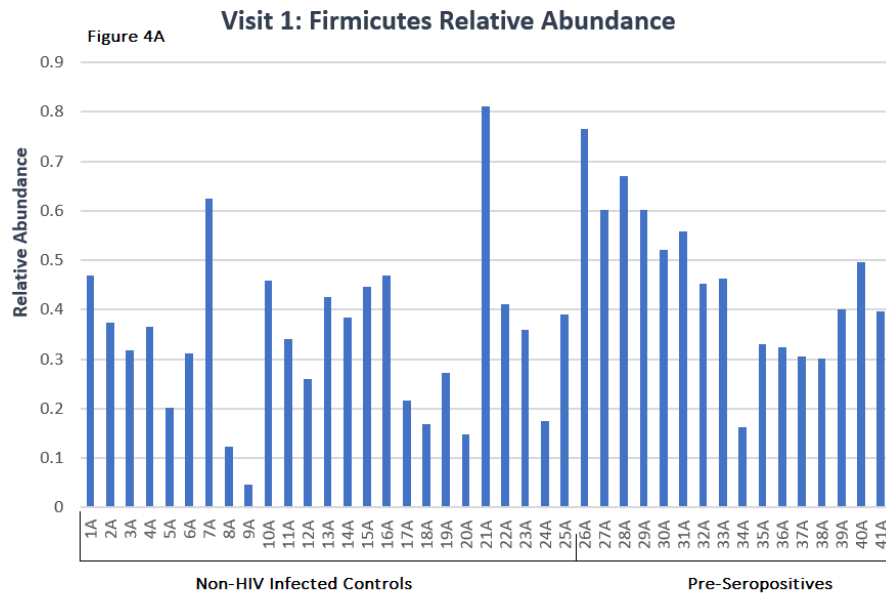
**A)** Visit 1 (1A-25A) Controls. **B)** Visit 2 (1B-25B) Controls. **C)** Visit 1 pre-seroconversion (26A-41A). **D)** Visit 2 post-seroconversion (26B-41B). *Phyla abundance is shown as a percentage of total bacterial sequences within the sample.*



**Figure 3 Bacteroidetes Relative Abundance**

**Figure 3. Bacteroidetes relative abundance.**

**A)** in Visit 1 non-HIV controls (*Seronegative*) and Pre-seropositive samples. **B)** in Visit 2 non-HIV controls (*Seronegative*) and Post-seropositive samples. SN: Seronegative, SC: Seroconverter.

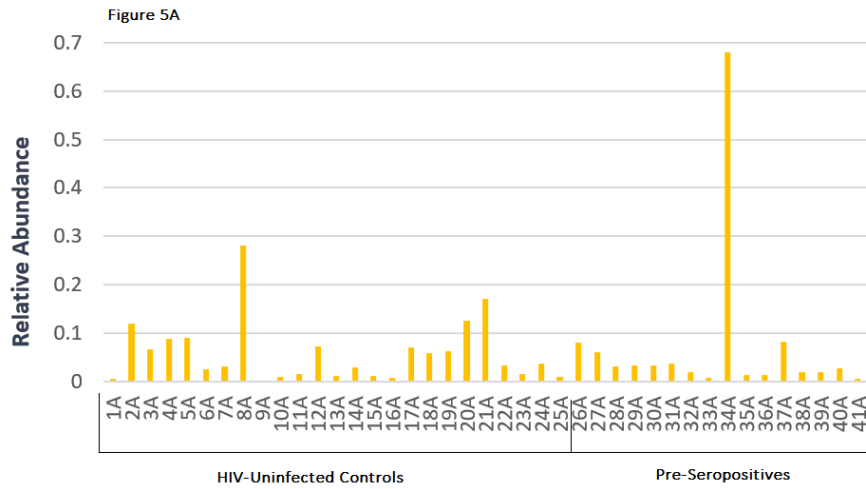


**Figure 4 Firmicutes Relative Abundance**

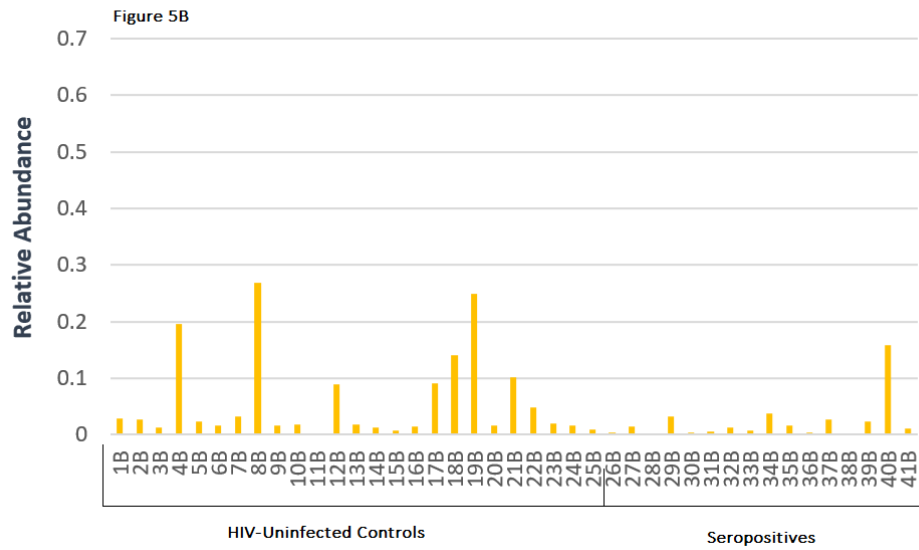
**Figure 4. Firmicutes relative abundance.**

**A)** in Visit 1 non-HIV controls (*Seronegative*) and Pre-seropositive samples. **B)** in Visit 2 non-HIV controls (*Seronegative*) and Post-seropositive samples. SN: Seronegative, SC: Seroconverter.

### Visit 1: Proteobacteria Relative Abundance



### Visit 2: Proteobacteria Relative Abundance



**Figure 5 Proteobacteria Relative Abundance**

**Figure 5. Proteobacteria relative abundance.**

**A)** in Visit 1 non-HIV controls (*Seronegative*) and Pre-seropositive samples. **B)** in Visit 2 non-HIV controls (*Seronegative*) and Post-seropositive samples. SN: Seronegative, SC: Seroconverter.

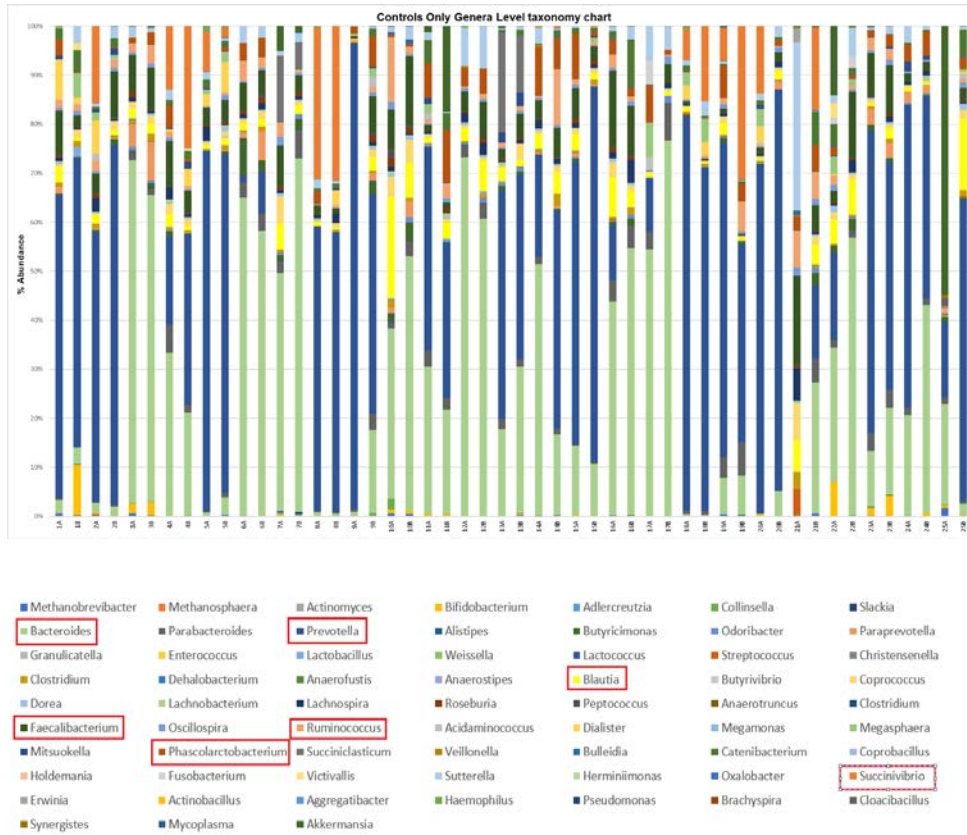
#### 4.4 GENUS LEVEL TAXONOMY ANALYSIS

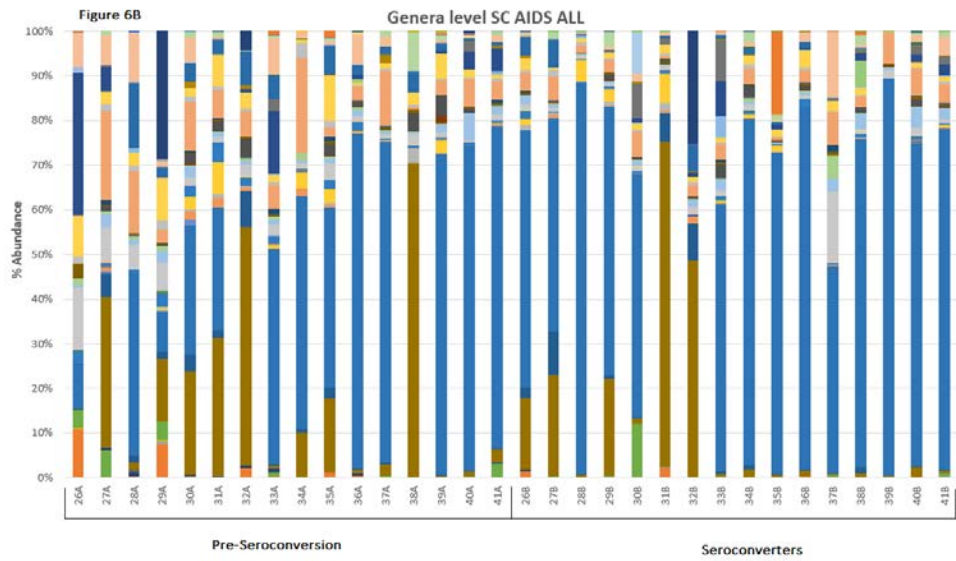
To examine differences in bacterial composition in samples, 100% stacked bar charts were created at the genus level. The four top dominant genera in both visits for non-HIV infected samples were *Prevotella* (38.0%), *Bacteroides* (27.11%), *Faecalibacterium* (4.68%), and *Succinivibrio* (4.60%) (**Figure 6A**). Visit 1 non-HIV controls constituted of *Prevotella* (39.49%), *Bacteroides* (25.50%), *Faecalibacterium* (4.32%), and *Succinivibrio* (3.96%). Visit 2 non-HIV controls constituted of *Prevotella* (36.50%), *Bacteroides* (28.80%), *Faecalibacterium* (5.06%), and *Succinivibrio* (5.20%) (**Figure 6A**). The three top dominant genera in both visits for SC AIDS is *Bacteroides* (14.1%), *Prevotella* (51.2%), *Faecalibacterium* (5.1%). Visit 1 SC AIDS constituted of *Bacteroides* (17.05%), *Prevotella* (40.32%), *Faecalibacterium* (6.78%) (**Figure 6C**). Visit 2 SC AIDS constituted of *Bacteroides* (14.04%), *Prevotella* (60.50%), *Faecalibacterium* (3.70%) (**Figure 6D**). Top 4 most abundant genus level bacterial composition in seroconverter groupings are shown in **Table 3**.

**Table 3 Percent Average Genus Bacterial Composition of SC AIDS groups**

Table 3.		% Average Genus Bacterial Composition in SC AIDS groupings							
# of Samples	AIDS developed Post SC	<i>Bacteroides</i> V1	<i>Bacteroides</i> V2	<i>Prevotella</i> V1	<i>Prevotella</i> V2	<i>Faecalibacterium</i>	<i>Faecalibacterium</i> V2	<i>Ruminococcus</i> V1	<i>Ruminococcus</i> V2
N= 3	2-5 years	6.22%	0.08%	8.15%	0.42%	3.57%	2.98%	2.21%	1.13%
N= 2	5-7 years	9.85%	8.33%	10.40%	42.11%	4.02%	3.10%	1.95%	1.05%
N= 4	7-10 years	15.02%	20.45%	15.62%	24.25%	4.51%	1.74%	2.29%	0.94%
N= 7	>10 years	9.03%	0.66%	39.39%	51.90%	3.57%	2.98%	2.20%	0.01%

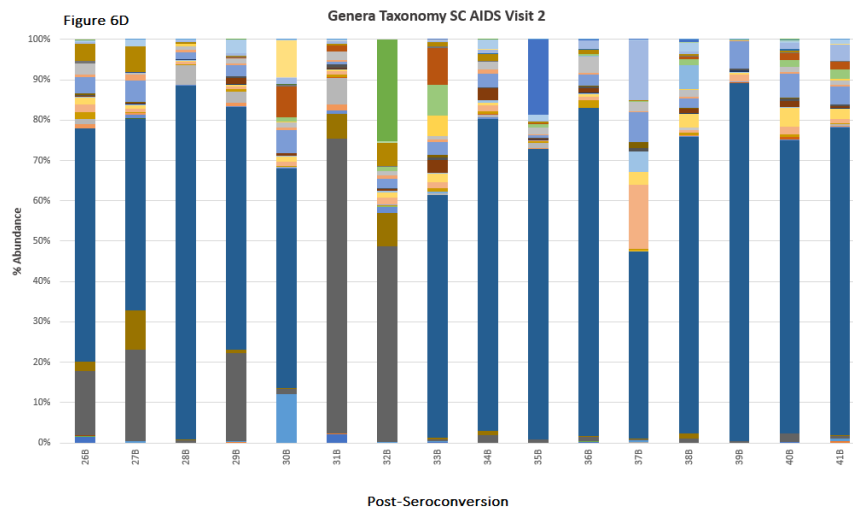
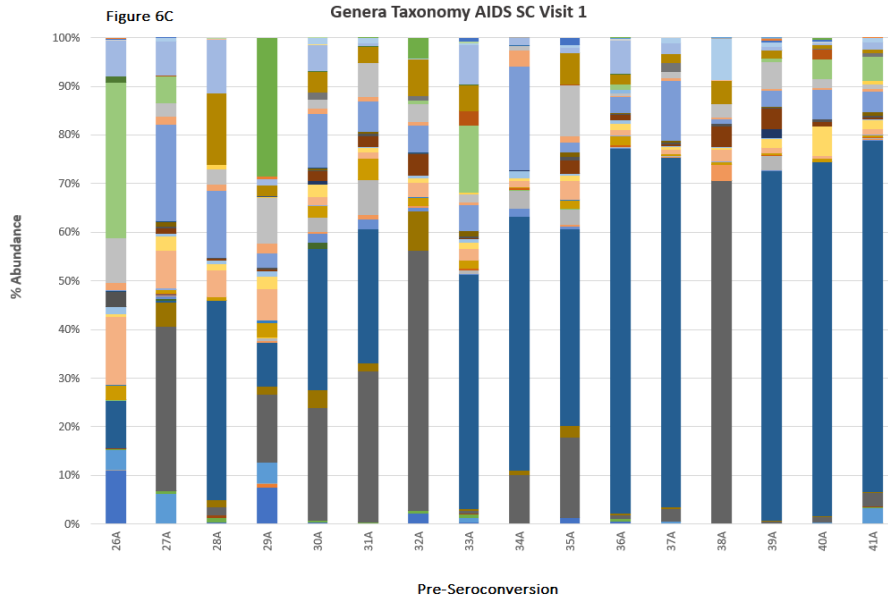
Figure 6A.





- |                      |                  |                   |                         |                   |                 |
|----------------------|------------------|-------------------|-------------------------|-------------------|-----------------|
| ■ Methanobrevibacter | ■ Methanosphaera | ■ Actinomyces     | ■ Alloscardovia         | ■ Bifidobacterium | ■ Collinsella   |
| ■ Eggertella         | ■ Slackia        | ■ Bacteroides     | ■ Parabacteroides       | ■ Prevotella      | ■ Alistipes     |
| ■ Butyrivibrio       | ■ Odoribacter    | ■ Paraprevotella  | ■ Granulicatella        | ■ Enterococcus    | ■ Lactobacillus |
| ■ Lactococcus        | ■ Streptococcus  | ■ Christensenella | ■ Clostridium           | ■ Dehalobacterium | ■ Anaerofustis  |
| ■ Anaerostipes       | ■ Blautia        | ■ Butyrivibrio    | ■ Coprococcus           | ■ Dorea           | ■ Epulopiscium  |
| ■ Lachnobacterium    | ■ Lachnospira    | ■ Roseburia       | ■ Peptococcus           | ■ Anaerotruncus   | ■ Clostridium   |
| ■ Faecalibacterium   | ■ Oscillospira   | ■ Ruminococcus    | ■ Acidaminococcus       | ■ Anaerovibrio    | ■ Dialister     |
| ■ Megamonas          | ■ Megasphaera    | ■ Mitsuokella     | ■ Phascolarctobacterium | ■ Veillonella     | ■ Bulleidia     |
| ■ Catenibacterium    | ■ Holdemania     | ■ Fusobacterium   | ■ Victivallis           | ■ Sutterella      | ■ Oxalobacter   |
| ■ Succinivibrio      | ■ Erwinia        | ■ Actinobacillus  | ■ Aggregatibacter       | ■ Haemophilus     | ■ Akkermansia   |



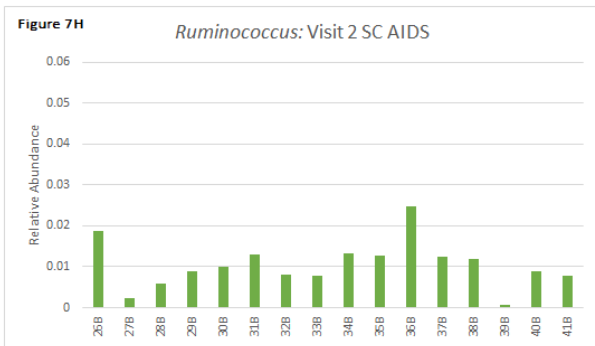
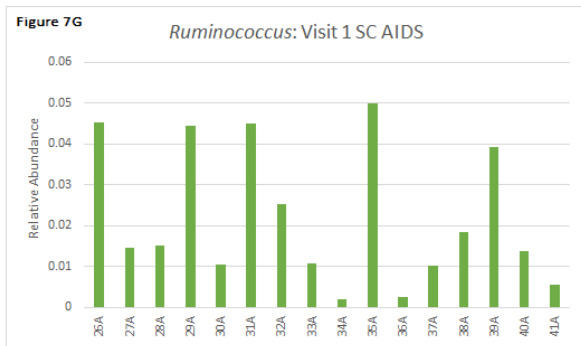
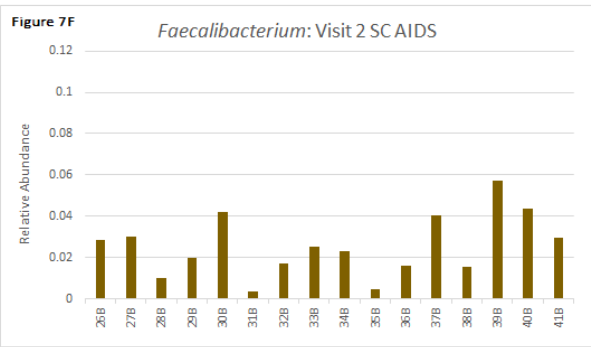
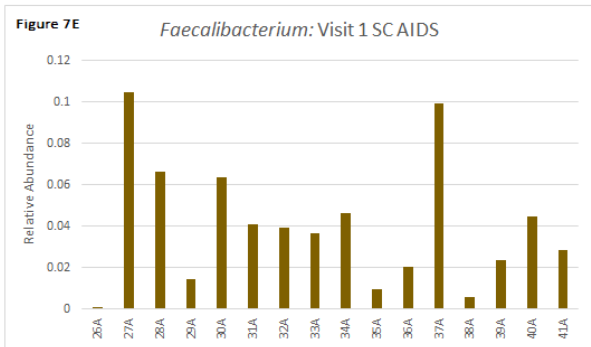
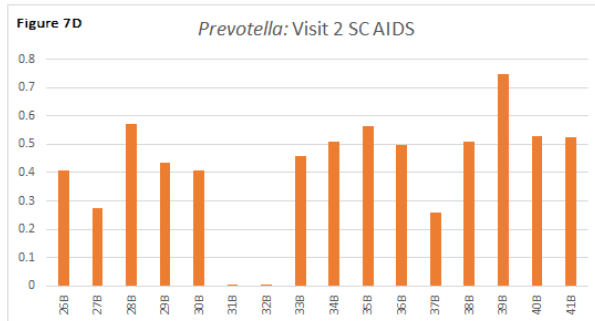
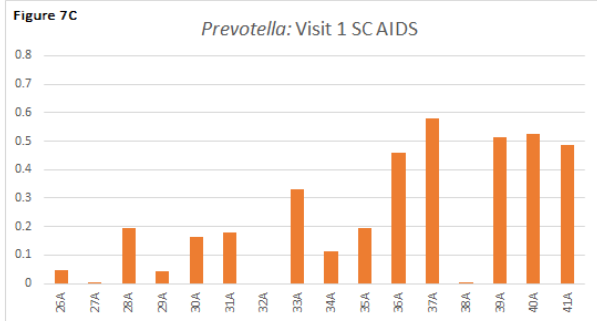
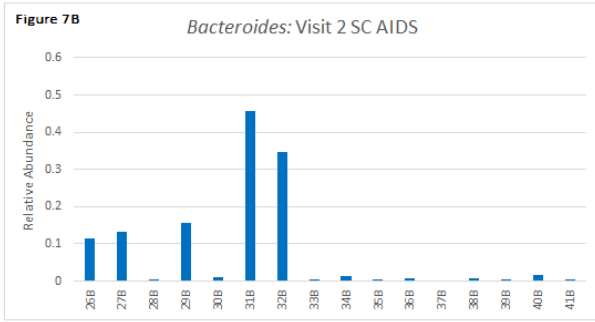
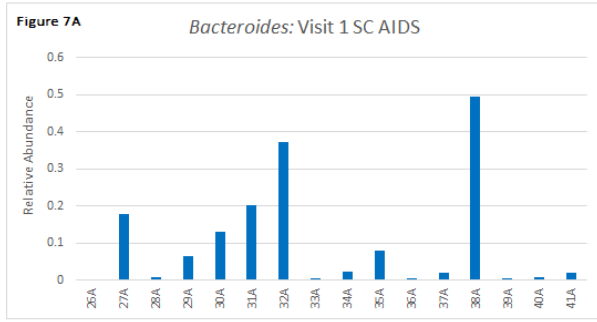


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|----------------------|------------------|-------------------|-------------------------|-------------------|-----------------|
| ■ Methanobrevibacter | ■ Methanosphaera | ■ Actinomyces     | ■ Alloscardovia         | ■ Bifidobacterium | ■ Collinsella   |
| ■ Eggerthella        | ■ Slackia        | ■ Bacteroides     | ■ Parabacteroides       | ■ Prevotella      | ■ Alistipes     |
| ■ Butyrivibrio       | ■ Odoribacter    | ■ Paraprevotella  | ■ Granulicatella        | ■ Enterococcus    | ■ Lactobacillus |
| ■ Lactococcus        | ■ Streptococcus  | ■ Christensenella | ■ Clostridium           | ■ Dehalobacterium | ■ Anaerofustis  |
| ■ Anaerostipes       | ■ Blautia        | ■ Butyrivibrio    | ■ Coprococcus           | ■ Dorea           | ■ Epulopiscium  |
| ■ Lachnobacterium    | ■ Lachnospira    | ■ Roseburia       | ■ Peptococcus           | ■ Anaerotruncus   | ■ Clostridium   |
| ■ Faecalibacterium   | ■ Oscillospira   | ■ Ruminococcus    | ■ Acidaminococcus       | ■ Anaerovibrio    | ■ Dialister     |
| ■ Megamonas          | ■ Megasphaera    | ■ Mitsuokella     | ■ Phascolarctobacterium | ■ Veillonella     | ■ Bulleidia     |
| ■ Catenibacterium    | ■ Holdemania     | ■ Fusobacterium   | ■ Victivallis           | ■ Sutterella      | ■ Oxalobacter   |
| ■ Succinivibrio      | ■ Erwinia        | ■ Actinobacillus  | ■ Aggregatibacter       | ■ Haemophilus     | ■ Akkermansia   |

**Figure 6 Genus Level Taxonomy Bar Charts**

*Bacteroides*, *Prevotella*, *Faecalibacterium*, and *Ruminococcus* are summarized from pre-seroconversion to post-seroconversion in **Figure 7A-7H** and fold change is assessed in **supplementary figure 2 (Supp. 2)**. *Bacteroides*, showed, on average, a decrease from pre-seroconversion (10.11%) to post-seroconversion (8%), correlating with previous studies who have shown a decrease in *Bacteroides* in HIV infected individuals compared to seronegative controls (**Table 4**). *Prevotella* showed an increase from pre-seroconversion (24%) to post-seroconversion (42%) (**Figure 7C-7D**). *Ruminococcus* and *Faecalibacterium* showed little to no change in composition between pre-and post-seroconversion groups (**Figure 7E-7H**).

Fold changes were assessed between visit 1 and visit 2 of Non-HIV infected individuals (**Supp. 3**). *Bacteroides*, *Prevotella*, *Faecalibacterium* and *Ruminococcus* fold changes among non-HIV infected individuals showed little to no difference from visit 1 to visit 2.



**Figure 7 Top Four Genus Level Bacterial Relative Abundances**

## 5.0 DISCUSSION

In this study, we focused on microbial composition differences among healthy non-HIV infected controls and those who developed HIV, as well as any microbial compositional changes between AIDS development timepoints post-seroconversion (i.e 2-5 years, 5-7 years, 7-10 years, >10 years). We compared the fecal bacterial microbiome among age-matched and sex-matched individuals. We found that both healthy controls and HIV-infected MSM predominantly were colonized by 3 main phyla: Bacteroidetes, Firmicutes and Proteobacteria. This confirmed previous gut phyla characterizations in HIV infection (66). Within these main phyla we saw the greatest microbial abundance among the gram-negative genera *Prevotella*, *Bacteroides*, *Faecalibacterium* and *Ruminococcus* in both our healthy controls and seropositive.

Previous research has shown that a switch from the genus *Bacteroides* to *Prevotella* can occur during the early stages of HIV-infection (23). In our non-HIV infected controls, we saw very little changes in *Bacteroides* and *Prevotella* genera from visit 1 to visit 2 (a six month interval); in the HIV-infected samples, we noticed a small decrease in the *Bacteroides* visit 1 (17%) to visit 2 (11%), and we did see an increase in the *Prevotella* genera between visit 1 (40%) and visit 2 (60%). This correlates with previous research showing a decrease in *Bacteroides* and an increase of *Prevotella* in HIV-infected individuals compared to seronegative controls (**Table 4**). Although not significant possibly due to the small number of samples tested, there was noticeable increase of *Prevotella* within the six months post-HIV seroconversion. Even though some studies have been elucidating that high *Prevotella* is correlated with diseased states, diets rich in carbohydrates and simple sugars (Western Diets), has been shown to be *Prevotella*-abundant in healthy US adults

(67). This is important because it shows that the microbiome varies among cultural and dietary regions, and that *Prevotella* is not necessarily related to disease state.

Overall our descriptive study revealed that pre-seroconversion (*before HIV-infection*) the microbiome showed an increase in the genus *Prevotella* and a decrease in genera *Bacteroides*, *Faecalibacterium*, and *Ruminococcus* compared to post-seroconversion (*i.e., within the first six months after HIV-infection*).

The changes we saw in our study are not broadly definitive primarily due to the relatively small sample size. There are still many questions that need to be addressed and further studied. Are the changes we see in the HIV seroconverters due to the changes of integrity in the gut? Are these changes driving HIV progression or dissemination? Or is HIV infection driving these changes?

**Table 4 Summary of HIV Microbiome Studies Findings**

<b>Table 4 Prevalent intestinal microbes found in HIV microbiome studies</b>			
<b>Phylum</b>	<b>Genera</b>	<b>Outcome</b>	<b>Reference</b>
<b>Firmicutes</b>	<i>Faecalibacterium</i>	Decreased abundance	34
		Increased abundance	Ling <i>et al</i> 2016
		Decreased abundance	33, 61
	<i>Roseburia</i>	Decreased abundance	27, 64
	<i>Ruminococcus</i>	Decreased abundance	64, Dubourg <i>et al</i> 2016
<b>Bacteroidetes</b>	<i>Alistipes</i>	Decreased abundance	64
		Increased abundance	61
	<i>Arabacteroides</i>	Increased abundance	61
	<i>Bacteroides</i>	Decreased abundance	(24, 30, 33, 34)
		Increased abundance	61, Noguera <i>et al</i> 2016
		Increased abundance	63
	<i>Prevotella</i>	Increased abundance	(23, 24, 30, 33, 34, 62, Pinto Cardoso <i>et al</i> 2017 Noguera <i>et al</i> 2016
Decreased abundance		63	
<b>Proteobacteria</b>		Increased abundance	31, 33, 61

*It is important to note that although the negative assay controls presented microbial composition, the DNA concentration was extremely low compared to the positive control. It is believed that the concentration is too low to influence the data (Table 2).*

## 5.1 FUTURE DIRECTIONS

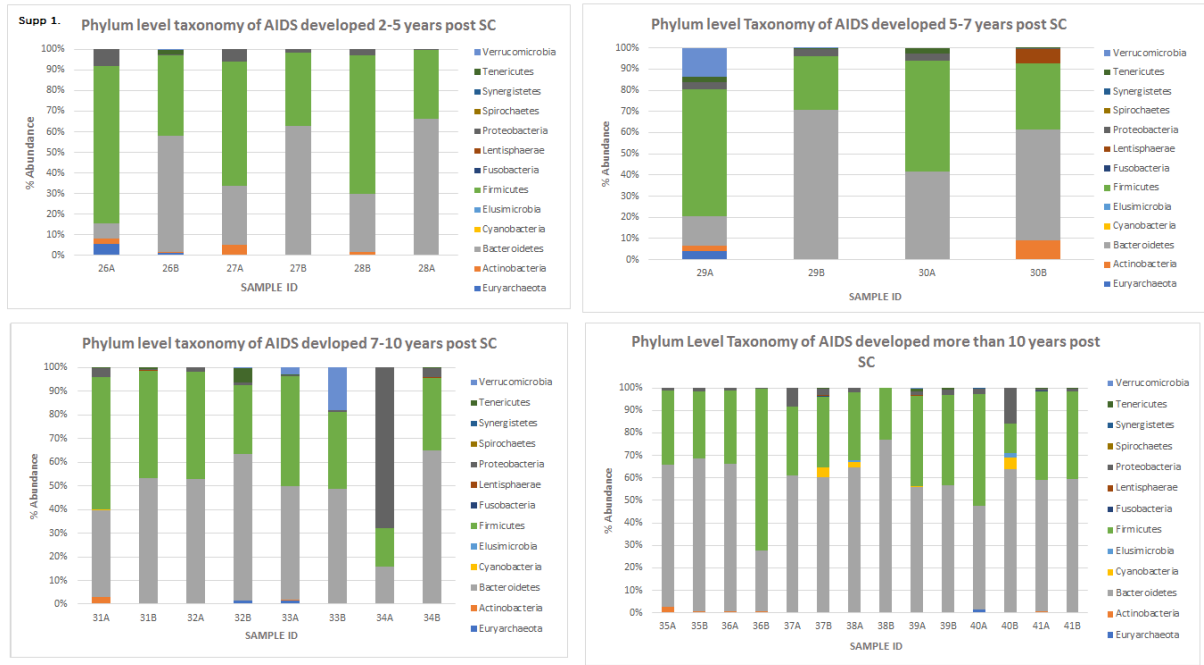
Since a beta-diversity analysis was not conducted, we do not have conclusive evidence to suggest correlation among changes in the gut microbiome of these participants. Beta-diversity should be a reliable statistical analysis. Future studies will look at these microbial changes and other demographics that may play a role (i.e., smoking and drinking behaviors, number of sexual partners, age). *Prevotella* showed an increase in post-seroconversion vs pre-seroconversion samples, and it could be possible that microbial translocation of *Prevotella* or its metabolites is occurring. Future studies should also assess potential biomarkers to define whether abundance or lack thereof a core gut microbiota species post-seroconversion leads to greater susceptibility to developing AIDS, and if there is an association with increases in serologic markers of inflammation related to translocation of microbial products, increases in HIV load, and decreases in CD4<sup>+</sup> T cell numbers.

The increase of *Prevotella* and decrease of other genera may indicate the possibility of metabolites being overproduced, underproduced, or newly produced. This inferred change in metabolites could indicate new biochemical pathways being activated or turned off. Future studies should focus on metagenomic analysis approaches, on MACS fecal samples (both 1984-1985 samples and contemporary samples), to identify any metabolites being lost or gained during microbiome dysbiosis that may lead to the development of AIDS sooner.

Another future aim will be to analyze seroconverters who developed AIDs to those who did not develop AIDS to assess any microbial compositional changes. It is inferred that microbial differences would be seen between those who developed AIDS vs those who did not.

The microbiome changes due to many factors. When analyzing the microbiome, one should keep in mind the natural microbiota changes when trying to determine which taxa are driving healthy and diseased states. For our study, we must also consider that our sample population, who tested positive for HIV within 6 months after visit 1, due to limited assays, may have produced false negatives.

## APPENDIX: SUPPLEMENTARY FIGURES

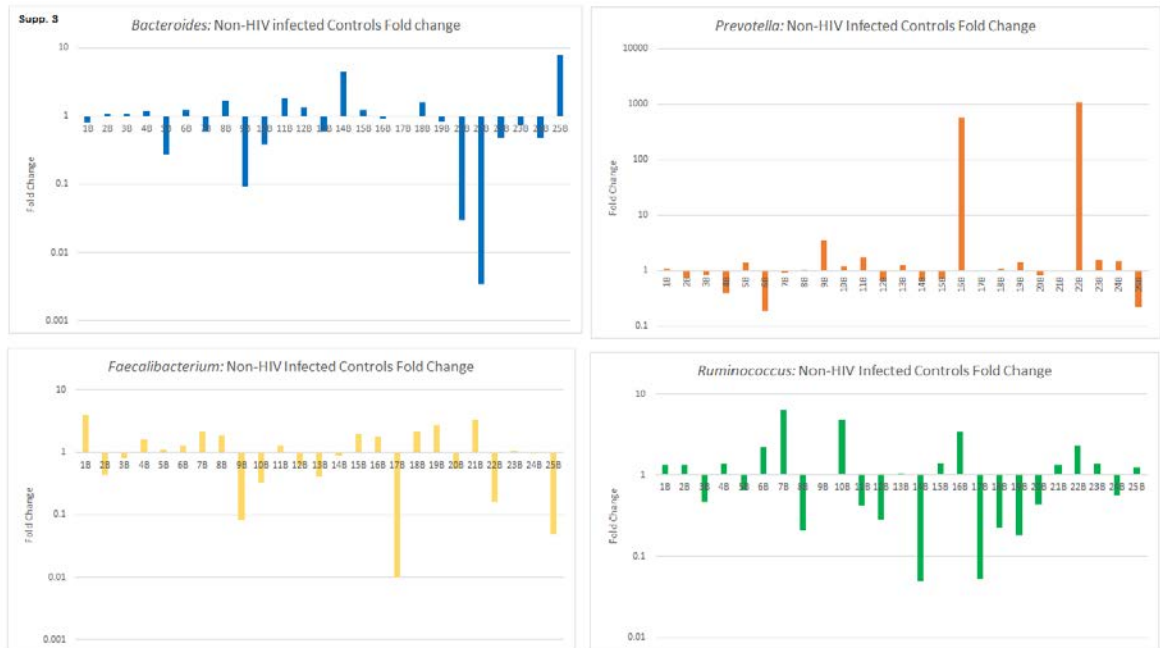


**Figure 8 Phylum level taxonomy of AIDS groupings**





**Figure 9** Fold changes of Top Four Genera from Pre- to Post Seroconversion



**Figure 10** Fold changes of Top Four Genera from Non-HIV infected controls

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