Determination of the impact of Antiretroviral therapy in the proportion and genetic diversity of diarrheal associated gut microbiota among HIV infected population.

A research report submitted in fulfillment of the requirements

For

The award of Master of science degree

Submitted by

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To

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DECLARATION

I, Musetsho Phumudzo Pertunia, hereby declare that this dissertation for the award of Master of Science degree in Microbiology at the University of Venda is my own work. It has not been submitted before for the degree examination at this or any other University. It is my own work at execution and the reference materials contained are therein have been duly acknowledged.

Signature:  Date: 31 January 2023

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DEDICATION

I would like to dedicate this Master of Science dissertation to

my daughter Ndivheni Tshilidzi Blessing and my mother Mukasi Yimisa Johannah.

ABSTRACT

Background: Human gut microbiota are microorganisms that reside in the gastrointestinal tract of humans. Gut microbiota provide various functions in the gut including protection from invasion of pathogenic microbes, shaping host immunity and substrate metabolism. HIV which targets the CD4+ T cells in the gut associated lymphoid tissue (GALT), causes gut microbiota alteration due to disruption of the gut barrier. This result in microbial translocation and pathogen invasion which lead to decreased diversity and proportion of gut microbial and increase in pathobionts and pathogens. As a result, HIV infected patients suffer from diarrhoea due to compromised gut microbiota. Antiretroviral therapy (ART) suppresses viral replication and lead to an undetectable viral load, thus restoring the immune system. Previous studies show that ART does not entirely restore the depleted gut microbiota, hence this study aims at determining the change in proportion and genetic diversity of diarrheal associated gut microbiota in HIV infected patients.

Hypothesis: Antiretroviral therapy causes changes in the proportion and diversity of diarrheal causing gut microbiota

Objective: The objective of the study was to determine the impact of ART in the proportion and genetic diversity of gut microbiota among HIV infected patients.

Methodology: Paired blood and stool samples were collected from 17 HIV infected and 11 HIV uninfected individuals (controls). Samples were collected during baseline (before ART initiation); and samples were collected after every three months thereafter, for a total of 12 months. Blood samples were used for CD4 and viral load measurements using BD FACSPresto machine (BD Biosciences) and HIV Qualitative PCR, respectively. Stool samples were used for extraction of total nucleic acid (TNA) using a modified Qiagen QIAamp Fast DNA Stool Mini Kit. Purification of TNA was done using Ampure XP bead. DNA library preparation kit (Illumina) was used for DNA library preparation. Illumina miniseq sequencing was used for sequencing and the obtained sequence reads were analysed using Geneious prime software for trimming and filtering low sequence reads and to determine the genetic diversity of diarrheal causing gut microorganisms. DRAGEN metagenomics was used for taxonomical classification to generate the proportion of diarrheal causing gut microorganism. GraphPad prism was used to generate graphs and for statistical analysis to generate the association between CD4+ T Cell/viral load and diarrheal causing gut microbiota.

Results: *Escherichia coli* (82.33%), *Bacteroides fragilis(2.46%)*, Shigella spp.(1.96%), *Salmonella enterica* (1.07%), *Clostridioides difficile*(0.99%), *Campylobacter jejuni* (0.20%) were

the most prevalent detected microoganisms from HIV infected individuals at prior ART initiation. Among HIV negative individuals, *Escherichia coli* (62.52%), *Bacteroides fragilis(18.43%)*, Shigella spp.(0.40%), *Salmonella enterica* (3.05%), *Clostridioides difficile*(15.06%), *Campylobacter jejuni* (0.54%) were found. The proportion of *Escherichia coli* (90.77%), *Clostridioides difficile* (2.20%), Shigella spp. (2.73%),and *Salmonella enterica* (1.28%) had increased with treatment at three months post treatment in HIV infected individuals. At six months post ART, there was an increase in the proportion of *Bacteroides fragilis* (88.65%) and *Vibrio cholera* (0.01%) when compared to HIV positive samples at three months post-treatment. *Escherichia coli* (9.74%), *Cryptosporidium parvum* (1.74%), and shigella spp. (0.35%) increased with treatment from six months to nine months post-treatment. *Escherichia coli* (58.58%), Shigella spp. (0.81%) and *Clostridioides difficile* (24.77%) had increased with ART at twelve months post treatment. The proportion of diarrheal causing gut microbiota were fluctuating throughout the intake of ART at different time points. There was no significant correlation between CD4/viral load and diarrheal causing microorganisms, as a result ART did not have an effect in the proportion of diarrheal causing gut microorganisms. Genetic diversity of diarrheal causing gut microbiota was higher in HIV positive individuals prior ART than in HIV negative individuals except for Salmonella typhi. Among HIV infected individuals, genetic diversity of most diarrheal causing gut microbiota was higher at three months post treatment. The fluctuation of diarrheal causing gut microbiota's genetic diversity from six months to twelve months post treatment may be due to inconsistent change in number of viral loads and CD4 counts at different time points of ART. However, this was statistically insignificant. As a result, the change in the genetic diversity of diarrheal causing gut microorganisms was not due to ART.

Conclusion: ART does not eradicate diarrheal causing gut microbiota. However, ART encourages a low genetic diversity of diarrheal causing gut microbiota.

Keywords: Gut microbiota, HIV, antiretroviral therapy, diarrhoea

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LIST OF ABBREVIATIONS

AIDS	:	Acquired immunodeficiency syndrome
ART	:	Antiretroviral therapy
CD4	:	Cluster of differentiation
Cp/mL	:	Copies per milliliter
C-section	:	Caesarean section
DGGE	:	Denaturing gradient gel electrophoresis
E.coli	:	Escherichia coli
EAEC	:	Enteroaggregative Escherichia coli
EHEC	:	Enterohaemorrhagic Escherichia coli
EIEC	:	Enteroinvasive Escherichia coli
EPEC	:	Enteropathogenic Escherichia coli
ETBF	:	Enterotoxigenic Bacteroides fragilis
ETEC	:	Enterotoxigenic Escherichia coli
GALT	:	Gut-associated lymphoid tissue
HIV	:	Human immunodeficiency virus
IL	:	Interleukin
INSTIs	:	Integrase Strand Inhibitors
LPS	:	Lipopolysaccharides
N/A	:	Not applicable
NGS	:	Next generation sequencing
NMEC	:	Neonatal Meningitis Escherichia coli
NNRTIs	:	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	:	Nucleoside reverse transcriptase inhibitors

NRTIs	:	Reverse Transcriptase Inhibitors
QC	:	Quality control
SCFAs	:	Short-chain fatty acids
SNP	:	Single nucleotide polymorphism
Spp.	:	Species
TAC	:	TaqMan Array Card
TGF-beta	:	Transforming growth factor beta
TGGE	:	Temperature gradient gel electrophoresis
Th17	:	T-helper 17
TNA	:	Total nucleic acid
Tregs	:	Regulatory T cells
UPEC	:	Uropathogenic Escherichia coli

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Microbiota is a group of microorganisms (bacteria, viruses, fungi, and parasites) that inhabit in a certain environment and do not cause any harm (Tomkovich and Jobin, 2015). Gut microbiota are the microbiota that reside in the gastrointestinal tract of humans (El-Far and Tremblay, 2018), and are acquired as early as during birth and sometimes in the womb during pregnancy as well as through breastfeeding and consumption of food (Quigley, 2017). Gut microbiota usually reside in the gut barrier, wherein they protect the gut against infectious microorganisms that need to cause infections in the gut (Passos and Moraes-filho, 2017).

The composition of the gut microbiota includes viruses, parasites, with bacteria being the most dominant with up to 50 different phyla (El-Far and Tremblay, 2018). However, only four phyla are the most common and these include *Firmicutes, Bacteroidetes,Actinobacteria, and Proteobacteria.* However*, Firmicutes* and *Bacteroidetes* are the most abundant phyla in the gut accounting for about 98% of all the gut microbiota (Liu et al., 2017).

Gut microbiota live in a mutual relationship with the gut, predominantly the bacterial flora wherein they plays a role in human's health. Hence, they provide metabolic, structural and protective functions, and gain nutrients from the gut (Flemer et al., 2017; D'Angelo, Reale and Costantini, 2017; Liu et al., 2017). The composition of gut microbiota is maintained and altered by different factors which include diet, type of delivery and breastfeeding, environmental and hygienic conditions, vaccination, antibiotic use, and infections (Liu et al., 2017; Quigley, 2017). Human immunodeficiency virus (HIV) infection is one of the infections that contribute to the alteration of gut microbiota (Chow, Tang and Mazmanian, 2011). HIV is an RNA virus that infects the immune cells which play a role in adaptive and innate immunity, including CD4+ T cells, monocytes, and macrophages (Lozupone et al., 2013). The primary impact of HIV infection is the depletion of CD4+ T cells which result in a compromised immune system (Koay, Siems and Persaud, 2018). This is mostly encountered in the gut-associated lymphoid tissue (GALT) since it contains a large number of lymphocytes (about 60% of lymphocytes) in the body, hence it results in the GALT weakening (Nwosu et al., 2014). HIV causes GALT weakening during early HIV infection; approximately 3-5 days after HIV first invades in human body (Nwosu et al., 2014).

Human immunodeficiency virus infection in the gut results in destruction of the protective gut microbiota in gut barrier and damage in gut barrier thus increasing intestinal permeability (Dinh et al., 2014). As a result, this encourages microbial translocation from the gut to the bloodstream and invasion of pathogenic microorganisms into the gut (Crum-Cianflone, 2010; Dubourg et al., 2017; Koay, Siems and Persaud, 2018). As a result, the microbial diversity in the gut is disturbed and this result in the imbalance of gut microbiota (dysbiosis), that is, increased number of normal gut microbiota that have the potential to be pathogenic (Tincati, Douek and Marchetti, 2016). The alteration of gut microbiota by HIV result in various gastrointestinal infections due to invasion of pathogenic microorganisms from the environment and overgrowth of diarrheal causing gut. HIV infection cannot be cured; however, it can be managed through the intake of antiretroviral therapy (ART) (Pinto-Cardoso et al., 2017).

Antiretroviral therapy is used worldwide by people living with HIV, improving their immune system, hence HIV has become manageable. Since 2016, ART is given to HIV infected patients immediately after diagnosis, and this has put HIV from a deadly to a chronic and manageable infection (Govere et al., 2021). However, ART does not entirely eradicate the virus in the peripheral circulation, instead, it results in the suppression of viral replication to an undetectable level, hence improving the immune function (Dinh et al., 2014). CD4+ T cells in the peripheral blood circulation are reconstituted leading to patients having increased life span.

1.2 LITERATURE REVIEW

1.2.1 GUT MICROBIOTA

The gastrointestinal tract is inhabited with a large number of microbes (over 10^{14} microbes) which form a symbiosis relationship with the body, hence they are not responsible for causing infections (Flemer et al., 2017; Pascale et al., 2018). However, some gut microbiota in unfavourable condition, become pathogenic, these are called pathobionts (Chow, Tang and Mazmanian, 2011). Gut microbiota may be acquired as early as in utero from the mother (Tomkovich and Jobin, 2015; Quigley, 2017). However, most of the gut microbes are acquired during birth wherein the proportion differs due to the type of delivery (Quigley, 2017; Pascale et al., 2018). Birth through vaginal delivery results in high number of gut microbes compared to delivery through caesarean section (C-section) (Sandhu et al., 2017).

The arrangement of gut microbiota differs in every person due to the genotype of the host and the initial settlement during birth (Passos and Moraes-filho, 2017). Although the arrangement of gut microbiota consist of viruses, parasites, and bacteria; its composition is inhabitant mostly by bacteria, and there are up to 50 different bacterial phyla in the gut (Rizzatti et al., 2017; Passos and Moraes-filho, 2017). However, only four phyla are the most common and these include *Firmicutes, Bacteroidetes* and *Actinobacteria, and Proteobacteria* (El-Far and Tremblay, 2018; Rizzatti et al., 2017).

1.2.1.1 FUNCTIONS OF GUT MICROBIOTA

Gut microbiota are essential for number of functions in humans body. They are important in the metabolism of substrates, as they harbour more metabolic enzymes than the host genome, and these enzymes independently or co-metabolize dietary or host-derived substrates (Xu, Chen and Li, 2017). They are also responsible for metabolism of bile acids from dietary substrates. The metabolism of bile acids by microbial metabolites is crucial since it results in the absorption of fat and fat-soluble vitamins, hence this prevent weight gain (Passos and Moraes-filho, 2017). Lipopolysaccharides (LPS) and short-chain fatty acids (SCFAs) are the most important metabolites produced by gut microbiota (Liu et al., 2017).

Gut microbiota that reside in the gut barrier offers protection to the gut (Flemer et al., 2017). They do this by inducing the production of antimicrobial peptides and proteins that inhibit microbial growth or kill microbes (Wells et al., 2017). For example, lactobacillus species produces lactic acid which disrupts the bacterial cell membranes (Jandhyala et al., 2015). Gut microbiota are responsible for competing with pathogenic microorganisms for nutrients, thus boosting and shaping host immunity (Xu, Chen and Li, 2017; Tomkovich and Jobin, 2015). Gut microbiota also induces the production of anti-inflammatory cells through induction of production of regulatory T cells via CD4+T cells differentiation. Some bacteria are responsible for the production of bacterial polysaccharide which encourages CD4+ T cells differentiation as well as lymphocytes development (Xu, Chen and Li, 2017).

1.2.1.2 FACTORS CONTRIBUTING TO THE COMPOSITION OF GUT MICROBIOTA

There are several factors that contribute to the composition of gut microbiota and these include; (1) diet, type of food that people consume contribute to the composition of microbiota in their gut (Quigley, 2017). Diet high in fat and proteins results in the reduction of *Bifidobacteria* (genus which falls under *Actinobacteria* phylum) and the intake of fermentable fibres or prebiotics can restore

the depleted *Bifidobacteria* (Sandhu et al., 2017). *Bifidobacteria* is responsible for the short-chain fatty acids production and inhibition of microbial invasion to the gut. In addition, diet influence three enterotypes (Bacteroides, Prevotella, and Ruminococcus) in the gut (Quigley, 2017). However, only two enterotypes are of importance, Bacteroides and Prevotella. Diet high in protein results in high levels of Bacteroides while a diet with high carbohydrates and starch result in high in prevotella species.

(2) Age can contribute to the composition of gut microbiota as well. The gut microbiota of an infant tend to differ from that of an adult. This is mostly because of the food intake that both the age group consume since infants only feed on breast milk while adults eat various kinds of food (Sandhu et al., 2017). (3) The use of antibiotics can result in the eradication of both pathogenic and commensals, thus reducing the number of gut microbiota (Langdon, Crook and Dantas, 2016). (4) Breastfeeding which is considered the best feeding type in children provide the baby with prebiotics, immunoglobulin A as well as cytokines. As compared to baby formula, breast milk has abundant oligosaccharides which are important substrates for *Bifidobacteria* hence *Bifidobacteria* are the most abundant in infants, and *Bifidobacteria* are important in reducing the PH in the gut thus inhibiting bacterial growth.

The above mentioned factors are mostly responsible for maintaining the gut microbiota, however, infection by HIV alters microbial composition and diversity. The alteration of gut microbiota result in dysbiosis (Villanueva-Millán et al., 2017).

1.2.2 DIARRHEAL CAUSING MICROORGANISMS IN THE GUT

A person is said to have diarrhoea if they have passage of three or more loose or watery stool in one day (Anand et al., 2016; Chu et al., 2020). Diarrhoea causes an increase in morbidity and mortality in the general population including both adults and children worldwide, however, it is the leading cause of death in children under the age of five years (Liu et al., 2013; Anand et al., 2016; Angarone and Snydman, 2019; Khurana et al., 2021). It is hard to differentiate diarrheal symptoms for different etiologic agents (Anand et al., 2016). Various microorganisms are responsible for causing diarrhoea and they range from bacteria, viruses, and parasites (Liu et al., 2013; Khurana et al., 2021).

Diarrheal causing microorganisms are differentiated into inflammatory (induce inflammatory reaction by cytokines activation after adhesion of intestinal mucosa) and non-inflammatory (cause infection through absorptive processes without inflammation) microorganisms (Anand et al., 2016). Non inflammatory diarrheal causing microorganisms include *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC) and rotaviruses (Khurana et al., 2021). *Salmonella spp*., *Shigella spp*., *Entamoeba histolytica* enterohemorrhagic *Escherichia coli*, enteroaggregative *Escherichia coli* and *Clostridium difficile* are inflammatory diarrheal causing microorganisms (Ajmera and Shabbir, 2021; Khurana et al., 2021). Diarrheal causing gut microorganisms are diverse in the human gastrointestinal tract, however, Salmonella spp., Escherichia Coli and Bacteroides fragilis are the most prevalent.

1.2.2.1 SALMONELLA SPP.

Salmonella species are Gram-negative, flagellated facultatively anaerobic bacilli characterized by O, H, and Vi antigens (Chu et al., 2020). They cause salmonellosis which ranges from common gastroenteritis to enteric fevers. The symptoms of gastroenteritis include abdominal pain, diarrhoea and fever, and an example of enteric fevers is typhoid fever. Non typhoidal salmonellosis is usually spread through ingestion of contaminated food, however, person to person transmission also occurs (Andino and Hanning, 2015; Magwedere et a., 2015; Ramatla et al.,2022). Typhoid fever occurs through person to person spread because these organisms lack a significant animal reservoir. Typhoid fever is a life threatening febrile systemic illness and the most important typhoid Salmonella is *Salmonella typhi*. Two species of Salmonella include *Salmonella bongori* and *salmonella enterica* (Ramatla et al.,2022). *Salmonella enterica* is subdivided into subspecies and these include subspecies enterica, salamae, arizonae, diarizonae, houtenae, and indica based on biochemical and genomic modification (Andino and Hanning, 2015).

1.2.1.2 ESCHERICHIA COLI

Escherichia coli (*E. coli*) is a gram negative, facultative anaerobe bacterium under Enterobacteriaceae family (Jang et al., 2017). They inhabit the gastrointestinal tract symbiotically (Anand et al., 2016; Jang et al., 2017). *E. coli* are found in the gastrointestinal tract few hours after birth. In addition to the mutual non-pathogenic *E. coli*, there are pathovars that cause infections mainly in immunocompromised individuals (Gomes et al., 2016). In addition, non-pathogenic E. coli can cause infections in conditions such as when the gastrointestinal tract is breeched. Pathogenic *E.coli* can be classified into diarrheagenic or extraintestinal E. coli (Gomes et al.,

2016). Enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli (*EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) are Diarrheagenic *E. coli* (Anand et al., 2016). The common extraintestinal *E. coli* include uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) (Gomes et al., 2016).

1.2.1.3 BACTEROIDES FRAGILIS

Bacteroides species are mutualistic in the gastrointestinal tract of humans (Wick and Sears, 2010). However, when the gastrointestinal tract environment has been compromised, *Bacteroides fragilis* becomes opportunistic and causes gastrointestinal infections (Wick and Sears, 2010). *Bacteroides fragilis* is a gram negative, anaerobic bacterium that falls under phylum Bacteroidetes (Hahnke et al., 2016). Only 1%-2% of Bacteroides species constitute *Bacteroides fragilis* in the gastrointestinal tract (Yekani et al, 2020). Enterotoxigenic *Bacteroides fragilis* (ETBF) are the common strains that cause diarrheal diseases in both children and adults (Wick and Sears, 2010; Amiri et al, 2022). In addition, ETBF is the most virulent species of Bacteroides species, and antibiotic resistance is common among this strain (Amiri et al, 2022).

1.2.3 HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND GUT MICROBIOTA

Human immunodeficiency virus is a member of Retroviruses and it is an RNA virus, spherical and enveloped (Ratnam et al., 2018). HIV target the immune cells including CD4+ T lymphocytes macrophages and monophages, however, CD4+ T lymphocytes are the most targeted (Brenchley et al., 2006; Nwosu et al., 2014). HIV infections are divided into three stages which include acute HIV infection, clinical latency and Acquired immunodeficiency syndrome (AIDS) (Klimas, Koneru and Fletcher, 2008). Acute HIV infection occurs within two to four weeks after the infection, and is characterised by flu like symptoms which is due to the body fighting against the virus (Cohen et al., 2010). Clinical Latency stage is ussually asymptomatic, however, if not treated the viral load starts to escalate and the CD4+ T cells decline. Furthermore, If not treated, clinical lactency progresses to AIDS. AIDS is the advanced stage of HIV which is due to weakened immune system, and it is usually characterised by the CD4+ T cells <200 cells/μL and opportunistic infections (Ratnam et al., 2018). South Africa is a leading country with the highest rates of HIV infections globally (Edet et al.,2019; Statistics South Africa, 2021).

The gastrointestinal tract is the primary site of HIV infection. In the gut, HIV targets the gut-associated lymphoid tissues (GALT) since most of the lymphocytes reside there (Marx, 2015).

HIV infection prompt the depletion of CD4+ T lymphocytes which elicit a compromised immune system (Nwosu et al., 2014). The CD4+ T cells in the GALT comprise of IL-17-producing T-helper 17 (Th17) and regulatory T cells (Tregs). HIV infection result in the loss of Th17 which is responsible for making microbial invasion less severe through the recruitment of neutrophils and this is crucial for antimicrobial defence (El-Far and Tremblay, 2018).

Th17 also play a role in maintaining the integrity of the gastrointestinal tract (Li et al., 2016). HIV also lead to loss of gut barrier function and microbial translocation (Dubourg et al., 2017). Tregs are responsible for promoting immune homeostasis and they produce IL-10 and TGF-beta which help to down-modulate the effector activity of other immune cells. HIV infection results in the loss of these cells, which give rise to chronic immune activation that can eventually lead to inflammation in the periphery and the gut (Li et al., 2016). Tregs cells are also important in the colonization of some bacteria in the gut, therefore their loss promote dysbiosis.

1.2.3 ANTIRETROVIRAL THERAPY (ART)

Patients who are HIV positive depend on ART for managing the infection (Pinto-Cardoso et al., 2017). As a result, ART prevent the progression of HIV infection to AIDS (Tincati, Douek and Marchetti, 2016). ART is given to all patients diagnosed with HIV infection immediately after diagnosis (Boyd et al., 2019; Govere et al., 2021). As a result, ART reduces morbidity and mortality due to HIV and improves the health of HIV infected patients (Mateo-Urdiales et al., 2018; Boyd et al., 2019). Although ART does not entirely eradicate HIV in the peripheral blood, it suppresses the virus reducing its viral particles and restricting viral replication thus prolonging people's lives (Hong and Mellors, 2015; Rajagopaul and Naidoo, 2021).

In addition, ART reduces person to person HIV transmission, prolonging the lives of HIV infected individuals, and result in increase in CD4+ T cells (improves the immune system) (Lederman et al., 1998; Pau and George, 2014). The restoration of CD4+ T cells may have a positive impact on the gut microbiota of HIV infected individuals (Flygel et al., 2019). It has been reported that ART has an effect in the composition of gut microbiota, the composition of gut microbiota changes with ART intake (Pinto-Cardoso et al., 2017; Nishijima et al.,2016). In addition, it has been reported that some ART, for example NRTIs, can exhibit antibacterial effects (Maier et al., 2018). Several studies have also reported that ART does not entirely restore the depleted gut microbiota to resemble that of HIV uninfected individuals (Noguera-Julian et al., 2016; Lozupone et al.,2013; Mutlu et al., 2014).

1.2.4 GUT MICROBIOTA AND NEXT GENERATION SEQUENCING (NGS)

Before the era of Next generation sequencing, culturing methods were used for assessing gut microbiota (Fouhy et al., 2012). However, this technique was only limited to culturable organisms. As a result, since gut microbiota is a highly diverse microbial community, only a small fraction of gut microbiota was isolated and assessed due to a large proportion being unculturable (Milani et al., 2017). However, the introduction of culture-independent methods such as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), and Dot-blot hybridization provided an overview of gut microbial composition (Fouhy et al., 2012). However, these techniques provide limited phylogenetic information of gut microbiota and can fail to provide data of gut microbiota as whole, while providing data of specific population (Gillilland et al, 2012).

The establishment of high throughput sequencing of gut microbiota prompted the assessment of a large proportion of gut microbiota (Milani et al., 2017). Metagenomics which is the study of all genetic materials in an environment, has become the widely used approach since it allows profiling of complex microbial communities (Gillilland et al, 2012). This approach is extensively used for identification and sequencing of gut microbiota, since it allows for sequencing of whole genome (Fouhy et al., 2012). The advantage of this approach is that it provides accurate and rapid taxonomic identification compared to the expensive and slow earlier approaches (i.e. Culture-dependent) (Arnold, Roach and Azcarate-Peril, 2016). Next generation sequencing is also important since it provides the proportion of each member of the gut microbiota, as compared to real time PCR which proportionate the gut microbiota as a whole without species identification (Fouhy et al., 2012).

1.3 STUDY RATIONALE

HIV targets CD4+ T cells of the immune system. The primary site for infection is the gut-associated lymphoid tissues (GALT) of the gut which contains the majority of CD4+ T cells in the body. HIV infection in the gut results in the depletion of CD4+ T cells and disruption of the gut barrier (Liu et al., 2017), which then lead to leakage and translocation of the gut microbiota (Dubourg et al., 2017). The translocation of gut microbes lead to the change in the microbial homeostasis. It also results in the loss of crucial microbes in the gut which have several functions including the protection against invasion of pathogenic microbes into the gut (Liu et al., 2017).

Microbial translocation from the gut to the peripheral blood circulation also causes a reduced microbial diversity in the gut, as a result, this encourages the invasion of pathogenic microbes and overgrowth of pathobionts (resident microbes with pathogenic capabilities) due to less or compromised protective gut microbiota (Chow, Tang and Mazmanian, 2011). As a result, about 40–80% of HIV infected patients suffer from diarrhoea which increases mortality and morbidity especially in developing countries (Elfstrand and Floren, 2010; Feasey, Healey and Gordon, 2011).

Enteric organisms, such as Campylobacter spp., Salmonella spp., Shigella spp., and different groups of enteropathogenic *Escherichia coli* are known to cause diarrhoea in the general population worldwide. In addition, they have also been isolated as the pathobionts of HIV infected patients in South Africa (Obi and Bessong, 2002; Obi et al., 2007; Samie, et al., 2010). Furthermore, enteric infections are more frequent and severe in HIV infected individuals than the general population. Previous studies show a decrease in the diversity of gut microbiota in HIV infected patients, with an increase in pathobionts/pathogens including groups of enterobacteriaceae (Monaco et al., 2016; Nowak et al., 2017).

The manifestations and duration of diarrhoea due to enteric microorganisms is influenced by immunity which is measured by the amount of CD4+ T cells. According to Sanchez et al., 2005, patients with lower CD4+ T cells are more likely to experience diarrheal manifestations. It has been documented that ART is able to improve the immune system due to the restoration of depleted CD4+ T cells leading to undetectable levels of the virus (Dinh et al., 2014). However, ART is not able to entirely restore the depleted gut microbiota, as a result, the gut microbiota of HIV infected patients treated with ART does not resemble that of HIV negative patients (Lozupone et al., 2013; Mutlu et al., 2014).

Previous studies show that patients taking ART still suffer from diarrhoea (Vujkovic-Cvijin et al., 2013; Mutlu et al., 2014). However, in South Africa, information about the proportion and genetic diversity of diarrheal causing gut microbiota throughout the intake of ART still lacks . This study aimed at determining the change in proportion and genetic diversity of diarrheal associated gut microbiota in HIV infected patients before and after taking ART. With the use of metagenomics by next generation sequencing (NGS), it was possible to analyse and compare microbial species that may not be found when using other traditional methods.

1.4 HYPOTHESIS

Antiretroviral therapy causes changes in the proportion and diversity of diarrheal causing gut microbiota.

1.5 OBJECTIVES OF THE STUDY

1.5.1 MAIN OBJECTIVE

To determine the impact of ART in the proportion and genetic diversity of gut microbiota of the HIV infected population.

1.5.2 SPECIFIC OBJECTIVES

1. To explore the proportion of gut microbiota before and after ART using metagenomics approach.
2. To determine the gut microbiota genetic diversity before and after ART.

CHAPTER 2: METHODOLOGY

2.1 ETHICAL CLEARANCE

This project is nested within a mother project to which ethical clearance and permissions to access health facilities were obtained from the University of Venda's Research Ethics Committee and the Provincial Department of Health, respectively (project number SMNS/17/MBY/24/1709).

The purpose of this study was explained to each participant and specific research codes were allocated to protect the patients' identity when they consent.

2.2 SAMPLE COLLECTION, PROCESSING AND STORAGE

A total of 56 paired blood and stool samples were collected from 17 HIV positive and 11 HIV negative participants from Donald Fraser hospital and the University of Venda Campus Health clinic, respectively. Samples were collected at baseline (before ART initiation) and at three months intervals for the period of twelve months. The total number of collected samples from HIV positive participants were sixteen for baseline, thirteen for first follow up, six for second follow up, three for third follow-up and one for fourth follow-up. Samples collected from HIV negative participants were eleven for baseline and five for first follow-up. Blood samples were processed for CD4 and viral load measurements. Stool samples were stored at -80°C freezer, prior to downstream processes.

2.3 VIRAL LOAD AND CD4 MEASUREMENTS

Whole blood samples were used for viral load and CD4 measurements. Viral load measurements (cp/mL) were performed at Lancet laboratories to determine the number of HIV viral copies in each patients' system. HIV Quantitative PCR was performed to test for viral load. CD4 measurements (cells p/μL) were done onsite at Donald Fraser Hospital and the University of Venda using Beckman Coulter equipment (PanLeucogating method) and BD FACSPresto machine (BD Biosciences, USA), respectively; to measure CD4 cells present per microliter of blood following the manufacturer's instructions.

2.4 TOTAL NUCLEIC ACID EXTRACTION AND QUANTIFICATION

Stool samples from -80°C freezer were allowed to thaw at room temperature for processing. Samples were aliquoted into 2 ml cryotubes for archiving and in one 1.5 ml screw-cap tube for

extraction of total nucleic acid (TNA). For extraction purposes, 200 mg and 200 µl were weighed for hard and loose stools, respectively, per tube. A modified Qiagen QIAamp Fast DNA Stool Mini Kit for extraction of TNA from stool samples was used (Liu *et al*, 2013, University of Virginia, USA).

Before extraction, the preparation of the intrinsic (internal) control was done. Twenty-five microliters of phage MS2 (Applied Biosystems, USA) was added into a tube containing one milliliter of InhiibitEx buffer (for removal of PCR inhibitors in stools) and mixed to form InhibitEX/MS2 solution. The solution was prepared for N+1 samples to be processed, meaning that if 10 samples were to be processed then the solution was prepared for 11 samples.

Two hundred milligram of hard stool samples or 200 µl of liquid stools was weighed into 2 ml microcentrifuge tubes. One epperndorf tube capful or 370 mg of acid-washed glass beads (Sigma Aldrich, SA) were added to the weighed stool samples. One milliliter of InhibitEX/MS2 solution was added into the tubes containing the stools and glass beads, then vortexed for 1 minute followed by bead beating at 13,400 rpm for 3 minutes using a cell disruptor. The suspensions were incubated at 95°C for 5 minutes, then vortexed for 15 seconds, followed by centrifugation at 13,400 rpm for 1 minute to pellet the stool particles. Six hundred microliter of the supernatant was added into the 2 ml microcentrifuge tube containing 25 µl of proteinase K, followed by the addition of 600 µl buffer AL then vortexed for 15 seconds. The formed homogeneous solution was incubated at 70°C for 10 minutes, then centrifuged briefly to remove the drops from the inside the tube lid. Addition of 600 µl of 100 % ethanol to the lysate was done and mixed by vortexing then centrifuged briefly to remove drops from the inside of the tube lid. The QIAamp spin column lid was labelled and placed on a 2 ml collection tube, then 600 µl of the lysate was carefully applied to the QIAamp spin column without moistening the rim, followed by centrifugation at 13,400 rpm for 1 minute.

The QIAamp spin column was retained and placed in a new 2 ml collection tube, discarding the collection tube containing the filtrate. To use all the lysate, this was repeated 2 more times: an additional 600 µl of the lysate was applied to the QIAamp spin column without moistening the rim, then centrifuged at 13,400 rpm for 1 minute, retaining the column and discarding the collection tube and the filtrate. The spin column was placed in a new collection tube and 500 µl buffer AW1 was added, then centrifuged at 13,400 rpm for 1 minute, the QIAamp spin column was placed into a new 2 ml collection tube and 500 µl buffer AW2 was added and centrifuged at 13,400 rpm for 3 minutes. The QIAamp spin column was retained and placed into a new 2 ml collection tube,

then centrifuged at 13,400 rpm for 3 minutes to eliminate the chance of possible buffer AW2 carryover. To elute the TNA, the QIAamp spin column was placed into a new labelled 2 ml microcentrifuge tube, and 200 µl of buffer ATE was added directly onto the QIAamp membrane then incubated at room temperature for 3 minutes followed by centrifugation at 13,400 rpm for 1 minute. The TNA was stored at -80°C freezer for subsequent experiments.

The extracted TNA was quantified for both DNA and RNA nucleic acids concentrations using Implen nanophotometer (Labotec, SA). Quantifying both nucleic acids was done to serve the sequencing purposes.

2.5 NEXT-GENERATION SEQUENCING

2.5.1 PURIFICATION OF EXTRACTED TOTAL NUCLEIC ACID

Extracted TNA was purified using AMPure XP beads (Beckman Coulter, USA). For 20 µl of TNA, 90 µl of AMPure XP beads were added followed by pipette mixing 10 times to bind the beads to the samples. Mixed samples were then incubated for 5 minutes at room temperature followed by placing the 96 well plate containing the samples into the magnet plate for 2 minutes to separate beads from the solution. The supernatant was aspirated when the solution became clear, followed by washing with 200 µl of 70 % ethanol to each well in the plate, and the solution was incubated for 5 minutes. Before the plate was removed from the magnet plate, 70 % ethanol was aspirated from the solution. Each well was added with 40 µl of elution buffer and mixed 10 times then incubated for 2 minutes at room temperature. The plate was placed into the magnetic plate to allow for the beads to separate from the solution and elute was transferred to a new plate to be used for sequencing.

2.5.2 DNA LIBRARY PREPARATION AND SEQUENCING

Purified TNA was used for metagenomics sequencing of gut microbiota by Illumina Miniseq (Illumina, USA) sequencing platform for identification of all diarrheal causing gut microbiota. Nextera DNA library preparation kit (Illumina, USA) was used for library preparation. A published protocol was used (Matume et al., 2019). Briefly, TNA was fragmented and tagmented with Illumina sequencing adapters and index primers from Nextera XT Index Kit (Illumina, USA). Index PCR amplification, purification by AMPure XP beads followed by quantification using Qubit ds high sensitivity kit (Thermofisher Scientific, USA) was done. Quantified libraries were normalized

and pooled in one tube. Pooled libraries were denatured and diluted to a loading concentration of 1.8 pM and loaded into the MiniSeq reagent cartridge, then subjected to a run for approximately eight hours using an Illumina Miniseq sequencing machine with a mid-output flow cell. After the run, sequence analysis was done.

2.5.3 SEQUENCE ANALYSIS

After sequencing, the obtained sequences were subjected to FastQC V0.11.8 for quality control. The sequences were then submitted to Geneious Prime software version 2019.3.0, where the sequences with low quality and error probability limit of less than 1% were trimmed and filtered.

For taxonomical classification from domain level to species level, the sequence reads were subjected to DRAGEN metagenomics, an Illumina BaseSpace online sequence analysis tool. For genetic diversity, the test sequences were mapped with reference sequences of diarrheal causing gut microbiota. Consensus sequences were generated and used for multiple alignments. The polymorphisms were found using the option “find variants”. Polymorphisms were used to determine the sequence variation (genetic diversity) of each diarrheal causing gut microbiota. GraphPad Prism V8.4.3 was used to generate bar graphs and for correlation of CD4+ T cells/viral load and diarrheal causing gut microbiota. Correlation between CD4+ T cells/viral load and diarrheal causing gut microbiota was determined by using spearman correlation test.

CHAPTER 3: RESULTS

3.1 CLINICAL AND DEMOGRAPHIC CHARACTERISTICS OF THE STUDY PARTICIPANTS

The demographic and clinical characteristics of the 17 HIV positive and 11 HIV negative participants are described in Table 1. Prior ART initiation, all HIV positive participants were ART-naïve with detectable plasma viral loads (median 81 050 copies/ml, range= 931–5600 000 copies/ml). After their enrolment, a total of four follow-ups were collected at three months interval for twelve months. After the first visit, the participants had decreased viral load (range = 0 - 276 778 copies/ml, median = 22 copies/ml for three months post-treatment; range = 0 – 203 copies/ml, median = 22 copies/ml for six months post-treatment; range = 19 – 276 778 copies/ml, median = 20 copies/ml for nine months post-treatment; and range = 19 – 4 260 copies/ml, median = 20 copies/ml for twelve months post-treatment), with three participants (23.1%) at three months post-treatment and 2 participants (33.3%) at six months post-treatment having undetectable viral load.

Table 1: Demographics and clinical characteristics of study participants

	HIV positive participants (n=17)	HIV negative participants (n=11)
Age (median)	3-60 years (median: 39)	18-28 (median: 23)
Gender:		
Male	7 (41.2%)	2 (18.2%)
Female	10 (58.8%)	9 (81.8%)
Marital status:		
Married	11 (64.7%)	0
Widower	1 (5.9%)	0
Single	4 (23.5%)	11 (100%)
N/A	1 (5.9%)	0
Risk of infection:		N/A
Sexual	14 (82.4%)	
Breastfeeding	1 (5.9%)	
Unknown	2 (11.8%)	
Year of treatment initiation		N/A
2018	14 (82.4%)	
Unknown	3 (17.6%)	
Complementary medicine		
Acne medicine		1 (11.1%)
Antibiotics:	2 (11.8%)	
Vitamin supplements	2 (11.8%)	
TB treatment	1 (5.9%)	
Viral load (counts per ml):		
Baseline (n= 17)	931 - 5 600 000	N/A
Three months (n= 13)	0 - 276 778	
Six months (n= 6)	0 – 203	
Nine months (n= 4)	19 - 47322	
Twelve months (n= 2)	19 – 4260	

n: number of participants, N/A: not applicable, 0: none

3.2 METAGENOMICS SEQUENCE ANALYSIS

3.2.1 SEQUENCE QUALITY CONTROL

The metagenomic sequence run generated sequences with an output yield of 4.5 Gb and QC of 85.3% ≥ Q30. The expected mid-output sequencing performance parameters are; output yield = 2.1-2.4 Gb, and minimum QC of 75% ≥ Q30. However, the output yield generated by this run was higher, this could be because of metagenomics sequencing as it was able to sequence the entire genomes in the samples. The range of generated sequence reads was from 72 264 reads to 590 566 reads, median = 290 474. The expected sequence reads are > 100 000 (Illumina, USA). Three samples from a total of 56 samples were excluded from the analysis because they did not pass QC of >100 000 sequence reads. FastQC was also used to plot per base sequence quality scores of the generated sequences (Figure 1). The background of the graph divides the y-axis into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). The base call of the sequences that passed the QC was very good, however, the quality degrades from orange to red as the run progresses. Hence the base calls were falling into the red area toward the end of the run. Moreover, it is common for the run to degrade towards the end of the run.

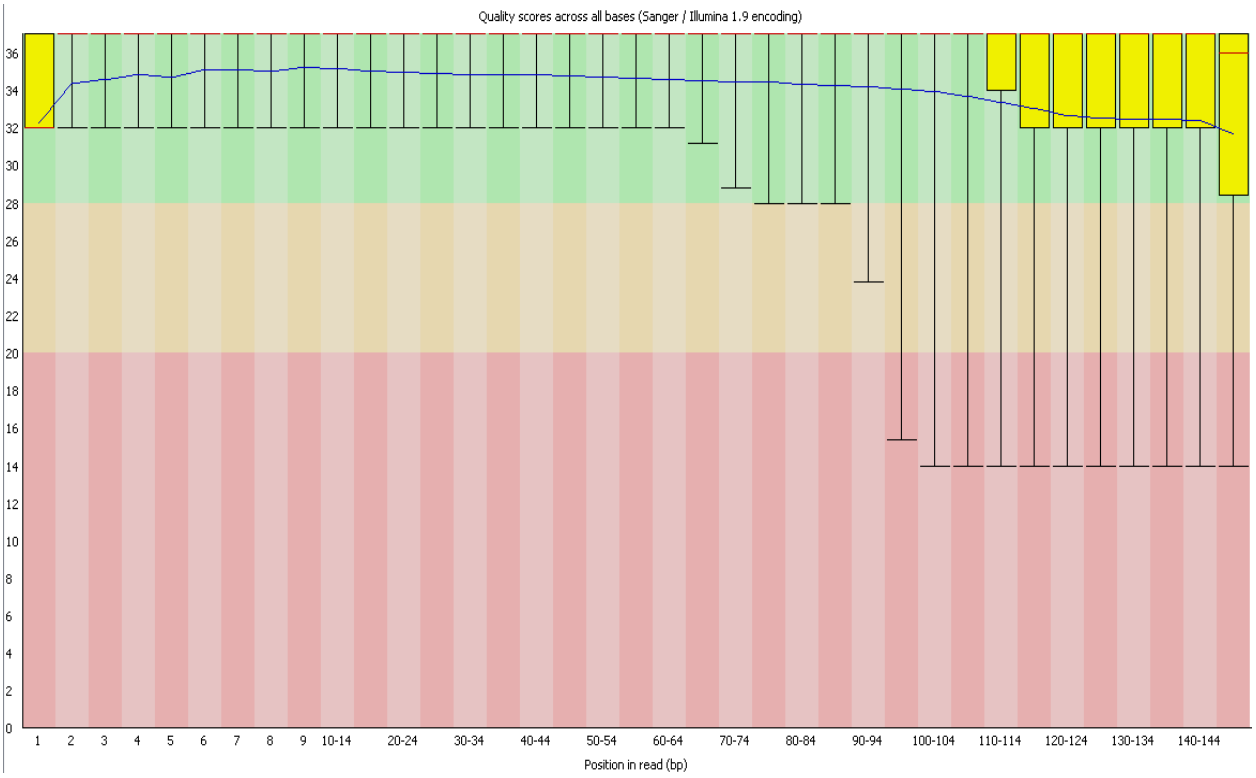

Figure 1: The representative quality scores of test samples per base sequence quality, generated from FastQC software. Y-axis represents the quality scores and the x-axis is the position in sequence read. The higher the score the better the base call.

3.2.2. DIARRHEAL CAUSING GUT MICROBIOTA COMPOSITION

3.2.2.1 HIV positive participants before ART

The composition of diarrheal causing gut microbiota was assessed from reliable DNA sequence of HIV infected samples. HIV infected participants prior ART exhibited various diarrheal causing gut microbiota, these include; *Escherichia coli*, *Bacteroides fragilis*, Shigella spp., *Salmonella enterica*, *Clostridioides difficile*, *Campylobacter jejuni*, *Helicobacter pylori*, *Campylobacter coli, Cryptosporidium parvum, Plesiomonas shigelloides, Vibrio cholera* and *Yersinia enterocolitica*. The composition of these diarrheal causing gut microbiota is shown in Figure 2A. *Escherichia coli* and *Bacteroides fragilis* were the most prevalent, whereas *Cryptosporidium parvum* and *Plesiomonas shigelloides* were the least prevalent in HIV positive samples prior ART.

3.2.2.2 HIV negative participants

Among HIV negative participants, reliable DNA sequence were used for the detection of diarrheal causing microorganisms . Diarrheal causing gut microbiota were; *Escherichia coli, Bacteroides fragilis, Clostridioides difficile, Salmonella enterica, Campylobacter jejuni* and Shigella spp.. The composition of diarrheal causing gut microbiota in HIV negative participants is shown in Figure 2B, with *Escherichia coli* been the most prevalent and shigella spp. the least prevalent. All the diarrheal gut microbiota that were detected from HIV negative samples were also found in samples from HIV positive prior ART initiation at different percentage of occurrence. *Helicobacter pylori, Campylobacter coli, Cryptosporidium parvum, Plesiomonas shigelloides, Vibrio cholera and Yersinia enterocolitica* were detected in HIV positive but not in HIV negative samples. *Escherichia coli* and Shigella had a high percentage of occurrence in HIV positive than in HIV negative. *Bacteroides fragilis, Salmonella enterica, Clostridioides difficile, and Campylobacter jejuni* had high percentage of occurrence in HIV negative than in HIV positive samples.

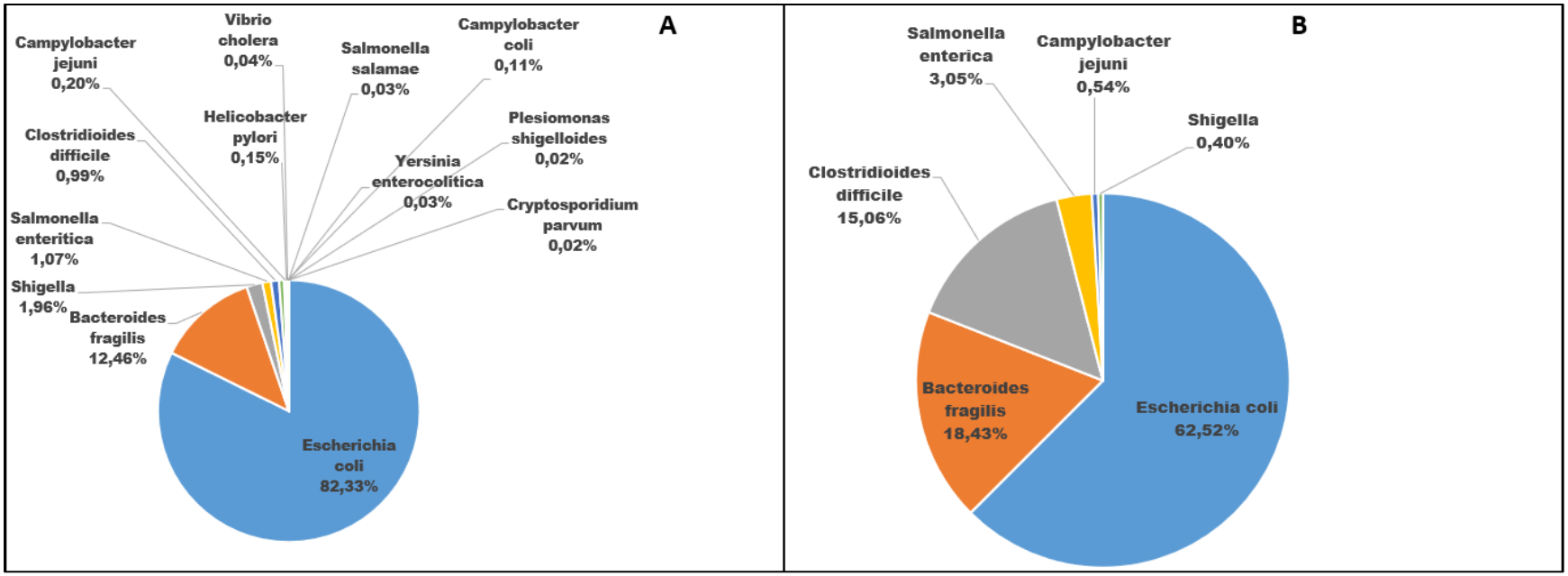

Figure 2: The composition of diarrheal causing gut microbiota. **(A)** Diarrheal causing gut microbiota in **HIV positive patients prior ART**. **(B)** Diarrheal causing gut microbiota in **HIV negative participants**. The number of samples used were 17 for HIV positive samples prior ART and 11 for HIV negative samples. More diarrheal causing gut microbiota were found in HIV positive than in HIV negative samples.

3.2.2.3 Three months post treatment initiation

From a total of 16 samples that passed the QC prior ART initiation, 12 samples were followed up at three months post-treatment initiation. Comparison of diarrheal causing gut microbiota prior ART and three months post-treatment was done for the 12 followed up samples (Figure 3). *Escherichia coli, Bacteroides fragilis, Clostridioides difficile, Shigella, Salmonella enterica, Cryptosporidium parvum, Helicobacter pylori, Campylobacter jejuni, Campylobacter coli, Yersenia enterocolitica, Plesiomonas shigelloides* and *Vibrio cholera* were detected in HIV samples at three months post-treatment initiation. The diarrheal causing gut microbiota that was found in HIV positive samples at three months post-treatment, were also found in HIV positive samples prior ART initiation at different percentage of occurrence. *Escherichia coli, Clostridioides difficile,* Shigella*, Salmonella enterica* and had increased with treatment. *Bacteroides fragilis, Helicobacter pylori, Campylobacter Jejuni, Campylobacter coli, Vibrio cholera, Plesiomonas shigelloides,Cryptosporidium parvum* and *Yersinia enterocolitica* had decreased with treatment initiation at three months post-treatment.

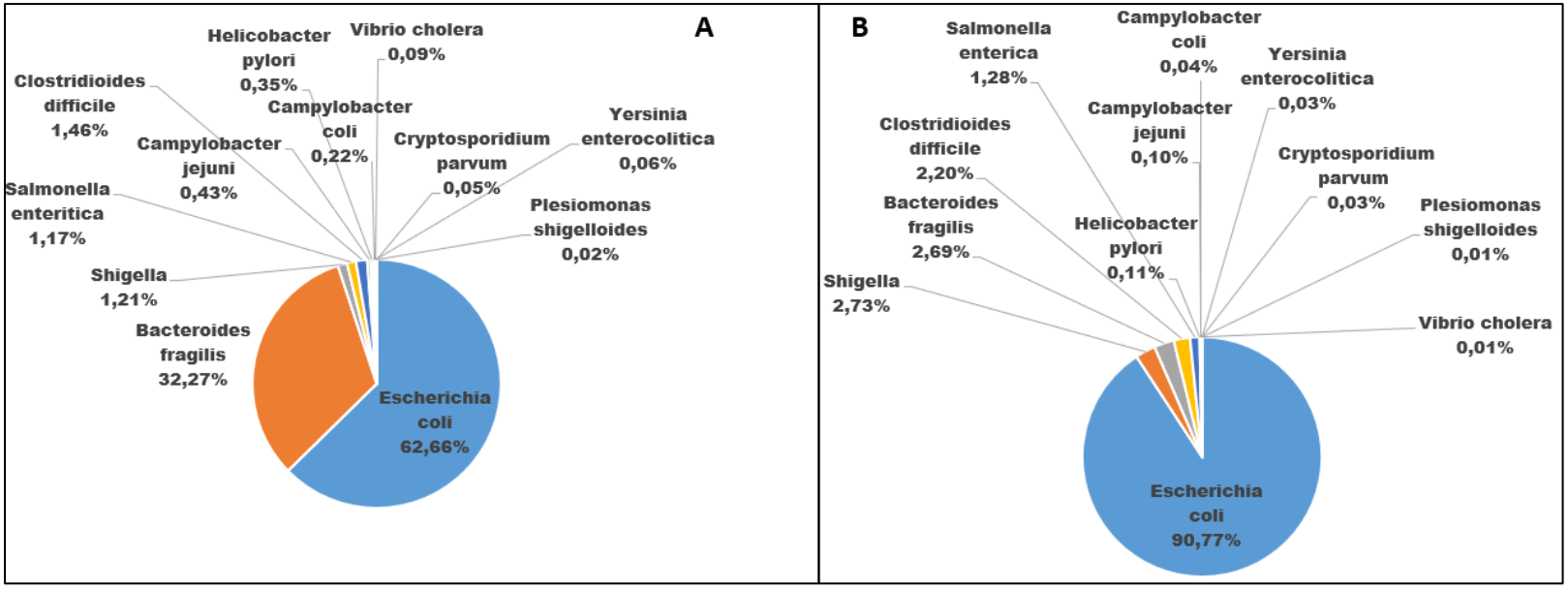

Figure 3: The comparison of diarrheal causing gut microbiota composition in HIV positive samples. **(A)** Composition of diarrheal causing gut microbiota in **HIV positive samples prior ART initiation**. **(B)** The composition of diarrheal causing gut microbiota in HIV positive samples at **three months post-treatment**. Only samples that were followed up at six months were used for composition of diarrheal causing gut microbiota at three months post-treatment (n= 12).

3.2.2.4 Six months post treatment initiation

Five samples were followed up at six months post-treatment from HIV positive individuals, there was no collection from HIV negative individuals. Diarrheal causing gut microbiota were compared at three month and six months post-treatment (Figure 4). At six months post-treatment in HIV positive individuals, *Escherichia coli, Bacteroides fragilis, Clostridioides difficile,* Shigella*, Cryptosporidium parvum, Helicobacter pylori, Campylobacter jejuni* and *Vibrio cholera* were detected. *Vibrio cholera* was detected at six months post treatment and not at three months post-treatment. *Salmonella enterica, Campylobacter coli*, and *Plesiomonas shigelloides* were

detected in HIV positive participants at three months post-treatment, however, they were not found at six months post-treatment. *Bacteroides fragilis* and *Vibrio cholera* had increased with treatment at six months post-treatment when compared to HIV positive participants at three months post-treatment. *Escherichia coli, Clostridioides difficile,* Shigella*, Cryptosporidium parvum, Helicobacter pylori,* and *Campylobacter jejuni* had decreased at six months post-treatment in HIV positive participants.

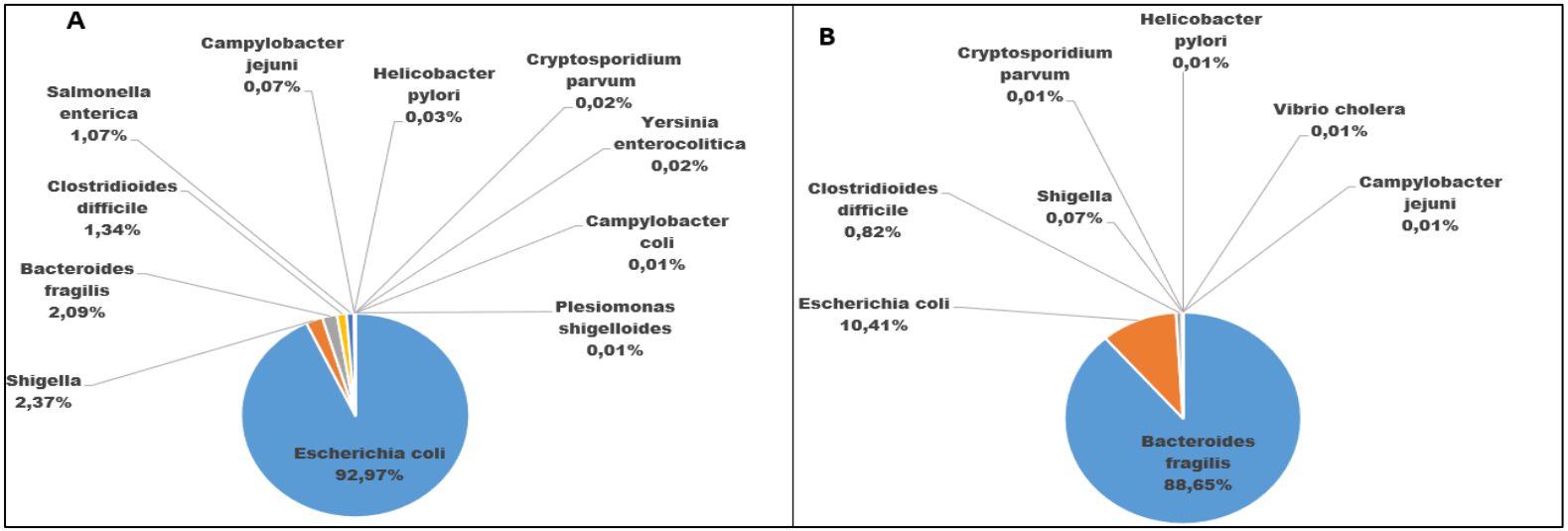

Figure 4: The comparison of diarrheal causing gut microbiota composition in HIV positive samples. **(A)** Composition of diarrheal causing gut microbiota in **HIV positive samples at three months post-treatment**. **(B)** The composition of diarrheal causing gut microbiota in **HIV positive samples at six months post-treatment**. Only samples that were followed up at six months were used for composition of diarrheal causing gut microbiota at three months post-treatment (n= 5).

3.2.2.5 Nine months post treatment initiation

Three samples were followed up from six months to nine months post-treatment. Comparison of diarrheal causing gut microbiota at six months and nine months post-treatment was done for the three followed up samples, this is depicted in Figure 5. Among HIV positive

samples at nine months post-treatment, *Bacteroides fragilis, Escherichia coli, Shigella, Clostridioides difficile, Cryptosporidium parvum, Plesiomonas shigelloides* and *Yersinia enterocolitica* were detected. From the diarrheal causing gut microbiota that were detected in HIV positive samples at nine months post-treatment, *Yersinia enterocolitica* and *Plesiomonas shigelloides* were not found in HIV positive samples at six months post-treatment. *Helicobacter pylori* was found in HIV positive samples at six months post-treatment, however, it was not detected at nine months post-treatment. *Bacteroides fragilis* and *Clostridioides difficile* decreased with treatment from six months post-treatment to nine months post-treatment. *Escherichia coli, Cryptosporidium parvum,* and shigella increased with treatment from six months to nine months post-treatment. *Salmonella enterica, Campylobacter coli, Campylobacter jejuni* and V*ibrio cholera* were not detected at both six and nine months post treatment.

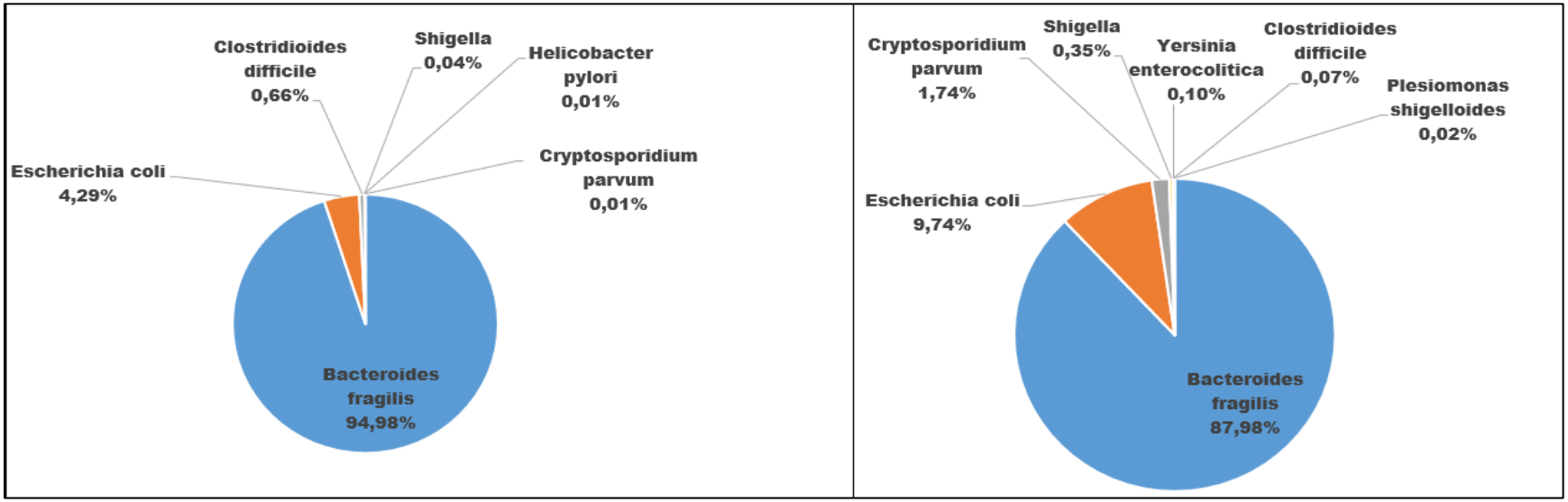

Figure 5: The comparison of diarrheal causing gut microbiota composition in HIV positive samples. **(A)** Composition of diarrheal causing gut microbiota in **HIV positive samples at six months post-treatment**. **(B)** The composition of diarrheal causing gut microbiota in **HIV positive samples at nine months post-treatment**. Only samples that were followed up at nine months were used for composition of diarrheal causing gut microbiota at six months post-treatment (n= 3).

3.2.2.6 Twelve months post treatment initiation

Two samples were able to be followed up in HIV positive samples from nine months to twelve months post-treatment. *Cryptosporidium parvum, Yersenia enterocolitica, Plesiomonas shigelloides* and *Helicobacter pylori* were detected at nine months post-treatment, however they were not found at twelve months post-treatment. *Campylobacter jejuni* and *Campylobacter coli* were not detected at nine months post-treatment, however, they were found at twelve months post treatment. *Escherichia coli,* Shigella and *Clostridioides difficile* had increased with treatment administration, this is depicted in figure 6. A decrease was observed in *Bacteroides fragilis* from nine months to twelve months post-treatment.

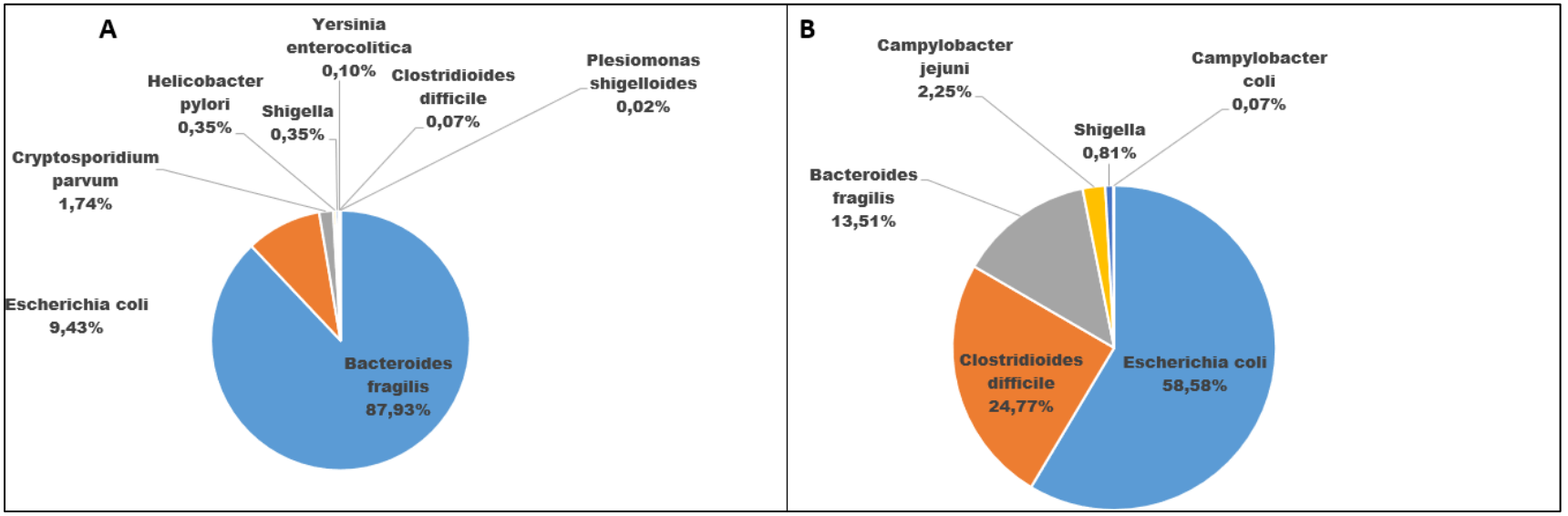

Figure 6: The comparison of diarrheal causing gut microbiota composition in HIV positive samples. **(A)** Composition of diarrheal causing gut microbiota in **HIV positive samples at nine months post-treatment**. **(B)** The composition of diarrheal causing gut microbiota in **HIV positive samples at twelve months post-treatment**. Only samples that were followed up at twelve months were used for composition of diarrheal causing gut microbiota at nine months post-treatment (n= 2).

3.2.3. ASSOCIATION BETWEEN CD4+ T CELLS/VIRAL LOAD AND DIARRHEAL CAUSING GUT MICROORGANISMS

CD4+ T cells and Viral load measurements were done using collected blood samples. CD4+ T cells were measured from HIV positive blood samples prior ART and at all time points after ART(three months to twelve months with three months interval). The association between proportion of diarrheal causing microorganisms and Viral load/CD4 count was assessed on most prevalent diarrheal causing gut microorganisms using Spearman's correlation. This was done to find if the level of CD4/Viral load had an impact in diarrheal causing microorganisms. There was no significant correlation between CD4 and diarrheal causing microorganisms; *E.coli* (r=-0.09575, *P* =0.8783), *Bacteroides fragilis* (r = 0.03085, *P* = 0.9607), Shigella spp. (r =0.1709, *P* = 0.7835), *Salmonella enterica* (r =-0.2106, *P* = 0.7339). The change in the level of viral load did not have an effect in the proportion of diarrheal causing gut microorganism. Hence, there was no significant correlation between viral load and diarrheal causing gut microorganisms; *E.coli* (r=0.6000, *P* =0.3500), *Bacteroides fragilis* (r = -0.8000, *P* = 0.1333), Shigella spp. (r = 0.5798, *P* = 0.3333), *Salmonella enterica* (r = 0.8660, *P* = 0.2000).

3.2.4 DIARRHEAL CAUSING GUT MICROBIOTA GENETIC DIVERSITY.

The genetic diversity of diarrheal causing gut microbiota was estimated by mapping the test sequences that were positive for a particular organism to its reference sequence. Mapped sequences were used in finding the variation in the genetic makeup of the reference sequence and test samples sequences. The variations were discovered by finding the single nucleotide polymorphisms (SNP) on the test sequences. Figure 7 is the representative of test sequences mapped to the reference sequence of ETEC with the generated SNPs. The diarrheal causing gut microbiota that were used for genetic diversity were those that had high proportions. Genetic diversity was determined at the species level from the diarrheal causing gut microbiota among HIV positive individuals prior ART to twelve months post-treatment initiation HIV negative participants.

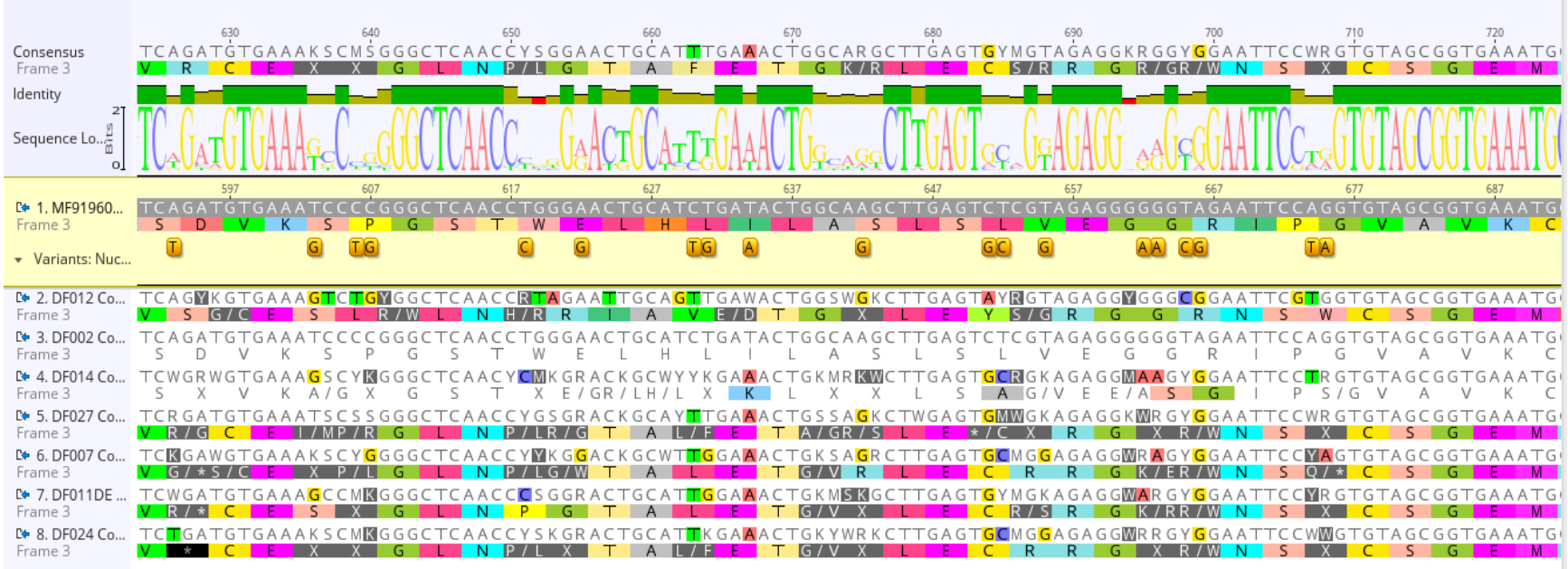

Figure 7: The representative genetic diversity of ETEC from HIV positive test samples. The consensus is the sequence that is generated from all the test samples mapped with the ETEC reference sequence. Identity is showing how identical the aligned sequences are; and green color represents 100% identity, Greeny-brown represents at least 30% and under 100% identity and red represent below 30% identity. The sequence logo shows the contribution of each nucleotide, the height of each nucleotide is proportional to its contribution to the information content. Sequence number 1 (MF91960) is the reference sequence; sequence number 2 (DF012) to sequence number 8 (DF024) are the test sequences. Variants are the SNPs polymorphisms detected. The sequences were translated using frame 3.

Campylobacter jejuni SNPs was 31.33% in HIV positive individuals prior ART and 22.07% in HIV negative individuals. Hence, the genetic diversity of *Campylobacter jejuni* was lower in HIV negative than HIV positive individuals. The SNPs were 31.87%, 34.4%, and 26.27% in HIV positive participants at three, six and twelve months post-treatment. The genetic diversity of *Campylobacter jejuni* was higher at three months and six months post-treatment in HIV positive participants. *Campylobacter jejuni* was not detected in HIV positive samples at nine months post treatment.

Genetic diversity of *Clostridioides difficile* was higher in HIV positive participants (20.4 % SNPs) than in HIV negative individuals' prior ART (17.87% SNPs). After ART initiation among HIV positive participants, 11.40%, 7.60%, 12.33% and 4.11% SNPs were found at three, six, nine and twelve months, respectively. The genetic diversity of *Clostridioides difficile* was higher before treatment when compared to after treatment.

Among HIV positive participants prior ART initiation, the rate of single nucleotide polymorphisms for Enteroaggregative *E. coli* (EAEC) was 18.07%. The rate of SNP for EAEC among HIV negative participants was 11.60%. Higher genetic variability (genetic diversity) of EAEC was revealed in HIV positive participants prior ART initiation than in HIV negative particpants. After ART initiation among HIV positive participants 27.40%, 1.80%, 14.73%, and 4.91% EAEC SNPs were found at three, six, nine and twelve months post-treatment initiation, respectively. Higher genetic diversity was exhibited at three months post-treatment, and lower genetic diversity was revealed at six months post-treatment.

Among HIV positive individuals prior ART, the rate of Enteropathogenic *E. coli* (EPEC) SNPs was 21.73%. Meanwhile, 12.07% SNPs were found in HIV negative individuals. Thus, high genetic diversity was revealed in HIV positive individuals at baseline than in HIV negative individuals. After ART initiation, 14.93%,10.60%, 25.13% and 12.57% EPEC SNPs were discovered at three, six, nine and twelve months, respectively in HIV positive individuals. Consequently, there was higher sequence variation (genetic diversity) at twelve months post-treatment, and lower genetic diversity at six months post-treatment in HIV positive individuals.

Enterotoxigenic *E. coli* (ETEC) had 15.8% SNPs in HIV positive individuals prior ART initiation. When compared to HIV positive, SNPs among HIV negative participants were lower (1.17% SNPs), hence a high genetic variability ETEC was revealed in HIV positive than in HIV negative individuals. after treatment initiation, SNPs at three, six, nine and twelve months were 31.27%,10.4%, 15.07% and 5.02%, respectively. Higher genetic diversity of ETEC was found at

three months post-treatment and lower genetic diversity was found at twelve months post-treatment in HIV positive participants.

High genetic diversity of *Salmonella bongori* was found in HIV positive individuals (18.73% SNPs) prior ART than HIV negative individuals (8.87% SNPs). After treatment initiation in HIV positive participants, 21.13%, 20.13%, 7.87% and 3.93% SNPs were found at three, six, nine and twelve months post-treatment, respectively. Higher genetic diversity of *Salmonella bongori* was revealed at three months post-treatment and lower genetic diversity was found at twelve months post-treatment.

Salmonella enterica subsp. enterica serovar Bareilly had 24.73% SNPs in HIV positive participants prior ART initiation and 13.40% in HIV negative participants. Consequently, high genetic diversity of *Salmonella bareilly* was observed in HIV positive than in HIV negative samples. At three months post-treatment in HIV positive participants the rate of SNPs was 23.93%. Higher genetic diversity was revealed prior ART than at three months post-treatment in HIV positive participants. *Salmonella bareilly* was not detected in HIV positive participants at six months, nine months and twelve months post treatment.

Among HIV positive participants prior ART initiation, *Salmonella enterica* subsp. enterica serovar Enteritidis had 21.47 % SNPs. The rate of SNPs in HIV negative participants was 18.60%. Hence, the genetic diversity of *Salmonella enteritidis* was higher in HIV positive participants at baseline than in HIV negative. At three months post treatment, 22.67% SNPs were found in HIV positive participants. Hence, genetic diversity of *Salmonella enteritidis* was higher at three post treatment than prior ART in HIV positive participants. *Salmonella enteritidis* was not detected in HIV positive samples at six, nine, and twelve months post-treatment.

Salmonella enterica subsp. salamae had 26.47% and 17.60% SNPs among HIV positive participants prior ART and HIV negative participants, respectively. Therefore, the genetic diversity of *Salmonella salamae* was higher in HIV positive individuals prior ART than in HIV negative individuals. After ART initiation in HIV positive participants, 22.67% SNPs found in HIV positive participants at three months pos-treatment. Genetic diversity of *Salmonella salamae* was higher in HIV positive participants prior ART than three months post-treatment in HIV positive participants. Salmonella salamae was not detected in HIV positive individuals at six months and twelve months post treatment.

Salmonella enterica subsp. enterica serovar typhi SNPs was detected in 15.27% in HIV positive participants prior ART and 20.93% in HIV negative participants. The genetic diversity of HIV

negative individuals was higher when compared to HIV positive individuals at baseline. After ART initiation,19.47% SNPs were found at three months post-treatment in HIV positive participants. Higher genetic diversity was found at three months post-treatment than prior ART in HIV positive participants. Salmonella typhi was not detected in HIV positive individuals at six months and twelve months post treatment.

CHAPTER 4: DISCUSSION, CONCLUSION, LIMITATION AND RECOMMENDATION

4.1 DISCUSSION AND CONCLUSION

The gastrointestinal tract of HIV infected individual is compromised due to breach of intestinal barrier and loss of mutualistic gut microbiota. This encourages the growth of pathogenic gut microbiota. Pathogenic and opportunistic microorganisms cause infections with the commonest manifestation being diarrhoea. Multiple infection by enteropathogens is common in HIV infected individuals. EPEC, *Clostridioides difficile*, EAEC and norovirus GI/GII are the most prevalent enteropathogens in HIV infected patients (Seid et al., 2018). This study aims at determining the proportion and genetic diversity of diarrheal causing gut microbiota.

In this study, the most prevalent enteropathogens in HIV positive patients prior to ART initiation were *Escherichia coli*, *Bacteroides fragilis*, Shigella, *Salmonella enterica*, Clostridioides difficile and *Campylobacter jejuni*. *Helicobacter pylori*, *Campylobacter coli, Cryptosporidium parvum, Plesiomonas shigelloides, Vibrio cholera* and *Yersinia enterocolitica* were the least prevalent. The predominance of Salmonella and Shigella in HIV positive individuals prior ART was also reported in (Belay et al., 2020). This was in contrast with the study by Kebede, Aragie, and Shimelis, 2017 who also found high prevalence of Salmonella spp. and Campylobacter spp. in HIV patients prior ART. In contrast with this study, Cryptosporidium parvum was one of the most common detected enteropathogens (Shah, Kongre, Kumar and Bharadwaj, 2016).

Among HIV negative patients the most prevalent diarrheal causing gut microbiota were *Escherichia coli, Bacteroides fragilis,* and *Clostridioides difficile,* while the least prevalent were; *Salmonella enterica, Campylobacter jejuni* and Shigella. *Escherichia coli* and Shigella had a high percentage of occurrence in HIV positive than in HIV negative. *Bacteroides fragilis, salmonella enterica, Clostridioides difficile, and campylobacter jejuni* had high percentage of occurrence in HIV negative than in HIV positive samples. The compromised gastrointestinal tract in HIV infected individuals encourages the invasion of more enteropathogens (Kebede, Aragie, and Shimelis, 2017) as a result, in this study there were more enteropathogens in HIV positive individuals as compared to HIV negative individuals. These enteropathogens in HIV positive individuals may lead to dysbiosis and increased chronic gut inflammation which in turn causes diarrhoea.

After ART initiation in HIV positive patients, Campylobacter coli and V*ibrio cholera* decreased with treatment from three months to twelve months post treatment. *Clostridioides difficile* and

Plesiomonas shigelloides had increased at three months and six months post treatment, however, they decreased at twelve months post treatment. *Campylobacter jejuni*, *Helicobacter pylori* and *Plesiomonas shigelloides* decreased with treatment from three months to six months post treatment and increased at twelve months post treatment. *E.coli*, shigella, *Clostridioides difficile*, *Yersenia enterocolitica* and *Cryptosporidium parvum* had increased at three months, decreased at six months, then increased at twelve months post treatment. *Bacteroides fragilis* had decreased at three months, increased at six months, and then decreased at twelve months post treatment. *Salmonella enterica* increased at six months post treatment, however, it was not detected at six- and twelve-months post treatment. *Yersenia enterocolitica* decreased at six months and increased at twelve months post treatment. The proportion of diarrheal causing gut microbiota were fluctuating throughout the intake of ART at different time points. This also support inconsistent change in number of viral loads and CD4 counts. However, this is statistically insignificant. In addition, this shows that patients were not taking their treatment regularly hence the virus was not suppressed. Furthermore, this may also be due to viral mutation or not taking the right ART dose.

Similarly to what has been found in this study, Shigella and *E.coli* were the most detected in HIV infected individuals on ART (Seid et al., 2018). In contrast, Shigella and *E.coli* were the least prevalent in HIV infected individuals on ART (Kebede, Aragie, and Shimelis, 2017). Salmonella was the least prevalent in HIV positive patients taking ART, and this correspond with what was found in this study at nine and twelve months post treatment among HIV positive individuals (Bayleyegn, Fisaha and Kasew, 2021). In contrast with the current study, Campylobacter spp. and Salmonella spp. were the most prevalent enteropathogens among HIV individuals taking ART (mean of 39 weeks post treatment) (Kebede, Aragie, and Shimelis, 2017). Cryptosporidium spp. had decreased with treatment in HIV infected individuals on ART (Kaniyarakkal et al.,2016; Nsagha et al., 2015).

SNP is a genomic variant on a single base at a position of a nucleotide in a DNA. SNPs are known to be caused by point mutation and are a common form of genetic variation which are used for strain identification (Rahman, Lim and Park, 2022). In this study, SNPs were used to determine the change in genetic diversity of diarrheal causing gut microbiota.

EAEC, ETEC, EPEC, *Salmonella bongori*, *Salmonella Bareilly, Salmonella Enteritidis, Salmonella salamae, Salmonella typhimurium, Clostridioides difficile,* and *Campylobacter jejuni* were used to find the change in genetic diversity of diarrheal causing gut microbiota. Genetic diversity was higher in HIV positive participants than in HIV negative participants in all the targeted diarrheal

causing gut microbiota except for *Salmonella typhimurium*, which had high genetic diversity in HIV negative than in HIV positive participants. The high genetic diversity of diarrheal causing gut microbiota in HIV positive individuals may lead to adaptation in the gastrointestinal tract environment, hence rendering them the ability to resist eradication by medication and immune activation.

At three months post treatment, genetic diversity increased in EAEC, ETEC, *Salmonella bongori, Salmonella enteritidis, Salmonella typhimurium*, and *Campylobacter jejuni*. However, a decrease in genetic diversity at three months post treatment was found in EPEC, *Salmonella bareilly, Salmonella salamae*, and *Clostridioides difficile*. At six months post treatment, genetic diversity of more enteropathogens decreased with treatment, and these include, EAEC, ETEC, EPEC, *Salmonella bareilly, Salmonella bongori, Salmonella salamae,* and *Clostridioides difficile*. There was no change in *Salmonella typhimurium* at six-month post treatment, when compared to three months post treatment. An increase in genetic diversity was found in *Salmonella enteritidis* and *Campylobacter jejuni* at six months post treatment. The decrease in genetic diversity of diarrheal causing gut microbiota at six months post treatment may encourage an immune respond.

At twelve months post treatment, *Salmonella salamae*, EAEC, ETEC, EPEC and *Clostridioides difficile* had increased genetic diversity. Moreover, *Salmonella bareilly, Salmonella bongori, Salmonella enteritidis, Salmonella typhimurium*, and *Campylobacter jejuni* had decreased genetic diversity. The fluctuation of diarrheal causing gut microbiota's genetic diversity from six months to twelve months post treatment may be due to inconsistent change in number of viral loads and CD4 counts at different time points of ART. However, was it not statistically significant, as a result, there was no correlation between CD4/viral load and diarrheal causing gut microbiota.

In conclusion, this study showed ART which improves the immune system of HIV infected individuals, could not eradicate the diarrheal causing gut microbiota in HIV infected individuals. As a result, ART does not affect the proportion of diarrheal causing gut microbiota in HIV positive population, therefore, this does not support the hypothesis of this study. In addition, this study revealed high genetic diversity of diarrheal causing gut microbiota in HIV positive individuals than in HIV negative individuals. Moreover, this study also revealed a decreased genetic diversity of diarrheal causing gut microbiota in HIV infected population with long term ART administration. As a result, this may indicate that even though ART could not eradicate diarrheal causing gut microbiota, it encourages low chances of genetic diversity.

4.2 LIMITATION AND RECOMMENDATIONS

In this study, diarrheal causing gut microbiota were detected with the use of a highly sensitive technique, i.e., Next generation sequencing (Shotgun metagenomics). Shotgun metagenomics allow the sequencing of all genomes in a sample. It was of importance to find the change in the proportion and genetic diversity of diarrheal causing gut microbiota from the start of ART to twelve months ART administration in HIV positive individuals. However, despite all that were found, there were some limitations, and these include (1) sample number: Collection of lower sample number resulted in comparing results of different sample sizes. Study participants who were willing to be part of the study were few to conclude the proportion and genetic diversity of diarrheal causing gut microbiota in HIV positive individuals. Most individuals did not want to be part, some died, and others dropped out of the study. (2) COVID-19 pandemic: sample collections were restricted during COVID-19 pandemic. As a result, no samples were collected in 2020 after the pandemic. Additionally, it would be advisable to use multiple hospitals and clinics as study sites for recruiting participants during sample collections to find valuable number of participants. This will help to maintain higher sample number even when some participants decide to drop from the study. Additional research would be of vast importance to reveal the proportion and genetic diversity of all diarrheal causing gut microbiota using targeted method like TaqMan Array Card (TAC) for detection of viruses, bacteria, and parasites. TAC is also cost effective and saves time, since it allows for simultaneous detection of different microorganisms at species and subtype level; hence, it does not require samples to be sequenced after testing.

CHAPTER 5: REFERENCES

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